# NEURAL MECHANISMS OF COGNITIVE CONTROL AND EMOTION IN BIRDS

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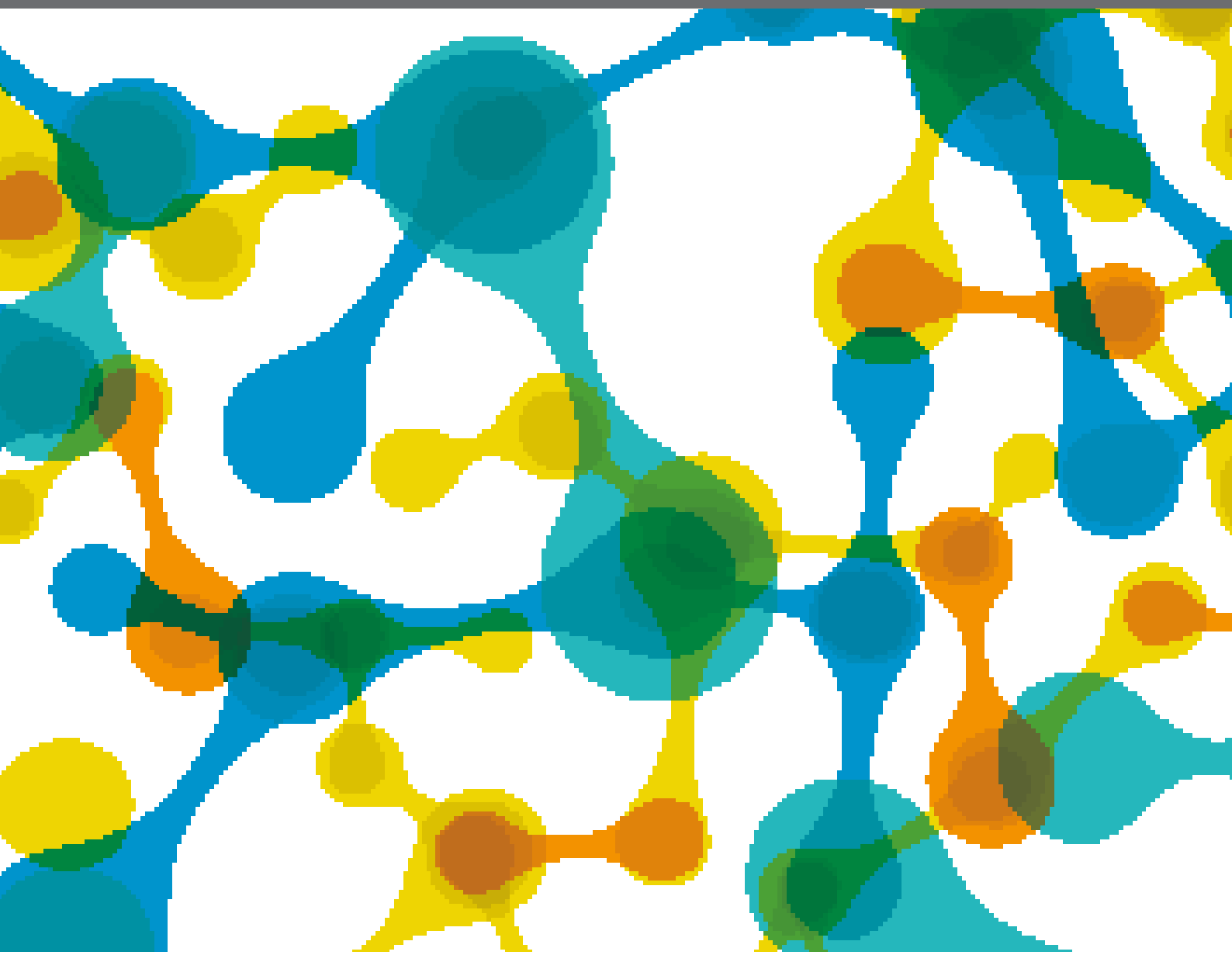

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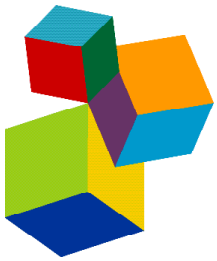

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# NEURAL MECHANISMS OF COGNITIVE CONTROL AND EMOTION IN BIRDS

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# Editorial: Neural mechanisms of cognitive control and emotion in birds

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KEYWORDS

**avian brain, learning, lateralization, reward, songbird**

Editorial on the Research Topic
Editorial Neural mechanisms of cognitive control and emotion in birds

In the present Research Topic, we have been successful in attracting contributions from a wide range of research centers around the globe. The new results gathered in a variety of biological disciplines represent important advances toward a better understanding of emotion and cognitive control in birds. Out of 21 articles by 67 authors (2 brief original research reports, 15 original research reports, and four review articles including one systematic review study), 10 studies focused on galliform precocial birds (and their hatchlings), nine on passerine birds (songbirds including crows and Java sparrows), and two on pigeons. By reviewing these studies, we hope to provide an update on cutting edge results hallmarking knowledge on avian brain and behaviors of today. We are grateful for the contributors, the guest editors and the reviewers for their efforts and intriguing ideas, which, so we believe, will set the foundation for future progress in avian physiology in a highly comprehensive manner, ranging across the fields of behavioral, anatomical, endocrinological, biochemical and evolutionary biology.

## Stress, emotion, and intrinsic reward system for singing

Animals are under continuous and endless barrages of stressors both from ambient physical world and social life. Kato et al. reports their novel finding on the molecular basis of stress responses and specified neurosecretory protein GL/GM (NPGL/NPGM), which acts as the stress mediator in the mediobasal hypothalamus. In particular, social isolation stress causes an acute increase in the expression of these proteins. Pross et al. examined the extended amygdala (division of the dorsal striatum) and found multiple types of enkephalin cells involved in stress regulation. Detailed neurochemical identification of the limbic network will constitute the critical basis for physiological specification of stress responses. Comparisons of songbirds (zebra finches ZFs and Bengalese finches BFs) led

Table of contributions

| title | first author | species | methods | main conclusion |
|---|---|---|---|---|
| Effect of Stressors on the mRNA Expressions of Neurosecretory Protein GL and Neurosecretory Protein GM in Chicks | Masaki Kato | chicks | gene expression of regulatory proteins | NPGM, but not NPGL or HDC, participates in several physiological responses to stress in chicks. |
| Developmental-Based Classification of Enkephalin and Somatostatin Containing Neurons of the Chicken Central Extended Amygdala | Alessandra Pross | chicken (adults) | gene expression of neuromodulators | Enkephalin cells likely derive from the dorsal striatal division (extended amygdala) as the basis for studying the role of these cells in stress regulation. |
| Effect of Darkness on Intrinsic Motivation for Undirected Singing in Bengalese Finch (Lonchura striata Domestica): A Comparative Study with Zebra Finch (Taeniopygia guttata) | Yunbok Kim | songbirds | behavioral study and blood corticosterone | Differences between two species of finch provide insights into the interactions among singing motivation, ambient light, and environmental stress. |
| The Role of the Endogenous Opioid System in the Vocal Behavior of Songbirds and Its Possible Role in Vocal Learning | Utkarsha A. Singh | songbirds | gene expression of transmitter receptors | Expression pattern of opioid receptors and vocal learning in songbird. |
| Birdsong and the Neural Regulation of Positive Emotion | Lauren V. Riters | songbirds | behavioral analysis and neuroanatomy | Nucleus accumbens contributes to positive emotional states that motivate and reward singing behavior and the responses to song. |
| Contribution of Endocannabinoids to Intrinsic Motivation for Undirected Singing in Adult Zebra Finches | Yunbok Kim | songbirds | behavioral pharmacology | Endocannabinoids are critically involved in the regulation of intrinsic motivation of undirected singing. |
| Activation of the Nucleus Taeniae of the Amygdala by Umami Taste in Domestic Chicks (Gallus gallus) | Francesca Protti-Sánchez | chicks | c-Fos imaging | Neuronal responses to umami and bitter tastes are lateralized in nucleus taeniae of the amygdala, a region processing reward information. |
| "Prefrontal" Neuronal Foundations of Visual Asymmetries in Pigeons | Qian Xiao | pigeons | *in vivo* single unit electrophysiology | The hemispheric asymmetries for visual discrimination are realized by a sequential buildup of lateralized neuronal responses in the avian forebrain. |
| Behavioral Training Related Neurotransmitter Receptor Expression Dynamics in the Nidopallium Caudolaterale and the Hippocampal Formation of Pigeons | Christina Herold | pigeons | gene expression of transmitter receptors | Patterns of neurotransmitter receptor expression in the avian prefrontal cortex and the hippocampal formation suggest association to learning and memory. |
| Behavioral and Evolutionary Perspectives on Visual Lateralization in Mating Birds: A Short Systematic Review | Masayo Soma | passerine and non-passerine birds | phylogenetic comparative methods | Passerine and non-passerine species showed opposite eye use for mating, which could have stemmed from a difference in altricial *vs.* precocial development. |
| Imprintability of Newly Hatched Domestic Chicks on an Artificial Object: A Novel High Time-Resolution Apparatus Based on a Running Disc | Naoya Aoki | chicks | behavioral study of imprinting | New apparatus was developed to detect behavioral changes during imprinting. |
| Src and Memory: A Study of Filial Imprinting and Predispositions in the Domestic Chick | Maia Meparishvili | chicks | biochemistry of learning | One pool of Src (tyrosine kinase) reflects the chick's predisposition to learn, while the second pool the inhibited condition as a result of learning. |
| GSK-3β Inhibition in Birds Affects Social Behavior and Increases Motor Activity | Stan Moaraf | zebra finch | behavioral pharmacology | Inhibition of GSK-3β acutely affected the social behavior and caused hyperactivity. |
| Suppressive Modulation of the Chick Forebrain Network for Imprinting by Thyroid Hormone: An *in Vitro* Study | Yuriko Saheki | chicks | slice electrophysiology, pharmacology | Thyroid hormone enhanced GABA-A action and suppressed the NMDA-R in IMM neurons, but the synaptic potentiation remained unchanged. |
| Proportional Cerebellum Size Predicts Fear Habituation in Chickens | Diego Stingo-Hirmas | chicken (adults) | neuroanatomy | Proportional cerebellum size does not predict an individual's fear response, but rather the habituation process to a fearful stimulus. |
| Chick Hippocampal Formation Displays Subdivision- and Layer-Selective Expression Patterns of Serotonin Receptor Subfamily Genes | Toshiyuki Fujita | chicks | *in situ* hybridization of receptor subtypes | Subfamilies of the serotonin receptor genes show subdivision- and layer-selective expression patterns in the hippocampus. |
| Serotonergic Neurons in the Chick Brainstem Express Various Serotonin Receptor Subfamily Genes | Toshiyuki Fujita | chicks | *in situ* hybridization of receptor subtypes | The expression pattern of 5-HT receptors in the serotonin neurons of chick DR and MR may vary, suggesting heterogeneity among and within the serotonin neurons of the DR and MR in the chick brainstem |
| The Acute Pharmacological Manipulation of Dopamine Receptors Modulates Judgment Bias in Japanese Quail | Katarína Pichová | Japanese quail | behavioral pharmacology | Dopamine D1 and D2 receptor blockade leads to a decrease in the reward expectation and the negative judgment of stimuli. |

(Continued on following page)

Table of contributions (*Continued*)

| title | first author | species | methods | main conclusion |
|---|---|---|---|---|
| American Crow Brain Activity in Response to Conspecific Vocalizations Changes When Food Is Present | Loma John T. Pendergraft | crows | FDG-PET imaging | The nucleus taenia (TnA) and caudal nidopallium show increased activity in response to conspecific calls if audio/visual stimuli are combined; a PET imaging study. |
| Mismatch Responses Evoked by Sound Pattern Violation in the Songbird Forebrain Suggest Common Auditory Processing with Human | Chihiro Mori | Java sparrow | event-related potential (ERP) | Violation of the triplet sequence pattern elicits mismatch negativity, indicating the ability to extract sound sequence in the auditory forebrain. |
| Song Preference in Female and Juvenile Songbirds: Proximate and Ultimate Questions | Tomoko G. Fujii | songbirds | Tinbergen's 4 questions | The current understanding of song preference in female and juvenile songbirds are summarized in the context of Tinbergen's four questions. |

Kim et al. to suggest that spontaneous singing (undirected songs, United States) could be associated with environmental stress. These two species of finches revealed striking differences in the frequency of "dark singing," where BFs emit United States much more than ZFs. Ambient light condition critically controls the singing motivation, but with a strong species difference.

Rich lines of evidence and ideas seem to converge on the positive emotional aspects of singing. Singh and Iyenga reviewed the opioid receptors in songbird brain and suggested that birds regulate singing and song learning by intrinsic motivational control. In accordance, the review by Riters et al. suggests that the nucleus accumbens in the medial striatum could be the site for intrinsic reward system that enables birds to proactively seek for mate. Kim and Kojima in their second paper, presented endocannabinoids as the activators of undirected spontaneous singing in songbirds. Study of singing behavior does not only tackle the issues of learned vocalization, but also those of its emotional/motivational background.

## Lateralization and its evolutionary background

Lateralized hemispheric control is widely observed in sensory perception, cognition, and executive control of behaviors. Protti-Sánchez et al. reports a unique case where the umami-tastes (tasting of amino acids) yield lateralized activation in the chick limbic nucleus taeniae. Xiao and Güntürkün further expand the scope toward an associative cortex, or "prefrontal"-like avian analogue, the nidopallium caudolaterale (NCL) of pigeons. They stress the importance of sequential build-up of lateralization through the cascade of signal processing in the visual system. The detailed study on transmitter receptors in NCL by Herold et al. should also be referred to in the light of functional lateralization. Readers of the subject often feel lost in the plethora of literature, unable to construct the framework for understanding these issues. The systematic review article by Soma provides us with such a framework, i.e., a comprehensive survey of visual lateralization. In particular, the finding of opposite lateralization patterns in passerine and non-passerine birds strikingly underlines the importance of evolutionary perspectives.

## Cellular and molecular substrates of learning

Studies of filial imprinting are still actively progressing. Aoki et al. propose a novel and quantitative method of imprinting that allows partial constraint of the body and head in awake chicks. Meparishvili et al. pointed out tyrosine kinase (Src) as a novel key player in innate predisposition and engram formation in the pallial network of domestic chicks. It is also noted that the kinase system plays a critical role in execution of social behaviors of adults, just as Moaraf et al. revealed the involvement of GSK-3β in the social behaviors of zebra finches; its inhibition caused birds to move closer to a stranger than to a familiar companion, furthermore, the treated birds showed hyperactivity. The cellular target of the thyroid hormone, another player critically involved in the sensitive period, was examined by Saheki et al., who revealed that the hormone acutely enhances GABA-AR mediated synaptic inhibition, supporting the idea that the excitation-inhibition balance is critical for the control of critical period.

## Structure and molecular architecture of the avian brain

Beside function, morphology of the avian brain is also subject to developmental control. Stingo-Hirmas et al. give us a unique example where the size of cerebellum could be a predictor of the habituation to fearful objects in adult chickens. Also, the importance of careful behavioral testing and control of genetic background are being stressed in this study. One should not overlook the importance of descriptive studies such as detailed

information on gene expression either. The avian hippocampus has been assumed to lack distinct subdivisions and layer structures. Fujita et al. present a significant contribution to our understanding of the avian hippocampus by detailed *in situ* hybridization analysis of serotonin receptor subtypes. In another paper, Fujita et al. were successful in the morphological dissociation between the dorsal and medial raphe in the chick brainstem by the same set of serotonin receptor subfamilies. Acute behavioral pharmacology in quails by Pichová et al. clearly showed functional control of reward expectation by the dopamine system. Notably, the manipulation of D1 and D2 receptors by antagonists yielded coherent rather than opposite results, meaning that both receptor types are likely involved in the given aspects of reward prediction.

### Comprehensive studies for prospect and future steps

Several new and powerful techniques have been applied to unravel the confounding issues of emotional control. Pendergraft et al. applied FDG-PET imaging of the brain in awake crows, and revealed that the activation of the nucleus taeniae amygdala (limbic) and nidopallium caudolaterale (NCL, medial portion) requires multimodal combination of multimodal stimuli of conspecifics, namely the sight of food and the sound of conspecific vocalization during foraging. Similarly innovative approach was successfully applied in the study of event-related mismatch responses reported in Java sparrows by Mori and Okanoya. Field potentials recorded from the auditory area of telencephalon (NCM) revealed significant effects of deviations from the repeated/habituated sounds in both pure tone and natural vocalization. Furthermore, the same team Fujii et al. contributes a comprehensive review on the question of why females are so choosy about male songs. As with all other biological disciplines, physiological mechanisms should be studied in the light of evolution/adaptive values, because evolutional thinking is a rich source of novel approaches in physiology.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

## Conflict of interest

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# Src and Memory: A Study of Filial Imprinting and Predispositions in the Domestic Chick

*Maia Meparishvili*[1], *Lela Chitadze*[1], *Vincenzo Lagani*[1], *Brian McCabe*[2]* *and Revaz Solomonia*[1,3]*

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Visual imprinting is a learning process whereby young animals come to prefer a visual stimulus after exposure to it (training). The available evidence indicates that the intermediate medial mesopallium (IMM) in the domestic chick forebrain is a site of memory formation during visual imprinting. We have studied the role of Src, an important non-receptor tyrosine kinase, in memory formation. Amounts of total Src (Total-Src) and its two phosphorylated forms, tyrosine-416 (activated, 416P-Src) and tyrosine-527 (inhibited, 527P-Src), were measured 1 and 24 h after training in the IMM and in a control brain region, the posterior pole of nidopallium (PPN). One hour after training, in the left IMM, we observed a positive correlation between the amount of 527P-Src and learning strength that was attributable to learning, and there was also a positive correlation between 416P-Src and learning strength that was attributable to a predisposition to learn readily. Twenty-four hours after training, the amount of Total-Src increased with learning strength in both the left and right IMM, and amount of 527P-Src increased with learning strength only in the left IMM; both correlations were attributable to learning. A further, negative, correlation between learning strength and 416P-Src/Total-Src in the left IMM reflected a predisposition to learn. No learning-related changes were found in the PPN control region. We suggest that there are two pools of Src; one of them in an active state and reflecting a predisposition to learn, and the second one in an inhibited condition, which increases as a result of learning. These two pools may represent two or more signaling pathways, namely, one pathway downstream of Src activated by tyrosine-416 phosphorylation and another upstream of Src, keeping the enzyme in an inactivated state via phosphorylation of tyrosine-527.

**Keywords: learning, recognition memory, IMM, intermediate medial mesopallium, memory formation, early learning**

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## INTRODUCTION

Src is a non-receptor protein tyrosine kinase that participates in a number of neuronal processes including neurotransmitter release, neurotransmitter receptor function, and synaptic plasticity. Autophosphorylation of tyrosine-416 in the activation loop of Src is thought to increase Src activity, whereas phosphorylation at tyrosine-527 by other kinases suppresses Src activity (Ohnishi et al., 2011). There is evidence that Src participates in learning and/or memory, possibly via phosphorylation of *N*-methyl-D-aspartate receptors, but its role is poorly understood.

Overexpression of Src affects excitatory synaptic transmission in area CA3 of the mammalian hippocampus, and impairs fear memory (Yan et al., 2017). After spatial maze learning, upregulation of Src mRNA was observed in area CA3 (Zhao et al., 2000). Increased autophosphorylation of Src enhances hippocampal long-term potentiation and spatial memory (Wang et al., 2018). To clarify the role of Src in memory, it would be helpful to simultaneously measure its activated form phosphorylated at tyrosine-416 (416P-Src) and its inactive form phosphorylated at tyrosine-527 (527P-Src) in a vertebrate brain region where memory is encoded, and for a type of learning for which a graded measurement of memory strength is available.

Visual filial imprinting in the domestic chick is a type of learning having these favorable characteristics for memory research. Imprinting is a process whereby, when exposed to a salient visual stimulus (training), a chick typically approaches, learns the features of, and subsequently recognizes the stimulus. Memory strength may be measured in terms of behavioral preference, namely approach to the training stimulus relative to approach to a stimulus not previously seen (Bolhuis, 1991; Horn, 2004; McCabe, 2019). The intermediate medial mesopallium (IMM) is a chick forebrain region of crucial importance for visual imprinting, and the available evidence indicates that this region stores information about the imprinting stimulus (Horn, 1985, 2004). Criteria used to infer that a change following training is learning-related have been formulated (Horn and Johnson, 1989; McCabe, 2013; Solomonia and McCabe, 2015; Margvelani et al., 2018a) (see also Discussion).

The IMM resembles mammalian association cortex (Horn, 1985) and, from evidence based on the distribution of cholecystokinin mRNA expression, homology with mammalian neocortical layers 2 and 3 has been proposed (Atoji and Karim, 2014). The IMM is also important in passive-avoidance learning (Rose, 2000), the nearby anterior medial mesopallium in auditory imprinting (Bredenkötter and Braun, 1997), and the caudal medial mesopallium in memory of tutor song in songbirds (Gobes et al., 2010).

The left and the right IMM are involved in imprinting in different ways (Horn, 2004). Shortly after training, learning-related molecular changes occur on both the left and the right sides of the IMM, whereas ∼24 h after training they are predominantly expressed in the left IMM. Learning-related changes also occur in the right IMM at 24 h, but in general are weaker than on the left side (Solomonia and McCabe, 2015).

Several proteins are changed in a learning-related manner following imprinting (reviewed in Solomonia and McCabe, 2015). The affected proteins include, among others, cell adhesion molecules, neurotransmitter receptors, protein kinases and their substrates, transcription factors, mitochondrial and membrane proteins, vesicle recycling components, and translation factors (McCabe and Horn, 1988, 1994; Sheu et al., 1993; Solomonia et al., 1997, 1998, 2000, 2003, 2005, 2008, 2011, 2013; Meparishvili et al., 2015; Margvelani et al., 2018a,b; Chitadze et al., 2020). Further results indicate that neurotransmitter release in the IMM is modulated with memory after imprinting (McCabe et al., 2001; Meredith et al., 2004).

Statistical analysis has indicated that certain biochemical quantities in the IMM that are correlated with the strength of learning are not the result of learning, but exist prior to training and reflect processes associated with a predisposition to learn well. Such processes thus give rise to rapid learning when training occurs. A microRNA with this property has been identified in the left IMM (Margvelani et al., 2018a).

In the present study, we have inquired whether the amounts of total Src, 416P-Src (putative activated form), and 527P-Src (putative inhibited form) are changed in a learning-related manner 1 h and 24 h after imprinting training. Four brain regions were analyzed, the left and the right IMM and two control forebrain regions, which are not involved in imprinting, the left and the right posterior pole of the nidopallium (PPN). We find a strong relation between the strength of learning and the level of 527P-Src at both times in the left IMM. In addition, there was evidence at both times implicating 416P-Src in the left IMM in a predisposition to learn readily. Twenty-four hours after training, Total-Src was dependent on strength of learning in both the left and the right IMM.

# MATERIALS AND METHODS

## Behavioral Training and Testing

Fertile eggs (Cobb 500) were obtained from Sabudara farm, Tbilisi, Georgia. Nineteen batches of eggs were incubated and hatched in darkness, and the chicks were reared in isolation in darkness and trained for 1 h. Chicks from 10 batches were used in experiments in which measurements were made 1 h after the end of training; in the remaining nine batches measurements were made 24 h after the end of training. In each batch, there were up to three trained chicks and a control chick from the same hatch. At 22–28 h post-hatch, each chick to be trained was exposed in a running wheel (1 revolution = 94 cm) to a training stimulus (a cuboidal red box rotating about a vertical axis) for 1 h. The box contained a light surrounded by a red filter (Lee Filters 106 Primary Red); the largest pair of sides of the box (18 × 18 cm) were translucent and vertical and the remaining sides (18 × 9 cm) were black. During training, the stimulus was turned on for 50 s and then off for 10 s each minute. The maternal call (70–75 dB) of a hen was played while the stimulus was on, a procedure that accelerates imprinting to a visual stimulus (Smith and Bird, 1963). As a chick attempted to approach the training stimulus, it rotated the running wheel and revolutions of the wheel were counted to provide a measure of approach activity ("training approach"). A preference test without the maternal call was performed 10 min after training, in which each chick in a running wheel was shown sequentially the training stimulus and an alternative stimulus that the chick had not previously seen, in the order training/alternative/alternative/training. Each period of exposure during the test lasted 4 min, making a total of 8 min for each stimulus. The alternative stimulus was a right circular cylinder (height 18 cm and diameter 15 cm) with a translucent wall and vertical axis, rotating about this axis at 28 revolutions

**Abbreviations:** DF, degrees of freedom; SE, standard error; SEM, standard error of the mean; t, Student's t.

per minute. The cylinder contained a light surrounded by a blue filter (Lee filters HT 118 Brilliant Blue). See Horn (1998) for illustrations of the training and alternative stimuli. A preference score (approach to training stimulus during test × 100/total approach during test) measured the strength of imprinting (i.e., learning). A preference score of ∼50 indicates poor learning, whereas a score of ∼100 indicates strong learning. There are individual differences in the preference scores of chicks after a fixed period of training. This variation was used to determine whether the amount of protein was related to preference score and, by means of subsequent analysis (see below and Discussion), whether a change in protein amount was attributable to learning that occurred during training. Chicks were decapitated either 1 or 24 h after the end of training. Four tissue samples were removed from each chick, from the left and the right IMM and from the left and the right PPN, and immediately frozen on dry ice. Thus in each batch, there were four samples from each of up to four chicks (one untrained, up to three trained), yielding up to 16 samples in all. The locations of the IMM and PPN are shown in a previous publication (Solomonia et al., 2013). For details of IMM removal, see Davies et al. (1985) and for PPN removal see Solomonia et al. (1998). Samples were coded after collection, and all further procedures were conducted blind. All behavioral experiments were carried out at the I. Beritashvili Centre of Experimental Biomedicine and performed in compliance with its approved animal care guidelines. The number of animals used was found sufficient in previous studies to permit reliable detection of correlations between biochemical measures in the IMM and preference score.

## Sample Preparation, SDS Electrophoresis, and Western Blotting

Samples were rapidly homogenized in standard Tris-HCl buffer containing phosphatase inhibitor and protease inhibitor cocktails, and sodium dodecylsulphate (SDS) solution was added at a concentration of 5% and the mixture was at 95°C for 3 min. Protein concentrations were determined in quadruplicate using a micro bicinchoninic acid protein assay kit (Pierce). Aliquots containing 30 μg of protein in 30 μl were subjected to SDS gel electrophoresis and Western blotting (Meparishvili et al., 2015). After protein had been transferred onto nitrocellulose membranes, the membranes were stained with Ponceau S solution to confirm transfer and uniform gel loading. In each batch, samples were run in triplicate, where one filter was stained with polyclonal antibody raised against total Src protein (see below), the second one with antibody against 416P-Src (Abcam, ab4816), and the third one with antibody against 527P-Src (Abcam, ab4817).

Rabbit polyclonal antibodies against chicken Src (UNIPROT P00523) were produced using an epitope the 19-mer peptide RRSLEPPDSTHHGGFPASC (amino acid residues 15–32 of chicken Src), which contained a terminal cysteine for conjugation to a carrier protein. Antibodies were purified on an antigen-affinity column. This epitope does not coincide with known phosphorylation sites, and thus recognizes both phosphorylated and non-phosphorylated forms of Src. The specificity of antibody reaction was confirmed by adsorption of the control peptide.

Standard immunochemical procedures were performed using peroxidase-labeled secondary antibodies and SuperSignal West Pico Chemiluminescent Substrate (Pierce-Thermo Fisher Scientific, Waltham, Massachusetts, USA). Blots were then exposed with intensifying screens to X-ray films preflashed with Sensitize (Amersham; GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). Optical density of protein bands was measured using LabWorks 4.0 software (Ultra-Violet Products Ltd., Cambridge, UK). Autoradiographs on each gel were calibrated using standard amounts of protein (15, 30, 45, and 60 μg total protein) obtained from homogenate fractions of the IMMs from a group of untrained chicks. For these standards, the optical densities of bands immunostained for the corresponding protein were linearly related to the amount of protein (**Supplementary Figure S1**). To obtain data for regression analysis the optical density of each band from each sample was divided by the optical density which, from the calibration of the same autoradiograph, corresponded to 30 μg of total protein in the standard (Meparishvili et al., 2015). This quantity is termed "relative amount of protein".

## Statistical Analysis

A linear mixed-effects regression model was fitted to the relative amount of protein, with fixed term preference score and random terms chick nested within batch. The analysis was conducted using the lme function in the nlme package (Pinheiro et al., 2021) of R (R Development Core Team, 2016). The R script used for statistical analysis was essentially that published by Margvelani et al. (2018a). Data from the left and the right IMM and the left and the right PPN were analyzed separately owing to the functional disparity of the IMM and PPN, where the IMM is essential for imprinting and the PPN is not (Solomonia et al., 1997, 1998, 2000, 2005, 2011, 2013; Margvelani et al., 2018a). The two sides were analyzed separately because there is a functional hemispheric asymmetry in IMM with respect to imprinting, reviewed in Solomonia and McCabe (2015), including neurobiological consequences of learning; to date, when bilateral effects of learning in the IMM occur, they have been found predominantly on the left side (reviewed in Solomonia and McCabe, 2015). Results are summarized in **Tables 1**, **2** and **Supplementary Tables S1**–**S6**. Approach during training and approach during testing were added to the regression model as covariates in a further series of analyses. When preference score and one or both approach terms were significant in these analyses, or when the addition of one or both significant covariates caused the preference score term to lose significance, this is additionally reported in Results. For the figures showing an association between protein amount and preference score, variation attributable to differences between batches has been removed from the relative amount of protein by subtracting the estimated effect of batch (batch mean – overall mean) from each value, as occurred in the statistical analysis. This quantity is referred to as "the standardized relative amount of protein." A regression line was fitted to the plot of the standardized relative amount of protein vs. preference score,

between the lowest and highest preference score achieved (see **Figures 1**–**4**). The regression line was used to estimate protein levels corresponding to (i) preference score 50 (no learning) and (ii) the maximum preference score attained in the experiment (indicating strong learning; in the present experiments this preference score was 100).

In interpreting the results, we first enquired whether protein level was significantly correlated with preference score, the measure of learning. This correlation was a necessary criterion for inferring learning-dependent change of protein level during training. However, a correlation can be interpreted in an alternative way, namely that protein level does not change during learning but reflects a predisposition to learn readily. In this second case, variation in protein level would contribute to the correlation/regression, thereby reducing the residual variance about the regression line (Margvelani et al., 2018a). A predisposition was therefore identified by (i) a significant correlation between protein level and preference score; (ii) no significant increase in the total variance of protein level in trained, relative to untrained chicks; (iii) a significant reduction of the residual variance of protein level in trained chicks relative to the variance in untrained chicks. If there were no significant reduction in residual variance, a learning-dependent association with preference score was inferred. See Discussion and Margvelani et al. (2018a) for further details.

Probabilities $<0.05$ were taken as significant and statistical tests are two-tailed, unless stated otherwise.

# RESULTS

## Behavior

Samples were taken 1 h after the end of training from 21 trained chicks and their associated untrained controls, and samples were taken 24 h after training from 19 trained chicks and their untrained controls. Mean preference scores were 72.1 ± 4.9 SEM and 72.0 ± 4.7 SEM, respectively. For both time points the mean preference score was significantly higher than the "no preference" score of 50 ($t$-test, $P < 0.0001$ in both cases). Mean approach during training and testing was 64.2 ± 15.0 and 13.0 ± 2.3 m, respectively for the 1-h experiments, and 105.0 ± 25.0 and 26.1 ± 7.1 m, respectively for the 24-h experiments.

## Immunostaining

All three antibodies reacted with a protein band of 60 kDa molecular weight corresponding to total Src (Total-Src), 416P-Src, and 527P-Src (**Supplementary Figure S1**). Four standards, 15, 30, 45, and 60 μg of total protein corresponding to 0.5, 1.0, 1.5, and 2.0 relative amounts of protein, respectively, were applied to each gel. For these standards the optical densities of the immunostained bands (Total-Src, 416P-Src, and 527P-Src) were plotted against the amount of protein; in all standards, least-squares regression showed a good fit to a straight line (**Supplementary Figure S1**).

## One Hour After the End of Training

### Left IMM

***Total-Src***

See **Figure 1A** and **Table 1**. No correlations were significant and thus no learning-related changes were detected.

***416P-Src***

See **Figure 2A** and **Table 1**. The correlation with preference score as the only predictor variable was significant. When both training approach and testing approach were added to the model, the significant correlation persisted and both approach terms were significant [preference score $F_{(1,8)} = 19.41$, $P = 0.0023$; training approach $F_{(1,8)} = 5.40$, $P = 0.049$, testing approach $F_{(1,8)} = 5.40$, $P = 0.044$]. The difference between the intercept at the maximum preference score and untrained mean was not significant and the variance about the regression line was significantly lower than the variance of the untrained chicks. The total variance in trained chicks was not significantly different from untrained values. Taken together, these results are consistent with a predisposition, namely a correlation between 416P-Src level and capacity to learn, rather than a consequence of learning during training (see Discussion).

***527P-Src***

See **Figure 3A** and **Table 1**. The correlation with preference score was significant. The difference between the intercept at the maximum preference score and untrained mean was also significant. The mean of the untrained chicks and the intercept at the preference score 50 were nearly identical. The variance about the regression plot was not significantly different from the variance of untrained chicks. Taken together, the results suggest that the correlation with preference score is attributable to learning that occurred during training (see Discussion).

***416P-Src/Total-Src***

See **Figure 4A** and **Table 1**. The correlation was not significant and there was thus no evidence of a learning-related effect.

***527P-Src/Total-Src***

See **Table 1** and **Supplementary Figure S2**. The correlation between this ratio and preference score was significant. However, the intercept at maximum preference was not significantly different from the mean of untrained chicks and the data were not sufficient to support inference of an effect of learning.

***527P-Src/416-P-Src***

See **Table 1**. The correlation between this ratio and preference score was not significant, providing no evidence for an association with learning.

### Right IMM

See **Supplementary Table S1** and, for Total-Src and 416P-Src/Total-Src, **Figures 1C**, **4C**, respectively. No correlations were significant and there was thus no evidence of learning-related changes.

**TABLE 1 |** Standardized relative amount of protein.

| Brain region | Left IMM | | | | | |
|---|---|---|---|---|---|---|
| **Protein** | **Total-Src** | **416P-Src** | **527P-Src** | **416P-Src/Total-Src** | **527P-Src/Total-Src** | **527P-Src/416P-Src** |
| **Untrained chicks** | | | | | | |
| Mean | 1.21 | 1.12 | 0.94 | 0.95 | 0.81 | 1.01 |
| SEM | 0.066 | 0.11 | 0.066 | 0.11 | 0.08 | 0.23 |
| *DF* | 9 | 9 | 9 | 9 | 9 | 9 |
| **Trained chicks** | | | | | | |
| Correlation protein amount vs. preference score | 0.084 | 0.65 (0.84) | 0.65 | 0.39 | 0.62 | 0.35 |
| *DF* | 10 | 10 (8) | 10 | 10 | 10 | 0.1 |
| *P* | 0.79 | 0.02 (0.022)* | 0.02* | 0.2 | 0.031* | 0.25 |
| y-intercept at preference score 100 | 1.22 | 1.43 (1.46) | 1.23 | 1.22 | 1.08 | 0.945 |
| SE of y-intercept | 0.11 | 0.16 (0.17) | 0.07 | 0.14 | 0.1 | 0.1 |
| **Comparison. y- intercept at preference score 100 vs. mean for untrained chicks** | | | | | | |
| *t* | 0.046 | 1.57 (1.61) | 2.85 | 1.50 | 2.01 | −0.26 |
| *DF* | 16.14 | 17.62 (14.26) | 18.92 | 18.54 | 18.58 | 12.67 |
| *P* | 0.96 | 0.13 (0.13) | 0.01* | 0.15 | 0.058 | 0.79 |
| y-intercept at preference score 50 | 1.18 | 1.24 (1.20) | 0.095 | 1.08 | 0.85 | 0.84 |
| SE of y-intercept | 0.09 | 0.15 (0.17) | 0.066 | 0.13 | 0.1 | 0.1 |
| **Comparison. y-intercept at preference score 50 vs. mean for untrained chicks** | | | | | | |
| *t* | −0.27 | 0.60 (0.36) | 0.11 | 0.75 | 0.32 | −0.67 |
| *DF* | 15.45 | 15.89 (12.65) | 17.58 | 16.74 | 16.85 | 18.97 |
| *P* | 0.78 | 0.55 (0.73) | 0.91 | 0.46 | 0.74 | 0.51 |
| Residual regression variance/variance untrained | 1.84 | 0.13 (0.08) | 0.88 | 0.29 | 0.41 | 0.048 |
| *P* | 0.81 | 0.002 (0.0008)* | 0.42 | 0.033* | 0.096 | 0.00002* |

*Summary of results for the left IMM 1 h after the end of training. For 416P-Src, approach during training and testing were significant; numbers in brackets give results when these approach terms were included in the regression model. Data for untrained chicks are in the upper part of the table and data from trained chicks below. y-Intercepts for preference scores 50 and 100 are given, together with results of comparisons of these intercepts with mean values for untrained chicks using t-tests. On the bottom line is given the probability (F-test) for a comparison of residual variance from the regression with the variance of untrained chicks. Asterisks indicate statistically significant results.*

### Left PPN

#### *527P-Src*

See **Supplementary Table S2**. There was a marginally significant correlation between preference score and the standardized amount of Src protein phosphorylated at tyrosine-527. The difference between the intercept at the maximum preference score and untrained mean was not significant and there was thus insufficient evidence of a learning-related process.

No other correlations were significant (see **Supplementary Table S2**).

### Right PPN

See **Supplementary Table S3**. There were no significant correlations with preference score and thus no evidence of learning-related changes.

### Summary of Changes 1 H After Training

According to our criteria for inference of learning-dependent changes (see Discussion), changes 1 h after the end of training attributable to learning were detected only for 527P-Src in the left IMM. The results indicate that the significant correlation of 416P-Src with preference score in the left IMM can be accounted for by a predisposition to learn (see Discussion).

## Twenty Four Hours After the End of Training

### Left IMM

#### *Total-Src*

See **Figure 1B** and **Table 2**. The total amount of Src increased significantly with preference score. The intercepts at both preference score 50 and maximum preference score were significantly higher than the mean value for untrained chicks. The residual variance from the regression with preference score was not significantly different from the variance of untrained chicks. Taken together, these results indicate that the correlation in the left IMM arose as a result of learning and that, in addition, training increased the amount of Total-Src when no learning had occurred (see Discussion).

#### *416P-Src*

See **Figure 2B** and **Table 2**. The correlation with preference score was not significant, giving no evidence of a learning-related change.

#### *527P-Src*

See **Figure 3B** and **Table 2**. The amount of Src protein phosphorylated at tyrosine-527 was significantly correlated with the preference score. The intercept at the maximum preference

**TABLE 2 |** Standardized relative amount of protein.

| Brain region | Left IMM | | | | | |
|---|---|---|---|---|---|---|
| **Protein** | **Total-Src** | **416P-Src** | **527P-Src** | **416P-Src/Total-Src** | **527P-Src/Total-Src** | **527P-Src/416P-Src** |
| **Untrained chicks** | | | | | | |
| Mean | 0.857 | 0.995 | 0.999 | 1.17 | 1.19 | 1.084 |
| SEM | 0.043 | 0.131 | 0.067 | 0.14 | 0.10 | 0.091 |
| *DF* | 8 | 8 | 8 | 8 | 8 | 8 |
| **Trained chicks** | | | | | | |
| Correlation protein amount vs. preference score | 0.748 | 0.009 | 0.86 | −0.75 (−0.83) | 0.02 | 0.86 |
| *DF* | 9 | 9 | 9 | 9 (7) | 9 | 9 |
| *P* | 0.008* | 0.97 | 0.0006* | 0.007 (0.005)* | 0.94 | 0.0006* |
| y-intercept at preference score 100 | 1.63 | 1.156 | 1.55 | 0.65 (0.69) | 0.95 | 1.399 |
| SE of y-intercept | 0.1 | 0.089 | 0.07 | 0.11 (0.13) | 0.084 | 0.093 |
| **Comparison. y- intercept at preference score 100 vs. mean for untrained chicks** | | | | | | |
| *t* | 6.94 | 1.01 | 5.59 | −2.86 (−3.97) | −1.84 | 2.40 |
| *DF* | 12.15 | 14.37 | 16.99 | 15.4 (14.94) | 15.96 | 16.97 |
| *P* | 0.00001* | 0.32 | 0.00003* | 0.011 (0.023)* | 0.08 | 0.027* |
| y-intercept at preference score 50 | 1.16 | 1.15 | 1.03 | 1.07 (1.13) | 0.94 | 0.90 |
| SE of y-intercept | 0.09 | 0.084 | 0.06 | 0.10 (0.12) | 0.074 | 0.087 |
| **Comparison. y- intercept at preference score 50 vs. mean for untrained chicks** | | | | | | |
| *t* | 3.02 | 1.01 | 0.34 | −0.58 (−0.24) | −1.98 | −1.42 |
| *DF* | 12.25 | 16.77 | 15.99 | 16.64 (13.52) | 16.63 | 15.97 |
| *P* | 0.01** | 0.32 | 0.73 | 0.56 (0.82) | 0.06 | 0.17 |
| Residual regression variance/variance untrained | 3.22 | 0.10 | 0.74 | 0.211 (0.18) | 0.42 | 0.336 |
| *P* | 0.94 | 0.001* | 0.33 | 0.016 (0.017)* | 0.11 | 0.06 |

*Summary of results for the left IMM 24 h after the end of training. Format and conventions as for* ***Table 1****. For 416P-Src/Total-Src, approach during training and testing were significant; numbers in brackets give results where these approach terms were included in the regression model.*
*Asterisks indicate statistically significant results.*

score significantly exceeded the mean level for untrained chicks. The intercept at preference score 50 and mean value of untrained chicks were not significantly different from each other. The residual variance from the regression plot and variance of untrained chicks were statistically homogeneous, indicating that, as with Total-Src, the increase in 527P-Src amount with preference score was a result of learning.

### *416P-Src/Total-Src*

See **Figure 4B** and **Table 2**. A significant negative correlation was found. The intercept of the maximum preference score was significantly lower than the mean for untrained chicks, which was not significantly different from the intercept value at preference score 50. Residual variance from the regression with preference score was significantly lower than the variance of untrained chicks, whereas the total variance in trained chicks was statistically homogeneous with the untrained chick values. These data support the hypothesis (see Discussion) that this correlation did not arise as a result of training but reflects a predisposition to learn existing in the absence of training.

### *527P-Src/Total-Src*

See **Table 2**. For this ratio, no correlation was significant, indicating that the learning-related increase in Total-Src was mainly or entirely attributable to the increase in amount of its 527P-Src form.

### *527P-Src/416P-Src*

See **Table 2**. The ratio of the two phosphorylated forms of Src increased significantly with preference score, predictably since 527P-Src increased and 416P-Src decreased as learning increased. The difference between the intercept at the maximum score was significantly higher than the mean of untrained chicks and the intercept at preference score 50 was not significantly different from the untrained mean. The residual variance from the regression plot and the variance of the untrained chicks were not significantly different from each other. It is concluded that, as in the case of Total-Src and 527P-Src, the ratio 527P-Src/416P-Src is a function of the strength of learning (see Discussion).

## Right IMM

### *Total-Src*

See **Figure 1D** and **Supplementary Table S4**. The amount of protein increased significantly with the preference score. Both intercepts, at preference score 50 and maximum preference score, were significantly greater than the mean of the untrained chicks. Statistical homogeneity of residual variance from the regression plot and variance of untrained chicks indicates that the increase in Total-Src amount with

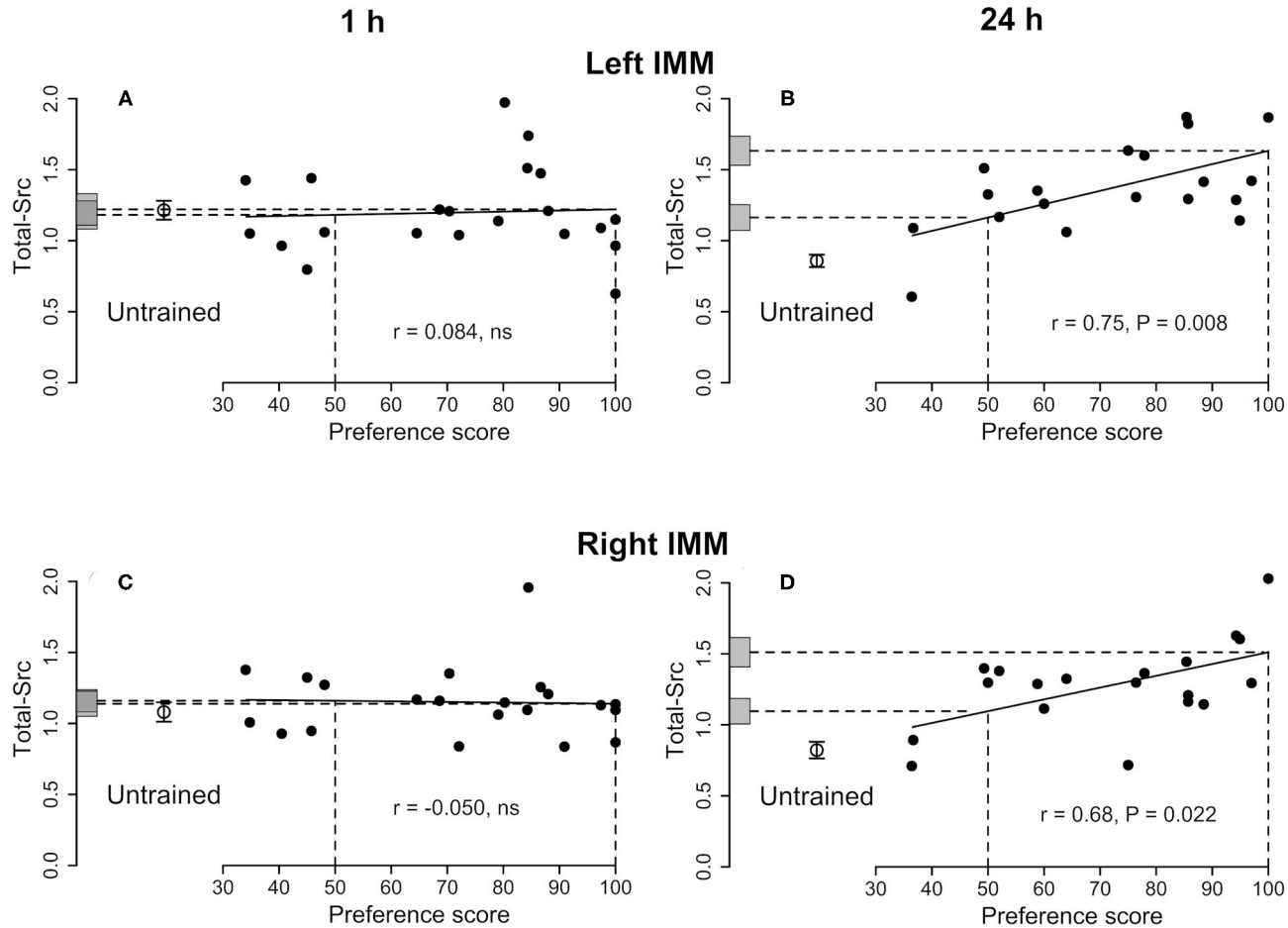


**FIGURE 1 |** Left IMM and right IMM, 1 and 24 h after the end of training. Standardized relative amount of Total-Src plotted against preference score. Filled circles, trained chicks. Open circles and associated bars, mean level in untrained chicks ± SEM. Vertical dashed lines, preference scores 50 (no preference/no learning) and 100 (approach only to the training stimulus /strong learning); horizontal dashed lines, *y*-axis intercepts for preference scores 50 and 100; gray bars on *y*-axis, ± SE of intercept. **(A)** Left IMM and **(C)** right IMM 1 h after the end of training. Correlations are not significant. **(B)** Left IMM and **(D)** right IMM 24 h after the end of training. For both sides, the correlations are significant and the differences between the untrained mean and the intercept at preference score 100 are also significant. The intercepts at preference score 50 are also significantly higher than the mean of untrained chicks, indicating an effect of training on Total-Src in addition to a learning-related effect.

preference score is attributable to learning. The significant difference between the mean of untrained chicks and preference score 50 indicates that, in addition, training increased the amount of Total-Src even when learning did not occur (see Discussion).

### *416P-Src/Total-Src*

See **Figure 4D** and **Supplementary Table S4**. There was no significant correlation between the amount of protein and preference score when the preference score was the only predictor variable. However, there was a significant correlation when training approach and testing approach were included as covariates: all three terms were significant [preference score $F_{(1,7)} = 6.67$, $P = 0.036$; training approach $F_{(1,7)} = 8.61$, $P = 0.022$, testing approach $F_{(1,7)} = 27.64$, $P = 0.0012$]. Residual variance about the regression line was significantly lower than the variance of untrained chicks, indicating that 416P-Src/Total-Src was associated with a predisposition to learn readily.

There were no further significant correlations between the amount of protein and the preference score (see **Supplementary Table S4**).

## Left PPN

See **Supplementary Table S5**. There were no significant correlations between the amount of protein and preference score and therefore no evidence of learning-related effects.

## Right PPN

See **Supplementary Table S6**. There were no significant correlations between the amount of protein and preference score and therefore no evidence of learning-related effects.

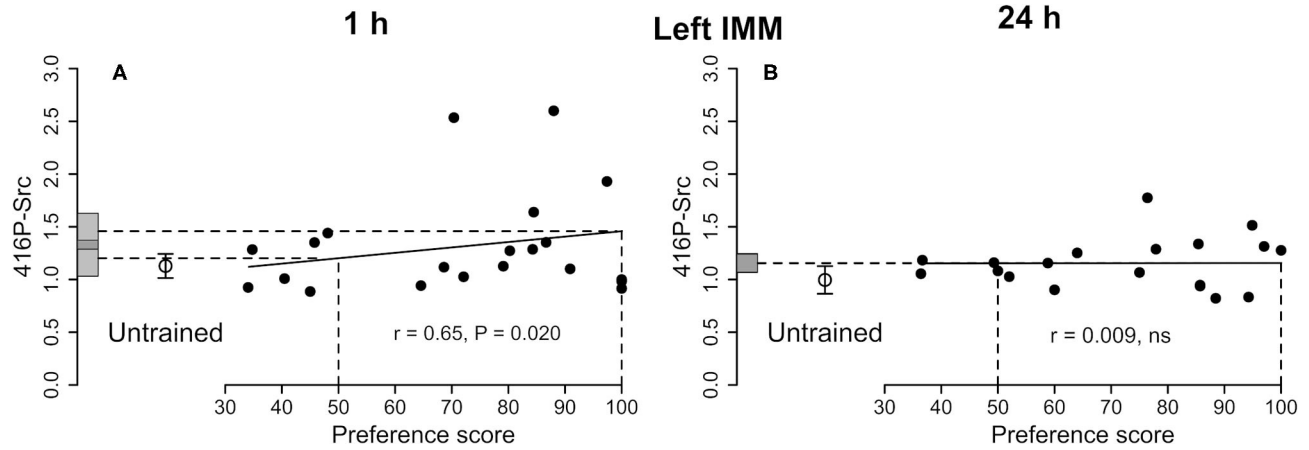


**FIGURE 2 |** Left IMM. Standardized relative amount of 416P-Src plotted against preference score 1 h **(A)** and 24 h **(B)** after the end of training. Conventions otherwise as for **Figure 1A**, the correlation is significant; the mean value for untrained chicks is not significantly different from either intercept on the *y*-axis. **(B)** Correlation not significant.

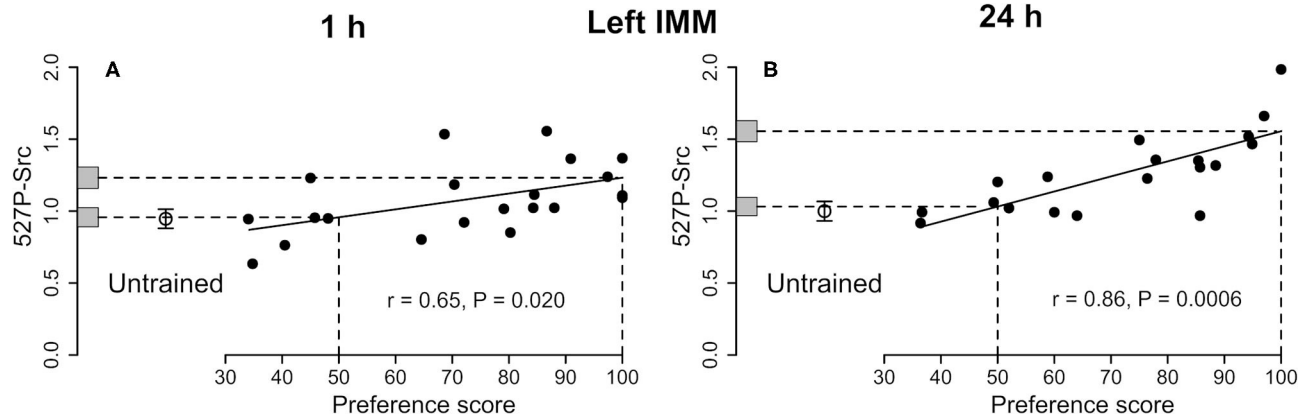


**FIGURE 3 |** Left IMM. Standardized relative amount of 527P-Src plotted against preference score 1 h **(A)** and 24 h **(B)** after the end of training. Conventions otherwise as for **Figure 1**. For both times the correlations are significant. In each plot, the mean value for untrained chicks is significantly lower than the intercept at preference score 100 and not significantly different from the intercept at preference score 50.

### Summary of Changes 24 H After Training

Twenty-four hours after the end of training a learning-dependent increase was found in the total amount of Src in both the left and right IMM. Furthermore, training in the absence of learning was associated with an increase in the amount of Total-Src. In the left IMM 527P-Src was increased in a learning-dependent way, whereas the ratio 527P-Src/Total-Src was not changed, indicating that the increase in the total amount of enzyme was attributable at least principally to changes in its inhibited form.

The results for 416P-Src/Total-Src in both left and right IMM (when the latter was corrected for approach during training and testing) indicate that these quantities reflected predispositions to learn.

## DISCUSSION

We have elucidated the role of Src and its two phosphorylated forms in the memory of visual filial imprinting. Our analysis has discriminated between (i) neurobiological changes specifically related to memory and (ii) a predisposition manifest in neural changes attributable to a capacity to learn, rather than a result of learning itself.

To determine whether an effect of training is specifically related to learning, we have used the criteria explained in previous papers (McCabe, 2013; Solomonia and McCabe, 2015; Margvelani et al., 2018a). Briefly, a significant correlation is required between the protein level and the preference score (i.e., learning), such that the protein level corresponding to the maximum preference score (strong learning) is significantly different from the mean for untrained chicks. The protein level for trained chicks at preference score 50 (no learning) indicates whether incidental correlates of learning, such as locomotion and vocalization, have generated side-effects unrelated to learning. If the protein level at preference score is 50 for trained chicks, then no side-effects are evident. A difference between these two levels plus a correlation is evidence for both learning-related

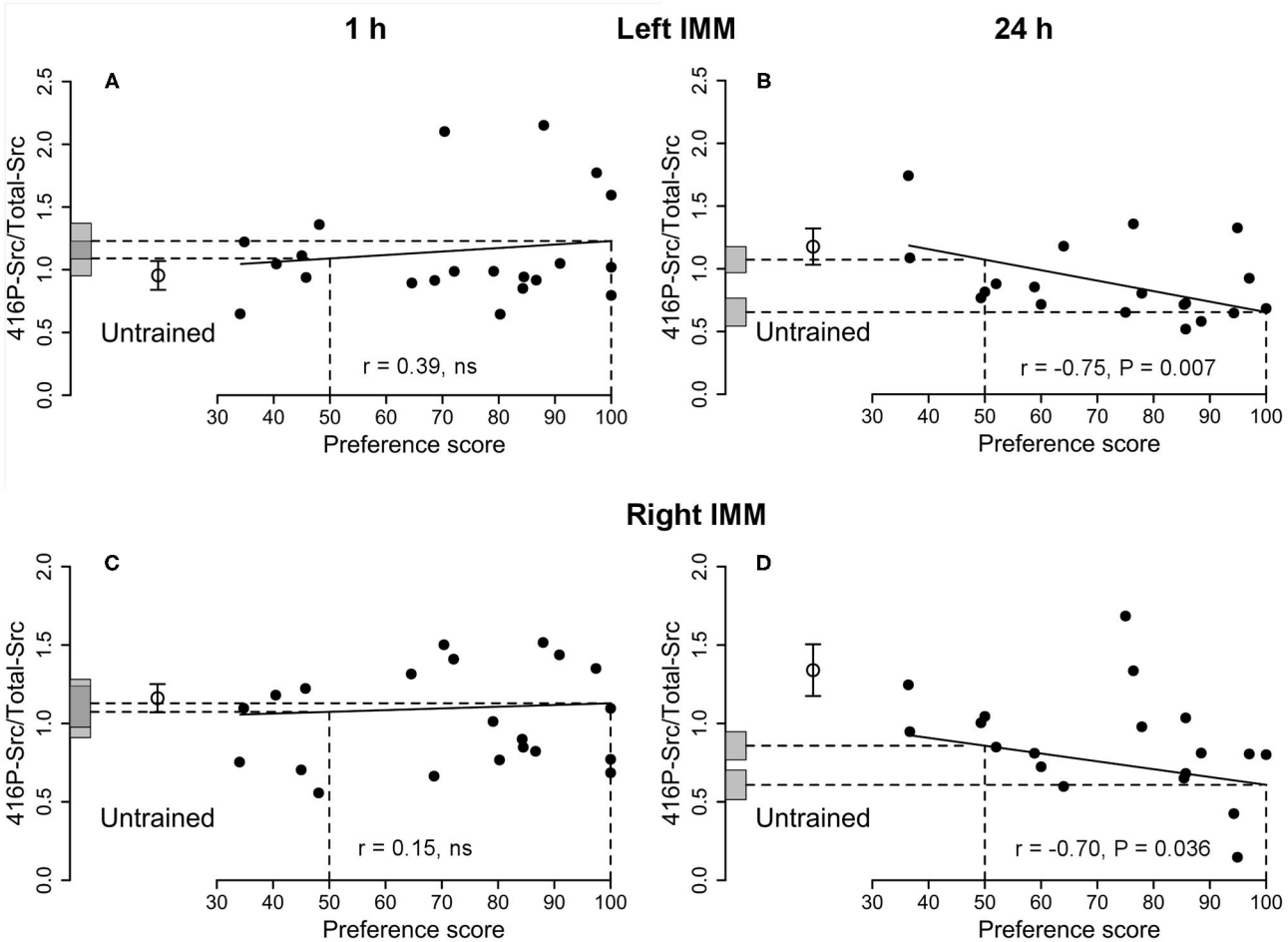


**FIGURE 4 |** Left and right IMM, 1 and 24 h after the end of training. 416P-Src/Total-Src plotted against preference score. Conventions as for **Figure 1**. **(A,B)** Left IMM; **(C,D)** right IMM; **(A,C)** 1 h after the end of training, correlations not significant. **(B,D)** Twenty four hours after the end of training, both correlations significant. In both left and right IMM at 24 h **(B,D)**, the intercept at preference score 100 is significantly different from the mean of untrained chicks. In the right IMM at 24 h **(D)**, the intercept at preference score 50 is also significantly different from the mean of untrained chicks. Data in **(D)** are corrected for effects of approach during training and testing, which were statistically significant (cf. **Supplementary Table S4**).

changes and side-effects unrelated to learning, which may then be distinguished from one another.

A further question is whether correlation with preference score is a result of learning or results from a predisposition to learn better, irrespective of training. To distinguish between these two possibilities, we have used an additional criterion, namely whether residual variance in the regression with preference score is at least as large as the variance in untrained chicks. If the correlation were a result of learning, one would expect the residual variance from the regression to be at least as large as the variance in untrained chicks, and learning would not be expected to lower this baseline variability. Conversely, if protein levels reflect a readiness to learn and are unaffected by training (a predisposition), one would expect the total variances of trained and untrained chicks to be similar, and in trained chicks some of the variance to be attributable to the correlation; this would lower the residual variance in trained chicks; see also Margvelani et al. (2018a).

## Properties and Pathways of Src

Phosphorylation of the tyrosine-527 residue of Src inactivates the enzyme by binding tyrosine-527 to the Src homology 2 domain, causing a conformational change that restricts substrate access to the kinase domain of Src (Xu et al., 1997; reviewed by Amata et al., 2014). Dephosphorylation of Tyr527 is followed by autophosphorylation at Tyr416, which leads to full activation of Src. Thus, the available data indicate that Src is not phosphorylated simultaneously on the 527 and 416 tyrosine residues and that changes in 416P-Src and 527P-Src therefore presumably reflect activated and inhibited forms, respectively, in different pools of Src. These pools could be in different cells or in different compartments of the same cell.

Phosphorylation of Src at Tyr527 is catalyzed by C-terminal Src kinase (Csk) (Okada, 2012), which attenuates NMDA-gated currents via the inhibition of Src (Socodato et al., 2017). Phosphorylation of Src at Tyr416 leads to activation of Src and phosphorylation of several synaptic proteins, including

components of the NMDA receptor complex (Rajani et al., 2021). The two pools of Src thus represent different states of critical neurobiological pathways.

In addition to phosphorylation at Tyr-416 and Tyr-527, Src is a substrate for serine/threonine kinases, including protein kinase C (at Serine-12), protein kinase A (at Serine-17), and CDK1/cdc2 (at Threonine-34, Threonine-46, and Serine-72) (Roskoski, 2005). The physiological importance of these phosphorylation sites in the brain are incompletely understood.

## Learning-Dependent Changes in the Left IMM

### One Hour After the End of Training

The above criteria for learning-dependence were satisfied for 527P-Src in the left IMM (**Figure 3A**). There was no significant correlation for Total-Src, and the ratio 527P-Src/Total-Src behaved similarly to 527P-Src alone, with the exception that the level of this ratio at maximum preference score was significantly greater than the mean for untrained chicks only at the $P < 0.1$ level (**Table 1**).

527P-Src is an inhibited form of the enzyme. Since there was no significant change in Total-Src, and since both 527P-Src and the ratio of 527P/Total-Src increased in learning-dependent ways in the left IMM, training evidently converted part of the pool of unphosphorylated Src to its inhibited form in proportion to the strength of learning. Learning and memory at 1 h are thus associated with a reduction of Src activity in the left IMM and may permit activity in pathways which are inhibited by active Src. A kinase strongly implicated in Tyr-527 phosphorylation, and which may have been responsible for generation of 527P-Src, is Csk (Okada, 2012; Amata et al., 2014).

### Twenty Four Hours After the End of Training

Total-Src in the left IMM satisfied the criteria for learning-dependence whereas the level corresponding to preference score 50 was significantly greater than the untrained value (**Figure 1B**). Therefore, the amount of Total-Src in the left IMM was evidently increased by learning-dependent processes and by separate processes unconnected with learning. The change in Total-Src level with increasing preference score was accompanied by a learning-dependent increase in 527P-Src (**Figure 3B**). The similar relation of each of these two measures to preference score resulted in their ratio not showing a significant correlation (**Table 2**).

The level of 416P-Src did not change significantly with preference score (**Figure 2B**), with the result that the ratio 527P-Src/416P-Src increased in the left IMM in a learning-dependent way (**Table 1**).

As at 1 h, the increase in 527P-Src with increasing preference score indicates more inhibition of Src activity as memory becomes stronger. At this later time, stronger memory is also associated with a greater amount of Total-Src, that is, Src available for activation and modulation via its phosphorylation sites.

## Results in the Left IMM Implying a Predisposition

### One Hour After the End of Training

A significant positive correlation was found for 416P-Src (**Figure 2A**), but the residual variance from the regression was significantly lower than the variance in untrained chicks (**Table 1**), implicating 416P-Src at this time after training in a predisposition reflects the readiness of chicks to learn.

### Twenty Four Hours After the End of Training

Although there was no significant correlation for 416P-Src alone, the ratio 416P-Src/Total-Src was negatively correlated with preference score in both the left and the right IMM (**Figures 4B,D**). The residual variance from the regression was significantly lower than the variance in untrained chicks (**Table 2** and **Supplementary Table S4**), implicating 416P-Src in a predisposition at 24 h as well as at 1 h. However, the pattern of variation with preference score was different at the two times, suggesting a time-dependent, possibly age-related, change in the role of Src in the predisposition. In addition, there was, in the right IMM at 24 h and after correction for approach, a significant difference between the intercept at preference score 50 and the mean of untrained chicks (**Figure 4D** and **Supplementary Table S4**). There was thus an additional effect of training, delayed and unrelated to learning, in the right IMM.

There is a precedent for a predisposition of the type discussed above, where the expression of the micro-RNA gga-miR-130b-3p in the left IMM was found to be inversely correlated with preference score, and its residual variance about the regression line was significantly lower than the variance of untrained control chicks (Margvelani et al., 2018a). The small, non-coding sequence of this micro-RNA regulates post-transcriptional expression of a number of proteins, including cytoplasmic polyadenylation element binding protein 3 (CPEB-3). Considering our previous findings together with the results of the present study, it appears likely that an array of biochemical conditions accompanies a predisposition to learn readily. Other predispositions, which can act in concert with imprinting, have been described (reviewed by Rosa-Salva et al., 2021) but the extent to which neurobiological mechanisms are shared by these processes is unknown.

It is of interest that learning-dependent processes and processes associated with a predisposition are associated with different patterns of Src phosphorylation. It is also of interest that processes reflecting both learning and a predisposition to learn occur in the left IMM, where there may be opportunities for interactions between the two types of process. Relevant to this result is the observation (Mayer et al., 2016), of a change in expression of the neuronal activity marker c-fos protein in the IMM in association with another predisposition—a preference for facial features of an adult bird (Johnson and Horn, 1988; Rosa-Salva et al., 2010). Interaction between predispositions and memory in the IMM might increase the adaptive advantage of behavior learned via imprinting, even though the IMM is not necessary for the predisposition to prefer faces (Horn and McCabe, 1984; Johnson and Horn, 1986).

Src activation increases NMDA currents in hippocampal cultured neurons (Yu et al., 1997) and Src inhibition reduces the surface expression of NR2B receptors and synaptic plasticity in the amygdala (Sinai et al., 2010). NMDA receptors and glutamate release in the left IMM are critically involved in the learning and memory of imprinting (McCabe and Horn, 1988, Meredith et al., 2004), NMDA receptor-dependent synaptic plasticity has been demonstrated in the IMM (Bradley et al., 1993; Matsushima and Aoki, 1995), and it is possible that Src contributes to these processes.

Overexpression of constitutively active Src suppresses $Ca^{2+}$-induced release of neurotransmitter (Ohnishi et al., 2011). Knockout of Src homolog domain-containing phosphatase 2 (Shp2) in hippocampal pyramidal neurons leads to the activation of Src with the disruption of excitatory synaptic transmission and impairment of remote fear memory in mice (Yan et al., 2017). Knockdown of cellular Src in primary cortical neurons protects cells against glutamate-induced loss of viability (Khanna et al., 2007). It appears, therefore, that excessive Src activity can be maladaptive. Given the importance of Src in memory demonstrated by the present results, it is possible that for the proper functioning of Src in synaptic plasticity it is necessary for a balance of activated and inhibited forms of the enzyme to be maintained, disturbance of which could lead to disruption of neuronal homeostasis.

### Regional Specificity

Learning-related changes in Src were found in the IMM and not in the PPN control forebrain region, reflecting a regional specificity found in all previous studies of imprinting in which measurements have been made on these two regions; see e.g., Solomonia and McCabe (2015).

### Hemispheric Asymmetry

The left and right IMM have different roles in imprinting, which are reflected in functional hemispheric asymmetries following training (reviewed by Horn, 1985; McCabe, 2013, 2019). Evidence from ablation experiments indicates that both the left and the right IMM have a storage function but that, in addition, the right IMM is necessary for subsequent storage without the IMM, in an, as yet, unidentified region termed S' (Cipolla-Neto et al., 1982); as shown in Solomonia et al. (2000) and Tiunova et al. (2019) for possible candidate regions for S'. Learning-related biochemical changes are usually more strongly expressed in the left IMM than in the right IMM, especially 24 h after imprinting (Solomonia and McCabe, 2015; see also Margvelani et al., 2018a; Chitadze et al., 2020). The present study provides additional evidence of hemispheric asymmetry. In the left IMM, significant learning-dependent changes in 527P-Src were observed at both 1 h and 24 h (**Figure 3** and **Tables 1**, **2**), but not in the right IMM (**Supplementary Tables S1**, **S4**).

A learning-dependent change in Total-Src was detected in both left and right IMM 24 h after the end of training, providing further evidence for the involvement of the right IMM in memory. Learning thus leads to a delayed increase in the amount of Total-Src in both hemispheres, evidently contributing to their different roles via differential phosphorylation.

### Conclusion

The present results demonstrate that Src protein contributes to memory formation and a predisposition to learn in ways that are time-dependent and regionally specific. The total amount of Src and its two phosphorylated conditions, one linked to activation and another to inhibition of the enzyme, have been shown to be involved. As far as we know, this is the first comprehensive study of Src and its phosphorylated forms in relation to learning and memory.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

This animal study was reviewed and approved by the I. Beritashvili Centre of Experimental Biomedicine.

## AUTHOR CONTRIBUTIONS

MM: major contribution to experimentation, experimental design, and data analysis. LC: experimentation, experimental design, and data analysis. VL and BM: data analysis and writing paper. RS: experimentation, experimental design, data analysis, and writing paper. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.736999/full#supplementary-material

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# American Crow Brain Activity in Response to Conspecific Vocalizations Changes When Food Is Present

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Social interaction among animals can occur under many contexts, such as during foraging. Our knowledge of the regions within an avian brain associated with social interaction is limited to the regions activated by a single context or sensory modality. We used 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) to examine American crow (*Corvus brachyrhynchos*) brain activity in response to conditions associated with communal feeding. Using a paired approach, we exposed crows to either a visual stimulus (the sight of food), an audio stimulus (the sound of conspecifics vocalizing while foraging) or both audio/visual stimuli presented simultaneously and compared to their brain activity in response to a control stimulus (an empty stage). We found two regions, the nucleus taenia of the amygdala (TnA) and a medial portion of the caudal nidopallium, that showed increased activity in response to the multimodal combination of stimuli but not in response to either stimulus when presented unimodally. We also found significantly increased activity in the lateral septum and medially within the nidopallium in response to both the audio-only and the combined audio/visual stimuli. We did not find any differences in activation in response to the visual stimulus by itself. We discuss how these regions may be involved in the processing of multimodal stimuli in the context of social interaction.

**Keywords: American crow, 18F-fluorodeoxyglucose PET imaging, social stimuli, brain activity, nucleus taenia of the amygdala (TnA), caudal nidopallium, vocalizations, multimodal stimulus**

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## INTRODUCTION

Social animals must filter, process, and act upon a variety of information when they assemble and interact with one another; they send and receive signals across multiple sensory modalities, observe interactions between conspecifics, and evaluate the intentions of others toward themselves, all the while remaining vigilant for danger and attempting to maximize their access to any resources in the area. This cognitive demand requires a brain with a high degree of processing power (Dunbar, 1998, 2009). Most species known to possess such a brain are mammals (such as primates or cetaceans) or birds (such as corvids or parrots). Despite convergently evolving advanced cognitive capabilities,

these two classes diverged approximately 300 million years ago (Burt et al., 1999), resulting in numerous structural differences between mammalian and avian brains (Shimizu, 2001).

Aside from regions and systems homologous to both clades, our understanding of the inner workings of the avian brain is limited relative to mammals, although a surge of research conducted over recent decades has made considerable progress in filling this gap (Reiner et al., 2004; Jarvis et al., 2005; Wada et al., 2017; Ksepka et al., 2020). Much of this work has focused on determining the functions of, and connectivity between, individual brain regions (see Cowan et al., 1961; Karten et al., 1973; Nottebohm et al., 1976; Wild et al., 1993; Shanahan et al., 2013 as examples). Fewer studies have examined how systems within the avian brain function holistically, though our extensive understanding of the avian song control system (Konishi, 1985; Brenowitz and Beecher, 2005; Brainard and Doupe, 2013) remains a notable exception. While scientists have uncovered numerous brain regions associated with the avian social network, such as the lateral septum, nucleus taenia of the amygdala (TnA), anterior hypothalamus, ventromedial hypothalamus, preoptic area, and potentially the dorsal arcopallium (Cooper and Erickson, 1976; Goodson, 2005; Atoji et al., 2006; Nishizawa et al., 2011; Ondrasek et al., 2018), how these regions interact with other areas of the brain under different social situations remains unclear. For example, counter-singing between neighboring rivals, fights over access to resources, courting a potential mate, and recruiting to food are all examples of social behavior, yet likely involve different regions/systems within the brain due to varying social contexts and sensory modalities. Scientists must also consider how the brain integrates multimodal sensory information, as animals regularly communicate social information using more than one modality (Horn, 1983; Gopher et al., 1996) and usually pay more attention to multimodal signals, regardless of whether each modality is transmitting redundant or non-redundant information (Partan and Marler, 1999).

American crows (*Corvus brachyrhynchos*) are songbirds noted for their intelligence and complex social dynamics; they guard territories and regularly fight among themselves (occasionally escalating to the death of one of the belligerents), yet they also cooperate to mob predators and roost communally in large numbers (Marzluff and Angell, 2005). Much of this complex social interaction can be observed when crows congregate around an ephemeral food source; while they certainly spend time obtaining food, they also use these occasions as opportunities to gauge their position within the local dominance hierarchy, search for prospective mates, and learn about potential rivals (Kilham, 1990; Marzluff and Angell, 2005, 2013). Crows exchange much information via vocalization; as a result, such gatherings can become quite noisy as crows communicate with one another (Pendergraft and Marzluff, 2019).

To supplement insights from behavioral observation, Positron Emission Tomography combined with the radiotracer 18F-fluorodeoxyglucose (FDG-PET) can be used to better understand the brain activity of animals from various stimulus conditions. In this brain imaging modality, the FDG, a glucose analog, is injected into the body and distributes systemically. The uptake of FDG within the brain is preferential to regions of increased activity; therefore, the levels of radioactivity in a brain region (as measured by PET) act as a surrogate marker of brain activity (Jonides et al., 1993). An advantage of FDG-PET over other *in vivo* imaging modalities is that the subject can be awake (unanesthetized) and free from restraints or attached apparatus (which can cause stress to an unhabituated animal, confounding the results) during the stimulation period, as the circulating FDG is trapped in the tissue of the active brain region but cannot be further metabolized by glucose-6-phosphotase within the glycolytic pathway (Newberg et al., 2002). The subsequent PET imaging can be performed under anesthesia to assess the brain activity during the prior stimulation period (Marzluff et al., 2012).

This methodology has been used for various studies to identify distinct regions within the crow's brain that respond to specific stimuli (Marzluff et al., 2012; Cross et al., 2013; Swift et al., 2020). For example, wild crows respond to a variety of dangerous stimuli by giving alarm calls and mobbing the threat, yet an innate fear (a red-tailed hawk, *Buteo jamaicensis*) caused increased activity in the caudal nidopallium, whereas a learned fear (human who was previously antagonistic) activated the amygdala and a novel fear (unfamiliar human holding a dead crow) stimulated the hippocampus (Cross et al., 2013). While other methods can reveal activity at the regional/neuronal level or the connectivity between regions, such as implanting microelectrodes (Kita and Wightman, 2008) or antegrade/retrograde degeneration (McGeer and McGeer, 1980), respectively, PET imaging allows one to examine brain activity holistically (albeit indirectly via FDG uptake) and make inferences about regional connectivity based on the active regions.

Here, we conducted a 18F-fluorodeoxyglucose PET imaging study with the objective of determining how the American crow brain holistically functions in response to different sensory modalities associated with communal feeding events, with the secondary objective of understanding how the avian brain processes multimodal sensory information. We compared the baseline brain activity (as measured by the relative FDG activity) in wild crows during a control condition (viewing an empty stage) to their brain activity when hearing conspecifics foraging, seeing a preferred food item, or both hearing foraging and seeing food. We selected four regions *a priori* that we hypothesized would be activated in response to specific conditions. Because vocalizations encode social information, we expected the audio stimulus to cause an increase in activity in regions of the brain associated with social interaction, such as the (1) amygdala (specifically TnA) or the (2) lateral septum; multiple studies point to these regions as being involved in the vertebrate social network (Cooper and Erickson, 1976; Goodson, 2005; Nishizawa et al., 2011). If the calls encode information about food (such as presence, quantity, or quality), we hypothesized that the (3) hypothalamus, which is involved with motivation and food regulation (Wright, 1968; Kuenzel, 1994; Kuenzel et al., 1999; Primeaux et al., 2013), would also increase in activity in addition to the regions associated with social interaction; for the same reasons, we predicted this region would also increase in activity in response to the sight of a preferred food item. Finally, because the (4) thalamus filters,

organizes, and relays information gathered by the senses to other brain regions (Bentivoglio et al., 1993), we expected to see increased activity here in response to the combined multimodal stimuli (sound of conspecific vocalizations and sight of food item), as this region will be processing additional information.

# MATERIALS AND METHODS

## Capturing and Housing Crows

We captured wild American crows near Woodinville, WA, United States, as they departed a large communal roost. We lured birds from flocks with bread, trapped them using a net launcher, and preferentially selected individuals that were likely adult males (determined by plumage color and wear, mouth color, and overall size, Emlen, 1936). We caught two groups of crows outside of the breeding season and held them for several months each (9 crows from October to December 2015 and 8 crows from January to March 2016) in a protected outdoor aviary at the University of Washington, Seattle. The crows were individually housed in adjacent cages (measuring 1.8 × 2.1 × 2.4 m) separated by wire mesh. We provided crows with a rotating diet of assorted meats, eggs, grain, fruit, and dried dog food *ad libitum*. After identifying the crows' most preferred food item (half of a fried chicken patty) by observing which food item was preferentially consumed first, we began wrapping it in plastic food film prior to giving it to them to match the food presentation during the imaging process. Crows easily removed the plastic film prior to consuming the chicken, and they habituated to receiving their favorite food item in this presentation.

## Imaging the Crows

We imaged up to three crows per day, using a Siemens Inveon PET/CT system. The scanning process consisted of a 20 min microPET scan, followed by a CT scan in the docked and coregistered microCT scanner. The scanners share a multimodality bed and have a bore diameter of ∼12 cm. The PET field of view was approximately 8 × 13 $cm^2$ while the CT field of view was 7.9 cm × 13.3 cm; both included the entire brain with a slice thickness of approximately 0.1 mm. The scanner bed contained a pressure pad, which we used to monitor the crow's breathing (and thus depth of anesthesia) during the scan process.

The evening before a bird was scanned (typically 1,600–1,700), we removed it from its aviary cage, placed it in a sock to keep it docile, and carried it across campus to the imaging laboratory, where we placed it in a small wire cage (1 × 0.5 × 0.5 m, hereafter imaging cage) within a fume hood to acclimate overnight. The imaging cage contained water but not food, ensuring crows fasted for at least 14 h prior to imaging to control for variable blood glucose levels influencing FDG uptake. We covered the cage with a blanket to keep the bird calm; it could hear the ambient noise of the equipment but could not see into the imaging lab.

On the morning of the experiment, we removed the acclimated crow from its cage, covered the bird's head with a cloth to calm it, and gave it an interperitoneal injection of approximately 1 mCi of [$^{18}$F] Flourodeoxyglucose (FDG) (exact volume adjusted to account for radioactive decay and the bird's weight, ranging from 0.05 to 0.10 mL). After injection, we returned the crow to the covered imaging cage. During the next 3 min, we positioned our stimulus stage (see section "Experimental Stimuli" below) in front of the covered cage and removed the blanket covering the wire cage- the crow remained in relative darkness because the fume hood blocked the view to the lab while the stimulus stage's closed sliding panels prevented the crow from seeing the illuminated stage interior (**Figure 1**). Three minutes post-injection, we opened the sliding panels to reveal the stimulus (see section "Experimental Stimuli" below). For the following 10 min (hereafter referred to as the "stimulus phase," see **Supplementary Table 1**), we used the sliding doors to alternatively reveal the stimulus to the crow for 60 s, then hide it for 30 s (seven exposures and six associated breaks total). After the stimulus phase ended at 13 min post-injection, we again removed the crow from the cage, covered its head with a cloth, and anesthetized it via a custom nose cone with 5% isoflurane in oxygen with a flow rate of 300–800 mL/min before placing it in the scanner (we reduced isoflurane concentration to 2.5–3% after the crow was fully induced). We used Velcro straps to secure the anesthetized crow to the scanner bed before starting the imaging process 26 min post-injection. After the scan was complete, we secured the crow in hand until it fully recovered from anesthesia (indicated when it regained the ability to grip with both feet), before returning it to the cage. We kept the crows in the imaging lab for 20 h (the time required for $^{18}$F radioactivity to decay to acceptable levels), after which we returned them to the aviary.

We imaged all crows twice- the first scan for all birds was the control while the second scan introduced one of three different stimuli (see section "Experimental Stimuli" below). This ordering was to prevent carryover of the crow's previous experience biasing the control scan (Swift et al., 2020). We waited at least 1 week between scans for most crows, although one crow received its second scan 4 days after the first due to logistical constraints.

## Experimental Stimuli

As part of the imaging process (see section "Imaging the Crows" above), we presented all experimental stimuli within a (1.2 × 0.6 × 0.45 m) wooden stage designed to block the crow's view of the imaging laboratory/personnel (thereby removing potential confounding sources of distraction) and to standardize the background color, light intensity, and light angle between trials (**Figure 1**). The stimulus stage always contained an LED light on the ceiling and a Bem wireless HL2022A speaker placed far enough to the side to be considered out-of-view for the experimental crow. The front of the stimulus stage had two overlapping sliding panels, which we used to reveal or hide the stage interior- in addition to blocking the crow's view of the stimulus, the panels also blocked nearly all the light from the internal LED, increasing the visual contrast between showing and hiding the stimulus. The panels opened from the center of the crow's view of the stage so that the crow's eyes received equal stimulation. We used this configuration as the control stimulus ($n$ = 13).

During the crows' second scan, we introduced either a visual stimulus ($n$ = 4), auditory stimulus ($n$ = 5), or combination of audio/visual stimulus ($n$ = 4) to the stimulus stage interior. For

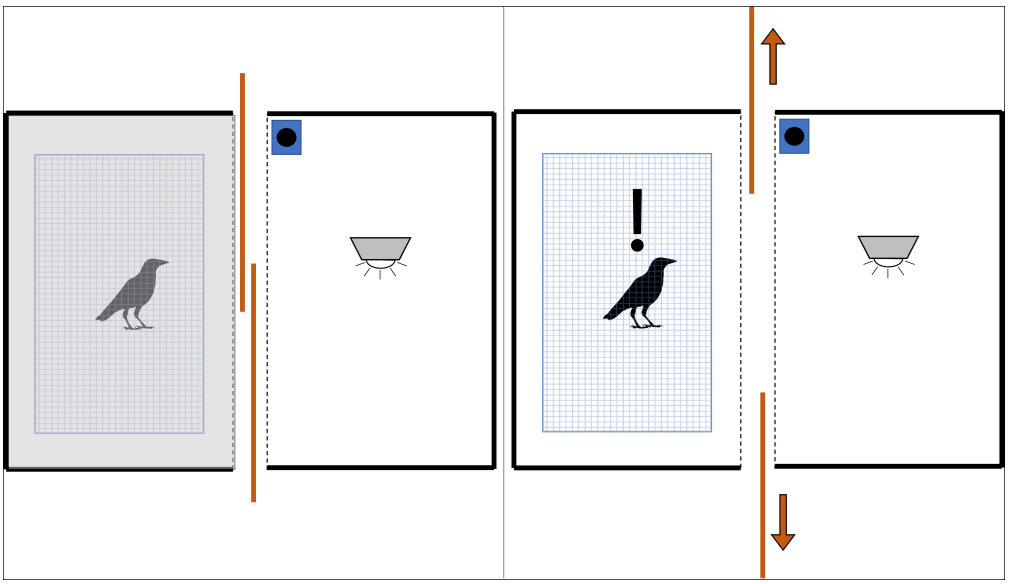

**FIGURE 1 |** Graphic of experimental layout during stimulus phase (top-down view). The fume hood surrounding the crow's cage blocked all view of the surrounding laboratory. When we closed the sliding panels between the crow's cage and the stimulus stage (left), the crow was plunged into relative darkness. We revealed the interior of the well-lit stage (and any stimulus within it) by opening the sliding panels (right).

the visual food-associated stimulus, we placed a fried chicken patty directly underneath the LED light in the center of the stage. We wrapped the food in plastic food film to reduce its scent profile, which matched how the crows received this item in the aviary. For the auditory food-associated stimulus, we used the internal wireless speaker to play a 60 s recording (see **Supplementary Figure 1** for a sample audio stimulus spectrogram) of roughly 30–40 crows vocalizing as they foraged around a food source (a pile of bread), which was synchronized to begin when the stimulus stage interior was revealed (see section "Imaging the Crows" above and **Supplementary Table 1**) and end when it was hidden. We recorded 22 min of crows vocalizing at the capture site (1 day prior to capture) in WAV format using a Marantz PMD-671 solid-state recorder and a Sennheiser MKH 20-P48 microphone contained within a Telinga Universal Parabolic Dish MK2 housing. From the 22-min master track, we selected ten 60 s duration intervals which were relatively free of other noises, such as passing cars. We controlled stimulus amplitude by normalizing the peak amplitude using Audacity (Audacity Team, 2015) and keeping the source and speaker volume consistent between trials (mean = 73 dB, SD = 3 dB). We reduced other potential acoustic confounding factors by excluding recordings with crow alarm vocalizations and randomly assigning a unique exemplar to each crow that received either an auditory or combined stimulus. For the combined A/V stimuli, we simultaneously showed the wrapped fried chicken while playing the assigned audio file of conspecifics vocalizing during each reveal of the stimulus stage interior.

## Behavior During Imaging

To better gauge their level of attention toward the stimulus stage and to control for factors which may influence FDG uptake within the brain (Marzluff et al., 2012; Cross et al., 2013), we used a GoPro Hero 4 camera to record (30 fps) the gaze time, blink rate, and amount of movement of each crow during the stimulus phase of the imaging process.

Avian brains are highly lateralized (Rogers and Anson, 1979), so we measured the gaze time from each eye to verify that any observed differences in hemispherical activity were not due to the bird preferentially using one eye to view the stimulus over the other (Mench and Andrew, 1986). We tracked each eye's gaze time independently from the other eye, e.g., we added gaze time to each eye if the bird binocularly gazed directly into the stage. We also used gaze time to measure a crow's level of interest in the stimulus being presented, and thus only recorded gaze when the stimulus stage's interior was revealed and visible to the crow (see section "Imaging the Crows" and **Supplementary Table 1**).

We measured blink rate to verify that the crows were not threatened by any of the presented stimuli or prior experience in the scanning apparatus, as previous studies have established a relationship between blink rate and the crow's perceived sense of danger; specifically, blink rate is negatively correlated with activity in fear-associated brain regions, and crows decrease blink rate when faced with a threatening stimulus compared to while foraging (Marzluff et al., 2012; Cross et al., 2013). Although the image resolution was sufficient to see the white flash of the crow's nictating membrane, the birds sometimes turned their heads such that their eyes were no longer visible, so we calculated an observed blink rate by dividing the number of observed blinks by the amount of time the eye was visible. The cage interior became too dark to observe blinks when the panels to the stimulus stage were closed, so we only calculated blink rate when the stimulus stage was visible during the seven reveals of the stimulus phase.

We measured the crows' movement because physiological activity can confound the amount and location of FDG uptake within the brain (Bhargava et al., 2011). We quantified the following actions as 1 unit of movement: crow moved 5–50 cm laterally along the perch (did not count if it moved < 5 cm, counted as 2 units if moved > 50 cm), crow rotated its body 180° to face the opposite direction, and crow hopping from the perch to the cage floor (or vice-versa). Because we measured movement to account for possible confounds to FDG uptake activity, we counted movement throughout the entire 10 min of the stimulus phase, including when the stimulus stage interior was hidden from the crow's view.

## Image Processing

After each crow was imaged, we conducted a 13 min attenuation scan, then reconstructed the image using the vendor-supplied 3D OSEM/MAP algorithm to an isotropic spatial resolution of 2.5 mm full width at half maximum, with attenuation and scatter corrections applied to the data. The image matrix was 128 × 128 × 159. We exported reconstructed images using DICOM for the statistical parametric analysis software.

We imported the raw DICOM data to ImageJ (Schneider et al., 2012), manually aligned their orientation to match the jungle crow (*Corvus macrorhynchos*) brain atlas established by Izawa and Watanabe (2007) and adapted for PET by Marzluff et al. (2012), and trimmed the images to include only the brain. We stereotactically aligned the scans by estimating and applying nine affine parameters to the images using algorithms originally designed for automated human brain analysis (NEUROSTAT, University of Utah; Minoshima et al., 1992), which have been adapted for crow brains analysis. We estimated alignment precision to be one-two pixels. Finally, we normalized all uptake values to a global brain FDG uptake.

Although we used Izawa and Watanabe's (2007) atlas as a guide to identify the regions significantly activated by each stimulus, we did not use it as the sole determinant. This was because jungle crows are larger than American crows (Jungle crow mean male weight: 680 g, American crow: 450 g, Kitagawa, 1980; Kilham, 1990). More importantly, the atlas was based on a sectioned brain, whereas our activation foci were based on *in vivo* imaging. Sectioned brains tend to "flatten" (reduced *Y*-axis length, increased *X*- and *Z*- axis lengths) after being extracted from the skull and are vulnerable to other artifacts which can further alter the original shape (Rolls et al., 2008). Therefore, we scaled Izawa and Watanabe's (2007) atlas for use with American crow brains and used it in conjunction with the shape and extent of the total activation (not just the focal coordinates) to determine the activated regions.

## Statistical Analyses

Due to the small sample size, we calculated differences between the first and second scan's stimulus phase behaviors (blink rate, gaze, and movement) using a paired samples *t*-test, correlation between FDG uptake and blink rate/movement using a Pearson correlation test, and differences between the different stimuli of the 2nd scan using a linear model, all in RStudio version 1.0.136 (RStudio Team, 2016). We determined significant differences in regional activity within the brain using an automated voxel-wise subtraction and Z-statistic mapping algorithm originally designed for automated human paired-brain analysis (NEUROSTAT, University of Utah; Minoshima et al., 1992). This algorithm conducts a paired *Z*-test comparing the study population's total difference in FDG signal strength between each individual subject's first (control) and second (stimulus) scans against the study population's pooled variance; it does this for each voxel coordinate throughout the entire brain. Due to the large number of comparisons made, the algorithm calculated a Z-threshold for statistical significance using a modified Bonferroni correction commonly utilized in imaging research (Friston et al., 1991). Because this threshold is conservative (Cross et al., 2013), we also report any regions with a Z-score more than 3.0, as these regions may be worth examining in greater detail in future studies. We verified the voxel-wise results by obtaining spherical volumes of interest (VOIs; 2-voxel radius) centered around the significant coordinates to determine if said results are driven by outlying individual scans.

## Ethical Note

We captured, housed, and tested all crows (including PET/CT scans) in accordance with the Institutional Animal Care and Use Committee of the University of Washington (IACUC; protocol number 3077-01), Federal Collecting Permit MB761139-0, and State of Washington Scientific Collection Permit 14-010. We released all crows back into the wild at the location where they were captured at the conclusion of the study.

# RESULTS

We were only able to obtain usable imaging data from 13 of the 17 captured crows; the other four individuals had one of their scans invalidated by mechanical/software issues with the imaging system. Additionally, we were unable to obtain blink rate data from one bird's 2nd scan (audio stimulus) due to it positioning itself with its eye remaining out of the camera's field of view throughout the stimulus phase.

## Differential Brain Activity

Crows that were exposed to the unimodal visual stimulus showed no notable increases in brain activity relative to their initial baseline scan, even at the coordinates of peak differential activity for the combined stimulus (**Supplementary Figure 2**). See **Supplementary Figure 3** for differential activity patterns throughout the entire brain in response to the visual stimulus.

By contrast, crows that were exposed to the unimodal audio stimulus showed significantly increased activity in the medial portion of the nidopallium in their left hemisphere compared to their baseline control scan (10.6% increase, $Z = 4.57$, $P < 0.001$, **Figures 2**, **3**); this region includes or is adjacent to Field L and possibly the lateral septum. This activity extends along the anterior-posterior axis with the most visible at A11.8. While no other regions' increase in activity in response to the audio stimulus exceeded the critical Z-threshold ($Z = 4.08$), there was a notable increase in FDG uptake within a part of the medial

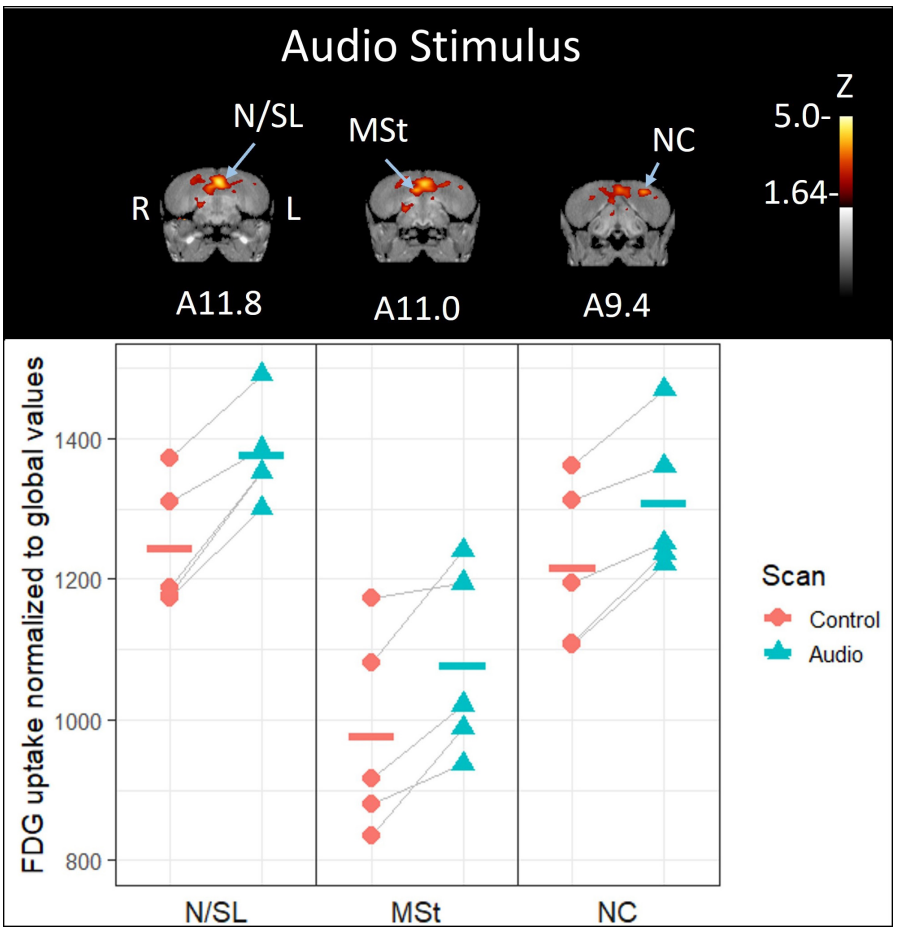


**FIGURE 2 | Top:** Coronal view of voxel-wise subtractions (converted to Z-scores) showing differential brain activity at the indicated region for all crows exposed to the vocalizations of feeding crows during their stimulus scan ($n$ = 5). Brain activity is superimposed atop a composite ($n$ = 4) structural MRI of the American crow brain. Slice coordinates (A11.8, A11.0, and A9.4) refer to Izawa and Watanabe (2007) jungle crow atlas. **Bottom:** individual normalized (global) uptake values obtained from VOI's centered on peak activation coordinates. Horizontal lines indicate group means. Note that only the nidopallium/lateral septum border (N/SL) showed significant increases in brain activity; the medial striatum (MSt) and caudal nidopallium (NC) did not meet the critical Z-threshold.

striatum ventral to the highly activated medial nidopallium, particularly in the right hemisphere (10.2% increase, $Z$ = 3.58, $P < 0.001$, at A11.0). We observed another low threshold (but notable) activity increase in the caudal nidopallium ventral to the HVC region in the left hemisphere (7.5% increase, $Z$ = 3.33, $P < 0.001$, at A9.4). See **Supplementary Figure 4** for differential activity patterns throughout the entire brain in response to the audio stimulus.

Simultaneous presentation of preferred food item and food-associated vocalizations induced higher FDG-uptake activity in those areas responding to audio stimuli in the left hemisphere (**Figures 4**, **5**), including the medial nidopallium (11.6% increase, $Z$ = 4.23, $P < 0.001$, at A11.4), lateral septum (11.8% increase, $Z$ = 4.00, $P < 0.001$ at A12.6), and the caudal nidopallium (13.1% increase, $Z$ = 3.66, $P < 0.001$, at A5.8) though the latter two were low threshold increases (Z-threshold = 4.14). We also observed significant increased activity in the TnA of the right hemisphere (14.7% increase, $Z$ = 4.27, $P < 0.001$, at A6.6). See **Supplementary Figure 5** for differential activity patterns throughout the entire brain in response to the combined A/V stimulus.

## Stimulus Phase Behavior

The crows visually attended to the stimulus stage whenever it was revealed, regardless of what was inside; they stared into the stage for a majority of the time (right eye: mean ± SD; 373.0 ± 48.0 s; left eye: 357.3 ± 87.3 s) that it was visible (420 s), with no significant difference in gaze time between their two scans (left eye: $t_{26}$ = 1.51, $P$ = 0.14; right eye: $t_{26}$ = 0.56, $P$ = 0.58) nor between any of the stimuli during their second scan [left eye: $F_{(2,\ 11)}$ = 1.35, $P$ = 0.30; right eye: $F_{(2,\ 11)}$ = 0.31, $P$ = 0.83]. Their mean blink rate remained steady (30.2 ± 8.3 blinks/min), with no significant change between the 1st and 2nd scans ($t_{11}$ = 0.83, $P$ = 0.43) nor any of the different stimuli presented during the 2nd scan [$F_{(2,\ 10)}$ = 0.07, $P$ = 0.93]. Most crows moved little during the stimulus phase, though there were several outlier individuals with a high degree of movement (3.4 ± 7.6 total movement, max = 39); there was no change in movement between the two

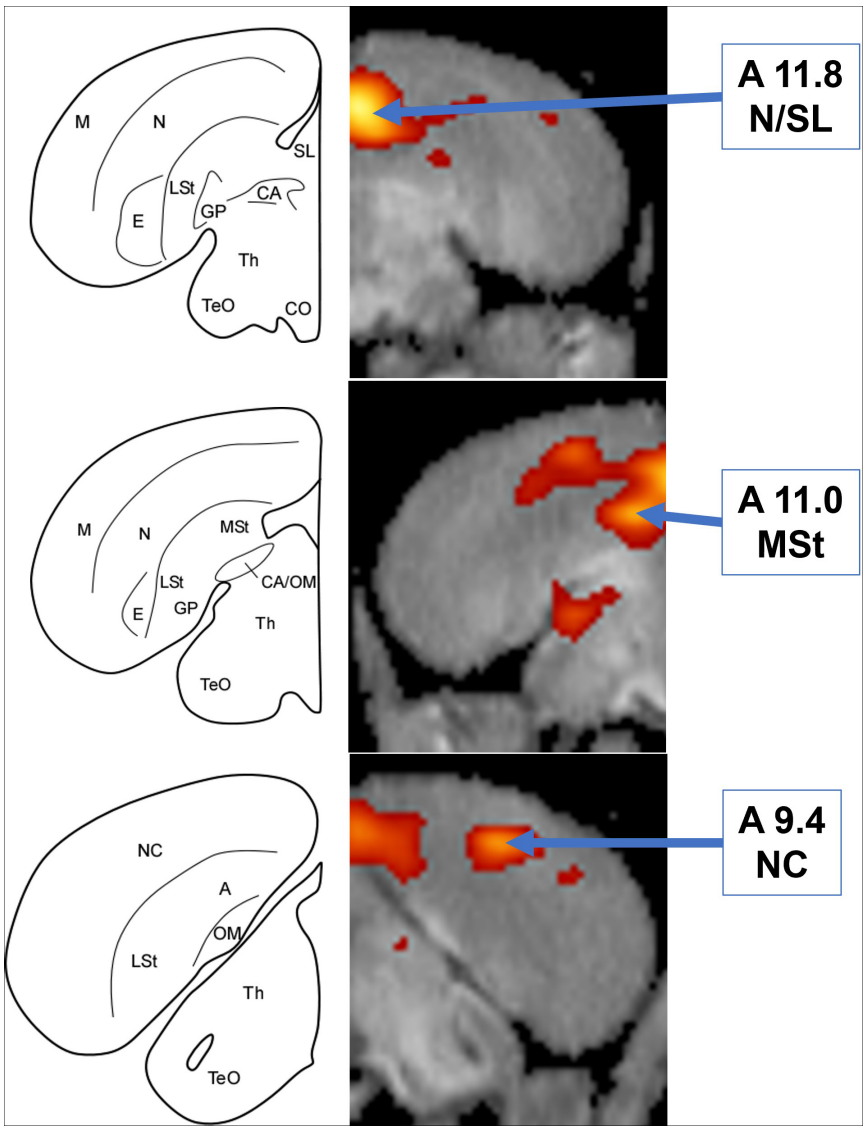


**FIGURE 3 | Left:** Schematic coronal hemisections of the American crow brain, drawn based on structural MRI of the American crow brain and Izawa and Watanabe jungle crow atlas. A, Arcopallium; CA, Anterior Commissure; CO, Optic Chiasm; E, Entopallium; GP, Globus Pallidus; LSt, Lateral Striatum; M, Mesopallium; MSt, Medial Striatum; N, Nidopallium; NC, Caudal Nidopallium; OM, Occipito-mesencephalic Tract; SL, Lateral Septum; TeO, Optic Tectum; Th, Thalamus. **Right:** Coronal view of voxel-wise subtractions (converted to Z-scores) showing differential brain activity at the indicated region for all crows exposed to the audio stimulus ($n$ = 5). Brain activity is superimposed atop a composite ($n$ = 4) structural MRI of the American crow brain.

scans ($t_{12}$ = 1.23, $P$ = 0.24) nor any of the different stimuli during the 2nd scan [$F_{(2,\ 11)}$ = 2.12, $P$ = 0.17]. See **Supplementary Figure 6** for details on behavioral changes between scans and stimuli. We did not observe any significant correlation between blink rate or movement with the rate of FDG uptake (see **Supplementary Figures 7**, **8** for details).

## DISCUSSION

When social animals gather around a food source, they must divide their attention between the activity of their fellow conspecifics and the food itself; the mental demand required to successfully navigate these situations likely necessitates increased neural activity in multiple regions and systems throughout the brain. Adult American crows have ample experience navigating the social milieu around a communal food source thus their relevant neural circuitry should be well-developed compared to other songbirds.

### Combined A/V and Emergent Activity

The combined A/V stimulus mimicked the conditions of a social feeding event; focal crows heard conspecifics vocalizing while simultaneously visually observing a preferred food item. These

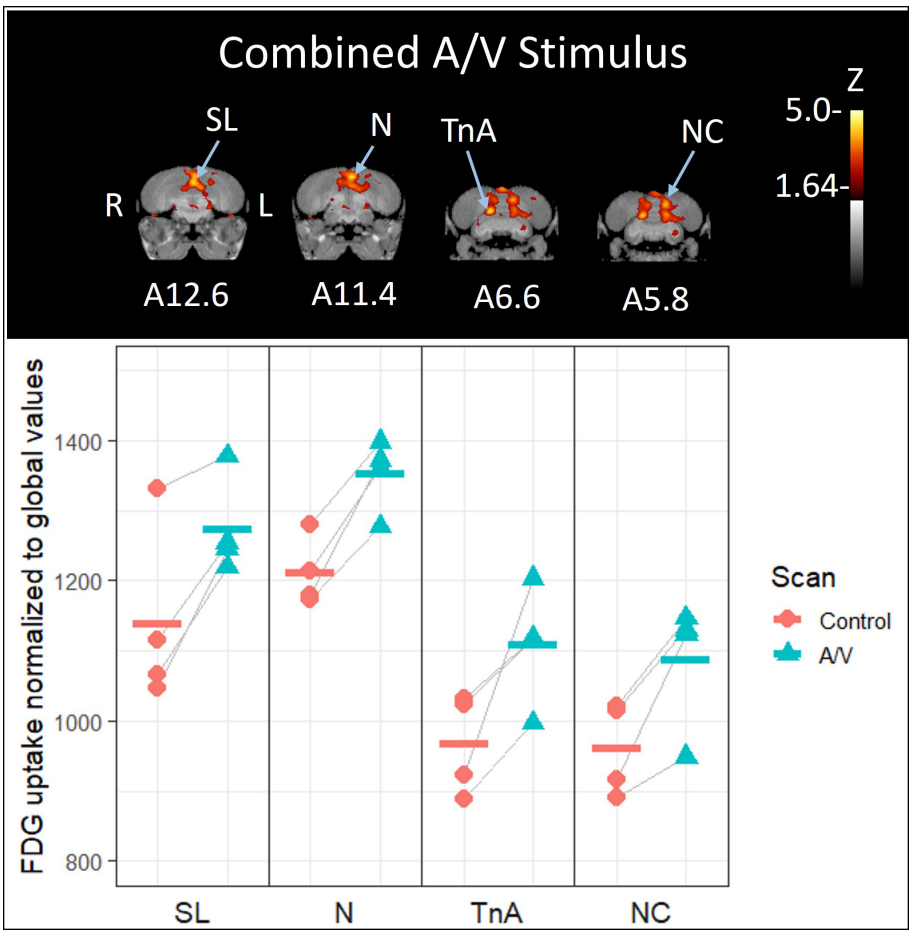


**FIGURE 4 | Top:** Coronal view of voxel-wise subtractions (converted to Z-scores) showing differential brain activity at the indicated region for all crows simultaneously exposed to the vocalizations of feeding crows and the sight of their preferred food item during their stimulus scan ($n$ = 4). Brain activity is superimposed atop a composite ($n$ = 4) structural MRI of the American crow brain. Slice coordinates (A12.6, A11.4, A6.6, and A5.8) refer to the Izawa and Watanabe (2007) jungle crow atlas. **Bottom:** individual values for normalized (global) uptake obtained from VOI's centered on peak activation coordinates. Horizontal lines indicate group means. Note that while the nucleus taeniae of the amygdala (TnA), and nidopallium (N) showed significant increases in FDG uptake, the lateral septum (SL) and caudal nidopallium (NC) did not meet the critical Z-threshold.

conditions triggered increased activity in two regions (the TnA and caudomedial nidopallium) that were not active in response to either unimodal stimulus, and further defined the activity of two regions (the lateral septum and medial nidopallium) that were active for the unimodal audio stimulus.

The largest increase in activity occurred in the TnA, a region associated with processing social information (Cheng et al., 1999; Mayer et al., 2019) and one of the regions we hypothesized would be active in response to hearing conspecific vocalizations. This suggests that the TnA is involved in integrating sensory information with social stimuli, which supports existing evidence linking the TnA with controlling social foraging behavior (Cheng et al., 1999, although see Xin et al., 2017). As the amygdala is also associated with processing a learned fear response (Cross et al., 2013), this result could alternatively be explained as the crows being especially frightened by some confounding factor associated with the combined A/V stimulus that was absent for the other stimuli. However, frightening stimuli usually decreases the blink rate of observing crows (Marzluff et al., 2012; Cross et al., 2013), which was not strong in our experiments (we observed only a slight reduction FDG uptake in the TnA; see **Supplementary Figure 7**). Finally, neither of the unimodal conditions prompted increased TnA activity. We therefore conclude that the TnA activity during the multimodal stimuli was not caused by a strong fear response.

The other region notably stimulated by the combined A/V stimulus, but not by either unimodal stimulus, was located medially within the caudal nidopallium. The caudomedial nidopallium is involved in avian auditory processing (Atoji and Wild, 2009; Moorman et al., 2012), and recent evidence suggests it may be a passerine-specific enlargement of the avian prefrontal area and involved with multimodal processing (von Eugen et al., 2020), which our results support.

The addition of the visual stimulus slightly enhanced the activity of the medial nidopallium and lateral septum; we observed their activity as a merged entity for the audio-only

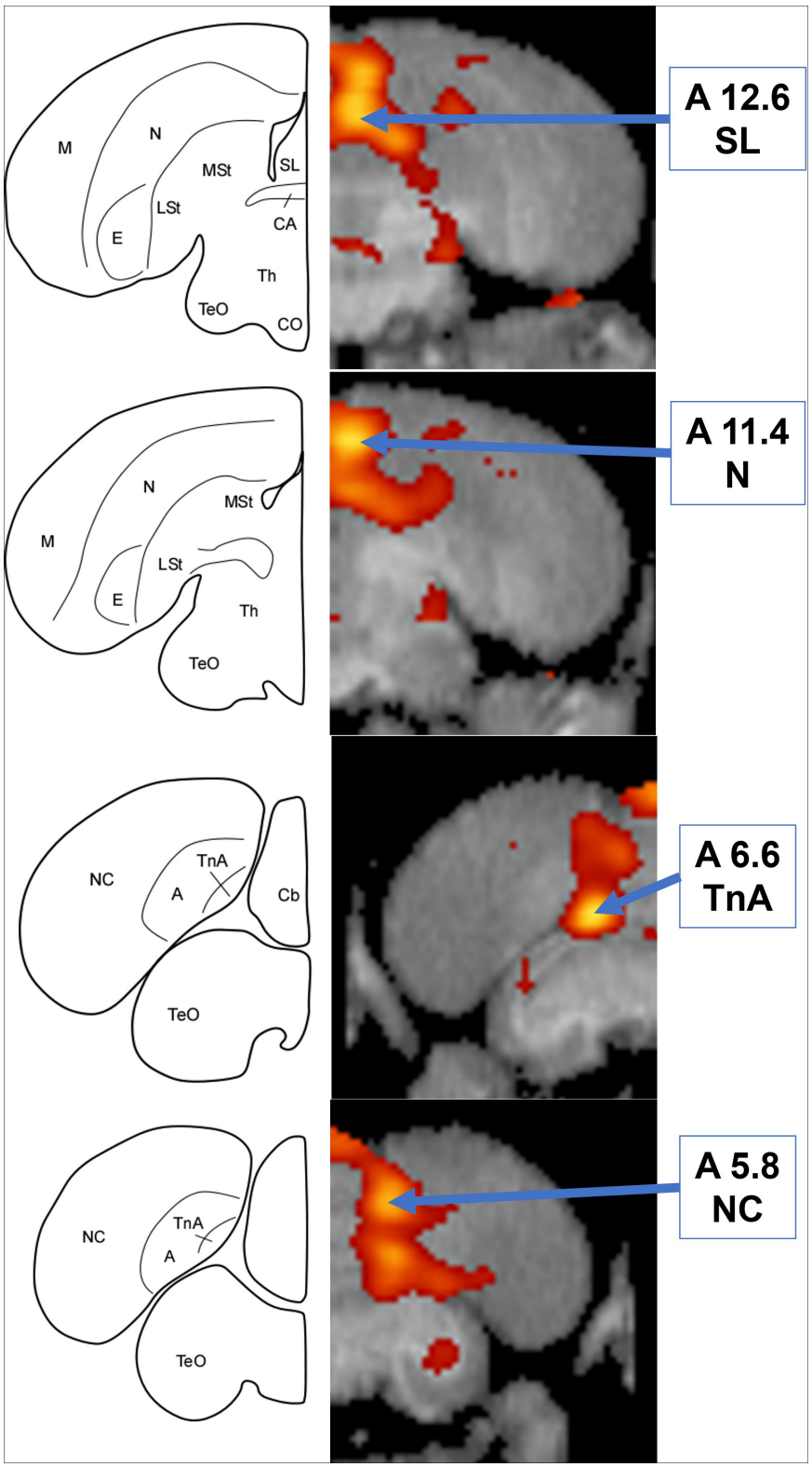


**FIGURE 5 | Left:** Schematic coronal hemisections of the American crow brain, drawn based on structural MRI and Izawa & Watanabe jungle crow atlas. Cb, Cerebellum; TnA, nucleus taeniae of the amygdala. For other abbreviations, see **Figure 3**. **Right:** Coronal view of voxel-wise subtractions (converted to Z-scores) showing differential brain activity at the indicated region for all crows simultaneously exposed to the audio and visual stimuli ($n$ = 4). Brain activity is superimposed atop a composite ($n$ = 4) structural MRI of the American crow brain.

stimulus and separately as two distinct entities for the combined A/V stimulus. As in mammals, the avian lateral septum is a part of descending connections from the hippocampus to the brainstem limbic centers and is involved in regulating aggression (Cooper and Erickson, 1976; Goodson et al., 1998; Goodson, 2005). This is consistent with the fact that fights and status displays were common among crows jockeying for access to food when we recorded the stimulus audio, so there is a high likelihood that the audio stimuli contained aggressive vocalizations. The other region stimulated by both audio-associated conditions, the medial nidopallium, has been implicated with A/V stimulus processing in imprinting learning (Wallhäusser and Scheich, 1987; Bredenkötter and Braun, 1997), though those studies focused on precocial chicks of a species belonging to a different taxonomic order. Further studies are needed to determine whether the medial nidopallium in different avian species are equivalent in function.

While we observed activity in two *a priori* hypothesized regions associated with social behavior (the TnA and lateral septum), we did not observe any activity in the thalamus, the region we predicted would be active during the multimodal stimulus. The thalamus is involved with filtering and relaying sensory information to other brain regions (Bentivoglio et al., 1993); it's possible that there was not enough contrast in sensory information presented between the control and stimulus scans. Although the audio stimulus increased the amplitude of the crow's sensory environment, we kept the amount of light, non-stimulus ambient sound, and temperature constant for all scans, potentially masking any effect the added stimulus sounds might have had on thalamus activity.

## Response to Unimodal Audio Stimulus

In contrast to the previous regions, the medial striatum was only notably stimulated by the unimodal audio stimulus; this activity was not present when food visually accompanied the vocalizations. The avian medial striatum, extending in the antero-posterior axis, corresponds to the mammalian ventral striatum. In birds, the anterior portion is involved in sensory processing (e.g., area X in songbirds), though the activated area observed in the present study is in the limbic posterior region including the avian nucleus accumbens (Husband and Shimizu, 2011).

The other region notably activated by the audio-only condition is located centrally between the nidopallium and caudal nidopallium, ventral to the HVC; this area is most likely a lateral area of Field L. Like the caudomedial nidopallium, Field L is heavily involved in songbird auditory processing and filtering conspecific vocal signals from other sounds (Zaretsky, 1978; Wild et al., 1993; Grace et al., 2003; Nagel et al., 2011). Despite its central role in the avian auditory network, Field L was not stimulated when the listening crows could also see food, which further emphasizes the importance of context in songbird auditory processing of a vocal signal.

## Lateralization of Activity

Avian brains are highly lateralized (Mench and Andrew, 1986), and we observed some bias in hemispherical activity in all observed regions. Most of the strongest observed activity occurred in the left hemisphere, though the TnA and medial striatum were most active in the right hemisphere. As there was no significant difference in gaze direction (see **Supplementary Figure 6**), this asymmetry cannot be explained because of directional sensory bias. Previous studies have found that the caudomedial nidopallium tends to be more active in the left hemisphere for passerine songbirds when processing audio information (Moorman et al., 2012; Ocklenburg et al., 2013), which is consistent with our findings.

## Lack of Response to Unimodal Visual Stimulus—Why More Activity for Vocalizations?

In contrast to the multiple active regions that we observed in response to conspecific vocalizations, we did not observe any areas of increased activity (*a priori* hypothesized hypothalamus or otherwise) in response to the unimodal visual stimulus of a preferred food item. This lack of activity was not the result of a single outlier individual's contrary activity masking a majority trend (**Supplementary Figure 4**). While it's possible that some unknown factor was occurring to reduce food-associated brain activity (such as imaging-associated stress inhibiting appetite) or the metabolic uptake of the radiotracer (such as increasing blood flow to the GI tract in anticipation of a meal), we believe this is unlikely for the simple reason that the food caused observable changes in brain activity when added to the auditory-associated stimuli.

Why did the food, something necessary for the crow's survival, evoke less activity throughout the brain than the vocalizations? We posit that the food was less interesting to the crows and required less cognitive power to neuronally process than the calls. The food did not add light to the crows' sensory environment (only a negligible increase in visual signals sent to the brain), remained static (less cognitive processing required), and, because they were fed after their previous control scan, was something they had already experienced in the context of the imaging lab (less stimulating). By contrast, the calls added sound to the crows' sensory environment (large increase in auditory signals sent to the brain), were dynamic (more cognitive processing required), and were likely much more surprising to hear within the setting of the imaging lab (more stimulating). When a more interesting stimulus (the vocalizations) was present to maintain the crows' attention, this may have had the secondary effect of encouraging the crows to pay more attention by proxy to the food than they would otherwise.

The social aspect of the vocalizations is another factor to consider. Although we recorded the stimulus vocalizations from crows as they gathered around a food source, crows in such contexts usually do not limit their communication to food-associated information; for example, they also vocalize to announce their presence, assert dominance, recruit allies, etc. (Pendergraft and Marzluff, 2019). In addition to the overt signals, their vocalizations also contained characteristics that can be used to identify the caller's sex and identity (Mates et al., 2015). The amount of social information contained within the vocalizations,

combined with the information being conveyed indirectly (they contained information *about* things, whereas the food *was* food) is a likely reason why the calls elicited significant increases in neural activity throughout the brain, yet the food did not.

The amount of neuronal processing required to extract all the social information from a vocal signal is supported by behavioral observations, as most birds closely attend to conspecific vocalizations (Dooling and Prior, 2017). Pinyon jays (*Gymnorhinus cyanocephalus*), another Corvidae species, pay most attention to conspecific vocalizations when they recognize the caller as belonging to their own flock (Marzluff and Balda, 2010), which may be applicable here: there's evidence that American crows can use acoustic properties of the vocalization to identify the caller (Mates et al., 2015) and we observed increased activity in the caudomedial nidopallium- a region associated with vocal recognition of known individuals (Chew et al., 1996; Bolhuis and Gahr, 2006). As we had pre-baited the capture site each day of the week preceding their capture and many of the crows had fallen into the habit of visiting the location each morning, it's possible that the experimental crows may have recognized some of the callers from the stimulus tracks, which would further motivate them to attend the audio stimulus.

### No Confounding Effects of Repeated Testing

In a prior study where we balanced the presentation of four stimuli to crows, we observed that after crows experienced a potentially dangerous stimulus in one trial, their subsequent responses were biased toward fearful reactions (Swift et al., 2020). We did not observe such carryover effects in the present study; neither blink rate nor movement varied from the first to the second scan. This is likely due to the benign, non-threatening stimulus presented during the control scan as well as the limited number of presentations (two) each bird received.

## CONCLUSION

Taken as a whole, the six distinct regions activated by the sound of conspecifics vocalizing at a food source are associated with either processing auditory sensory data or social information. This would suggest that all the identified regions are either involved in a larger socio-auditory processing system or, more likely, are components of two or more brain systems that are triggered when the bird needs to process additional modalities or contextual information. Although multiple studies have examined avian brain activity in response to social interactions, this study is the first to use functional imaging to holistically measure activity in response to social signals under the context of communal foraging, one of many social contexts that birds regularly encounter in nature. The visual presence of food, despite not causing any notable changes in activity on its own, significantly alters the neural activity triggered by the sound of conspecifics vocalizing and even stimulates activity in regions not activated by either modality alone. This demonstrates that the context associated with a stimulus matters to the neuronal processing of that information, especially for something as varied as social interaction.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Washington (IACUC; protocol number 3077-01).

## AUTHOR CONTRIBUTIONS

LP: conceptualization, funding acquisition, animal care, investigation, data curation and analysis, and writing—original draft. JM: conceptualization, investigation, funding acquisition, project administration, supervision, and writing—review and editing. DC: methodology, supervision, data curation and analysis, and writing—review and editing. TS: methodology, validation, and writing—review and editing. CT: methodology, funding acquisition, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.766345/full#supplementary-material

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# Serotonergic Neurons in the Chick Brainstem Express Various Serotonin Receptor Subfamily Genes

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Serotonin (5-hydroxytryptamine, 5-HT) is a phylogenetically conserved modulatory neurotransmitter. In mammals, 5-HT plays an important role in the regulation of many mental states and the processing of emotions in the central nervous system. Serotonergic neurons in the central nervous system, including the dorsal raphe (DR) and median raphe (MR) nuclei, are spatially clustered in the brainstem and provide ascending innervation to the entire forebrain and midbrain. Both between and within the DR and MR, these serotonergic neurons have different cellular characteristics, developmental origin, connectivity, physiology, and related behavioral functions. Recently, an understanding of the heterogeneity of the DR and MR serotonergic neurons has been developed at the molecular level. In birds, emotion-related behavior is suggested to be modulated by the 5-HT system. However, correspondence between the raphe nuclei of birds and mammals, as well as the cellular heterogeneity in the serotonergic neurons of birds are poorly understood. To further understand the heterogeneity of serotonergic neurons in birds, we performed a molecular dissection of the chick brainstem using *in situ* hybridization. In this study, we prepared RNA probes for chick orthologs of the following serotonin receptor genes: *5-HTR1A*, *5-HTR1B*, *5-HTR1D*, *5-HTR1E*, *5-HTR1F*, *5-HTR2A*, *5-HTR2B*, *5-HTR2C*, *5-HTR3A*, *5-HTR4*, *5-HTR5A*, and *5-HTR7.* We showed that the expression pattern of 5-HT receptors in the serotonin neurons of chick DR and MR may vary, suggesting heterogeneity among and within the serotonin neurons of the DR and MR in the chick brainstem. Our findings regarding the molecular properties of serotonergic neurons in the bird raphe system will facilitate a good understanding of the correspondence between bird and mammalian raphes.

**Keywords: chick, dorsal raphe nucleus, median raphe nucleus, serotonin receptor, heterogeneity, optic tectum, serotonergic neuron**

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## INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is a phylogenetically conserved modulatory neurotransmitter (Marin et al., 2020). Many studies suggest that the influence of the 5-HT system on cognition, behavior, and emotion is evolutionarily conserved in the animal kingdom (Kandel et al., 2000; Bacque-Cazenave et al., 2020). To improve the understanding of the

phylogenetic continuity of the neural basis for cognition and emotion, it is important to elucidate the neural circuits that process cognitive and emotional behaviors in animals other than mammals. Birds are model animals of interest for understanding the evolutionary continuity of the neural basis of cognition and emotion (Rosa Salva et al., 2015; Papini et al., 2019). It has been suggested that the 5-HT system controls aggressive behavior in pigeons (Fachinelli et al., 1989), feather pecking behavior (Kops et al., 2017; de Haas and van der Eijk, 2018) and foraging behavior in chickens (Matsunami et al., 2012). In addition, 5-HT transporter systems have been suggested to modulate fear-related behaviors in chickens using a combination of behavioral and functional polymorphisms (Krause et al., 2017, 2019; Phi Van et al., 2018). However, the neural circuits that are modulated by 5-HT and responsive 5-HT receptors (5-HTRs) in avian brains are largely unknown.

In mammals, the serotonergic neurons in the central nervous system are spatially clustered raphe nuclei in the brainstem, classically designated as groups B1–B9 (Dahlström and Fuxe, 1964; Soiza-Reilly and Gaspar, 2020). Among them, the dorsal raphe (DR: B6 and B7) and median raphe (MR: B5 and B8) are of special importance and have been intensively studied because they provide ascending innervation to the entire forebrain and midbrain (Olivier, 2015; Cohen and Grossman, 2020; Soiza-Reilly and Gaspar, 2020). A considerable heterogeneity of serotonergic neurons among and within DR and MR with respect to multiple aspects, such as developmental origin, connectivity, electrophysiological properties, and behavioral function exists (Calizo et al., 2011; Alonso et al., 2013; Okaty et al., 2019; Soiza-Reilly and Gaspar, 2020). The diversity of 5-HT neurons in the DR and MR has received particular attention in mammals (Okaty et al., 2019). Recently, the rapid development of single-cell RNA sequencing technology has provided a powerful tool for understanding the heterogeneity of serotonergic neurons at single-cell resolution among and within the DR and MR (Huang K. W. et al., 2019; Ren et al., 2019; Okaty et al., 2020). These studies have laid a molecular foundation for investigating the heterogeneity of serotonergic neurons in the DR and MR.

In contrast, the distribution and diversity of avian serotonergic neurons in the raphe are poorly understood currently. Only a few studies have reported the histological position of the avian raphe (Kuenzel and Masson, 1988; Puelles et al., 2007) and have investigated the distribution of 5-HT neurons in the avian brainstem, including the raphe. 5-HT neurons in the avian brainstem are divided into several groups based on histological features, such as the location, size, and shape and immunoreactivity (Yamada et al., 1984) or fluorescence chemical reactivity for the detection of monoamines (Ikeda and Gotoh, 1971; Dube and Parent, 1981). However, there are no studies on the distribution and diversity of 5-HTRs in the avian raphe. Furthermore, the correspondence between the mammalian and avian raphe is not yet clear.

In the present study, we investigated the chick brainstem at the molecular level using *in situ* hybridization (ISH) to improve the understanding of heterogeneity of 5-HT neurons in the avian raphe. In mammals, serotonergic neurons exhibit a high level of within-system communication *via* feedback where serotonin activates serotonin receptors on serotonergic neurons and a considerable heterogeneity of serotonin receptors exist among and within the serotonergic neurons of DR and MR. We selected chick orthologs of serotonergic neuron marker genes: tryptophan hydroxylase 2 (TPH2) and serotonin transporter (SERT), and the following serotonin receptor genes: *5-HTR1A*, *5-HTR1B*, *5-HTR1D*, *5-HTR1E*, *5-HTR1F*, *5-HTR2A*, *5-HTR2B*, *5-HTR2C*, *5-HTR3A*, *5-HTR4*, *5-HTR5A*, and *5-HTR7*. We found that the chick DR and MR serotonin neurons vary in the expression patterns of *5-HTR*s, suggesting heterogeneity of serotonin neurons among and within the DR and MR. Understanding the molecular properties of 5-HT neurons in the bird raphe will elucidate the correspondence between bird and mammalian raphes.

# MATERIALS AND METHODS

## Animals

Fertilized eggs of domestic chicks of the Cobb strain (*Gallus gallus domesticus*) were purchased from a local dealer (3-M, Aichi, Japan) and incubated at the Teikyo University (Kaga, Itabashi-ku, Tokyo, Japan). Animal experiments were performed as described previously by Yamaguchi et al. (2008a; 2008b). Newly hatched chicks (P0) were transferred to dark plastic enclosures in a dark warm cage at 30°C for 1 day (P1). Previous studies showed that embryonic development of the serotonergic system is almost completed before hatching (Okado et al., 1992; Huang X. et al., 2019), suggesting that the distribution of serotonin neurons in the brainstem of P1 chick was almost same as that in the adult chick. We used P1 chick because we do not need to take care of rearing environment that might affect the level of gene expression. In this study, we used six chicks for tryptophan hydroxylase 2 (*TPH2)*, four for serotonin transporter (*SERT)*, four for the 5-*HTR1A* condition, two for the 5-*HTR1B*, five for the 5-*HTR1D*, four for the 5-*HTR1E*, two for the 5-*HTR1F*, one for the 5-*HTR2A*, one for the 5-*HTR2B*, one for the 5-*HTR2C*, one for the 5-*HTR3A*, one for the 5-*HTR4*, four for the 5-*HTR5A*, and two for the 5-*HTR7* (**Supplementary Table 1**). All the procedures were reviewed and approved by the Committee on Animal Experiments of Teikyo University and conducted in accordance with the guidelines of the national regulations for animal welfare in Japan.

## Histological Sample Preparations

P1 chicks were anesthetized by an intraperitoneal injection (0.40 mL/individual) of a 1:1 solution of ketamine (10 mg/mL, Ketalar-10, Sankyo Co., Tokyo, Japan) and xylazine (2 mg/mL, Sigma, St. Louis, MO, United States). The anesthetized chicks were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.5, PFA-PBS). Whole brain specimens were dissected up to the spinal cord level and immediately immersed in PFA-PBS for 1 or 2 day(s) at 4°C and placed in an 18% sucrose/PFA-PBS solution for cryoprotection for 2 days at 4°C. Subsequently, brains with sucrose substitution were embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan), frozen immediately on dry ice, and stored at −80°C until further use.

**TABLE 1 |** Comparison of the terminology regarding the serotonergic neuron cell groups and possible correspondence between birds and mammals.

| Birds (chicken) | | | | Mammals (rat) |
|---|---|---|---|---|
| **This study, 2021** | **Ikeda and Gotoh, 1971** | **Yamada et al., 1984** | **Puelles et al., 2007** | **Dahlström and Fuxe, 1964** |
| Dorsal raphe | B5 | Group 5 | DR | B6 |
| | | | | B7 |
| Median raphe | B6 | Group 6 | r1MnR | B5 |
| | | | r2MnR | |
| | B7 | Group 10 | CLi | B8 |

*CLi, caudal linear nucleus of the raphe; DR, dorsal raphe nucleus; r1MnR, rhombomere 1 median raphe nucleus; r2MnR, rhombomere 2 median raphe nucleus.*

The frozen brain blocks were cut into 18 μm-thick sections using a cryostat (Leica CM3050S or Leica CM1850, Leica Biosystems, Nußloch, Germany). The level of the serial coronal sections (A2.6 to A0.8) corresponded to those of the atlas by Kuenzel and Masson (1988).

## cDNA Cloning and RNA Probe Preparations

Total RNA was extracted from the chick brain using TRIzol Reagent (Invitrogen, Carlsbad, CA, United States) and reverse-transcribed using the SuperScript III kit (Invitrogen, Carlsbad, CA, United States) using an oligo (dT) primer, according to the manufacturer's protocol. Reverse-transcription polymerase chain reaction (RT-PCR) was performed using the following gene-specific primer (forward and reverse) pairs: *TPH2:* 5′-TTTTGTGGACTGTGACTGCA-3′ and 5′-GATGCTCCCAATGAAGCGAG-3′, respectively; *SERT*: 5′-TACCCGCCAAGTTCTACAGG-3′ and 5′-CCTCCAA AAGTCAGGGTTGC-3′, respectively; 5-*HTR1D:* 5′-CCGTGG ATGAAAGGACACTG-3′ and 5′-TTCGTGAAGGCTTGCG TTAA-3′, respectively; 5-*HTR1E:* 5′-ACAACCCTGACT ATGCTGCT-3′ and 5′-AGCCAGGTCCAAGTCATTCT-3′, respectively; 5-*HTR1F:* 5′-TCCATTACCCTGTCTGTGCT-3′ and 5′-ATTCAGATCTGGGGCTTCGT-3′, respectively; 5-*HTR2B:* 5′-TGGAGCTGGCAATGGATCTT-3′ and 5′-CTTTTCTGGTGAGCAGGCAG-3′, respectively; 5-*HTR5A:* 5′-AGTAATCGGAGCGGGTCATC-3′ and 5′-CCGGACAGTGAAGACCATCT-3′, respectively; and 5-*HTR7:* 5′-CAACGGCAGCCACCTCTA-3′ and 5′-AAGAGCCACACACACAGGAT-3′, respectively. PCR products were subcloned into the pGEM-T easy vector (Promega, Madison, WI, United States). The sequences were confirmed using Sanger sequencing. For 5-*HTR1A, 5-HTR1B, 5-HTR2A, 5-HTR2C, 5-HTR3A*, and 5-*HTR4* probes, we used the plasmids which had already prepared in our previous study (Fujita et al., 2020). Plasmids containing the cDNA fragments for *TPH2, SERT, 5-HTR1A, 5-HTR1B, 5-HTR1D, 5-HTR1E, 5-HTR1F, 5-HTR2A, 5-HTR2B, 5-HTR2C, 5-HTR3A, 5-HTR4, 5-HTR5A*, and 5-*HTR7* were amplified by PCR using the M13 primer pair. Amplicons containing the T7 and SP6 promoter sites were purified using a PCR purification kit (Qiagen, Valencia, CA, United States). Digoxigenin (DIG)-labeled sense and antisense RNA probes were prepared by *in vitro* transcription using a DIG RNA labeling kit (Roche, Basel, Switzerland). For the double ISH analysis, *TPH2* fluorescein-labeled sense and antisense RNA probes were prepared by *in vitro* transcription using a fluorescein RNA labeling kit (Roche, Basel, Switzerland).

**TABLE 2 |** Overview of the serotonergic neuron marker and *5-HTR*s probes used in this study.

| Accession number | Gene symbol | Molecular characteristics | Probe preparation |
|---|---|---|---|
| NM_001001301.1 | *TPH2* | Enzyme | This study |
| NM_213572.1 | *SERT* | Membrane transporter | This study |
| NM_001170528.1 | *5-HTR1A* | GPCR | Fujita et al., 2020 |
| XM_015284634.2 | *5-HTR1B* | GPCR | Fujita et al., 2020 |
| XM_015297583.1 | *5-HTR1D* | GPCR | This study |
| XM_015284709.2 | *5-HTR1E* | GPCR | This study |
| XM_004938334.3 | *5-HTR1F* | GPCR | This study |
| XM_025151250.1 | *5-HTR2A* | GPCR | Fujita et al., 2020 |
| XM_025153310.1 | *5-HTR2B* | GPCR | This study |
| XM_004940651.3 | *5-HTR2C* | GPCR | Fujita et al., 2020 |
| XM_004948063.3 | *5-HTR3A* | Ligand-gated ion channel | Fujita et al., 2020 |
| XM_015293658.2 | *5-HTR4* | GPCR | Fujita et al., 2020 |
| XM_425970.3 | *5-HTR5A* | GPCR | This study |
| XM_015288394.2 | *5-HTR7* | GPCR | This study |

*5-HTR, 5-hydroxytryptamine receptor; GPCR, G protein-coupled receptor; SERT, serotonin transporter; TPH, tryptophan hydroxylase 2.*

## *In situ* Hybridization

*In situ h*ybridization was performed as described previously by Fujita et al. (2019), with some modifications. Specimens of brain sections were re-fixed in 4% PFA-PBS, pre-treated, and hybridized with DIG-labeled RNA probes at 70°C. After stringent washes, hybridized probes were detected *via* an immunohistochemical examination with an alkaline phosphatase-conjugated anti-DIG antibody (1:1,000; Roche, Basel, Switzerland). To visualize the signals, a chromogenic reaction with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) was performed at room temperature (about 25°C) for the following durations: *TPH2* and *SERT*, 16–16.3 h; 5-*HTR1A*, 18–18.5 h; 5-*HTR1B*, 18.5–19.2 h; 5-*HTR1D*, 16.5–18 h; 5-*HTR5A*, 19.2 h; and 5-*HTR5A*, 18 h. Sense probes were used as negative controls for every experiment.

## Double *in situ* Hybridization

The process from re-fixation of sections to the chromogenic reaction with NBT/BCIP was performed as described above, except for hybridization. During hybridization, DIG-labeled RNA probes and fluorescein-labeled RNA probes were mixed and mounted simultaneously. After the first chromogenic reaction with NBT/BCIP, the anti-DIG antibody was detached using 100 mM glycine (pH 2.2). After washing in PBS, fluorescein-labeled probes were detected immunohistochemically with an alkaline phosphatase-conjugated anti-fluorescein antibody (1:1,000; Roche, Basel, Switzerland). To visualize the signals, the second color chromogenic reactions were performed at room temperature with SIGMAFAST Fast Red TR/Naphthol AS-MX tablets (Sigma-Aldrich, St. Louis, MO, United States cat#F4523) for the following durations: *5-HTR1A* and *TPH2*, 3 h and 18 h; *5-HTR1B* and *TPH2*, 3–16 h and 18–18.5 h; *5-HTR1D* and *TPH2*, 16 h and 19.3 h; *5-HTR1E* and *TPH2*, 2.5 h and 17.3 h; *5-HTR5A* and *TPH2*, 2.5 h and 17.3 h. Sense probes were used as negative controls for every experiment.

## Image Acquisition and Data Processing

Digital photographs of sections on each slide glass were acquired semi-automatically using NanoZoomer 2.0HT or NanoZoomer XR systems (Hamamatsu Photonics, Shizuoka, Japan). The microscopic fields of interest were cropped using NDP.view2 software (ver. 2.7.25[1], Hamamatsu Photonics, Shizuoka, Japan). For the ISH images, the cropped images were converted to 8-bit images, and their brightness and contrast were adjusted using ImageJ (ver. 1.52a, National Institute of Health, United States[2]).

[1]https://www.hamamatsu.com/

[2]https://imagej.nih.gov/ij/

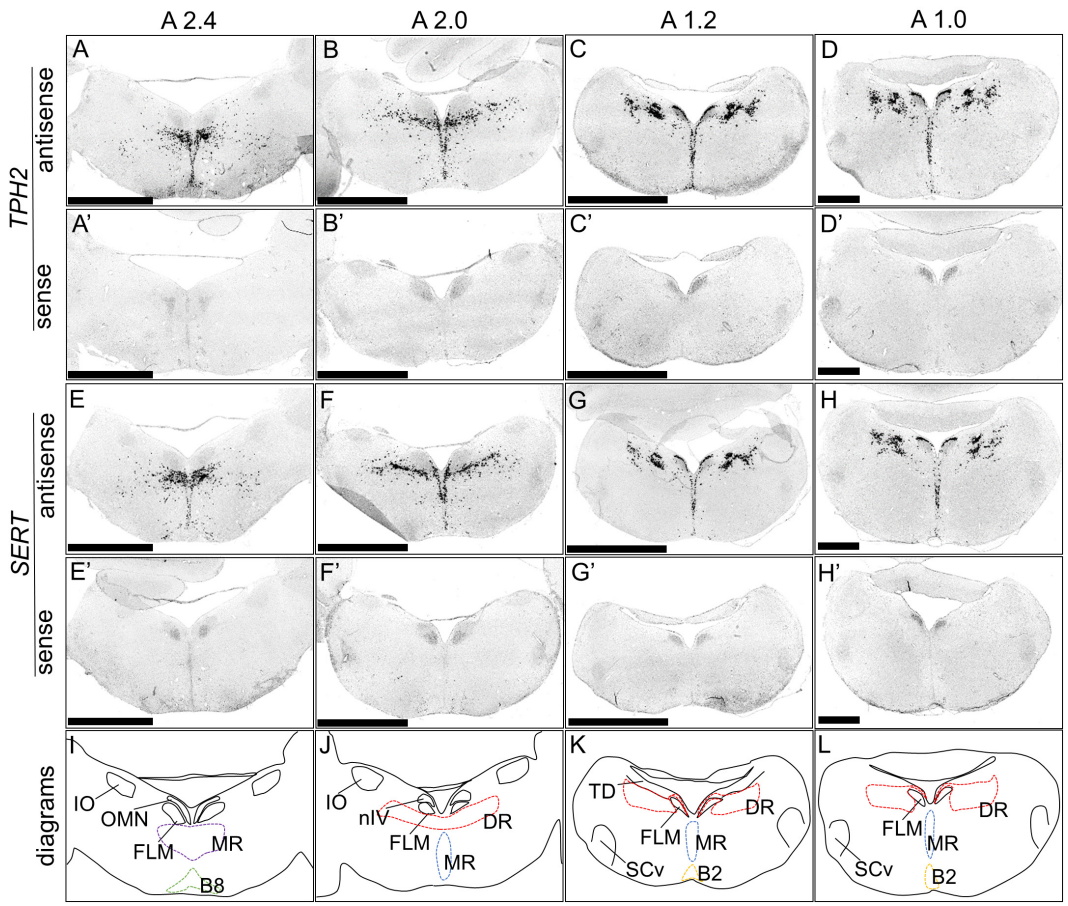


**FIGURE 1 |** *In situ* hybridization of *TPH2* and *SERT* in the P1 chick brainstems. Digoxigenin-labeled RNA antisense [*TPH2* **(A–D)**, and *SERT* **(E–H)**] and sense [*TPH2* **(A'–D')** and *SERT* **(E'–H')**] probes were used for *in situ* hybridization in P1 chick brain coronal sections. To evaluate the expression patterns of *TPH2* and *SERT*, sections of six chicks for *TPH2* and four chicks for *SERT* were analyzed, and the representative levels of sections (A2.4, A2.0, A1.2, and 1.0) are shown. The levels of the sections are in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). **(I–L)** Diagrams of coronal sections shown in panels **(A–D)**. B2, B2 cell group; B8, B8 cell group; DR, dorsal raphe; FLM, fasciculus longitudinalis medialis; IO, nucleus isthmo-opticus; MR, median raphe; nIV, nucleus nervi trochlearis; OMN, oculomotor nucleus; SCv, nucleus suboeruleus ventralis; P1, post-hatch day 1. Scale bars = 2.5 mm **(A–C,A'–C')** and 1 mm **(D,D',H,H')**.

## Terminology of the Bird Raphe

All the histological terminologies used in this study followed the atlas of Kuenzel and Masson (1988) and the description of the avian brain nomenclature consortium (Reiner et al., 2004), except for the histological position of the raphe and the terminologies of serotonergic neuron cell groups. The histological position of the raphe follows the nomenclature by Puelles et al. (2007). As for serotonergic neuron cell groups, several studies determined the distribution of chicken serotonergic neuron cell groups and proposed possible correspondence to mammalian groups (Ikeda and Gotoh, 1971; Dube and Parent, 1981; Yamada et al., 1984). Based on previous studies (Ikeda and Gotoh, 1971; Yamada et al., 1984; Puelles et al., 2007), we used the terms DR and MR for the serotonergic neuron cell groups. The relationship between the terminologies used in this study and that in previous studies are summarized in **Table 1**.

# RESULTS

## Selection of the Chick Orthologs of Mammalian Serotonergic Neuron Marker Genes and 5-Hydroxytryptamine Receptor Genes

We selected chick orthologs of the following mammalian serotonergic neuron marker genes: *Tph2*, *Sert*, and all the chick *5-HTR* genes (*5-HTR1A, 5-HTR1B, 5-HTR1D, 5-HTR1E, 5-HTR1F, 5-HTR2A, 5-HTR2B, 5-HTR2C, 5-HTR3A, 5-HTR4, 5-HTR5A*, and *5-HTR7*; except *5-HTR6*, for which we could not obtain a subclone). TPH2 is a key enzyme for serotonin biosynthesis (Walther and Bader, 2003; Walther et al., 2003). SERT is a transporter responsible for serotonin uptake from the synaptic cleft to the presynaptic neuron and is encoded by the

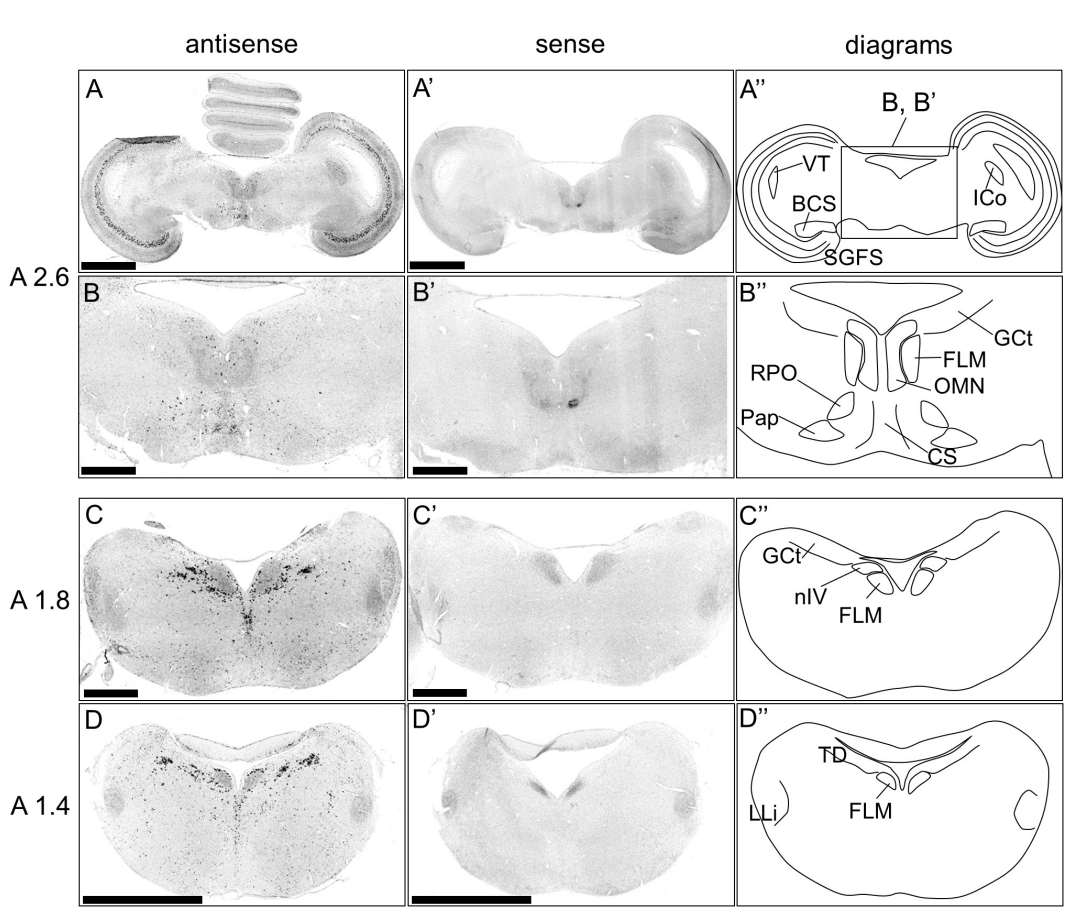


**FIGURE 2 |** *In situ* hybridization of *5-HTR1A* in the P1 chick brainstems. Digoxigenin-labeled RNA antisense **(A–D)** and sense **(A'–D')** *5-HTR1A* probes were used for *in situ* hybridization in coronal sections of P1 chick brains. To evaluate the expression patterns of *5-HTR1A*, sections of four chicks were analyzed, and representative images of two chick brain sections are shown. **(B,B')** Magnified views of brain areas in the box in panel **(A")** are shown. **(A"–D")** Diagrams of coronal sections are shown in the rightmost panels. The levels of the sections (A 2.6, A 1.8, and A 1.4) are in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). BCS, brachium colliculi superiors; CS, nucleus centralis superior; FLM, fasciculus longitudinalis medialis; GCt, griseum centrale; ICo, nucleus intercollicularis; LLi, nucleus ventralis lemnisci lateralis; nIV, nucleus nervi trochlearis; OMN, oculomotor nucleus; Pap, nucleus papillioformis; RPO, nucleus reticularis pontis oralis; SGC, stratum griseum centrale; TD, nucleus tegmenti dorsalis; VT, ventriculus tecti mesencephali; P1, post-hatch day 1. Scale bars = 2.5 mm **(A,D,A',D')** and 1 mm **(B,C,B',C')**.

solute carrier family 6 member 4 (*Slc6a4*) gene (Hoffman et al., 1991; Lesch et al., 1993). The orthologs exhibited the following sequence similarities (protein and DNA) between chicks and humans: *TPH2*, 91.6% and 81.1%, respectively; *SERT*, 81.9% and 76.9%, respectively; *5-HTR1D*, 79.8% and 71.6%, respectively; *5-HTR1E*, 86.8% and 75.7%, respectively; *5-HTR1F*, 83.2% and 79.1%, respectively; *5-HTR2B*, 79.5% and 73.9%, respectively; *5-HTR5A*, 80% and 77.6%, respectively; and *5-HTR7*, 84.6% and 79.2%, respectively. We searched for sequence similarities of *5-HTR1A, 5-HTR1B, 5-HTR2A, 5-HTR2C, 5-HTR3A*, and *5-HTR4* with other animals in a previous study (Fujita et al., 2020). The characteristics of these ortholog gene probes are summarized in **Table 2**. We designed probes to detect multiple transcript variants of the orthologs registered in the database. We performed ISH and analyzed the expression patterns of orthologs in the brainstem of chicks.

## *TPH2* and *SERT* Expression in the Chick Brainstem

We performed an ISH analysis to reveal the expression patterns of the serotonergic neuron markers *TPH2* and *SERT* with neighbor sections around A2.4 to A 1.0 in the brainstems of naive chicks on post-hatch day 1 (P1). We detected cells showing strong signals of *TPH2* and *SERT* in the MR (B7 cell cluster) and B8 cell cluster (**Figures 1A,E,A',E'**), DR (B5 cell cluster), and MR (B6 cell cluster, **Figures 1B–D,F–H,B'–D',F'–H'**), and B2 cell clusters (**Figures 1C,D,G,H,C',D',G',H'**), respectively. Serotonergic neurons are widely distributed in the MR and DR

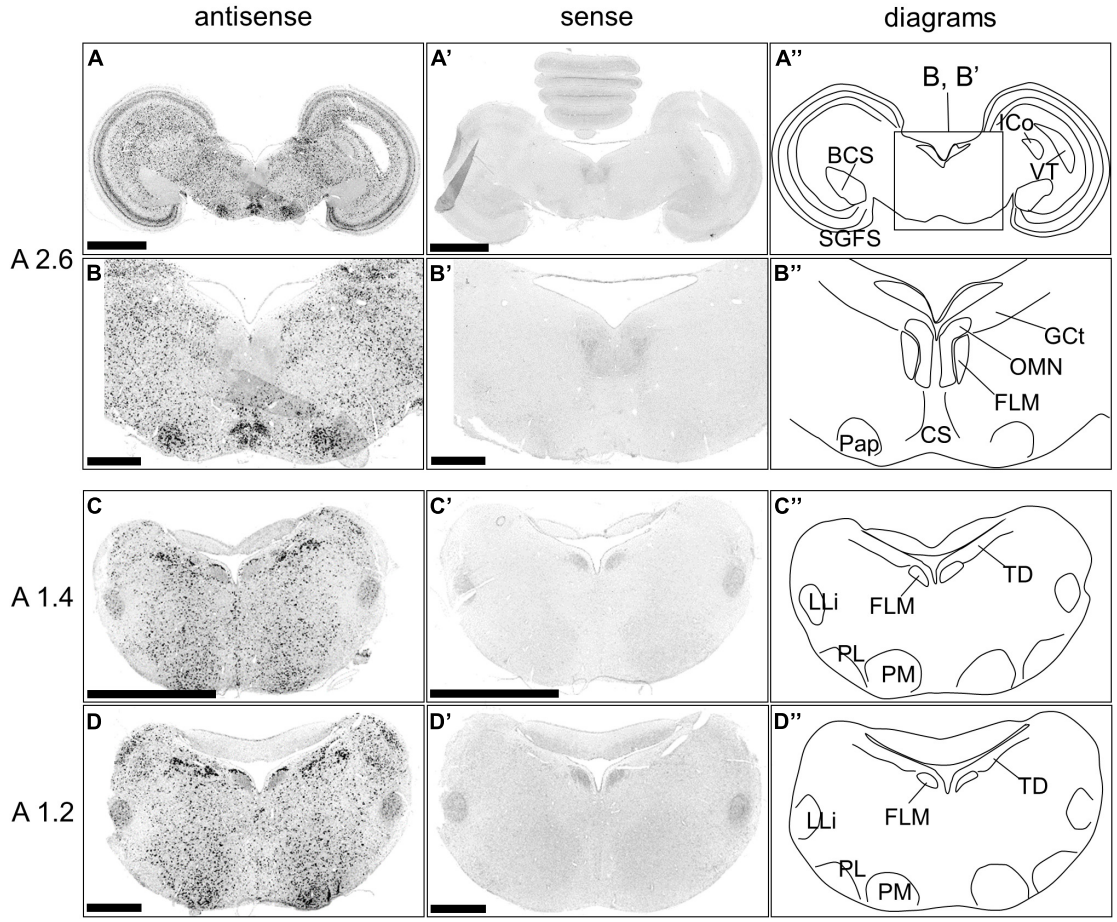


**FIGURE 3 |** *In situ* hybridization of *5-HTR1B* in the P1 chick brainstems. Digoxigenin-labeled RNA antisense **(A–D)** and sense **(A'–D')** *5-HTR1B* probes were used for *in situ* hybridization in coronal sections of P1 chick brains. To evaluate the expression patterns of *5-HTR1B*, sections of two chicks were analyzed, and representative images of chick brain sections are shown. **(B,B')** Magnified views of brain areas in the box in panel **(A")** are shown. **(A"–D")** Diagrams of coronal sections are shown in the rightmost panels. The levels of the sections (A 2.6, A 1.4, and A 1.2) are in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). BCS, brachium colliculi superiors; CS, nucleus centralis superior; FLM, fasciculus longitudinalis medialis; GCt, griseum centrale; ICo, nucleus intercolicularis; LLi, nucleus ventralis lemnisci lateralis; OMN, oculomotor nucleus; Pap, nucleus papillioformis; PL, nucleus pontis lateralis; PM, nucleus pontis medialis; SGFS, stratum griseum et fibrosum superficiale; SGP, stratum griseum periventriculare; TD, nucleus tegmenti dorsalis; VT, ventriculus tecti mesencephali; P1, post-hatch day 1. Scale bars = 2.5 mm **(A,C,A',C')** and 1 mm **(B,D,B',D')**.

(Dahlström and Fuxe, 1964; Soiza-Reilly and Gaspar, 2020). The distribution of serotonergic neurons in chick brainstems with *TPH2* and *SERT* signals was similar (Ikeda and Gotoh, 1971; Dube and Parent, 1981; Yamada et al., 1984), showing that these two chick ortholog genes are useful markers for visualizing serotonergic neurons in the avian brainstem.

## *5-HTR1A* Expression in the Chick Brainstem

We examined the expression pattern of 5-*HTR1A* in sections A2.6 to A1.4 in the P1 chick brainstems (**Figure 2**). Strong signals were detected in a layer of the stratum griseum centrale (SGC; **Figures 2A,A'**). We detected cells showing strong signals in the nucleus intercolicularis (ICo; **Figures 2A,A'**), nucleus centralis superior (CS), fasciculus longitudinalis medialis (FLM), oculomotor nucleus (OMN), nucleus papillioformis (Pap), and nucleus reticularis pontis oralis (RPO; **Figures 2B,B'**). In addition, we detected cells with strong signals in the B5 cell cluster and B6 cell cluster and cells with relatively weak signals in other areas (**Figures 2C,D,C',D'**).

## *5-HTR1B* Expression in the Chick Brainstem

We examined the expression pattern of 5-*HTR1B* in sections A2.6 to A1.2 in the P1 chick brainstems (**Figure 3**). Strong signals were detected in a wide area of the Optic tectum (OT), except for the brachium colliculi superiors, *5-HTR1A* positive layers, and ICo (**Figures 3A,A'**). We also detected strong signals in a wide area of the brainstem, including the Pap, except for the FLM and OLM (**Figures 3B,B'**). In addition, we detected cells with strong signals in a wide area of the brainstem, including the B5 cell cluster, B6 cell cluster, nucleus pontis medialis, and nucleus ventralis lemnisci lateralis (LLi), except for the FLM and nucleus pontis lateralis and around the LLi (**Figures 3C,D,C',D'**).

## Comparison of the *5-HTR1A* and *5-HTR1B* Expression Patterns in the OT

To compare the expression patterns of *5-HTR1A* and *5-HTR1B*, we show a magnified view of the OT (**Figure 4**). We detected strong *5-HTR1A* signals in the SGC (layer 13) and sparse signals in the stratum griseum et fibrosum superficiale (SGFS; layers 2, 3, 5, and 7). On the contrary, we detected strong *5-HTR1B* signals in the stratum album centrale (layer 14), stratum griseum periventriculare (layer 15), and SGFS (layers 4, 8, 10, 11, and 12). The expression patterns of *5-HTR1A* and *5-HTR1B* in the OT layers were mutually exclusive.

## *5-HTR1D* Expression in the Chick Brainstem

We examined the expression level of 5-*HTR1D* in sections A1.8 to A1.4 in the P1 chick brainstem (**Figure 5**). Strong signals were detected in the nucleus semilunaris (SLu; **Figures 5A,B,A',B'**). In addition, cells with strong signals were distributed in the DR (B5 cell cluster), and cells with relatively weak signals were distributed in the MR (B6 cell cluster; **Figures 5A–C,A'–C'**).

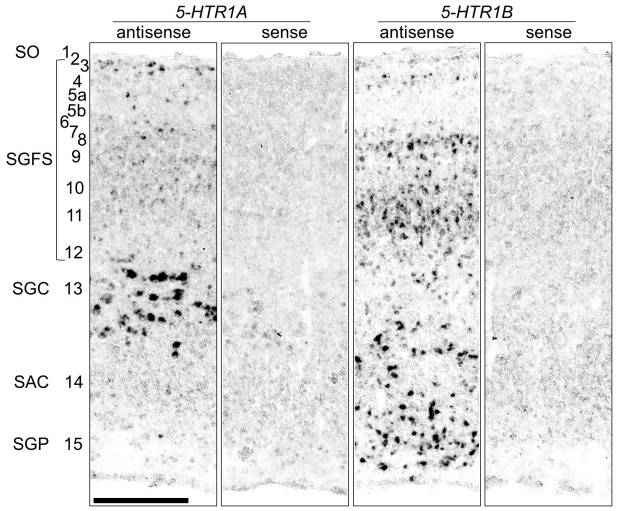


**FIGURE 4 |** Comparison of the *5-HTR1A* and *5-HTR1B* expression patterns in the optic tectum in the P1 chick brainstem sections. Magnified views of the regions of the optic tectum to compare layers of *5-HTR1A*-expressing and *5-HTR1B*-expressing areas. Numbers represent tectal layers according to Ramón y Cajal (1911). The leftmost alphabetical system of nomenclature is in accordance with that mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). SAC, stratum album centrale; SGC, stratum griseum centrale; SGFS, stratum griseum et fibrosum superficiale; SGP, stratum griseum periventriculare; SO, stratum opticum; P1, post-hatch day 1. Scale bars = 250 μm.

## *5-HTR1E* Expression in the Chick Brainstem

We examined the expression level of *5-HTR1E* in the sections of the brainstem and did not detect clear expression patterns (**Figures 6A,A',A"**). However, when we observed the sections in detail, we detected cells with relatively weak signals in a part of the DR (B5 cell cluster; **Figures 6B,B',B"**) and MR (B6 cell cluster; **Figures 6C,C',C"**), respectively.

## *5-HTR5A* Expression in the Chick Brainstem

We examined the expression level of *5-HTR5A* in the brainstem sections, and we did not detect clear expression patterns when the entire sections of brainstem were observed (**Figures 7A,A',A"**). When we observed the sections in detail, we detected cells with relatively weak signals in a part of the DR (B5 cell cluster; **Figures 7B,B',B"**) and MR (B6 cell cluster; **Figures 7C,C',C"**).

## *5-HTR1F, 5-HTR2A, 5-HTR2B, 5-HTR2C, 5-HTR3A, 5-HTR4*, and *5-HTR7* Expression Patterns in the Chick Brainstem

We examined *5-HTR1F, 5-HTR2A, 5-HTR2B, 5-HTR2C, 5-HTR3A, 5-HTR4,* and *5-HTR7* expression levels in sections around A2.4 to A0.8 in the P1 chick brainstem and did not detect any signal, suggesting that the expression levels of the *5-HTR* genes were very low or the cells expressing the *5-HTR*

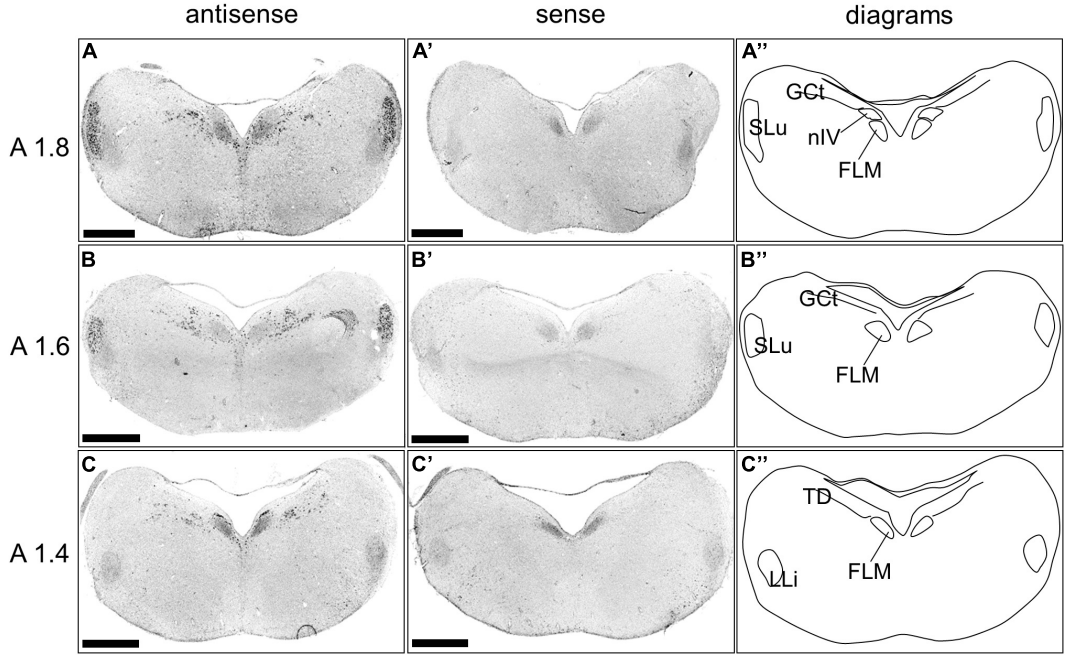


**FIGURE 5 |** *In situ* hybridization of *5-HTR1D* in the P1 chick brainstems. Digoxigenin-labeled RNA antisense **(A–C)** and sense **(A'–C')** *5-HTR1D* probes were used for *in situ* hybridization in coronal sections of P1 chick brains. To evaluate the expression patterns of *5-HTR1D*, sections of five chicks were analyzed, and representative images of two chick brain sections are shown. **(A"–C")** Diagrams of coronal sections are shown in the rightmost panels. The levels of the sections (A1.8, A1.6, and A1.4) are in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). FLM, fasciculus longitudinalis medialis; GCt, griseum centrale; LLi, nucleus ventralis lemnisci lateralis; nIV, nucleus nervi trochlearis; SLu, nucleus semilunaris; TD, nucleus tegmenti dorsalis; P1, post-hatch day 1. Scale bars = 1 mm.

genes were very rare. In mammals, the 5-HT2B, 2C or 5-HT7 receptors can control the activity of raphe neurons (Beyeler et al., 2021) but their expression level is very low (Huang K. W. et al., 2019; Okaty et al., 2019; Ren et al., 2019). This is consistent with our results that the expression level of the 5-HT2B, 2C or 5-HT7 receptors was too low to detect by our *in situ* hybridization system in this study.

## Double *in situ* Hybridization Analysis of the 5-Hydroxytryptamine Receptor and Serotonergic Neuron Marker Genes in the Chick Brainstem

We found that five 5-HTRs (*5-HTR1A, 5-HTR1B, 5-HTR1D, 5-HTR1E*, and *5-HTR5A*) were expressed in the DR (B5 cell cluster) and MR (B6 cell cluster), as mentioned above. Subsequently, we examined whether the *5-HTR*-expressing cells were serotonergic neurons. We performed a double ISH analysis to reveal that the *5-HTR*-expressing cells expressed a serotonergic neuron marker gene, *TPH2*, at the section level around A1.6 to A1.4 for the DR (B5 cell cluster) and A1.6 to A 1.2 for the MR (B6 cell cluster; **Figures 8**, **9**). We detected double positive cells of *5-HTR1A* and *TPH2*, *5-HTR1B* and *TPH2*, and *5-HTR1D* and *TPH2*, and a small number of double positive cells of *5-HTR1E* and *TPH2;* and *5-HTR5A* and *TPH2* in the DR (B5 cell cluster; **Figures 8A–E,A'–E'**). In the B6 cell cluster of the MR, we detected double positive cells of *5-HTR1A* and *TPH2*, *5-HTR1B* and *TPH2*, *5-HTR1E* and *TPH2*. We also detected double-positive cells of *5-HTR1D* and in a part of the MR (B6 cell cluster), and weakly double-positive cells of *5-HTR5A* and *TPH2* in a part of the MR (B6 cell cluster; **Figures 9A–E,A'–E'**).

# DISCUSSION

## Distribution of Serotonergic Neurons in the Brainstem of Chicks

In the present study, we described the distribution of serotonergic neurons in the brainstem of chicks, which expressed the chick orthologs of mammalian serotonergic neuronal marker genes *TPH2* and *SERT*, including the chick DR and MR (**Figure 1**). The expression patterns of *TPH2* and *SERT* in the avian brainstem appears to correspond the distribution of serotonergic neurons in chick brainstems (Ikeda and Gotoh, 1971; Dube and Parent, 1981; Yamada et al., 1984), showing that these two chick ortholog genes are useful markers for visualizing serotonergic neurons in the avian brainstem. Our ISH analysis shows that serotonergic neurons are distributed laterally in the avian brainstem, especially in the DR, which is consistent with the finding of previous studies (Ikeda and Gotoh, 1971; Dube and Parent, 1981; Yamada et al., 1984; Wallace, 1985; Sako et al., 1986; Okado et al., 1992; Huang X. et al., 2019). In

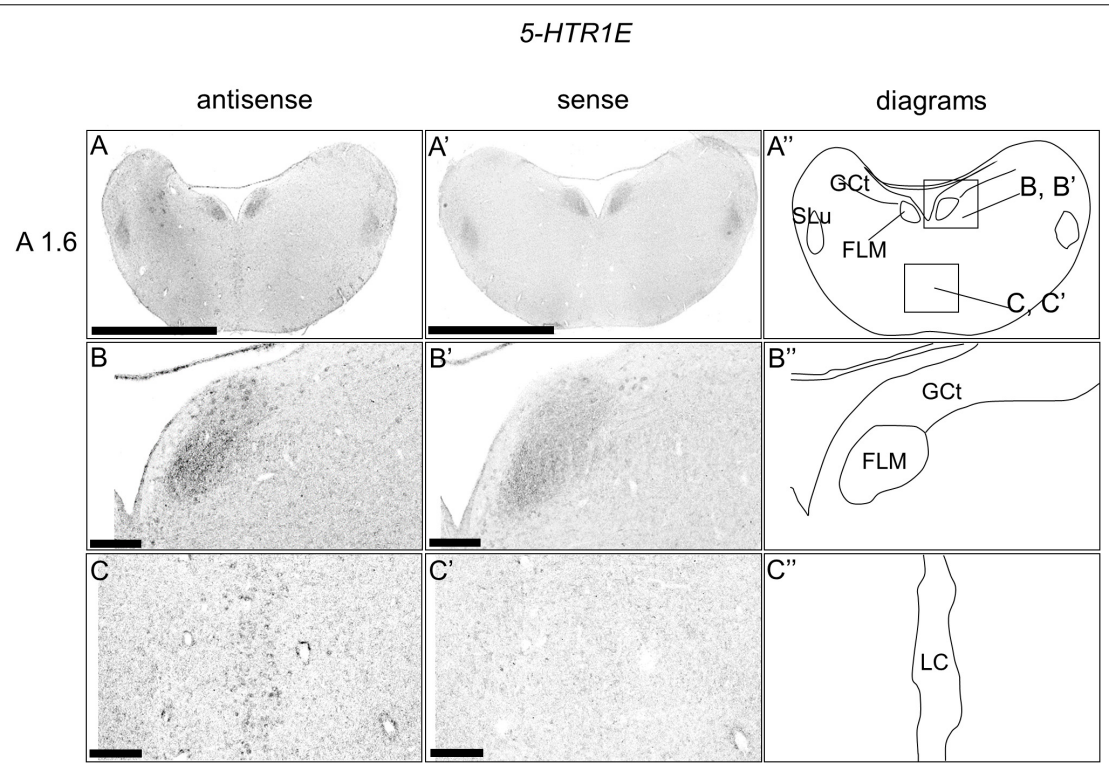


**FIGURE 6 |** *In situ* hybridization of *5-HTR1E* in the P1 chick brainstems. Digoxigenin-labeled RNA antisense **(A–C)** and sense **(A'–C')** *5-HTR1E* probes were used for *in situ* hybridization in coronal sections of P1 chick brains. To evaluate the expression patterns of *5-HTR1E*, sections of four chicks were analyzed, and the representative levels of sections (A 1.6) are shown. **(A"–C")** Diagrams of coronal sections shown in the rightmost panels. The levels of the sections (A 1.6) is in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). **(B–C,B'–C')** Magnified views of brain areas shown in the boxes in panel **(A")**. FLM, fasciculus longitudinalis medialis; GCt, griseum centrale; MR, median raphe; SLu, nucleus semilunaris; P1, post-hatch day 1. Scale bars = 2.5 mm **(A,A')** and 250 μm **(B–C,B'–C')**.

previous studies, chick serotonergic neurons in the brainstem were detected using histochemical (Ikeda and Gotoh, 1971; Dube and Parent, 1981) or immunohistochemical methods with anti-5-HT antiserum (Yamada et al., 1984); however, the authors did not clearly detect the distribution of serotonergic neuronal cell bodies alone because of the limitations of their methods. Generally, cell bodies can be clearly detected using ISH. In the future, a good understanding of the strict correspondence between the raphe nuclei of birds and mammals should be elucidated by a comprehensive analysis of the expression patterns of *TPH2* and *SERT* in the bird brainstem.

## Heterogeneity of Chick Serotonergic Neurons in the Chick Brainstem

We also found that the expression patterns of *5-HTR1A, 5-HTR1B, 5-HTR1D, 5-HTR1E,* and *5-HTR5A* were different but overlapped partially in the DR and MR of the chick brainstem (**Figure 10**). Cells expressing *5-HTR1A* and/or *5-HTR1B* were widely distributed in the DR; however, the number of cells that expressed *5-HTR1D* or *5-HTR5A* in the DR was limited. Cells expressing *5-HTR1E* were not detected in the DR, except in the dorsal region of the FLM. In the MR, cells expressing *5-HTR1A* and/or *5-HTR1B* and/or *5-HTR1D* were widely distributed but those expressing *5-HTR1E* or *5-HTR5A* were limited. Moreover, cells expressing 5-HTRs also expressed the serotonergic neuronal marker gene, *TPH2*, at least partially (**Figures 8**, **9**). Taken together, the serotonergic neurons in the chick DR and MR vary in their expression pattern of 5-HTRs, suggesting a heterogeneity of serotonergic neurons among and within the DR and MR in the chick brainstem. On the contrary, in mammals, single-cell RNA sequencing studies recently revealed that *5-Htr1a 5-Htr1b*, and *5-Htr1d* are widely expressed in the DR and MR of the mouse brainstem; however, *5-Htr5b* is expressed in limited clusters of the DR and MR. Given the absence of *5-Htr1e* in the mouse genome and the absence of *5-HTR5b* in the chicken genome (International Chicken Genome Sequencing Consortium, 2004; Vilaró et al., 2020), it seems likely that the *5-HTR* subtypes expressed in the DR and MR serotonergic neurons are similar in chicks and mice. Collectively, we assume that the molecular properties of serotonergic neurons present in the DR and MR of birds and mammals are similar in terms of *5-HTR* subtypes, raising the possibility that with a few exceptions, the heterogeneity of serotonergic neurons is evolutionarily conserved. Our assumption is consistent with the previous reports about the development of the brainstem and the pattern of gene expression in chicks, which resembles that of mice, even

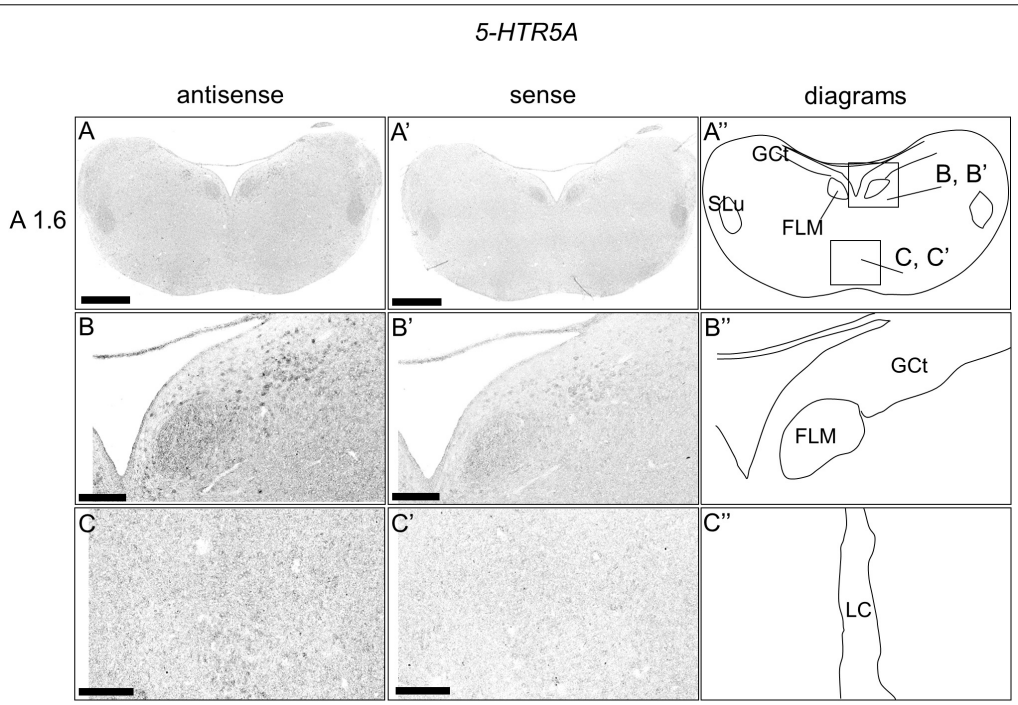


**FIGURE 7 |** *In situ* hybridization of *5-HTR5A* in the P1 chick brainstems. Digoxigenin-labeled RNA antisense **(A–C)** and sense **(A'–C')** *5-HTR5A* probes were used for *in situ* hybridization in coronal sections of P1 chick brains. To evaluate the expression patterns of *5-HTR5A*, sections of four chicks were analyzed, and the representative levels of sections (A1.6) are shown. **(A''–C'')** Diagrams of coronal sections shown in the rightmost panels. The levels of the sections (A 1.6) is in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). **(B–C,B'–C')** Magnified views of brain areas shown in the boxes in panel **(A'')**. FLM, fasciculus longitudinalis medialis; GCt, griseum centrale; MR, median raphe; SLu, nucleus semilunaris; P1, post-hatch day. Scale bars = 1 mm **(A,A')** and 250 μm **(B–C,B'–C')**.

though the species are separated by approximately 300 million years of evolution (Cambronero and Puelles, 2000; Alonso et al., 2013; Watson et al., 2019).

## Expression Patterns of *5-HTR1A* and *5-HTR1B* in the OT Layers

During the process of detecting the expression of 5-*HTR*s in raphe, we happened to find that the expression patterns of *5-HTR1A* and *5-HTR1B* in the OT layers were mutually exclusive (**Figures 2A,A'**, **3A,A'**, **4**). The avian brain has extensively developed optic lobes and a high degree of lamination. The OT is a component of the tectofugal pathway, comprising the retina, OT, nucleus rotundus, and entopallium, which is thought to be the most important visual pathway in birds (Wylie et al., 2009). In the OT, there are 15 recognizable layers and a strict separation of visual input and output; generally, the input from the retina terminates in the superficial layer, while the major output from the OT to the nucleus rotundus is derived from the projection neurons in the deep layer, especially layer 13 (SGC) (Ramón y Cajal, 1911; Luksch, 2003). The cells expressing *5-HTR1A* were distributed mainly in layer 13 and sparsely in layers 2, 3, and 5 (**Figure 4**). Conversely, cells expressing *5-HTR1B* were not distributed in layer 13 and existed in layers 4, 8, 10, 11, 12, 14, and 15 (**Figure 4**). However, a previous study using radioligand autoradiography revealed that the density of 5-HTR1A densities was the strongest in layer 5 and decreased stepwise until layer 14 in the OT of pigeons (Herold et al., 2012). The difference in the 5-HTR1A signal density among different layers may reflect the density of the axonal terminals of 5-HT neurons projecting to the OT. Moreover, it is known that serotonergic neuron projections to the OT are widely distributed (Yamada and Sano, 1985). Collectively, *5-HTR1A* and *5-HTR1B*, which have characteristic expression patterns in the OT, might play important roles in the modulation of neurons, including visual processing in the tectofugal pathway. Note that we did not perform a comprehensive search for the expression of 5-HTR subtypes in the avian OT. In this study, we focused on the expression of *5-HTR*s in raphe rather than in the OT. In fact, a previous study that used immunohistochemistry reported that 5-HTR2A was strongly expressed in layer 13 cells and was widely detected in the OT (Metzger et al., 2006). This must be addressed in the future through a comprehensive search for the expression of 5-HTR subtypes in the avian OT.

## Possible Functions of Chick 5-Hydroxytryptamine Receptors in Serotonergic Neurons

As for the expression pattern of *5-HTR1A*, cells expressing *5-HTR1A* were distributed densely in the DR and MR and sparsely in the FLM, OMN, Pap, and RPO of the chick brainstem. We

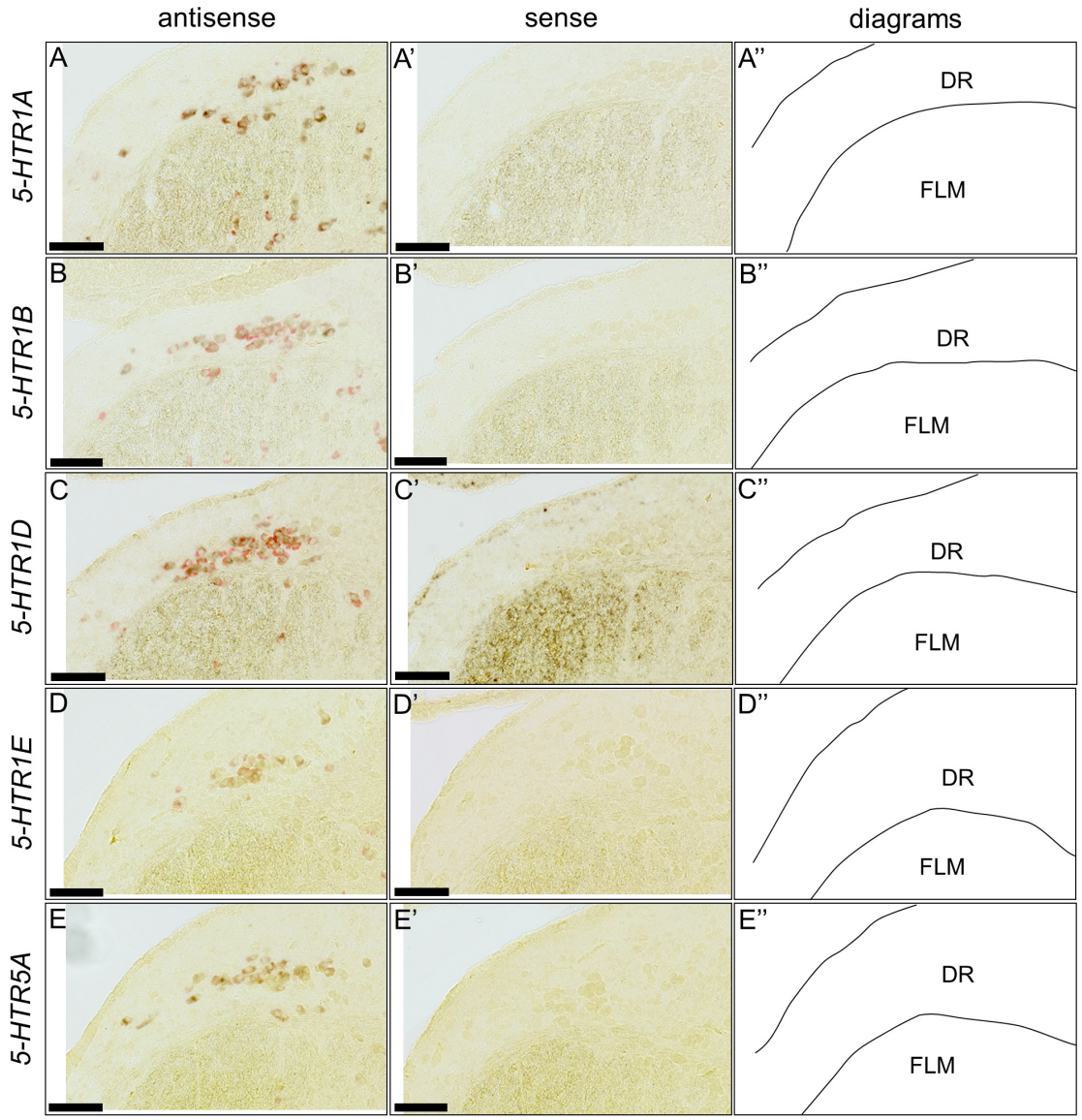


**FIGURE 8 |** Double *in situ* hybridization of *5-HTRs* and *TPH2* in the DR (B5) of the P1 chick brainstems. Digoxigenin-labeled (*5-HTRs*) and fluorescein-labeled *TPH2* RNA antisense **(A–E)** and sense **(A'–E')** probes were used for double *in situ* hybridization in coronal sections of P1 chick brains. **(A"–E")** Diagrams of coronal sections are shown in the rightmost panels. DR, dorsal raphe; FLM, fasciculus longitudinalis medialis; P1, post-hatch day 1. Scale bars = 100 μm.

found that the distribution pattern of cells expressing *5-HTR1A* seemed to represent the overall distribution of serotonergic neurons, suggesting that *5-HTR1A* is expressed in most of the serotonergic neurons in the bird raphe. This is consistent with the findings of previous reports on the raphe in rodents, which showed that *5-Htr1A* is expressed in all serotonergic neurons (Day et al., 2004; Bonnavion et al., 2010; Vilaró et al., 2020). In mammals, 5-Htr1A is the major receptor involved in feedback inhibition of serotonergic neurons *via* direct and indirect (multisynaptic) feedback inhibition (Commons, 2020). The function of 5-HTR1A in serotonergic neuron feedback systems may be evolutionarily conserved in birds and mammals.

As for the expression pattern of *5-HTR1B*, cells expressing *5-HTR1B* were distributed widely in the brainstem of chicks, including the DR and MR, except for the FLM and OLM. Consistent with our results regarding the expression pattern of *5-HTR1B* in the brainstem of chicks, previous studies using mammals revealed that *5-Htr1B* is expressed widely, including

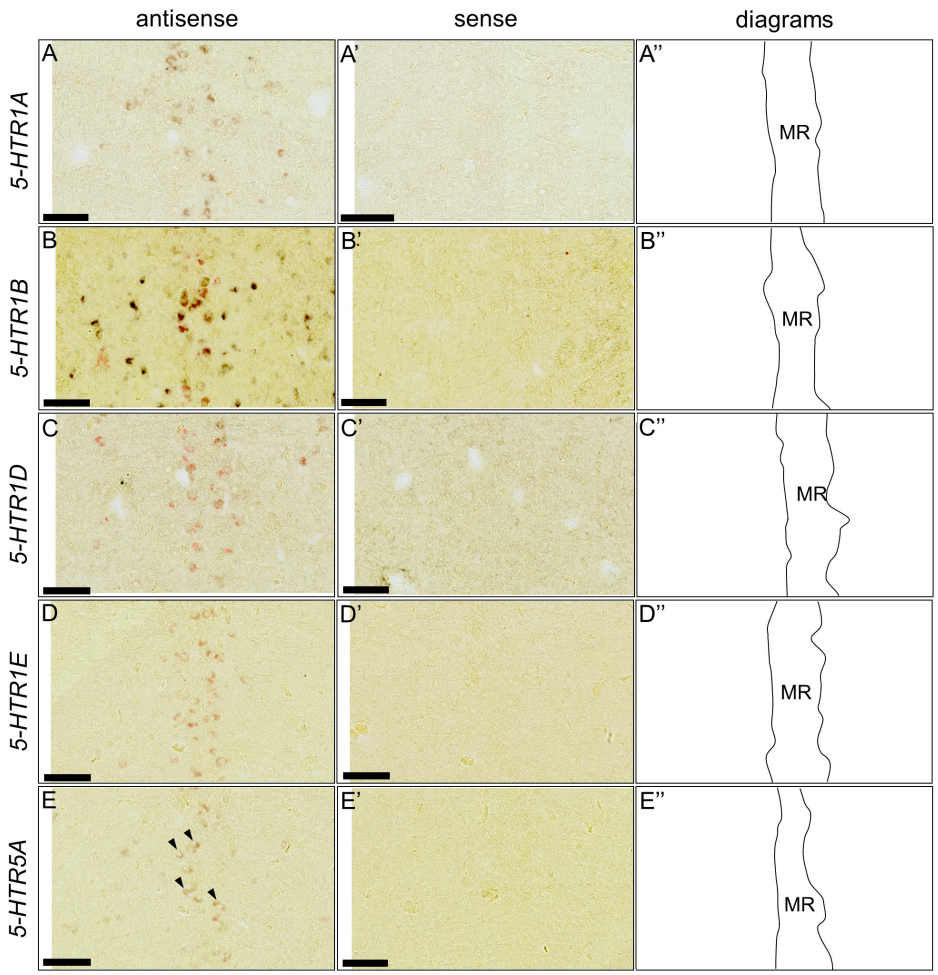


**FIGURE 9 |** Double *in situ* hybridization of *5-HTRs* and *TPH2* in the MR (B6) of the P1 chick brainstems. Digoxigenin-labeled (*5-HTRs*) and fluorescein-labeled *TPH2* RNA antisense **(A–E)** and sense **(A'–E')** probes were used for double *in situ* hybridization in coronal sections of P1 chick brains. **(A"–E")** Diagrams of coronal sections are shown in the rightmost panels. Arrowheads indicate signals. MR, median raphe; P1, post-hatch day 1. Scale bars = 100 μm.

the raphe (Bruinvels et al., 1994; Varnäs et al., 2005). In mammals, the localization of the 5-Htr1B receptor protein does not frequently correspond to that of the mRNA in the brain areas, suggesting that 5-Htr1B also functions as presynaptic auto- or heteroreceptors and modulates the release of various neurotransmitters (Vilaró et al., 2020). The presynaptic feedback mechanisms mediated by 5-Htr1B, located on serotonergic boutons, are expected to finely regulate local axonal excitation in a synapse-specific manner (Commons, 2020). Considering the similarity of expression patterns between the raphe of birds and mammals, we assumed that the modulatory function of 5-HTR1B in serotonergic neurons may be conserved among birds and mammals.

*5-HTR1D* was densely expressed in the SLu, which is one of the isthmic nuclei in birds and has reciprocal connections with the ipsilateral OT (Luksch, 2003). It is possible that *5-HTR1D* is also related to the regulation of visual function in birds. We also found that *5-HTR1D* was expressed in serotonergic neurons in the DR and MR of chicks. In terms of the mode of action in mammals, *5-Htr1D* is considered to act as an auto- or heteroreceptor and modulate the release of various neurotransmitters, since protein and mRNA localization does not correspond in some brain regions (Bruinvels et al., 1994). Bird *5-HTR1D* may act as an auto- or heteroreceptor for serotonergic neurons and might be involved in the regulation of the serotonergic system in birds.

As for the expression pattern of *5-HTR1E*, cells expressing *5-HTR1E* were serotonergic neurons, and they were distributed in the DR and MR. In mammals, the distribution of *5-ht1e* has been described in guinea pigs, monkeys, and the human brain (Bruinvels et al., 1994; Mengod et al., 2006) and by using antibodies against the 5-ht1e receptor in guinea pigs (Klein and Teitler, 2012). However, previous studies have not clearly

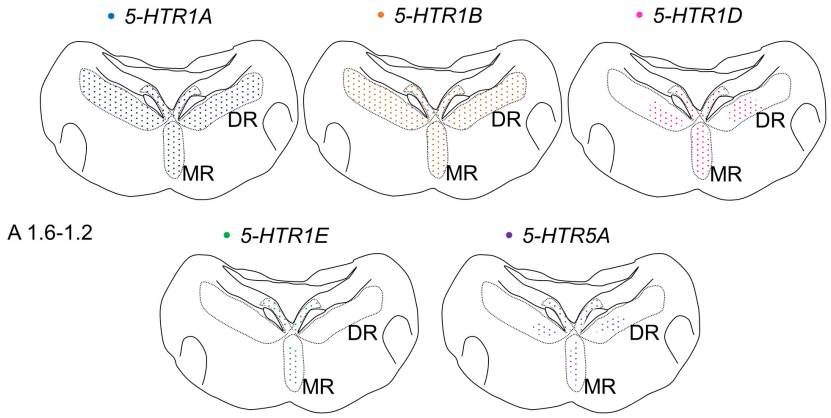


**FIGURE 10 |** Schematic summary of the expression patterns of *5-HTR1A, 5-HTR1B, 5-HTR1D, 5-HTR1E*, and *5-HTR5A* in the DR and MR in the P1 chick brainstems. Representative expression patterns in sections around A 1.6. A 1.2 are exhibited in colored areas (blue, *5-HTR1A*; orange, *5-HTR1B*; magenta, *5-HTR1D*; green, *5-HTR1E*; purple, *5-HTR5A*). The dotted pattern indicates that the expressed cells are sparsely distributed. The levels of the sections are in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). DR, dorsal raphe; MR, median raphe; P1, post-hatch day 1.

demonstrated the expression of *5-ht1e* in the raphe nuclei. Since the *5-Htr1E* gene is absent in the genomes of both rats and mice, there is no previous study on the distribution of *5-Htr1E* in these animals even though they are the most common model animals. Here, we clearly showed that *5-Htr1E* was expressed in serotonergic neurons in the DR and MR in the chick brainstem. In the serotonergic neurons of the DR and MR in birds, *5-HTR1E* can be involved in the regulatory function of the serotonergic system.

Further, cells expressing *5-HTR5A* were distributed in the DR and MR and co-expressed the serotonergic neuron marker *TPH2*. The function of *5-HTR5A* has not been well characterized, even in mammals (Vilaró et al., 2020); however, there are a few studies on the distribution of *5-HTR5A* in mammalian brains (Plassat et al., 1992; Erlander et al., 1993; Matthes et al., 1993; Kinsey et al., 2001; Mengod et al., 2006). The distribution patterns suggested that *5-HTR5A* might be involved in the regulation of cognition, anxiety, and sensory perception. In addition, immunohistochemistry showed that *5-HTR5A* is localized in serotonergic neurons in the raphe nuclei of the rat brain (Oliver et al., 2000), suggesting that *5-Htr5A* might be an auto- or heteroreceptor that responds to a neurotransmitter released from the serotonergic neurons. Considering the similarity of expression patterns in raphe nuclei among birds and mammals, we assume that *5-HTR5A* might have a conserved modulatory function as an auto- or heteroreceptor in serotonergic neurons of birds and mammals.

## CONCLUSION

We have comprehensively described the serotonergic neurons in the DR and MR of the chick brainstem and found that *5-HTR1A, 5-HTR1B, 5-HTR1D, 5-HTR1E,* and *5-HTR5A* were expressed in serotonergic neurons. These receptors may be involved in the regulation of bird serotonergic neuron activity to control the serotonergic system. In addition, we found that *5-HTR1A* and *5-HTR1B* were expressed in some layers of the OT in a mutually exclusive manner. Our findings can be used as a basis for understanding the serotonergic modulation of serotonergic neurons themselves and the visual pathway in birds.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on Animal Experiments of Teikyo University.

## AUTHOR CONTRIBUTIONS

TF and SY designed the study and performed the research. TF, NA, CM, EF, KH, and SY analyzed the data. TF, NA, CM, TM, KH, and SY wrote the manuscript. All authors have reviewed the manuscript and contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.815997/full#supplementary-material

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# Behavioral and Evolutionary Perspectives on Visual Lateralization in Mating Birds: A Short Systematic Review

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The division of cognitive processing between the two hemispheres of the brain causes lateralized eye use in various behavioral contexts. Generally, visual lateralization is shared among vertebrates to a greater extent, with little interspecific variation. However, previous studies on the visual lateralization in mating birds have shown surprising heterogeneity. Therefore, this systematic review paper summarized and analyzed them using phylogenetic comparative methods. The review aimed to elucidate why some species used their left eye and others their right to fixate on individuals of the opposite sex, such as mating partners or prospective mates. It was found that passerine and non-passerine species showed opposite eye use for mating, which could have stemmed from the difference in altricial vs. precocial development. However, due to the limited availability of species data, it was impossible to determine whether the passerine group or altricial development was the primary factor. Additionally, unclear visual lateralization was found when studies looked at lek mating species and males who performed courtship. These findings are discussed from both evolutionary and behavioral perspectives. Possible directions for future research have been suggested.

**Keywords: mating, social cognition, visual lateralization, evolution, sexual selection, courtship**

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## INTRODUCTION

Neural mechanisms controlling social cognition and sexual interactions in birds cannot be fully understood without knowledge of asymmetries in hemispheric functions. Because avian brain has an asymmetrical structure and lacks major interhemispheric commissure, lateralization of cognitive processing can be seen in every aspect of life of birds (Rogers, 2012), such as sleeping during flight (Rattenborg, 2017), foraging (Alonso, 1998), and social interactions (Vallortigara and Andrew, 1991). This means that looking into lateralized visual behaviors can potentially elucidate both behavioral evolution and its underlying physiological mechanisms.

Lateralized brain function, evident in visual information processing, can have a great impact on how individuals behave in particular situations (Bisazza et al., 1998; Vallortigara et al., 1999; Wiper, 2017; Güntürkün et al., 2020). However, its adaptive significance is still debated (Ghirlanda and Vallortigara, 2004; Rogers et al., 2004; Vallortigara, 2006; Corballis, 2009). Thus far, accumulated evidence has indicated that birds, like many other vertebrates, showed visual lateralization, with biased eye use for particular behavioral tasks or differential behavioral performance based on which eye was used (Vallortigara et al., 1999; Rogers, 2012; Manns and Ströckens, 2014; Wiper, 2017).

Specifically, it is well-known that birds generally use the left eye-right hemisphere system for anti-predator search and the right eye-left hemisphere system for foraging, which is consistent with other vertebrates (e.g. Mench and Andrew, 1986; Alonso, 1998; Rogers et al., 2004, 2018). This gives an impression that the pattern of visual lateralization is evolutionarily conserved among avian species.

However, if we focus on the mating context, such as courting, copulation, and pair-bonding interactions between sexes, there is a striking heterogeneity in the previous findings. Some studies reported on right-eye dominance, others on left-eye dominance, or even the lack of clear lateralization (**Table 1**). For example, males of the black-winged stilt (*Himantopus himantopus*) tend to use their left eye to see a potential mate when performing courtship display (Ventolini et al., 2005), whereas zebra and gouldian finches (*Teniogygia guttata* and *Erythrura gouldiae*) males rely more on their right eye and fail to discriminate appropriate female partners when it is covered with an eyepatch (Templeton et al., 2012, 2014). In contrast, in the greater sage-grouse (*Centrocercus urophasianus*), it is less evident whether the left or right eye is predominantly used by males performing courtship (Krakauer et al., 2016). Such mixed results could partially be due to the differences in experimental designs or specific behavioral contexts. Leliveld et al. (2013) indicated in their review that vertebrates share more or less similar lateralization patterns in emotional processing, including those for inter-sexual interactions, and that right hemisphere generally controls copulation. Based on that, it is suggested that switching from left to right hemisphere would occur in association with the transition from courtship to copulation, as inhibition of the right hemisphere by the left hemisphere takes place during courtship but not copulation (Rogers, 2012; Rogers and Kaplan, 2019). If so, dominant eye is expected to be reversed between courtship and copulation phases. In addition, there could be among-species variations in visual information processing. It should also be noted that previous results might have been overlooked or confounded by sex differences in visual lateralization, if any, as most of them looked at only the male sex (**Table 1**).

To explain the above interspecific variations, a number of ultimate and proximate factors can be applied. First, phylogenetic relatedness itself is a crucial predictor of the presence/absence or direction of visual lateralization in mating contexts (cf. Vallortigara et al., 1999; Brown and Magat, 2011). Given that hemispheric asymmetries are widely conserved among animal species (Güntürkün et al., 2020), it is plausible that evolutionary constraints limit their development to an extent. Second, sexual selection, another ultimate factor, may have an influence. In general, the evolution of sexual signals is inseparable from the sensory systems that process the signals in each species (e.g. Lind and Delhey, 2015; Hiyama et al., 2018; Heffner et al., 2020). If visual lateralization has an advantage in recognizing "good" mates, as shown for foraging tasks (Güntürkün et al., 2000), those species experiencing intense sexual selection (e.g. polygyny) may exhibit strong lateralization, especially in females. Lastly, as a proximate factor, the diversity in the developmental mode, known as precocial-altricial spectrum, can play a role. Due to the asymmetrically turned head position in the egg, the left and right eyes of chicken embryos experience imbalanced light exposure, which is known to determine the ontogeny of lateralized visual behaviors (reviewed in Rogers, 2012; Güntürkün and Ocklenburg, 2017). Similarly, ontogenetic light experience is important in pigeons, although in a different way from that in chickens, which is argued to be caused by differences in altricial (pigeon) and precocial (chicken) neural development (Rogers, 2012; Manns and Ströckens, 2014) (see also Templeton and Gonzalez, 2004).

This review aimed to offer new perspectives by synthesizing past research findings on visual lateralization in mating contexts of a variety of bird species by relying on quantitative interspecific comparative approaches called phylogenetic comparative methods (PCMs) (Garamszegi, 2014; Cornwell and Nakagawa, 2017). Specifically, this approach can allow the estimation of the strength of phylogenetic constraints (i.e., phylogenetic signal) for visual lateralization, the tendency that closely related species resemble each other (Münkemüller et al., 2012). More importantly, the effects of the aforementioned proximate and ultimate factors (mating system and developmental mode) can be statistically evaluated using phylogenetic regression models. Additionally, it was predicted that the methodology (e.g., focal behavioral contexts and the use of an eyepatch) may be partially accountable for the heterogeneity in previous findings.

# METHODS

## Literature Data

Data were collected from published research literatures that reported on visual lateralization in the contexts of courting, mate assessment, copulation and pair-formation or -bonding. A search was conducted on Google Scholar using the keyword search bar and terms such as "laterality" "mating" "courtship" and "sexual." Cross-reference searches were conducted by checking all the cited literature of the papers included. **Table 1** presents the data collected from the literature.

Based on the results from each study, the presence of visual lateralization and its direction at both the specific behavioral context level ($n = 23$; **Table 1**) and the species level ($n = 11$; **Figure 1A**) was determined. Whether one study looked at multiple behavioral contexts, or multiple studies looked at the same species, there was no contradiction regarding the direction of laterality, except for the reports on domestic chickens (Rogers et al., 1985; Workman and Andrew, 1986; Bullock and Rogers, 1992); more repeatable findings that contained species' laterality data were prioritized (**Table 1**; **Figure 1A**).

## Phylogeny

For the phylogenetic comparative analyses below, multiple candidate trees were obtained from the Global Phylogeny of Birds database (Jetz et al., 2012). In particular, 1,000 trees were downloaded, the analyses was repeated for each tree, and the model-averaged outcomes were obtained, which have been commonly conducted in recent studies with PCMs (for details see Garamszegi and Mundry, 2014).

**TABLE 1 |** Summary of the previous findings of visual lateralization in mating birds.

| Species | Eye | | Behavioral context | Eyepatch | Focal sex | Sex effect | |
|---|---|---|---|---|---|---|---|
| **Non-passerine** | | | | | | | |
| White-fronted geese (*Anser albifrons*) | **L** | V | following partner | - | MF | Not tested | [d] |
| Barnacle geese (*Branta leucopsis*) | **L** | V | following partner | - | MF | Not tested | [d] |
| Indian peafowl (*Pavo cristatus*) | Unclear[a] | C | wing shaking (courtship display) | - | M | - | [e] |
| | Unclear[a] | C | train rattling (courtship display) | - | M | - | |
| Domestic chicken (*Gallus gallus domesticus*) | **R** | V | fixating a model (block)[c] | - | M | - | [f] |
| | **L** | V | fixating a conspecific (male)[c] | - | M | - | |
| | **L** | M | copulation (toward human hand)[c] | ✓ | M | - | [g] |
| | **L** | M | copulation (toward human hand)[c] | ✓ | M | - | [h] |
| Japanese quail (*Coturnix coturnix japonica*) | **L** | V | approaching opposite sex | ✓ | MF | No | [i] |
| Wild turkey (*Meleagris gallopavo*) | NS | C | strutting (courtship display) | ✓ | M | - | [j] |
| Greater sage-grouse (*Centrocercus urophasianus*) | Unclear[b] | C | strutting (courtship display) | - | M | - | [k] |
| Black-winged stilt (*Himantopus himantopus*) | **L** | C | bill shaking (courtship display) | - | M | - | [l] |
| | NS | C | preening (courtship display) | - | M | - | |
| | NS | C | time spent for courtship | - | M | - | |
| | **L** | M | mounting | - | M | - | |
| | **L** | M | time spent for copulation | - | M | - | |
| **Passerine** | | | | | | | |
| Gouldian finch (*Erythrura gouldiae*) | **R** | V | viewing potential mates | ✓ | M | - | [m] |
| | **R** | C | singing | ✓ | M | - | |
| Zebra finch (*Taeniopygia guttata*) | **R** | V | fixating a female | - | M | - | [f] |
| | NS | V | orienting toward potential mates | - | MF | No | [n] |
| | **R** | C | singing frequency | ✓ | M | - | [o] |
| | **R** | C | singing time | ✓ | M | - | [p] |
| Java sparrow (*Lonchura oryzivora*) | **R** | C | courtship dancing | - | MF | No | [q] |

[a] *Inferred based on relative male-female positioning reported.*
[b] *Left-eye for frontal view and right-eye for lateral view.*
[c] *Hormone-induced sexual behaviors in young males.*
[d] *Zaynagutdinova et al. (2021).*
[e] *Dakin and Montgomerie (2009).*
[f] *Workman and Andrew (1986).*
[g] *Rogers et al. (1985).*
[h] *Bullock and Rogers (1992).*
[i] *Gülbetekin et al. (2007).*
[j] *Vernier (2016).*
[k] *Krakauer et al. (2016).*
[l] *Ventolini et al. (2005).*
[m] *Templeton et al. (2012).*
[n] *ten Cate et al. (1990).*
[o] *George et al. (2006).*
[p] *Templeton et al. (2014).*
[q] *Endo (2018).*

*Left (L) or right (R) eye bias is shown with corresponding behavioral context (C, courting, including performing courtship display or singing; M, mounting or copulation; V, viewing, or fixating a potential mate/pairing partner outside the courtship or copulation phase). The use of an eyepatch and the focal sex (M, male; F, female) are also indicated. NS indicates statistically non-significant eye bias.*

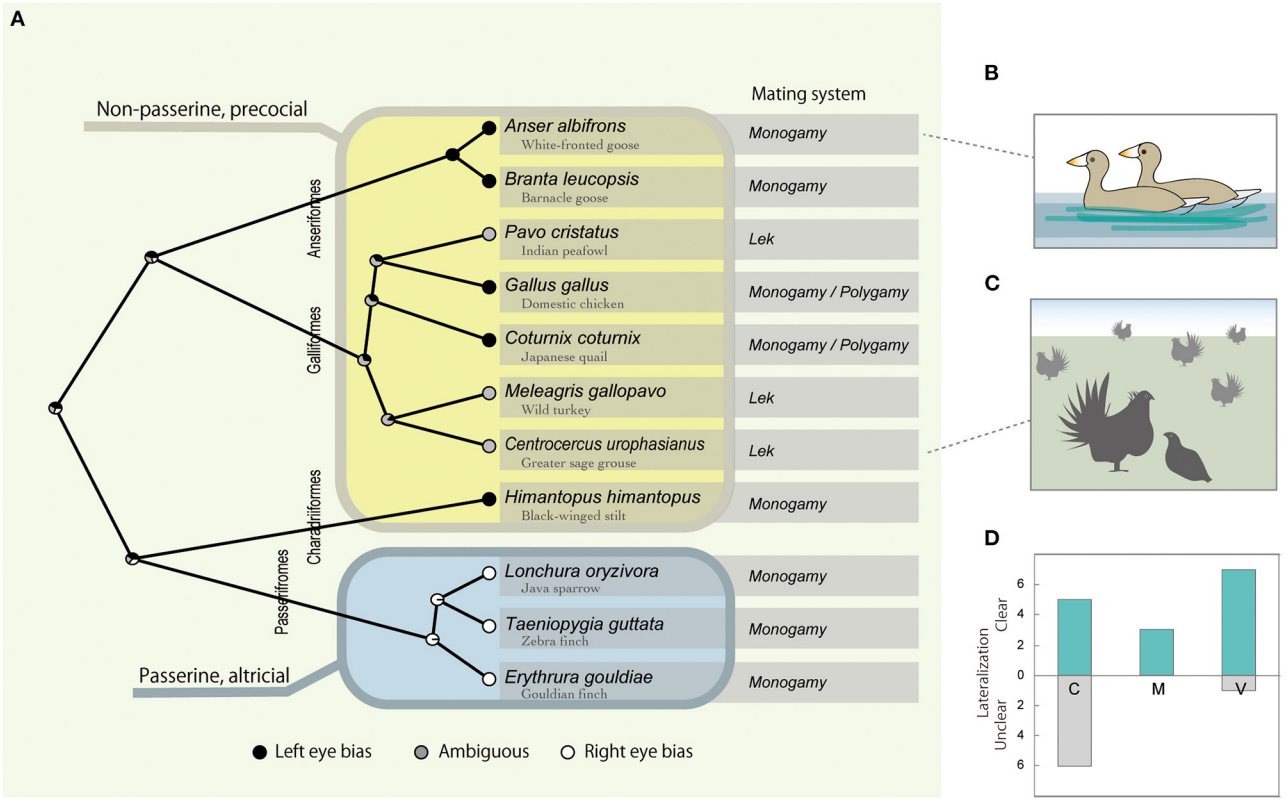


**FIGURE 1 |** Interspecific variation of the visual lateralization in mating context, with left and right eye bias plotted black and white and ambiguous gray, respectively at each tip of the phylogenetic tree **(A)**. Node pie charts in **(A)** represent the relative probability of left, right, or ambiguous eye bias, inferred by ancestral state reconstruction. Typical examples of the behavioral contexts are given as illustrations, partner viewing in monogamous geese in **(B)**, and courtship display of lekking grouse in **(C)**. **Table 1** data are summarized as the number of cases that found clear or unclear lateralization under C (courting), M (mounting) or V (viewing) context in **(D)**.

## Species-Level Analyses

To assess the evolutionary history of visual lateralization, a maximum-likelihood ancestral state reconstruction was performed and the phylogenetic signal (Pagel's λ) was quantified, which ranged from 0 (no phylogenetic signal) to 1 (strong signal). To examine the effects of factors that covaried with visual lateralization, phylogenetic regression analyses (phylogenetic generalized least-squares, PGLS) was conducted, where the dependent variable was entered as 1: left-eye dominance, −1: right-eye dominance, or 0: unclear. As independent variables, the mating system was scored and entered as 0: monogamy, 1: mixed monogamy, and polygamy, 2: lek breeding, along with the developmental mode binary-categorized as precocial or altricial, based on literature (Goodwin, 1982; Madge and McGowan, 2002) and birds of the world online (https://birdsoftheworld.org/bow/home). The present data did not contain species with semi-precocial or semi-altricial development.

## Behavioral Context-Level Analyses

Under the prediction that the methodological aspects of each study would have an influence on whether clear visual lateralization could be found, another set of phylogenetic regression analyses using the data in **Table 1** was performed. To deal with multiple entries per species, generalized linear mixed models (GLMM) were used using the Markov Chain Monte Carlo technique (MCMCglmm, Hadfield, 2010), where the dependent variable was entered as 1: presence or 0: absence of clear visual lateralization in each behavioral context of each species. As explanatory factors, the focal behavioral contexts were considered and categorized as C (courting, which included courtship display or singing, **Figure 1C**), M (mounting or copulation), or V (just viewing, or fixating a potential mate/pairing partner outside the courtship or copulation phase, **Figure 1B**), and whether an eyepatch was used to control the available hemifield.

All statistical analyses were conducted using the R ver. 4.1.0, and its package phytools (Revell, 2012), caper (Orme, 2012), phylolm (Ho et al., 2018), and MCMCglmm (Hadfield, 2010).

# RESULTS

## Phylogenetic Signal

Although it should be noted that the previously studied species were limited and belonged to only four avian

orders (Anseriformes, Galliformes, Charadriiformes, and Passeriformes), a strong and statistically significant phylogenetic signal for the direction of visual lateralization (average $\lambda = 1.0$, $p < 0.02$) with passerine species that showed right-eye bias was found, while the rest showed left -or obscure left/right dominance (**Figure 1A**). Additionally, the phylogenetic signal was tested for the presence/absence of visual lateralization, but it was relatively weak and not statistically significant (average $\lambda = 0.34$, $p < 0.55$).

## Factors Responsible for Visual Lateralization at Species Level

The outcomes from the PGLS indicated that visual lateralization was associated with both the mating system and developmental mode (**Supplementary Table S1**). Specifically, right-eye bias tended to be observed in monogamous and altricial species and left-eye bias in polygynous and precocial species (**Figure 1A**). However, this result can be interpreted in different ways. The binary categorization of developmental modes (altricial vs. precocial) also completely matched with passerine vs. non-passerine distinctions, at least for the present species data (**Figure 1A**). While all passerine birds are altricial, non-passerines show a range of developmental patterns from altricial to precocial. Without the data from altricial non-passerine species (e.g., pigeons, cormorants, and parrots), it was difficult to determine whether being passerine or altriciality was the key factor.

## Factors Responsible for Visual Lateralization at the Behavioral Level

As predicted, focal behavioral contexts had an effect on whether clear visual lateralization was observed (**Figure 1D**). In particular, compared with V (viewing) and M (mounting and copulation) contexts, clear lateralization was less likely to be observed in the C (courtship) context (**Table 1**; **Supplementary Table S2**). The use of an eyepatch did not have a significant influence (**Supplementary Table S2**).

In addition, **Table 1** showed that the left/right eye dominance was not necessarily dependent on particular behavioral context, which was against the prediction that dominant eye would be reversed between courtship and copulation phases (i.e., right eye for courtship and left eye for copulation, Rogers, 2012; Rogers and Kaplan, 2019).

# DISCUSSION

Despite numerous past reviews and empirical studies on avian visual lateralization, to my best knowledge, the present paper was the first attempt to apply phylogenetic comparative methods (PCMs) to systematically integrate previous findings focused on heterogeneous results regarding mating context. Although the outcome interpretations should be done with caution due to the limited availability of species data, the PCMs have revealed overlooked aspects of the evolution of avian visual lateralization. As predicted, the phylogenetic signal for the direction of visual lateralization was quite strong, and passerines (songbirds) and non-passerines (non-songbirds) showed opposite trends in biased eye use, with less variability depending on behavioral contexts. This could potentially be seen as differences in altricial and precocial developments and was also associated with interspecific variations in the mating system. In addition, whether research could find clear visual lateralization was likely to be dependent on the focal behavioral context, as males who performed courtships tended to show less clear laterality, presumably using both eyes.

Reversed visual lateralization between passerine and non-passerine species, as shown in the present study (**Figure 1A**), may not be as simple as everything being reversed between them, but could be seen as part of complex interspecific variations of visual information processing among birds. For example, previous studies that looked at visual behaviors sensing predator-like stimuli reported that both zebra finches and chicken chicks relied on the left eye-right hemisphere system (Rogers, 2000; Rogers et al., 2018). However, two closely related passerine species showed opposite lateralization within a study (Franklin and Lima, 2001). In contrast, in visual discrimination tasks, passerines and non-passerines were contrasting, as European starling (*Sturnus vulgaris*) relied on the left eye (Templeton and Gonzalez, 2004) while non-passerines, which included not only chicken chicks but also pigeons (*Columba livia*), used the right eye (Mench and Andrew, 1986; von Fersen and Güntürkün, 1990): these are instances where the passerine vs. non-passerine rather than the altricial vs. precocial distinction may apply. Above all, the biggest lesson learnt is that investigating model species, especially chicken chicks alone, may not be sufficient for a synthetic understanding, as Galliformes reflect the features of basal avian species, from which more recently evolved species (e.g., Passeriformes) would have been greatly diverged. Hence, its corresponding mechanisms and evolutionary selective forces are unclear.

It should also be noted that visual behaviors in the mating context are trickier than those in others, such as foraging or detouring, due to the nature of bilateral communication between the sexes. In essence, seeing or being seen is not easily separable during mating. For both males and females, visual information of prospective mates is important, but in different ways. Generally, females carefully assess male quality by relying on morphological or behavioral sexual traits (Andersson, 1994; Byers et al., 2010; Soma and Garamszegi, 2011). Similarly, males may assess females but would rather monitor their responses to adjust their behaviors depending on the feedback from females for better success in copulation or pair formation (e.g. Balsby and Dabelsteen, 2002; Patricelli et al., 2006; Barske et al., 2015). However, this could be reversed in species with sex role reversals (Edward and Chapman, 2011). In such mating interactions, the female's mate choice, or female choosiness usually plays a major role as a driver of sexual selection. This means that most of the past visual lateralization studies summarized in this paper focused on the side that was subject to scrutiny (i.e., males). Indeed, some studies revealed how indiscriminating males could be and showed that testosterone treated chicken chicks normally showed copulatory behaviors, even toward a human hand or a block covered with a yellow towel (**Table 1**) (Rogers et al., 1985;

Workman and Andrew, 1986; Bullock and Rogers, 1992). Due to the scarcity of evidence (**Table 1**), it is difficult to discuss sex differences in visual behaviors during mating interactions, if any. However, males and females may not differ dramatically in visual lateralization (Güntürkün and Kischkel, 1992; Gülbetekin et al., 2007).

Given the above-mentioned seeing and being seen interaction between the sexes, it makes sense that clear visual lateralization tends to be lacking, particularly for males performing courtship display and not for those viewing or mounting females. In general, courtship displays are highly ritualized and stereotyped within species, which limits the flexibility of the body head orientation and mobility of the males. More importantly, as the nature of sexual signals conveying the physical quality of individuals, courtship behaviors, expressed as vocalizations and/or physical movements, are energetically costly (Vehrencamp et al., 1989; Zollinger et al., 2011; Clark, 2012). Therefore, it might be too challenging to cope with both, showing off the best courtship performance and adjusting positions and postures to trace a female moving around trying to assess the quality of the potential mates (Krakauer et al., 2016).

In addition, seeing and being seen interactions could be more complicated in the social environment of lekking species, which might explain the rather unexpected heterogeneity associated with mating systems found in previous research. As shown in **Figure 1**, the three previously studied lekking species did not show clear visual lateralization. For example, males of the greater sage-grouse showed left-eye bias when they used the frontal field but not they used the lateral field (Krakauer et al., 2016), which suggested the possibility that they might be monitoring individuals other than the courtship target using the frontal view at times. In lek, by definition, many males gather and are visited by multiple females, where both male-male competition and female attraction affect the mating success of individuals (**Figure 1C**) (Andersson, 1993; Loyau et al., 2005). Unlike monogamous mating species, where one male—one female interaction is common, lekking males pay attention to rival males and potential courtship targets that are visiting other males. For this, they could rely on the left eye-right hemisphere system, as shown for visual lateralization concerning social information processing in Galliformes and other animals (Deng and Rogers, 2002; Daisley et al., 2009; Salva et al., 2012).

For future directions of research in this area, exploring the following three domains is suggested: courtship vs. copulation, females, and pigeons. Although reversed eye-use is predicted between courtship and copulation phases (Rogers, 2012; Rogers and Kaplan, 2019), only a few studies contrasted the two behavioral contexts within the same species, which limits our understanding of cognitive control of sexually motivated behaviors. Moreover, as already mentioned, the evolution of visual lateralization in mating contests is still unclear due to a lack of species data and less focus on females. In particular, clarifying whether the passerine vs. non-passerine or the altricial vs. precocial distinction is responsible for the interspecific variations in lateralization by studying non-passerine altricial species would provide valuable insights. In addition, it should be noted that the passerine songbirds previously studied were all Estrildid finches (family: Estrildidae), implying that the observed phenomena were specific to a taxonomic group. Lastly and most importantly, I would like to emphasize that all the above viewpoints would lead us to deeper insights into physiological mechanisms of sexual/social behaviors and their evolution in birds. This would be critically important when we try to answer how much cognitive mechanisms related with sexual/social interactions are conserved in birds even while they evolved to diversify developmental patterns, mating systems, and the ways to communicate.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

MS collected and analyzed the data and wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

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# Proportional Cerebellum Size Predicts Fear Habituation in Chickens

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The cerebellum has a highly conserved neural structure across species but varies widely in size. The wide variation in cerebellar size (both absolute and in proportion to the rest of the brain) among species and populations suggests that functional specialization is linked to its size. There is increasing recognition that the cerebellum contributes to cognitive processing and emotional control in addition to its role in motor coordination. However, to what extent cerebellum size reflects variation in these behavioral processes within species remains largely unknown. By using a unique intercross chicken population based on parental lines with high divergence in cerebellum size, we compared the behavior of individuals repeatedly exposed to the same fear test (emergence test) early in life and after sexual maturity (eight trials per age group) with proportional cerebellum size and cerebellum neural density. While proportional cerebellum size did not predict the initial fear response of the individuals (trial 1), it did increasingly predict adult individuals response as the trials progressed. Our results suggest that proportional cerebellum size does not necessarily predict an individual's fear response, but rather the habituation process to a fearful stimulus. Cerebellum neuronal density did not predict fear behavior in the individuals which suggests that these effects do not result from changes in neuronal density but due to other variables linked to proportional cerebellum size which might underlie fear habituation.

**Keywords: emergence test, behavioral predictability, domestication, neural density, isotropic fractionation**

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## INTRODUCTION

The cerebellum, the most neuron dense brain region (Herculano-Houzel et al., 2014; Olkowicz et al., 2016), is highly conserved in its anatomy and circuitry among jawed vertebrates (Voogd and Glickstein, 1998) but varies widely in both absolute and relative sizes (Eager, 1967; Yopak et al., 2020). This variation in cerebellar size has been extensively associated with differences in motor skills across species (Iwaniuk et al., 2007, 2009; Yopak et al., 2007; Hall et al., 2013). For example, in bower building birds the complexity of these structures correlates positively with cerebellum size (Day et al., 2005). While traditionally the cerebellum has mainly been seen as a sensory motor control system (Strick et al., 2009), recent findings from neuroanatomical, neuroimaging, and behavioral studies propose a paradigm shift on this view (Schmahmann, 1991; Tedesco et al., 2011; Koziol et al., 2014; Hoche et al., 2018). By demonstrating that the cerebellar output targets several non-motor cortical areas (see review Strick et al., 2009), the range of functions associated with

the cerebellar activity has been remarkably expanded, including associative learning, memory, and emotional processing (Schmahmann, 1991; Ferrucci et al., 2012; Buckner, 2013; Lupo et al., 2015; Adamaszek et al., 2017).

Fearfulness behavior is one of the many functions recently suggested to be linked with the cerebellar anatomy and activity across different vertebrate species (Yoshida and Hirano, 2010; Møller and Erritzøe, 2014; Symonds et al., 2014; Moreno-Rius, 2018; Katajamaa et al., 2021). In birds, for instance, the relative size of the cerebellum appears to predict predator-prey interactions, such as the flight initiation distance of a species (Møller and Erritzøe, 2014; Symonds et al., 2014). However, whether cerebellum size is positively or negatively correlated with fearfulness responses across species remains a matter of debate (Møller and Erritzøe, 2014; Symonds et al., 2014). At least within species, in the case of the red junglefowl (*Gallus gallus*), the size of the cerebellum is positively associated with the memory of fear-like stimuli (Katajamaa et al., 2021). When repeatedly exposed to the same fearful stimuli, junglefowl chicks with reduced fearfulness reactions (faster habituation) had larger cerebellum when adult than chicks with stronger fearfulness reactions (Katajamaa et al., 2021). To what extent, however, this behavioral pattern of habituation to fear is consistent throughout the development of the chicken, and whether the increase in cerebellum size reflects proportionally more neurons is still unknown.

Here, to test if the link between cerebellum size and fear habituation is consistent across development, we performed repeated behavioral testing in a fear inducing test (the emergence test) early in life and after sexual maturity (eight trials per age group) in a population of 36 chickens bred from a unique advanced intercross between domestic chickens (White Leghorn) and their wild ancestor, the red junglefowl. Domestic chickens have a much larger cerebellum (both absolute and proportionally to the rest of the brain, Henriksen et al., 2016) with proportionally more neurons (Racicot et al., 2021) than red junglefowl, and differ significantly in their response to fearful stimuli (Campler et al., 2009). By interbreeding these two chicken populations, we were able to generate a population with large phenotypic variation in (1) cerebellum size (both absolute and proportional to the rest of the brain) and (2) fearfulness. With this population, we could test the link between fear habituation and cerebellum size down to the neural level. Based on previous findings, we hypothesized that both young and adult chickens habituating more rapidly to the emergence tests have larger cerebellum than chickens that require more time to habituate. We also predicted that size differences in the cerebellum among chickens would reflect proportional shifts in the numbers of cerebellar neurons.

# MATERIALS AND METHODS

## Animal Rearing and Housing

We raised 36 individuals from a highly advanced intercross (F18) chicken population that began with one red junglefowl male originated from Thailand (*Gallus gallus*) and three White Leghorn females (*Gallus gallus domesticus*) (Höglund et al., 2020). All individuals were group housed indoor and were kept in day-night cycles of 12/12 h until 10 weeks of age, after which they had access to an outdoor area. Perches, food, and water were provided *ad libitum*. During behavioral testing early in life the chicks were kept in groups of six per cage, while during the adult testing the individuals were kept in single cages within the same room during the testing days. At 229 days of age all individuals were culled by neck dislocation and subsequently decapitated.

### Ethics Statement

The study was approved by the local Ethical Committee of the Swedish National Board for Laboratory Animals.

## Emergence Test

The emergence test is an adaptation of the "Hole-in-the-wall box test" (as reported by Bryan Jones and Mills, 1983) and is commonly used for assessing a general fear response in chickens (Forkman et al., 2007; Archer and Mench, 2014, 2017; Ericsson et al., 2016). We tested the latency of the birds to emerge from a box at 5 to 6 weeks of age and again after sexual maturity at 26 to 27 weeks of age. The recorded tests were used for scoring the latencies of the individuals to (a) emerge their head outside the box (head-emergence) and (b) emerge their whole body outside of the box (body-emergence).

### Young Testing Round

All chicks were tested once per day during eight consecutive days. The chicks were individually placed in boxes of 19.5 by 39.5 by 21cm (W*L*H) with the bottom covered with absorbent paper that was replaced between trials. The box was placed up against the shorter end of the arena and had an opening of 7 cm × 17 cm toward the open arena. Eight individuals were tested simultaneously in individual arenas, and both the boxes and arenas were in complete darkness until the filming of the test started; lights were then turned on only inside the arena, leaving the box in gloom. The tests lasted until the bird had fully emerged from the box or a maximum of 5 min if the bird did not emerge. Recordings were done using video cameras connected to a computer. Tests were carried out from 9am to 12pm and the order of testing for each individual was randomized every day.

### Sexual Mature Testing Round

Half of the individuals ($n$ = 18) were tested during 4 consecutive days the first week of testing and the other half ($n$ = 18) was tested during 4 consecutive days during the second week of testing. Each individual was tested twice a day (morning and afternoon). The chickens were placed in a box (58L:35W:46H) and let to acclimatize for 30 s. A light was then turned on outside of the box, and a hatch (17 by 21 cm) was opened. The hatch gave access to an arena of 2.79 m long and 93 m wide.

Both the box and the arena floors were covered with a bedding of wood shavings that was cleaned from feathers and feces after each test. Three individuals were tested simultaneously in separate arenas of identical conditions, with the test lasting until the chicken emerged the box or a maximum of 5 min if the bird did not emerge. Tests were recorded using cameras GoPro Black 7© (2021 GoPro Inc.) and were carried out from

9am to 11:30 (morning), and a second time from 12pm to 14:30 (afternoon). The order in which individuals were tested was maintained between mornings and afternoons but was randomized across the days.

## Brain Measurements

For all the individuals, brains were extracted just after the culling, and dissected into cerebrum (telencephalon), cerebellum, optic tectum, and brainstem region (thalamus, remaining midbrain, and hindbrain) (sensu Henriksen et al., 2016). The brain regions were immediately weighed after dissection and then the right cerebral hemisphere as well as the right optic tectum and the right half of the cerebellum (cut down the vermis, and each half weighed) was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for at least 72 h. The left-half of the cerebrum, optic tectum, cerebellum as well as the brainstem regions were flash-frozen in liquid nitrogen and stored at −80°C for later studies. For the cerebellum, we estimated numbers of total cells, neurons, and non-neuronal cells following the isotropic fractionator technique (Herculano-Houzel and Lent, 2005). The right half of the cerebellum was analyzed for cell counting and the numbers obtained were multiplied by 2. As described elsewhere, the isotropic fractionator consists in mechanically dissociating the brain tissue (e.g., cerebellum) in 40 mM sodium citrate with 1% Triton X-100 using Tenbroeck tissue homogenizers (Herculano-Houzel and Lent, 2005; Olkowicz et al., 2016). Once the brain is transformed into a suspension of free cell nuclei with a defined volume and kept homogeneous by agitation, the total number of cells was estimated by using fluorescent DNA marker 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI). At least four aliquots (10 μL) per individual were sampled and counted using Neubauer improved chamber under a fluorescent Nikon eclipse 80i microscope at 400× magnification. For the cell counts, the coefficients of variation among the four aliquots were lower than 0.15. To determine the proportion of neurons across our samples, we used immunocytochemical detection of neuronal nuclear antigen NeuN, expressed in the nuclei of most neuronal populations within the brain (Mullen et al., 1992). Although NeuN is not expressed by Purkinje cells (Mullen et al., 1992), these cells do not represent the largest fraction of cells in the cerebellum (Cunha et al., 2020, 2021), and therefore not sampling them should not be a major issue for our comparative dataset. We used mouse monoclonal antibody anti-NeuN 488 AlexaFluor conjugated (1:300 in phosphate-buffered saline; clone A60, Chemicon; MAB377X), incubated at 10°C overnight. A minimum of 500 nuclei was counted to estimate the proportion of neurons (immunolabeled in Dapi and NeuN +) in the sample. The number of non-neuronal cells in the cerebellum was obtained through subtraction.

## Statistical Analysis

All analyses were performed using R version 4.1.1 (R Core Team, 2021). Data on the time taken for the chickens to head-emerge or body-emerge during the 16 trials (8 trials early in life and eight trials after sexual maturity) included multiple censored observations, in which individuals failed to emerge. Cox proportional hazards models were therefore used to analyze the effect of cerebellum size and other explanatory variables on the time taken to emerge. Cox proportional hazards models were built with the package "survival" (Therneau, 2021). Covariates of the four brain regions size [both absolute (g) and in proportion (%) to total brain size (g)], trial number, sex, and cerebellar neuronal density (CND) were tested using the *coxph* function. Cerebellar neural number and absolute mass are strongly correlated (see **Supplementary Figure 2**) but the correlation varies between individuals and therefore both variables were included in the model. The data was clustered by individual to account for correlations among repeated observations of the same bird and inter-individual variation in initial fear response (trial 1). In Cox proportional hazard models, estimated coefficients are log hazard ratios. Hence, coefficients that are $> 0$ correspond to hazard ratios $> 1$ and increased risk of emergence. For class variables it is an increased risk compared to the reference class, whereas for continuous variables the risk increases with increased values of the covariate. Behavioral observations were grouped by both the day (1 to 8 for young, 1 to 4 for mature) and the number of tests (1 test per day for chicks and 2 tests within a day for chickens). For observations where the animal did not head- or body-emerge, a maximum value of 300 s was given. The time to head-emerge and body-emerge was highly correlated across all observations (Spearman's correlation coefficient = 0.956, $P < 0.001$). Because of this strong relationship between head- and body emergence, we chose to use head emergence as our main response variable for the proportional hazards models, as it is deemed to fairly represent the results with a reduced number of censored observations (i.e., chickens that did not head-emerge) in the data. Since behavioral testing was done during an eight-day period for the chickens early in life and during a four-day period when they reach sexual maturity, the cox proportional hazards models were fitted separately for each age. For further visualization of the results, all individuals were divided into four quartiles of nine individuals in each age group based on proportional cerebellum size, and Kaplan-Meier curves (Kassambara et al., 2021) were plotted for each trial (see **Figure 1**), and log-rank tests were used to estimate the significance of the differences between the curves. Because Kaplan-Meier estimates commonly represent $y$ as the probability of survival at time $t$ (chance of the event not occurring), survival curves in the graphics were transformed such that $f(y) = 1-y$. Therefore, **Figure 1** represent the likelihood of the individual emerging at time t.

# RESULTS

The between-individual variation in time taken for the head to emerge during the emergence test ranged from 16.7% on the first day to 61.1% on the last day when tested early in life, and from 75 to 83.3% when tested after sexual maturity. For the adult individuals, the time of day (morning vs. afternoon) had no effect on the latency for head emergence, and this variable was therefore removed from the model, making the final model used for each age group the same. There was a significant effect of proportional cerebellum size on the latency to head emergence in

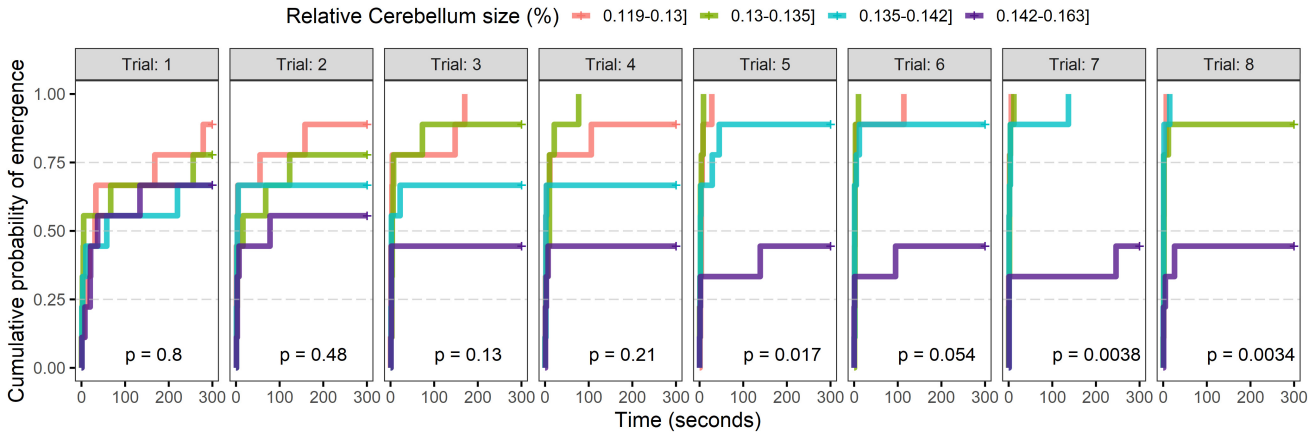


**FIGURE 1 |** Kaplan-Meier survival curves for adult individuals showing the effect of proportional cerebellum size (%cerebellum) on habituation during repeated trials (eight trials in total) in an emergence test. Proportional cerebellum size is divided in quartiles with equal number of individuals in each group. Significance for differences in survival curves between groups are calculated with a log-rank test represented by the p-value in each graphic.

adult chickens during the eight trials with increasing significance as the trials progress (see **Table 1**). Absolute cerebellum size as well as the size (absolute and proportional) of the other three brain regions had no significant effect on latency to emerge ($p > 0.05$) in adult chickens and was therefore removed from the final model.

The latency to emerge when the individuals were tested early in life was not significantly affected by the interaction between trial number and cerebellum size (both absolute and proportional, $p < 0.05$), and the interactions were therefore removed from the final model. Similarly, none of the three other brain regions sizes (absolute and proportional) had any significant effects on latency to emerge early in life ($p > 0.05$) and were therefore removed from the final model. Latency for head emergence was, however, significantly affected by trial number early in life, with latency being significantly different on all trials compared to trial 1 (see **Table 2**). Sex had no effect on latency to head-emerge neither in young ($P = 0.99$) or adult birds ($P = 0.70$), and neither did cerebellum neural density (adult: $p = 0.93$, young: $p = 0.313$); the later variable was therefore removed from the final models (see **Tables 1**, **2**). The Kaplan-Meier survival curves for all adult individuals divided into quartiles based on proportional cerebellum size (**Figure 1**) showed that proportional cerebellum is negatively correlated with an individual's likelihood to emerge as the trials progress in adult individuals (see **Figure 1**).

**TABLE 1 |** Adult test rounds: Hazard ratios of proportional cerebellum size (%cerebellum), trial number, and the interaction effect between both variables (denoted by ":"), when controlling for sex, for the latency to head-emerge.

| Covariate | Coefficient | Hazard ratio | Robust se | *p*-value |
|---|---|---|---|---|
| Sex (female) | 0.15 | 1.16 | 0.39 | 0.70 |
| %Cerebellum size | −0.17 | 0.85 | 0.20 | 0.41 |
| Trial 2 | 2.01 | 7.47 | 3.50 | 0.57 |
| Trial 3 | 5.11 | 165.21 | 3.31 | 0.12 |
| Trial 4 | 5.09 | 162.63 | 3.01 | **0.09** |
| Trial 5 | 5.56 | 260.07 | 2.92 | **0.06** |
| Trial 6 | 7.82 | 2492.73 | 3.26 | **0.02** |
| Trial 7 | 9.40 | 12080.87 | 3.14 | **0.003** |
| Trial 8 | 9.03 | 8327.15 | 2.93 | **0.002** |
| %Cerebellum: Trial 2 | −0.14 | 0.87 | 0.26 | 0.59 |
| %Cerebellum: Trial 3 | −0.36 | 0.70 | 0.25 | 0.15 |
| %Cerebellum: Trial 4 | −0.35 | 0.70 | 0.22 | 0.11 |
| %Cerebellum: Trial 5 | −0.37 | 0.69 | 0.22 | **0.09** |
| %Cerebellum: Trial 6 | −0.52 | 0.59 | 0.25 | **0.03** |
| %Cerebellum: Trial 7 | −0.62 | 0.54 | 0.23 | **0.007** |
| %Cerebellum: Trial 8 | −0.60 | 0.55 | 0.22 | **0.007** |

*Trial 1 used as reference level in Cox proportional hazards model.*
*Bold indicates statistical significance.*

## DISCUSSION

We find that proportional cerebellum size is linked to the behavioral response of adult chickens tested repeatedly in an emergence test. While proportional cerebellum failed to predict

**TABLE 2 |** Young test rounds: Hazard ratios for latency to head-emerge, as affected by the number of trials.

| Covariate | Coefficient | Hazard ratio | robust se | *p*-value |
|---|---|---|---|---|
| Sex (female) | $5 \times 10^{-4}$ | 1.00 | 0.35 | 0.999 |
| %Cerebellum size | 0.21 | 1.23 | 0.16 | 0.2 |
| Trial 2 | 0.91 | 2.48 | 0.33 | **0.006** |
| Trial 3 | 1.23 | 3.43 | 0.40 | **0.002** |
| Trial 4 | 1.50 | 4.49 | 0.42 | **0.0003** |
| Trial 5 | 1.43 | 4.17 | 0.48 | **0.003** |
| Trial 6 | 1.80 | 6.07 | 0.47 | **0.0001** |
| Trial 7 | 1.89 | 6.59 | 0.49 | **0.0001** |
| Trial 8 | 1.71 | 5.54 | 0.45 | **0.0002** |

*Trial 1 used as reference level in Cox proportional hazards model.*
*Bold indicates statistical significance.*

fearfulness during the initial trials, the proportional size of this brain region predicted fearfulness as the trials progressed. The correlation was unaffected by sex and no other brain region (proportional or absolute) correlated with fearfulness. This indicates that, while proportional cerebellum size is not linked to a chicken's initial fear response in an anxiety test, it is linked to the change in behavior that occurs when an individual is repeatedly exposed to the same anxiety stimuli. In **Figure 1** we can see that although most individuals seem more likely to emerge faster from the emergence box as the trials progress, adult individuals with the largest proportional cerebellum (upper 25%) tended to stay slightly longer in the emergence box as the trials progressed. When looking at the behavior of these individuals early in life, there was no significant correlation between time to emerge and adult proportional cerebellum or any other brain measurement.

Our findings are comparable to previous finding on red junglefowl in which adult cerebellum size, but no other brain region, correlated with habituation to fear measured early in life (Katajamaa et al., 2021). In this study the authors found that chicks with the largest proportional cerebellum as adult seem to emerge sooner (we found a similar non-significant correlation for the chicks in our study, see **Supplementary Figure 1**). Yet, as we can see from our study this pattern is reversed when testing fear habituation in adult chickens. While most of the adult individuals appear to habituate and thereby reduce their fear response in the emergence test, the individuals with the largest proportional cerebellum seem to dishabituate (sensitize) by increasing their fear response as the trials progress. Habituation is a form of simple learning in which the magnitude of the response to a specific stimulus decreases with repeated exposure to that stimulus (Rankin et al., 2009). It is believed that habituation allows animals to filter out irrelevant stimuli and focus selectively on important stimuli (Rankin et al., 2009). According to the *dual-process theory*, habituation and dishabituation (sensitization) processes act independently and then sum together to produce the observed behavioral effects of repeated stimulation (Groves and Thompson, 1970), indicating that slightly different neural pathways might be involved in these two behavioral processes. Although habituation is well studied across species, very little is known about the neural mechanisms underlying this behavioral response (Rankin et al., 2009). As one of the key functions of the cerebellum is to identify and store sequences of actions (Popa and Ebner, 2019), the cerebellum's involvement in fear response, reported in this study (and the study by Katajamaa et al., 2021) could be due to its ability to predict future outcome when exposed to the same stimuli multiple times (*sequence detection hypothesis* by Leggio and Molinari, 2015).

It is interesting that the adult proportional size of the cerebellum to some extent predicts habituation behavior of chickens both early in life and after sexual maturity, but it is baffling as to why the link is reversed during development. The size of the cerebellum, as well as other major brain regions, changes in absolute as well as proportional size during post-hatch development in chickens (Henriksen et al., 2016). For example, the cerebellum exhibits its largest proportional size in the brain around four weeks of age in domestic chickens and red junglefowl and has a much smaller proportional size both before and after this age (Henriksen et al., 2016). The adult chicken brain is therefore not just a scaled-up version of the chick brain, which makes it difficult to correlate adult cerebellum size with chick behavior and to predict the brain composition of these chicks early in life. Furthermore, although the proportional size of all major brain regions follows the same growth trajectories in red junglefowl and domestic chickens, the proportional size of these brain regions still shows substantial variation at any given age between wild and domestic chickens (Henriksen et al., 2016). Since the population used in our study was an intercross between red junglefowl and domestic chickens this further increases the range of possible variation in brain composition between these individuals early in life.

Our study is not the first to demonstrate a link between the cerebellum and the behavioral process of habituation. Previous brain positron emission tomography (PET) studies in humans (Timmann et al., 1998) and lesion studies in rodents (Leaton and Supple, 1991) have reported the cerebellum to be involved in the habituation of individuals startle response to fearful acoustic stimuli, but our results are the first to indicate that the link between adult cerebellum and habituation could be influenced by the proportional size of this brain region. To further explore the correlation between fear habituation and the functional significance of cerebellum size we measured neural density in the cerebellum. Neural density, however, did not predict fear habituation, demonstrating that the proportional size of the cerebellum is a better predictor for habituation behavior in chickens than neural number and density. This finding indicates that factors other than neural density, linked to proportional cerebellum size, could be responsible for affecting the degree of habituation. For example, our results suggest that the observed effects are not simply due to proportionally larger cerebellum having greater or lesser neuronal density, but rather is representative of different mechanisms that are linked with proportional size. The advanced intercross population used in this study was based on red junglefowl and domestic White Leghorn chickens. We have previously demonstrated that the cerebellum has increased during domestication, both proportional to the brain and in absolute size (Henriksen et al., 2016). Additionally, this increase in cerebellar size in domestic chickens is associated with an increase in the foliation of the cerebellum, granule cell layer size, granule cell size and number, and Purkinje cell number (Racicot et al., 2021). The intercross population used in this study therefore have the potential to vary in all these brain parameters. Two limitations to the isotropic fractionation technique used in this study is that it does not include Purkinje cells in the cell counting sample, nor can it be used to assess the size of the different neurons in the cerebellum. Additionally, it was outside the scope of this study to measure cerebellar foliation. We can therefore only speculate about the significance of these variables.

Our finding that increased proportional cerebellum size is linked to dishabituation in adult chickens and thereby higher fear response in a fear inducing emergence test, is in accordance with a previous study by Møller and Erritzøe (2014). Here the authors demonstrate that across bird species increased proportional cerebellum is linked to increased antipredator response, and

thereby fear as this behavior is generally defined as a reaction to the perception of danger (Boissy et al., 1998). This result, combined with ours suggests that both within and among species the proportional size of the cerebellum is linked to fear response in birds, but as suggested by our findings (and previous finding by Katajamaa et al., 2021) this link might have more to do with the ability of the cerebellum to store sequences of actions and thereby the ability of this brain region to modify the behavioral fear response when repeatedly exposed to the same type of fear inducing stimuli.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Local Ethical Committee of the Swedish National Board for Laboratory Animals.

## AUTHOR CONTRIBUTIONS

RH and DW conceived the study. DS-H, FC, RC, LC, DW, and RH collected data. DS-H and LR performed the analysis. DS-H, FC, and RH wrote the initial manuscript. All authors revised and contributed to the initial and final draft.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.826178/full#supplementary-material

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REVIEW
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# The Role of the Endogenous Opioid System in the Vocal Behavior of Songbirds and Its Possible Role in Vocal Learning

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The opioid system in the brain is responsible for processing affective states such as pain, pleasure, and reward. It consists of three main receptors, mu- (μ-ORs), delta- (δ-ORs), and kappa- (κ-ORs), and their ligands – the endogenous opioid peptides. Despite their involvement in the reward pathway, and a signaling mechanism operating in synergy with the dopaminergic system, fewer reports focus on the role of these receptors in higher cognitive processes. Whereas research on opioids is predominated by studies on their addictive properties and role in pain pathways, recent studies suggest that these receptors may be involved in learning. Rodents deficient in δ-ORs were poor at recognizing the location of novel objects in their surroundings. Furthermore, in chicken, learning to avoid beads coated with a bitter chemical from those without the coating was modulated by δ-ORs. Similarly, μ-ORs facilitate long term potentiation in hippocampal CA3 neurons in mammals, thereby having a positive impact on spatial learning. Whereas these studies have explored the role of opioid receptors on learning using reward/punishment-based paradigms, the role of these receptors in natural learning processes, such as vocal learning, are yet unexplored. In this review, we explore studies that have established the expression pattern of these receptors in different brain regions of birds, with an emphasis on songbirds which are model systems for vocal learning. We also review the role of opioid receptors in modulating the cognitive processes associated with vocalizations in birds. Finally, we discuss the role of these receptors in regulating the motivation to vocalize, and a possible role in modulating vocal learning.

**Keywords: songbirds, endogenous opioids, opioid receptors, learning, reward, basal ganglia**

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## INTRODUCTION

Acoustic communication is important for the survival of animals living in large social groups. Many species of animals utilize innate vocalizations to relay information to others. The context for such innate vocalizations may be acquired from the environment (DeVries et al., 2015; Wegdell et al., 2019) but is not affected by changes in auditory input during early development (Cheney et al., 1992; Hammerschmidt et al., 2012). In contrast, some animals have elaborate vocal repertoires which are learnt during the course of development and even in adulthood (**Figure 1**; Harcus, 1977;

Eens, 1997; Hardy and Parker, 1997; Dowsett-Lemaire, 2008; Gammon and Altizer, 2011; Balsby et al., 2012; Janik, 2014; Mello, 2014; Reichmuth and Casey, 2014; Stoeger and Manger, 2014; Favaro et al., 2016; Mori et al., 2018; Johnson and Clark, 2020; Vernes and Wilkinson, 2020; Dalziell et al., 2021). Neuroethologists study these vocal learners to understand the intricacies and origins of human speech acquisition.

One such avian species which learns to produce complex vocal signals are songbirds. Like humans, these birds live in intricate social groups wherein vocal communication is essential (Zann, 1996; Fernandez et al., 2017; Tchernichovski et al., 2017). Birdsong is used to attract mates, mark territories and is the defining feature of songbirds, refined across generations by sexual selection (Zann, 1996). One of the first studies that hinted at song learning in passerine birds was performed by Barrington (1773). In a letter to the Royal Society of London, he wrote about the stages of song learning in young birds, differentiating begging calls for food, distance calls for communication and learnt vocalizations. He stated that the initial phase of song production in young birds was very similar to babbling in human babies, which was strongly influenced by the social environment (Barrington, 1773). Furthermore, active tutors were found to be essential for both young songbirds and human babies. Earlier studies had demonstrated that human babies did not learn language from audio/video recordings (Kuhl et al., 2003). Similarly, Baptista and Petrinovich (1984, 1986) showed that white crowned sparrows (*Zonotrichia leucophrys*) tutored with taped songs of conspecific adult males until 50 days post hatch chose to learn the songs of a live heterospecific tutor (strawberry finches, *Amandava amandava*) presented to them after the sensitive period for learning ended. These findings suggested that vocal learners chose to learn from a live heterospecific tutor rather than from the taped vocalizations of conspecifics, showing that social interactions were important for vocal learning.

The presence of a strong social influence and an internal reward guiding vocal learning [reviewed in Riters (2011) and Riters et al. (2019)] hints at the involvement of neuromodulators in vocal learning during the sensitive period. One of the most potent neuromodulator groups that are associated with social reward and motivation is the endogenous opioid system. It is composed of opioid receptors (ORs) and their ligands, the endogenous opioid peptides. The four primary subtypes of opioid receptors include μ (mu)-ORs encoded by the OPRM1 gene (Chen et al., 1993a; Fukuda et al., 1993; Wang et al., 1993), δ (delta)-ORs encoded by the OPRD1 gene (Evans et al., 1992; Kieffer et al., 1992), κ (kappa)-ORs encoded by OPRK1 gene (Chen et al., 1993b; Meng et al., 1993; Minami et al., 1993; Yasuda et al., 1993) and Nociceptin/Orphanin FQ receptors encoded by the OPRL1 gene (Fukuda et al., 1994; Mollereau et al., 1994; Wang et al., 1994; **Table 1**). The present review focuses mainly on the two most studied subtypes, that is, the μ- and δ-ORs. Besides being activated by their respective endogenous ligands (Pleuvry, 1991), endorphins and enkephalins, respectively, the ORs also bind to a lesser degree to the other opioid ligands (Jordan et al., 2000). The endorphins are synthesized after post-translational modification of the precursor prohormone preproopiomelanocortin (POMC; Smyth, 1983), whereas δ-ORs are activated by enkephalins derived from the peptide precursor preproenkephalin (PENK; Hughes et al., 1975). A third type of opioid ligand, the dynorphins, synthesized from prodynorphin bind to κ-ORs (Goldstein et al., 1979, 1981). By binding to these receptors, opioids influence a variety of physiological activities including analgesia, hunger, motivation, anxiety and even learning (Bodnar, 2004; Wilson and Junor, 2008; Kibaly et al., 2019). Whereas research on opioid addiction has largely overshadowed the role of these receptors in higher cognition, recent studies have shown their involvement in learning (Meilandt et al., 2004; Bertran-Gonzalez et al., 2013).

Both μ- and δ-ORs can act to modulate different kinds of learning, with some researchers hypothesizing that these receptors help in learning the association between drug and reward in addiction (Klenowski et al., 2015). In the present review, we will discuss the anatomical distribution of opioid ligands and receptors in the brain of songbirds and how these receptors may modulate vocalization and vocal learning.

## VOCAL LEARNING AND THE UNDERLYING NEURAL CIRCUITRY

The process of vocal learning begins with the perception of adult vocalizations. Human babies are exposed to language *in utero* and newborns respond more to their mother's voice and language (DeCasper and Fifer, 1980; Moon et al., 1993). This suggests that before learning semantics and grammar, babies learn to identify phonetic arrangements specific to their native language. Songbirds too begin to learn parental vocal signals early in development. The superb fairy wren (*Malurus splendens*) learns its mother's incubation calls *in ovo* and uses a similar vocal structure in its own begging calls for food (Colombelli-Negrel et al., 2014). This has been tested by showing that the embryos of this species of birds show an increased heart rate in response to a playback of tutor songs *in ovo* (Colombelli-Negrel and Kleindorfer, 2017).

After hatching, the young birds begin an early phase of learning is called the *sensory phase*, during which they memorize their tutor's songs. An increased response to the playback of a known song in white-crowned sparrows, trained using songs taped from the tutor, suggests the presence of a memory of the imitated song (Nelson, 1997). Once the song template is learnt, young birds begin to sing a soft and immature subsong (Immelmann, 1969). This is the sensorimotor phase during which the bird tries to match its own song to the "mental template" it had acquired during the sensory phase. Auditory feedback helps in matching the bird's own vocalizations to that of their fathers/tutors (Konishi, 1965; Brainard and Doupe, 2000). With practice, the vocalizations of the young bird become more structured, but still possess the ability to undergo change. These vocalizations are called plastic songs, which finally develop into a fully structured unchangeable vocal pattern in adulthood (Eales, 1985; Slater and Jones, 1998). For closed-ended learners such as zebra finches (*Taenopygia guttata*) and white crowned sparrows (*Zonotrichia leucophrys*), the adult song does not undergo further change and is aptly referred to as "crystallized

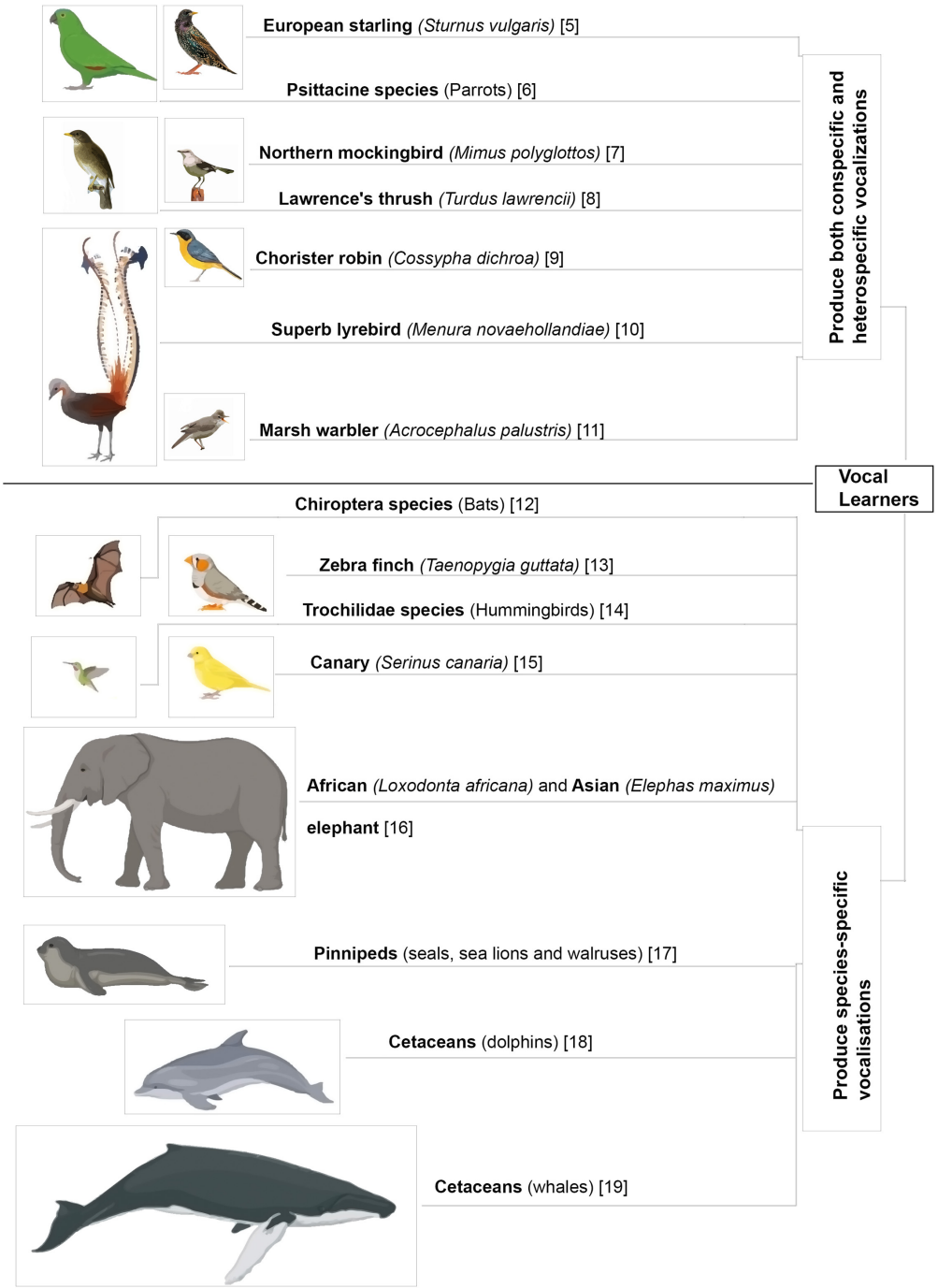


**FIGURE 1 |** Different animal species that learn to vocalize. Vocal mimics further enhance their vocal repertoires by including heterospecific and environmental sounds in their vocalizations (Harcus, 1977; Eens, 1997; Hardy and Parker, 1997; Dowsett-Lemaire, 2008; Gammon and Altizer, 2011; Balsby et al., 2012; Janik, 2014; Mello, 2014; Reichmuth and Casey, 2014; Stoeger and Manger, 2014; Favaro et al., 2016; Mori et al., 2018; Johnson and Clark, 2020; Vernes and Wilkinson, 2020; Dalziell et al., 2021).

song" (Immelmann, 1969; Marler and Peters, 1982; Böhner, 1990; Zann, 1990).

For songbirds, the process of vocal learning and production is controlled by specific brain areas called song control nuclei. Nottebohm et al. (1982) showed the presence of five such nuclei in the songbird brain that were associated with vocal control. These nuclei included the pallial sensorimotor nucleus HVC (used as a proper name) which projects to a pallial motor nucleus RA (robust nucleus of the arcopallium) in the caudal part of the brain and forms the vocal motor pathway (VMP), which

**TABLE 1 |** Opioid peptide receptors and their ligands.

| Name* | Endogenous ligand(s) | Action | pKi |
|---|---|---|---|
| **μ, mu or MOP** | β-Endorphin | Full agonist | 9 (Raynor et al., 1994) |
| | Leu-Enkephalin | Partial agonist | 8.1 (Toll et al., 1998) |
| | Met-Enkephalin | Full agonist | 9.2 (Raynor et al., 1994) |
| | Endomorphin-1 | Potential full agonist | 8.3 (Zadina et al., 1997; Gong et al., 1998) |
| | Endomorphin-2 | Potential full agonist | 8.5 (Zadina et al., 1997) |
| | Dynorphin A | Full agonist | 8.3 (Toll et al., 1998) |
| | Dynorphin B | Full agonist | 8.5 (Toll et al., 1998) |
| **δ, delta or DOP** | Leu-Enkephalin | Full agonist | 8.4 (Raynor et al., 1994)–8.7 (Toll et al., 1998) |
| | β-Endorphin | Full agonist | 8.3 (Toll et al., 1998)–9 (Raynor et al., 1994) |
| | Met-Enkephalin | Full agonist | 6.0 (Meng et al., 1993) |
| | Dynorphin A | Full agonist | 7.8 (Toll et al., 1998) |
| | Dynorphin B | Full agonist | 7.8 (Toll et al., 1998) |
| | Endomorphin-1 | Potential full agonist | 6.1 (Zadina et al., 1997) |
| **κ, kappa or KOP** | Dynorphin A | Full agonist | 8.3–10.8 (Simonin et al., 1995; Zhu et al., 1995, 1997; Toll et al., 1998) |
| | Dynorphin B | Full agonist | 8.1–9.9 (Meng et al., 1993; Simonin et al., 1995; Toll et al., 1998) |
| | Leu-Enkephalin | Full agonist | 6.8 (Meng et al., 1993) |
| | Met-Enkephalin | Partial agonist | 6.3 (Simonin et al., 1995)–7.9 (Toll et al., 1998) |
| | α-Neoendorphin | Full agonist | 8.3–10.2 (Li et al., 1993; Meng et al., 1993; Simonin et al., 1995; Zhu et al., 1995) |
| **NOP** | Nociceptin/ orphanin FQ | Full agonist | 8.4–10.4 (Adapa and Toll, 1997; Dooley et al., 1997; Bigoni et al., 2002) |

**NC-IUPHAR (Nomenclature and Standards Committee of the International Union of Basic and Clinical Pharmacology) -approved nomenclature.*
*DOP, delta opioid receptor; KOP, kappa opioid receptor; MOP, mu opioid receptor; NOP, nociceptin opioid receptor.*

is important for vocalization since it controls the movements of the syrinx (Nottebohm and Arnold, 1976; Nottebohm et al., 1982). A nucleus in the avian basal ganglia, Area X, also receives projections from HVC and projects to the thalamic nucleus DLM (dorsolateral nucleus of the medial thalamus), which in turn projects to LMAN (lateral magnocellular nucleus of the anterior nidopallium, LMAN). The pathway connecting Area X, DLM and LMAN forms a thalamocortical basal ganglia loop called the anterior forebrain pathway (AFP; Nottebohm et al., 1976; Bottjer et al., 1989), responsible for vocal learning (Bottjer et al., 1984; Scharff and Nottebohm, 1991). The two pathways are interconnected via projections from LMAN to RA (**Figure 2**; Herrmann and Arnold, 1991).

## SIGNALING AND NEURAL EXPRESSION OF THE ENDOGENOUS OPIOID SYSTEM

The opioid receptors are members of the G-protein coupled receptor (GPCR) family. They are composed of seven hydrophobic transmembrane domains connected via intra and extra-cellular loops and possess N- or amino and C or carboxylic groups at the end terminals. Structurally, the μ- and δ-ORs have a binding pocket that interacts with the respective ligand and specifically recognizes the morphinan group (**Table 1**; Mansour et al., 1997; Granier et al., 2012). Following activation, μ-ORs inhibit adenyl cyclase and voltage-gated $Ca^{2+}$ channels, and stimulate G protein-activated inwardly rectifying K+ channels (GIRKs) and phospholipase Cβ by the activation of the $G_{\alpha i/o}$ and $G_{\beta\gamma}$ subunits (Childers, 1991). Similarly, for δ-ORs, the activation of the $G_{\alpha i/o}$ and $G_{\beta\gamma}$ subunits results in modulation of the activity of calcium channels (P/ Q-, N-, and L-type), GIRKs, and inhibition of adenylyl cyclase which reduces the level of intracellular calcium via inhibition of cAMP-dependent calcium channels. Together, these events result in the inhibition of neural activity (Kieffer and Evans, 2009; Al-Hasani and Bruchas, 2011; Gendron et al., 2016). Once activated, both μ- and δ-ORs are internalized and δ-ORs are specifically degraded via the lysosomal pathway (Whistler et al., 2002). In contrast, μ-ORs may continue to be involved in signal transduction even after internalization and only unbound μ-ORs are recycled (Al-Hasani and Bruchas, 2011).

### μ-ORs and Their Ligands

In mammals, μ-ORs and their ligands (enkephalins and endorphins) are mostly concentrated in the hippocampus, thalamic nuclei, amygdala, locus coeruleus, parabrachial nucleus, and the nucleus of the solitary tract (Bloom et al., 1978; Di Giulio et al., 1979; Gall et al., 1981). Within the striatum, these receptors are concentrated in patches, which are embedded in a matrix intensely stained for acetylcholine and rich in Substance P (Pert et al., 1976; Brimblecombe and Cragg, 2017). Although the patch and matrix organization of the mammalian striatum is absent in birds, levels of μ-ORs are uniformly high across the striatum in birds including pigeons (*Columba livia*) (Reiner et al., 1989), chick (*Gallus gallus*) (Csillag et al., 1990), juncos (*Junco hyemalis*) (Gulledge and DeViche, 1995; Gulledge and Deviche, 1999) and zebra finches. Furthermore, μ-ORs are present across development in some of the song control regions of juncos HVC, RA, LMAN, and Area X (Gulledge and DeViche, 1995; Gulledge and Deviche, 1999), especially in RA, and in all song control nuclei including HVC, RA, LMAN, Area X, and DLM in adult male zebra finches (**Figure 2**; Khurshid et al., 2009).

Comparatively fewer studies have been conducted to detect opioid ligands in songbirds. Initial studies used specific antibodies to detect the presence of leu-enkephalin (Ryan et al., 1981) and met-enkephalin (Ryan et al., 1981; Bottjer and Alexander, 1995) in different song control nuclei. Both reports demonstrated the presence of enkephalinergic fibers and somata in components of the VMP and AFP in adult male zebra finches. Additionally, Carrillo and Doupe (2004) found that medium spiny neurons as well as large DLM-projecting neurons in Area X were immunoreactive for leu-enkephalin. These findings were confirmed by Xie et al. (2010), who used mass spectrometry and MALDI-TOF to demonstrate that both proopiomelanocortin (POMC), the precursor peptide of

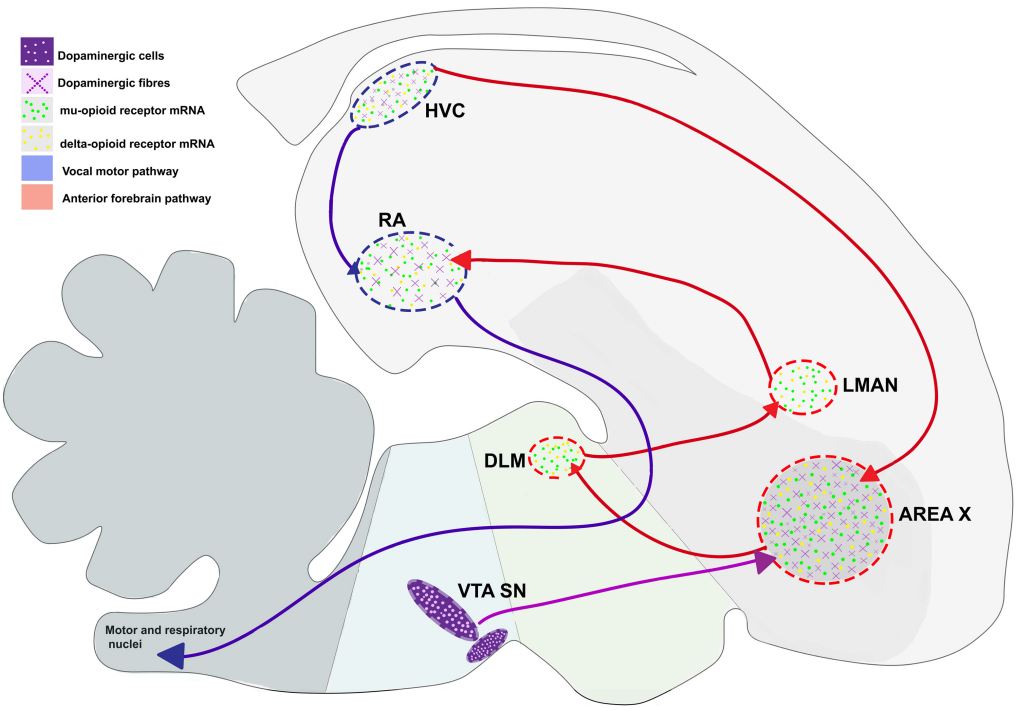


**FIGURE 2 |** Expression pattern of μ- and δ-opioid receptors and the dopaminergic system in the vocal control nuclei in the songbird brain, based on data from Bottjer (1993) and Khurshid et al. (2009). Blue arrows represent connections of HVC connections, red arrows represent connections of AFP, and violet arrows represent dopaminergic input. DLM, dorsolateral nucleus of the medial thalamus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium; SN, substantia nigra; VTA, ventral tegmental area.

β-endorphin, and preproenkephalin (PENK, the precursor of enkephalin) were present in Area X, LMAN, HVC, and RA.

The presence of both POMC and PENK as well as μ-ORs in components of the VMP (HVC→RA) and AFP (the neural circuit connecting LMAN, Area X, and DLM) in songbirds suggests that they may be able to modulate both vocalization and vocal learning and/or singing in different social contexts. In particular, the localization of μ-ORs in the avian striatum suggests that these receptors may be important for the control of reward-guided behavior, since undirected singing and song learning (cf., Scharff and Nottebohm, 1991) during practice is thought to be internally rewarding (Riters et al., 2019).

## δ-ORs and Their Ligands

The neural expression of δ-ORs has been well-documented in rodents. Developmentally, δ-OR expression begins in the pons and the hypothalamus at embryonic day 13 (E 13.5), whereas μ- and κ-ORs are detected in the basal ganglia and midbrain at E 11.5. The prenatal expression of δ-ORs at day E 17.5 and E 19.5 is very low and restricted to the caudate putamen, parabrachial nucleus and olfactory tubercle (Zhu et al., 1998). In contrast, in adult rodents, δ-ORs are distributed in the olfactory tubercle, cerebral cortex, amygdala, nucleus accumbens, and striatum (Mansour et al., 1987, 1994) and have a low, yet detectable presence in the hippocampus and VTA (Erbs et al., 2015). Since this pattern of expression is absent in the prenatal stages, it is possible that they may influence the development of neural circuits developing after birth and modulate the associated cognitive processes. Furthermore, the expression of δ-ORs in areas regulating reward, motivation, learning, memory, and emotional processing (Jutkiewicz, 2018) suggests that these receptors may be involved in cognitive processes such as vocal learning.

In songbirds (zebra finches), the expression of enkephalin is similar to that of δ-ORs. Neuronal fibers immunoreactive for met-enkephalin are distributed across the pallium and are concentrated in song control areas including HVC, RA, LMAN, Area X, and DLM (Ryan et al., 1981; Bottjer and Alexander, 1995). Patterns of δ-OR expression mirrored these results, with δ-OR mRNA being localized to the song control nuclei (LMAN, HVC, RA), all parts of the pallium and hippocampus, and Area X expressing the highest levels of these receptors in adult male zebra finches (Khurshid et al., 2009; Parishar et al., 2021).

As in rodents (see above), it is possible that the expression of δ-ORs and their ligands (enkephalins) may be developmentally regulated in songbirds as well. An earlier study has found higher levels of δ-OR expression in Area X and RA of juvenile juncos (Gulledge and DeViche, 1995), whereas Carrillo and Doupe (2004) have shown that higher levels of leu-enkephalin are present in Area X in juvenile zebra finches, compared to those in adult birds of both species. Another study by Wada et al. (2006) demonstrated that singing for a 30-min duration led to the expression of preproenkephalin (PENK, the precursor of enkephalin) in the song control regions HVC and Area X in adult

and juvenile male zebra finches. Furthermore, the expression of PENK (which primarily binds δ-ORs) as well as mRNA for μ-ORs was upregulated in the mPOA (medial preoptic nucleus) in male European starlings (*Sturnus vulgaris*) during fall, a season marked by increased undirected singing (Riters et al., 2014). The mPOA is connected to the ventral tegmental area (VTA) in male starlings, and lesions of this nucleus lead to deficits in the motivation to sing to females and other courtship-associated behaviors (Riters and Ball, 1999; Alger et al., 2009).

Taken together, these findings suggest that the endogenous opioid system may be involved in modulating song-induced reward associated with positive affect [reviewed in Riters (2011) and Riters et al. (2019)] and may also be involved in guiding vocal learning during development.

## THE ROLE OF THE ENDOGENOUS OPIOID SYSTEM IN VOCALIZATION AND THE MOTIVATION TO SING

Besides their involvement in reward and motivation in mammals, μ-ORs are also important for socialization and singing in birds. Female-directed (FD) singing is a highly motivated behavior in which both the mesolimbic dopaminergic systems as well as the opioid system are involved. Recent studies have shown that blocking dopamine receptors using antagonists leads to a decrease in courtship singing in zebra finches (Schroeder and Riters, 2006; Rauceo et al., 2008). The expression of endogenous opioids has been demonstrated in song control nuclei and areas important for motivation and reward in zebra finches (Bottjer and Alexander, 1995; Carrillo and Doupe, 2004). Furthermore, systemic administration of high doses of naloxone (a general opioid receptor agonist with higher affinity for μ- compared to δ-ORs) led to small increases in the number of FD songs in male starlings (Riters et al., 2005), whereas there was a significant decrease in their number following injections of the μ-OR agonist, fentanyl (Schroeder and Riters, 2006). In addition to the endogenous opioids, μ-ORs are expressed in the song control nuclei as well as the VTA-SNc complex in adult male zebra finches (Khurshid et al., 2009, 2010). In contrast to their effects on male starlings, systemic administration of low doses of the opioid antagonist naloxone leads to a decrease in both FD and undirected (UD) singing in adult male zebra finches. Despite different results in the two species, both sets of findings suggest that ORs are involved in the motivation to sing. In naloxone-treated birds, the decrease in the motivation to sing was accompanied by changes in the quality of song: spectral features (including goodness of pitch, frequency, and amplitude modulation) decreased, whereas the duration of songs and intersyllable intervals (temporal features) increased in length, compared to vehicle-treated controls (Khurshid et al., 2010).

Studies on starlings by Kelm-Nelson et al. (2013) and Riters et al. (2014) have demonstrated that mPOA is involved in the motivation to sing. Kelm-Nelson and Riters (2013) have demonstrated that high levels of μ-ORs and enkephalin are present in mPOA in birds which are poor singers (Kelm-Nelson et al., 2013). As mentioned above, Riters et al. (2014) have reported an increase in the expression of PENK and μ-OR mRNA in the mPOA of male starlings during undirected singing in fall. More recently, research on male European starlings suggests that there is a correlation between reward associated with singing behavior and opioid-related gene expression in mPOA. These findings have been confirmed by Stevenson et al. (2020), wherein blocking μ-ORs in mPOA leads to a significant decrease in undirected song and hinders the association between singing and a positive affective state.

Recent studies have shown that blocking ORs with naloxone specifically in components of the AFP, as opposed to systemic injections, led to changes in the motivation to sing as well as those in the acoustic features of FD songs in adult male zebra finches (Kumar et al., 2019, 2020). Infusions of naloxone into LMAN (Kumar et al., 2019) resulted in a significant decrease in the number of FD songs (Kumar et al., 2020). Blocking ORs in both LMAN and Area X led to significant decreases in the length of motifs produced during FD song. Whereas blocking ORs in LMAN led to significant decreases in the amplitude modulation of motifs at a specific dose (100 ng/ml) of naloxone (Kumar et al., 2019), the same manipulation in Area X led to significant decreases in frequency and amplitude modulation and pitch goodness as well as a significant increase in pitch (Kumar et al., 2020). Additionally, blocking ORs in LMAN and Area X led to changes in the spectral quality of individual syllables in directed songs. Furthermore, naloxone infusion into Area X resulted in a local increase in dopamine. These results suggest that altering opioid modulation in LMAN and Area X may lead to changes downstream at the level of the ventral tegmental area (VTA) which sends dopaminergic projections to Area X, among other targets (Gale et al., 2008).

## THE ROLE OF OPIOID RECEPTORS IN LEARNING

### μ-ORs and Learning

Besides playing a role in modulating the motivation to sing as well as the spectro-temporal features of song, both μ- and δ-ORs may play a role in modulating song learning since they are present in components of the AFP, including Area X, LMAN, and DLM (Khurshid et al., 2009; Parishar et al., 2021). These findings are supported by earlier reports demonstrating that these receptors are involved in different kinds of learning.

### Studies in the Rodent Model System

Aloyo et al. (1993) showed that μ-ORs could modulate associative learning. Using Pavlovian conditioning in rabbits, they conditioned the nictitating membrane reflex to an audio tone using an air puff. Whereas intraventricular administration of saline did not interfere with the learning process, intraventricular injections of D-Ala, Me- Phe-, Gly-ol enkephalin (DAMGO), a μ-OR agonist, impaired conditional learning in experimental animals. This effect was blocked by μ-OR antagonist naloxone (Aloyo et al., 1993). Furthermore, Loh and Galvez (2014) demonstrated that opioid modulation regulated associative learning in a rodent model system. They used a trace

paradigm – whisker–trace–eye blink (WTEB) conditioning, wherein an eye blink, elicited using a periorbital electric shock, was conditioned to whisker stimulation. They observed that if naloxone was administered before the conditioning, it was capable of significantly impairing associative learning. However, administration of naloxone after learning did not have any effect on the conditional association in this paradigm (Loh and Galvez, 2014).

To further establish that μ-ORs are involved in motor learning, Lawhorn et al. (2009) used the dermorphin-saporin toxin which specifically targets and destroys neurons in the striosomes which express μ-OR in the basal ganglia. When these mice were tested on motor tasks, they showed specific impairments only on the rotarod test. Striosomes project to substantia nigra pars compacta, a major source of dopaminergic input to the striatum, and ablating μ-OR-positive neurons in this region would lead to a decrease in dopamine release. Since dopaminergic feedback from the midbrain to the striatal and cortical circuits may provide the necessary reinforcement needed to learn and perform on the rotarod, its absence would lead to deficits in motor learning (Lawhorn et al., 2009). In contrast to this study, Cominski et al. (2014) has shown that the loss of μ-ORs leads to an increase in hippocampal neurogenesis which in turn facilitates spatial learning. Furthermore, the μ-OR antagonist naltrexone is known to facilitate spatial learning and memory formation in mice by increasing AMPA receptor phosphorylation and membrane insertion (Kibaly et al., 2016). Yet another study (Laurent et al., 2015) has shown that μ- and δ-ORs play important roles in incentive learning and in value-based and stimulus-based decision-making in mice.

Although these learning paradigms cannot be compared directly to vocal learning in birds, striatal-based learning involves different aspects of social association (Carouso-Peck and Goldstein, 2019), timing (Gobes et al., 2019) and cued dopaminergic input (Gadagkar et al., 2016), each of which are important factors for vocal learning.

## Studies on δ-ORs and Learning in the Chicken Model System

Initial studies on the involvement of δ-ORs in learning were performed on chicks (*Gallus gallus*) by training them on a passive avoidance task. In this experiment, birds were provided with steel beads coated with a bitter tasting chemical called methyl anthranilate (MEA), which they are averse to. A different set of birds, used as controls, were presented with steel beads coated with water. Both experimental and control birds were presented with a single steel bead and their latency to peck and aversive behavior after pecking at the bead was measured to estimate their behavioral response. Experimental birds learned to associate the steel bead with the bitter taste and avoided pecking at the other bead whereas control birds did not avoid the bead. Administration of leu-enkephalin and D-Pen–2, L-Pen–5 enkephalin (DPLPE), a δ-OR selective agonist, into the intermediate medial hyperstriatum ventrale 5 min before training resulted in poor performance on this task. This study also demonstrated that the amnesia caused by δ-OR agonists was reversed by administration of δ-OR antagonists (Patterson et al., 1989). Csillag et al. (1993) also used a passive avoidance task similar to that used by Patterson et al. (1989) to establish the role of these receptors in learning. In their paradigm, 1-day old chicks were trained to peck at a chrome bead coated with methyl anthranilate or water (control). Neural tissue from the trained birds was tested for binding with radio-labeled ligands specific for δ-, μ-, and κ-ORs. Interestingly, there was higher binding for the δ-OR ligand 3H-DPDPE in the striatal areas medial striatum (MSt) and lateral striatum (LSt) (Csillag et al., 1993) in birds which performed well on the avoidance task. In another study, site-specific injections of δ-OR antagonist ICI-174,864 in MSt abolished the avoidance learning for the bitter-tasting bead in 1-day old chicks (Freeman and Young, 2000).

## Studies on δ-ORs and Learning in the Rodent Model System

Recent studies on δ-ORs and learning have provided further evidence that these receptors show changes in expression patterns based on the learning experience. Bertran-Gonzalez et al. (2013) trained mice on a Pavlovian instrumental transfer protocol (PIT) in which associations learnt following the delivery of a reward influences the behavior toward two external cues. In this study, a sound was linked to a food reward, followed by training on a second task where a lever press delivered the food reward. A successful test session of the instrumental transfer comprised of the mouse pressing the lever when the sound stimulus was presented. The researchers observed an increase in the expression of δ-ORs in cholinergic interneurons within the shell of nucleus accumbens in mice that had learnt the reward association as well as the instrumental transfer (Bertran-Gonzalez et al., 2013). Interestingly, the level of learning determined the extent of δ-OR expression. These results were similar to those shown in the earlier study by Csillag et al. (1993), which demonstrated an increased binding for δ-ORs in the striatum of chicks trained on an avoidance task.

Another study explored the function of δ-ORs in the hippocampus and their role in learning by using δ-OR gene knockout mice (*Oprd1*$^{-/-}$). These gene-deficient mice were poor in place recognition (hippocampal-based learning). Interestingly, they performed better on tasks involving the striatum, such as balancing on an accelerating rotarod, compared to wild type mice (controls). Peripheral injections of the δ-OR antagonist naltrindole in normal mice were able to produce learning deficits similar to the *Oprd1*$^{-/-}$ mice. This study concluded that δ-ORs are involved in hippocampal-based learning and possibly modulate parvalbumin interneurons that regulate long term potentiation (Le Merrer et al., 2013). Recently, Leroy et al. (2017) has demonstrated a more specific role for hippocampal δ-ORs. They injected naltrindole into the CA2 region of the hippocampus of young mice interacting with their mates. The study revealed that blocking δ-ORs impairs social memory formation by a failure to induce plasticity in the CA2 region (Leroy et al., 2017).

Despite these findings, learning and memory can never be separated from internally reinforcing reward signals, making it difficult to isolate the role of opioid receptors in learning and memory formation from reward related processes in the brain.

It is therefore possible that opioid receptors might modulate learning and memory not just via inhibition of local circuits but also by regulating dopaminergic signaling.

## MODULATION OF DOPAMINERGIC SIGNALING VIA δ-ORs: EFFECTS OF THE OPIOID SYSTEM ON THE REWARD PATHWAY

The interaction between μ-ORs and dopaminergic signaling has been extensively studied in association with addiction and pain pathways (Van Vliet et al., 1990; Li et al., 2016; Burns et al., 2019). Recently, Galaj et al. (2020) have shown that μ-ORs are highly expressed by the GABAergic neurons of the substantia nigra pars reticulata (SNr), that sends dense innervations to the dopamine rich substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) in mice. They observed that optogenetic activation of SNr GABAergic neurons increased heroin intake and reduced heroin-primed drug seeking, whereas inhibition of these neurons induced optical cranial self-activation and place-preference. These results hint at the opioid modulation of reward via inhibitory projections of the SNr to SNc and VTA (Galaj et al., 2020).

Earlier research shows that δ-OR signaling also influences dopaminergic circuitry and vice versa. Dopaminergic afferents acting via D2 receptors are known to inhibit the production of enkephalin which preferentially binds δ-ORs in striatal neurons (Normand et al., 1988; Jiang et al., 1990; Llorens-Cortes et al., 1991). Conversely, blocking D2 receptors increases the production of enkephalin in the striatum (Steiner and Gerfen, 1999). This coupling between δ-OR and dopaminergic signaling may be responsible for memory retrieval and increased retention of information in mice with induced amnesia (Dubrovina and Ilyutchenok, 1996). Dopaminergic release in the striatum also regulates predictive learning by signaling for error in performance (Iordanova, 2009).

Interestingly, the pharmacological activation of μ-ORs in the ventral striatum induces the activation of δ-OR subtypes, which further enhances dopamine release within the area (Hirose et al., 2005). Opioids can exert control over dopaminergic signaling via different mechanisms. The ORs expressed by GABAergic neurons in VTA or on medium spiny neurons in the striatum lead to the disinhibition of dopaminergic neurons thereby causing an increased release of dopamine. Furthermore, ORs present on dopaminergic neurons in the VTA can inhibit the release of dopamine in the striatum [reviewed in Xi and Stein (2002)]. Taken together, a fine balance between dopaminergic and opioid signaling is required for behavioral reinforcement.

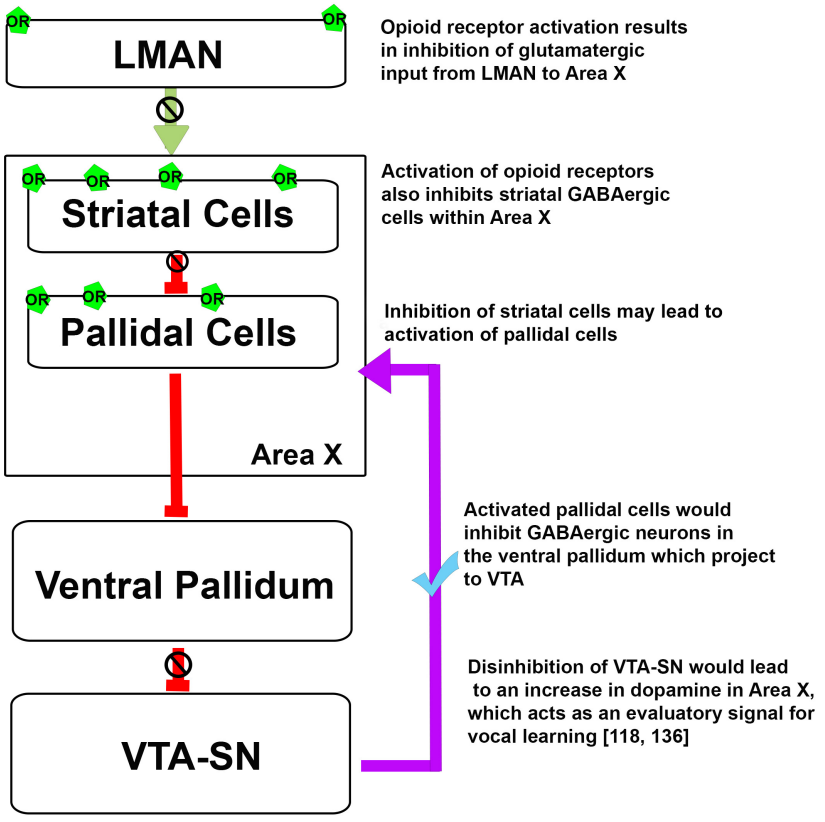


**FIGURE 3 |** Possible mechanism for the opioid modulation of dopaminergic input to Area X and the regulation of vocal learning. Green arrow, Glutamatergic projection; red arrows, GABAergic projections; violet arrow, dopaminergic projection, OR, opioid receptors.

The error prediction and behavioral reinforcement through dopaminergic signaling from the midbrain is essential for vocal learning as well. Evaluative signals in the form of bursts of dopamine released in Area X in zebra finches shape the spectral features of song according to the desired template (Gadagkar et al., 2016; Xiao et al., 2018). Since μ- and δ-ORs are present in components of the AFP (such as LMAN and Area X) which are linked to the VTA-SN complex in birds (Ding and Perkel, 2002; Kumar et al., 2019, 2020), it is possible that the complex interplay between OR activation and dopamine release plays a role in the structuring of vocal patterns during song learning, as discussed below.

## OPIOID REGULATION OF SOCIALLY REWARDING BEHAVIOR AND VOCALIZATIONS MAY ALSO BE INVOLVED IN THE REGULATION OF VOCAL LEARNING

Vocal learning is a social process (Tchernichovski et al., 2017) which is rewarding in itself (Riters and Stevenson, 2012). Whereas rodents do not display socially guided vocal learning, certain vocalizations are associated with socially rewarding behavior (Humphreys and Einon, 1981). One such behavior is social play, wherein rodents emit short bursts of high frequency vocalizations (<0.5 s; ∼ 50 Hz) while playing. A play-associated place preference can be established in rodents (Normansell and Panksepp, 1990), suggesting that it is intrinsically rewarding. Similarly, singing-induced place preference can be established in European starlings and zebra finches singing undirected songs (Riters and Stevenson, 2012), also indicating that undirected song, which is produced during learning and for song maintenance, is intrinsically rewarding [reviewed in Riters et al. (2019)]. This play-associated vocal behavior is also demonstrated when young rodents are placed in a spatial location associated with play behavior (Knutson et al., 1998). Opioid agonists such as morphine increase these play-associated vocalizations when administered chronically (Hamed and Boguszewski, 2018). Furthermore, response to play vocalizations is enhanced by opioid agonists and reduced by antagonists (Wohr and Schwarting, 2009). These findings suggest that opioids enable behaviors associated with social activity. In case of a social learning process, like vocal learning, this could help in shaping vocal structure by directing attention toward adult vocalizations produced by tutors and/or other members of the flock (Chen et al., 2016).

Earlier studies (Khurshid et al., 2010; Kumar et al., 2019, 2020) have demonstrated that the opioid system can modulate different aspects of singing. Based on the neuroanatomical localization of ORs in the song control areas of oscines, it is possible that these receptors may be involved in vocal learning (Gulledge and Deviche, 1999). As mentioned above, findings from Wada et al. (2006) have demonstrated that there is an increase in the level of the opioid ligand pre-proenkephalin in Area X after singing in juvenile male birds. An increase in the activation of ORs in Area X could potentially lead to a decrease in the activity of MSNs and a disinhibition of pallidal neurons. This would ultimately lead to the disinhibition of the VTA-SN complex and an increase in DA release in Area X, which acts as an evaluatory signal, shown to play an important role in vocal learning (**Figure 3**; Gadagkar et al., 2016; Xiao et al., 2018). Additionally, ORs are involved in associative learning, modulate dopaminergic signaling, and are differentially expressed in the brain in the developmental phase rather than in the adulthood in mammals and songbirds, which suggests that ORs may influence cognitive processes such as vocal learning.

## CONCLUSION

A number of studies on different species of mammals and birds have demonstrated that the endogenous opioid system is involved in higher cognitive functions including learning. Whereas the endogenous opioid system has been shown to modulate the motivation to vocalize and also effects the acoustic properties of song in different species of songbirds, recent anatomical findings demonstrate that it is present in components of the AFP. Given that the AFP is involved in learning, is connected to the VTA-SN complex and can influence dopamine release, the endogenous opioid system may potentially modulate vocal learning during the sensitive period. Experiments wherein the ORs are blocked or activated in young songbirds at different time points during the sensitive period would provide further insights into the role of the endogenous opioid system in vocal learning.

## AUTHOR CONTRIBUTIONS

UAS and SI contributed to scientific discussions and writing leading to this review. SI approved the final manuscript. Both authors contributed to the article and approved the submitted version.

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# Mismatch Responses Evoked by Sound Pattern Violation in the Songbird Forebrain Suggest Common Auditory Processing With Human

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Learning sound patterns in the natural auditory scene and detecting deviant patterns are adaptive behaviors that aid animals in predicting future events and behaving accordingly. Mismatch negativity (MMN) is a component of the event-related potential (ERP) that is reported in humans when they are exposed to unexpected or rare stimuli. MMN has been studied in several non-human animals using an oddball task by presenting deviant pure tones that were interspersed within a sequence of standard pure tones and comparing the neural responses. While accumulating evidence suggests the homology of non-human animal MMN-like responses (MMRs) and human MMN, it is still not clear whether the function and neural mechanisms of MMRs and MMN are comparable. The Java sparrow (*Lonchura oryzivora*) is a songbird that is a vocal learner, is highly social, and maintains communication with flock members using frequently repeated contact calls and song. We expect that the songbird is a potentially useful animal model that will broaden our understanding of the characterization of MMRs. Due to this, we chose this species to explore MMRs to the deviant sounds in the single sound oddball task using both pure tones and natural vocalizations. MMRs were measured in the caudomedial nidopallium (NCM), a higher-order auditory area. We recorded local field potentials under freely moving conditions. Significant differences were observed in the negative component between deviant and standard ERPs, both to pure tones and natural vocalizations in the oddball sequence. However, the subsequent experiments using the randomized standard sequence and regular pattern sequence suggest the possibility that MMR elicited in the oddball paradigm reflects the adaptation to a repeated standard sound but not the genuine deviance detection. Furthermore, we presented contact call triplet sequences and investigated MMR in the NCM in response to sound sequence order. We found a significant negative

shift in response to a difference in sequence pattern. This demonstrates MMR elicited by violation of the pattern of the triplet sequence and the ability to extract sound sequence information in the songbird auditory forebrain. Our study sheds light on the electrophysiological properties of auditory sensory memory processing, expanding the scope of characterization of MMN-like responses beyond simple deviance detection, and provides a comparative perspective on syntax processing in human.

**Keywords: songbird, mismatch negativity, mismatch response, caudomedial nidopallium (NCM), local field potentials**

## INTRODUCTION

Mismatch negativity (MMN) is a component of the scalp-recorded event-related potential (ERP) that occurs in human when they are exposed to unexpected or rare sensory stimuli (Näätänen et al., 1978). Auditory MMN is typically measured by the presentation of an oddball task in which infrequent, deviant sounds are embedded in a sequence of frequent, standard sounds. Typically, the deviant sound has one acoustic feature (such as pitch or duration) that differs from the standard sound. Several studies on human MMN suggest that the main sources of MMN are located in the auditory cortex, with some contribution from the temporal, frontal, and parietal regions (Scherg and Picton, 1988; Csépe et al., 1992; Alho, 1995; Molholm et al., 2005). MMN can be elicited without attention through an automatic, pre-attentive process in the auditory cortex (Näätänen et al., 2001). This pre-attentional process is considered a useful tool to investigate the cognitive function related to recognition categories, abstract patterns, sound localization, and psychiatric and developmental disorders (Näätänen, 1995, 2003; Lopez et al., 2003; Nagai et al., 2013).

Several animals, including non-human primates (Javitt et al., 1992; Komatsu et al., 2015), dogs (Howell et al., 2011), cats (Csépe et al., 1987), rodents (Ruusuvirta et al., 1998; Umbricht et al., 2005; Ehrlichman et al., 2008; Shiramatsu et al., 2013; Christianson et al., 2014; Harms et al., 2014), and pigeons (Schall et al., 2015), show MMN-like responses, called mismatch responses (MMRs). Previous studies demonstrated that MMRs are generated from the higher-order areas of the auditory cortex in cats (Pincze et al., 2001) and rats (Shiramatsu et al., 2013). While accumulating evidence suggests the homology of non-human animal MMR and human MMN, it is still not clear whether the function and neural mechanisms of MMR and MMN are comparable. There is controversy as to whether MMR reflects sensory memory-based responses, such as MMN, or neural adaptation, such as stimulus-specific adaptation (SSA) (May and Tiitinen, 2010). Repeated exposure to the standard sound results in attenuation of neural responses in the auditory cortex, whereas rare deviant sounds elicit larger neural responses. To resolve this controversy, a number of studies have applied several control paradigms and violations of abstract rules in an attempt to elicit and record responses reflecting genuine deviance detection (Schröger and Wolff, 1996; Ruusuvirta et al., 2007; Wild et al., 2009; Ruhnau et al., 2012; Astikainen et al., 2014; Harms et al., 2014; Attaheri et al., 2015).

In this study, we investigated intracranial MMR from the songbird caudomedial nidopallium (NCM), a forebrain structure considered to be analogous to the higher-order auditory cortex in mammals (Bolhuis and Gahr, 2006). Songbirds communicate with each other by vocalization and develop a complex vocal pattern through vocal learning. The Java sparrow (*Lonchura oryzivora*), a species of songbird, is highly social and maintains communication with flock members using frequently repeated contact calls. They use seven types of calls (Goodwin and Woodcock, 1982) and emit similar but distinct calls in disparate situations of aggressiveness or affinity, which are composed of short syllables with narrow intervals repeated in quick succession (Baptista and Atwood, 1980; Goodwin and Woodcock, 1982; Furutani et al., 2018). In addition, male birds learn the song from their fathers and coordinate bill-click sound with song sequences, suggesting that non-vocal sounds are integrated with vocal courtship signals (Soma and Mori, 2015). It is crucial for the Java sparrow to process repeated sound signals and detect novel ones.

The NCM receives input from the primary area field L, which has inputs from the auditory thalamus (Vates et al., 1996). A substantial body of evidence suggests that the NCM is involved in the processing of complex natural vocalizations and memorizing of tutor song to imitate during vocal learning (Chew et al., 1996; Bolhuis and Gahr, 2006; Yanagihara and Yazaki-Sugiyama, 2016). It has been reported that neurons in NCM show stimulus-specific habituation to natural sounds such as conspecific and heterospecific vocalizations (Mello et al., 1995; Chew et al., 1996). In addition, neurons in the secondary auditory areas, i.e., NCM and caudomedial mesopallium (CMM), show response bias to deviant sounds in an oddball paradigm during multiunit and single-unit recordings in both anesthetized birds (Beckers and Gahr, 2012; Ono et al., 2016) and awake-restrained birds (Dong and Vicario, 2018). These studies suggest that the NCM integrates auditory information over both long- and short-time windows, and focal attention to auditory stimuli is not required for this process.

Furthermore, NCM neurons have been reported to show sensitivity to differences in sound sequence order (Lu and Vicario, 2014; Ono et al., 2016). The ability to extract rules from sound sequences is considered to be important for vocal learning. Human infants demonstrated the ability to extract grammatical rules in an artificial language task using made-up words (e.g., ABA grammar, such as "ga ti ga" and ABB grammar, such as "ga ti ti") (Marcus et al., 1999). Recently, some animal studies measuring MMR reported that the auditory cortex of rats

(Astikainen et al., 2014) and frontal cortex of macaques (Attaheri et al., 2015) respond to changes in a pattern of sound sequences. This suggests that some cognitive elements related to language learning, such as the ability to extract grammatical rules, might have originally evolved for more general behavioral skills.

In this study, we recorded local field potentials (LFPs) from the NCM of Java sparrows *via* chronically implanted electrodes with a wireless transmitter under freely moving conditions. We measured MMR in response to the deviant sounds in the single sound oddball task and in response to the sound element order in a triplet sequence oddball task. We expect that the songbird is a potentially useful animal model that will both further our understanding of the neural mechanisms of auditory sensory memory processing and provide a comparative perspective on syntax processing in human.

# MATERIALS AND METHODS

## Subjects

Nine adult Java sparrows (5 male and 4 female birds) over 200 days post-hatch were used. Five birds were purchased from local suppliers, and the rest were birthed and raised in a breeding colony at the University of Tokyo. Birds were housed in our aviary under a 14:10 h light/dark cycle. Food and water were provided *ad libitum*. All animal experiments and housing conditions were approved by the Institutional Animal Care and Use Committee at the University of Tokyo.

## Experiment Design and Auditory Stimuli

Auditory stimuli were presented to the subjects from a speaker positioned at 15 cm from where the subjects sat on the perch. The sound pressure level was approximately 70 dBA when measured around the bird's head (NL-27; Rion). Subjects remained on the perch for the majority of the time when auditory stimuli were played. Sounds were synthesized using sound analysis software (SASLab Pro; Avisoft Bioacoustics). To acclimate subjects to the experimental setup, they were placed in a plastic cage (150 mm × 305 mm × 220 mm) in the experimental chamber (600 mm × 500 mm × 500 mm) at least 30 min before the first session.

## Oddball Paradigm

Three types of sound pairs were used for ascending and descending oddball sequences (**Figures 1A,B**): (1) two pairs of pure 2 vs. 3 kHz and 5 vs. 6 kHz tones (duration: 50 ms, rise/fall times: 8 ms), (2) two types of song elements (duration: 75 ms, peak frequency: 2.0 and 4.3 kHz, and entropy: 0.297 and 0.339) from a bird, and (3) two calls from two individuals (duration: 26 ms, peak frequency: 2.9 and 3.7 kHz, and entropy: 0.309 and 0.484). Two sequences were presented for each sound pair with one stimulus serving as the frequent standard stimulus (87.5%) the other as the rare deviant stimulus (12.5%), and vice versa. Sound stimuli were pseudo-randomly delivered in sequences with a 600-ms inter-onset interval (IOI) between sounds. Standard and deviant stimuli presented a total of 4,400 times in each sequence using MATLAB (The MathWorks, Inc., Natick, MA, United States). In addition, two control procedures using pure tone oddball sequences were conducted to verify the influences of the probability and predictability of stimuli (**Figure 1A**). To investigate whether changing the probability affects the response, eight frequencies (1, 2, 3, 4, 5, 6, 7, and 8 kHz) were presented with a probability of 12.5% in a pseudorandom order as a sequence that prompted the same level of adaptation as the deviant (termed "randomized standards sequence"). To investigate the effect of predictability on the response, five frequencies (2, 3, 4, 5, and 6 kHz) were presented in a regular pattern from low to high frequency and then back down to low frequency, repetitively (termed "regular pattern sequence"). In this sequence, the highest and lowest frequency stimuli corresponded to the deviant in the oddball sequence (pure 2 kHz tones of descending oddball sequence in a pair of 2 vs. 3 kHz tones and 6 kHz tones of ascending oddball sequence in a pair of 5 vs. 6 kHz tones) and were presented with the same probability as in the oddball sequence. The second highest and lowest frequency stimulus corresponded to the standard in the oddball sequence.

## Triplet Sequence Paradigm

Triplet sound sequences (**Figure 1B**) were synthesized using five calls (duration: 21.2 ± 3.1 ms, peak frequency: 2.84 ± 0.48 kHz, entropy: 0.33 ± 0.03, and mean ± SD) from five Java sparrows (2 male and 3 female birds). The IOI between each call was 125 ms and between each triplet sequence was 225 ms, which was presented in groups of three based on the paradigm suggested by Astikainen et al. (2014). AAB types, which consisted of two identical calls followed by a different call, were presented as the standard sequences (16 different variants, 90%). The deviants consisted of two different types as follows: (1) "pattern-obeying deviants" that had the same AAB type pattern as the standards but differed physically (two different variants, 5%) and (2) "pattern-violating deviants" that were ABB type and thus differed from the standard sequences both physically and in pattern type (two different variants, 5%). All stimulus types were pseudo-randomly presented 3,600 times.

## Surgery and Electrophysiological Recordings

The birds were anesthetized with pentobarbital (6.48 mg/ml; 60 μl/10 g body weight) by intraperitoneal injection, and their heads were fixed in a stereotaxic apparatus with ear bars and a beak holder. Then, recording electrodes (Platinum-Iridium wires coated with Teflon with bare tips, 0.127 mm diameter, impedance 5 MΩ) of a wireless dual-channel transmitter (weight: 2.3 g, EPOCH-T2; Biopac Systems Inc., Goleta, CA, United States) were stereotactically implanted into bilateral NCM. The birds were allowed to recover for at least 3 days after surgery before the first recordings. We recorded LFP in freely behaving birds. Each channel of LFP signals was amplified 800-fold, sampled at 100 Hz (voltage range: ± 2.5 mV) with the transmitter, sent to a receiver (EPOCH-RAT-EEG-SYS; Biopac Systems Inc.), and stored on a PC using an acquisition unit (CED Power 1401) and software (Power 1401

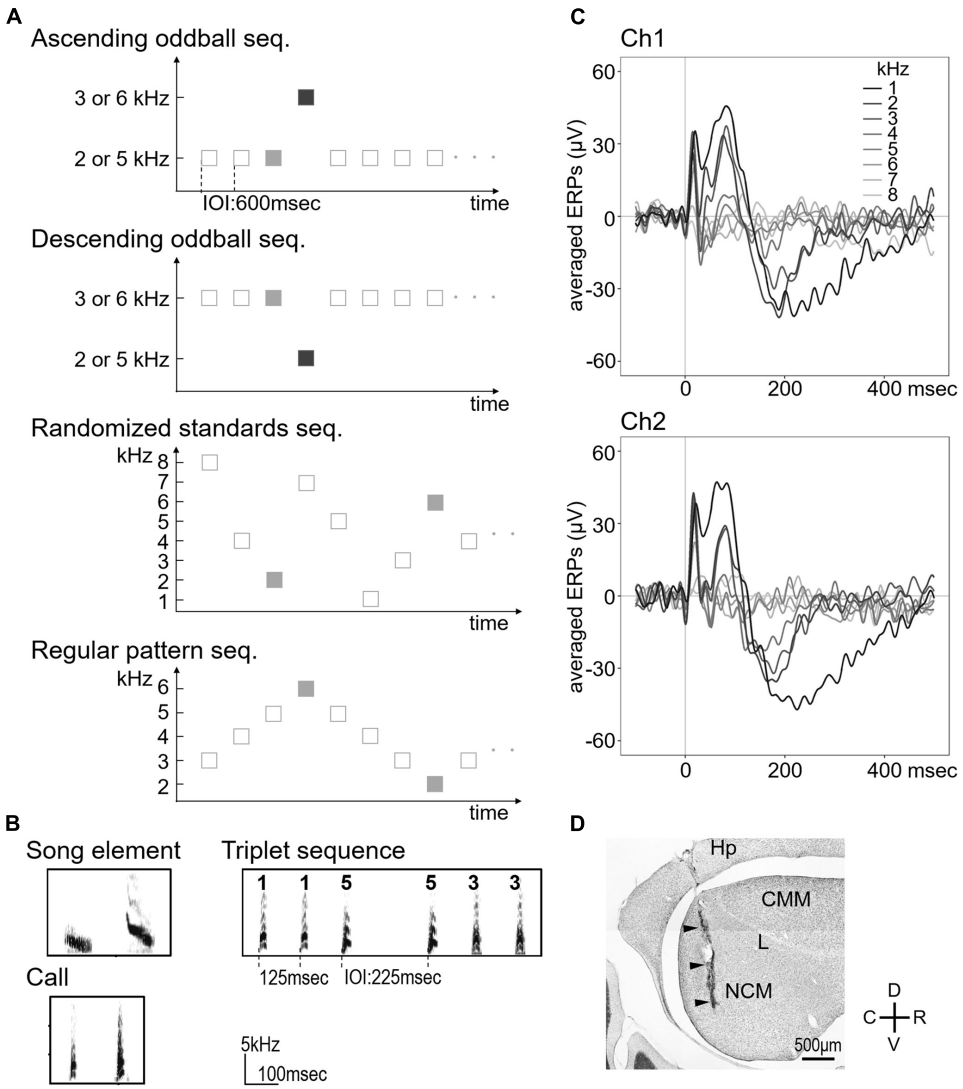


**FIGURE 1 |** Sequence designs used in the passive auditory paradigm and averaged event-related potentials (ERPs) to pure tones. **(A)** Schematic diagram of the oddball paradigm. Deviant stimuli (dark gray squares) were presented randomly in ascending and descending oddball sequences. Light gray squares indicate standard/control stimuli to be analyzed for neural responses. **(B)** Sound spectrograms of the song elements and contact calls used as auditory stimuli (left). Examples of the triplets used in the triplet sequence (right). **(C)** Averaged ERPs were recorded from each hemisphere in response to the randomized standard sequence ($n$ = 7, 1–8 kHz presented at an equal probability of $p$ = 0.125). There was no significant difference in ERPs between the two channels in all experiments. **(D)** Representative Nissl-stained parasagittal section showing the location of the electrode. ERP, event-related potential; Hp, hippocampus; CMM, caudomedial mesopallium; L, field L; NCM, caudomedial nidopallium; D, dorsal; V, ventral; R, rostal; C, caudal. Arrowheads indicate the electrode tract.

and Spike2; Cambridge Electronic Design Ltd., Cambridge, United Kingdom). **Figure 1C** shows the ERPs elicited by each of the different frequencies in the randomized standard sequence. After all of the experiments, the birds were deeply anesthetized by an overdose of pentobarbital and perfused with 1 × PBS and then 4% paraformaldehyde/1 × PBS. Sagittal sections, 40 μm thick, were cut on a freezing microtome and stained with cresyl violet to verify the electrode position (**Figure 1D**).

## Electrophysiological Data Analysis

Local field potential data processing was performed offline using MATLAB and the open-source toolbox EEGLAB (Delorme and Makeig, 2004). LFP signals were band-pass filtered at 0.1–50 Hz and were analyzed in 600-ms epochs (100 ms before and 500 ms after stimulus onset) using baseline correction over a 100-ms pre-stimulus interval. Epochs with body movement artifacts were excluded manually. Raw recording data were averaged across all recording conditions for each channel. The mean amplitudes of ERP components were analyzed using repeated-measures ANOVAs. We treated each channel as independent recording data because electrode positions, such as anterior-posterior axes and depth, within an individual were varied in the NCM. Therefore, we investigated the difference of the data recorded from left and right hemispheres as a

between-subject factor to consider laterality. For the single sound oddball paradigm, the between-subject factor was channel (left and right). The within-subject factors were stimulus type (standard and deviant) and frequency of sound (2, 3, 5, and 6 kHz), or song element type or call type. For the triplet sequence paradigm, the between-subject factor was channel (left and right). The within-subject factors were stimulus type (standard "AAB," pattern-obeying deviants "AAB," and pattern-violating deviants "ABB") and time window (95–120 ms, 215–240 ms). The significance level was set at $p < 0.05$.

# RESULTS

## Event-Related Potentials to Deviant Stimuli Negatively Shifted in the Oddball Sequence

For the pure tone oddball paradigm, the group averaged auditory evoked potential consisted of an initial positive peak at approximately 20 ms, a negative peak at 50 ms followed by a positive peak latency of approximately 80 ms, and a broad negative component with a peak at approximately 150–200 ms (**Figure 2A**). A difference waveform was obtained by subtracting the standard-evoked ERP from the deviant-evoked ERP. A mean amplitude measure was extracted over a 165–190 ms latency window corresponding to the negative peaks of the difference waveform. To evaluate the differences between the ERPs, the mean amplitudes of ERP components were analyzed using repeated-measures ANOVAs. The ERPs to the deviant significantly shifted ($F_{1,12} = 18.0290$, $p = 0.0011$). In addition to the main effects of stimulus type, 2 and 3 kHz pure tones elicited larger amplitude ERPs than the 5 and 6 kHz tones ($F_{3,36} = 8.9812$, $p = 0.0001$). There was no significant difference in ERPs between the two-channel positions ($F_{1,12} = 0.0606$, $p = 0.8097$).

For the song element oddball paradigm, the group averaged auditory evoked potential was characterized by a positive peak at approximately 10 ms, a negative peak at 30 ms followed by a positive peak latency of approximately 45 ms, and a broad negative component with a peak at approximately 150–300 ms (**Figure 2B**). Difference waveforms consisted of a negative peak at 245–270 ms. We analyzed the differences between the mean ERP amplitudes extracted over a 245–270 ms latency window using repeated-measures ANOVA. Significant differences between standard and deviant ERPs were confirmed ($F_{1,10} = 14.7813$, $p = 0.0032$). Differences in ERPs between channels were not detected ($F_{1,10} = 0.0077$, $p = 0.9317$).

For the call oddball paradigm, the averaged auditory evoked potential consisted of an initial positive peak at approximately 20 ms, a second positive peak at 35 ms, followed by a third peak latency of approximately 50 ms, and a broad negative component with a peak at approximately 150–250 ms (**Figure 2C**). The mean ERP amplitude at 195–220 ms, corresponding to the negative peaks of the difference waveform, was analyzed by repeated-measures ANOVA. We found significant differences in waveform amplitude between deviant and standard stimuli ($F_{1,10} = 21.2892$, $p = 0.0010$). In addition to the main effects of stimulus type, there was a significant effect of call type ($F_{1,10} = 33.4938$, $p = 0.0002$). We did not find significant differences in ERPs between channels ($F_{1,12} = 1.5952$, $p = 0.2352$).

The significant differences between deviant and standard ERPs, both to pure tones and natural vocalizations, were observed in the negative component ranging from 165 to 270 ms after stimulus onset. This is comparable with MMR in human and other species.

## Possible Cause of the Negative Shift of Event-Related Potentials to Deviant Stimuli Is Stimulus-Specific Adaptation

In the oddball sequence, the probability differed between the standard and deviant stimuli. Repeated exposure to a sound causes SSA and decreased neural responses to repeated playback of a sound (Chew et al., 1995; Stripling et al., 1997; Kozlov and Gentner, 2014). To examine the effect of the probability, we compared responses to the deviant stimulus in the oddball paradigm and the control stimulus in the randomized standard sequence (in both cases, it is the same physical stimulus) (**Figure 2D**). If MMR in the oddball sequence reflects sensory memory-based responses, MMR will be detected using the randomized standard sequence as a control. In contrast, if SSA is the principal cause of the significant difference in response to the deviant and standard stimuli in the oddball sequence, MMR will not be detected using the randomized standard sequence. The mean amplitude of the ERPs at 165–190 ms was analyzed by repeated-measures ANOVA. No significant differences in ERPs were observed in stimulus type or channels (stimulus type: $F_{1,12} = 1.9127$, $p = 0.1919$; channel: $F_{1,12} = 0.0763$, $p = 0.7871$). ERPs were significantly different between frequencies of sound ($F_{3,36} = 11.4555$, $p < 0.0001$). Furthermore, in addition to equally probable stimuli, we compared responses to predictable tones in the regular pattern sequence and unpredictable deviant tones in the oddball sequence to examine the effect of predictability (**Figure 2E**). If MMR reflects prediction error elicited by the deviant, MMR will be detected using the regular pattern sequence as a control. The mean amplitude of the ERPs at 165–190 ms was analyzed by repeated-measures ANOVA. There were no significant differences in ERPs between stimulus type or channels (stimulus type: $F_{1,12} = 1.9291$, $p = 0.1901$; channel: $F_{1,12} = 0.2818$, $p = 0.6052$). ERPs were significantly different between frequencies of sound ($F_{1,12} = 6.6616$, $p = 0.0241$).

Considering that there were significant differences in the ERPs between the deviant and standard stimuli in the oddball sequence but no significant differences between the deviant stimulus in the oddball sequence and control stimuli in the randomized standard sequence, these results indicate that stimulus probability affected the waveform of ERPs. High stimulus probability reduced the amplitude of later negative peaks of ERPs. In contrast, the predictability of the stimuli did not affect ERP amplitude, as no mismatch response was seen when regular pattern sequences were presented as a control. However, another possibility for this result is that the rules of regular pattern sequence were

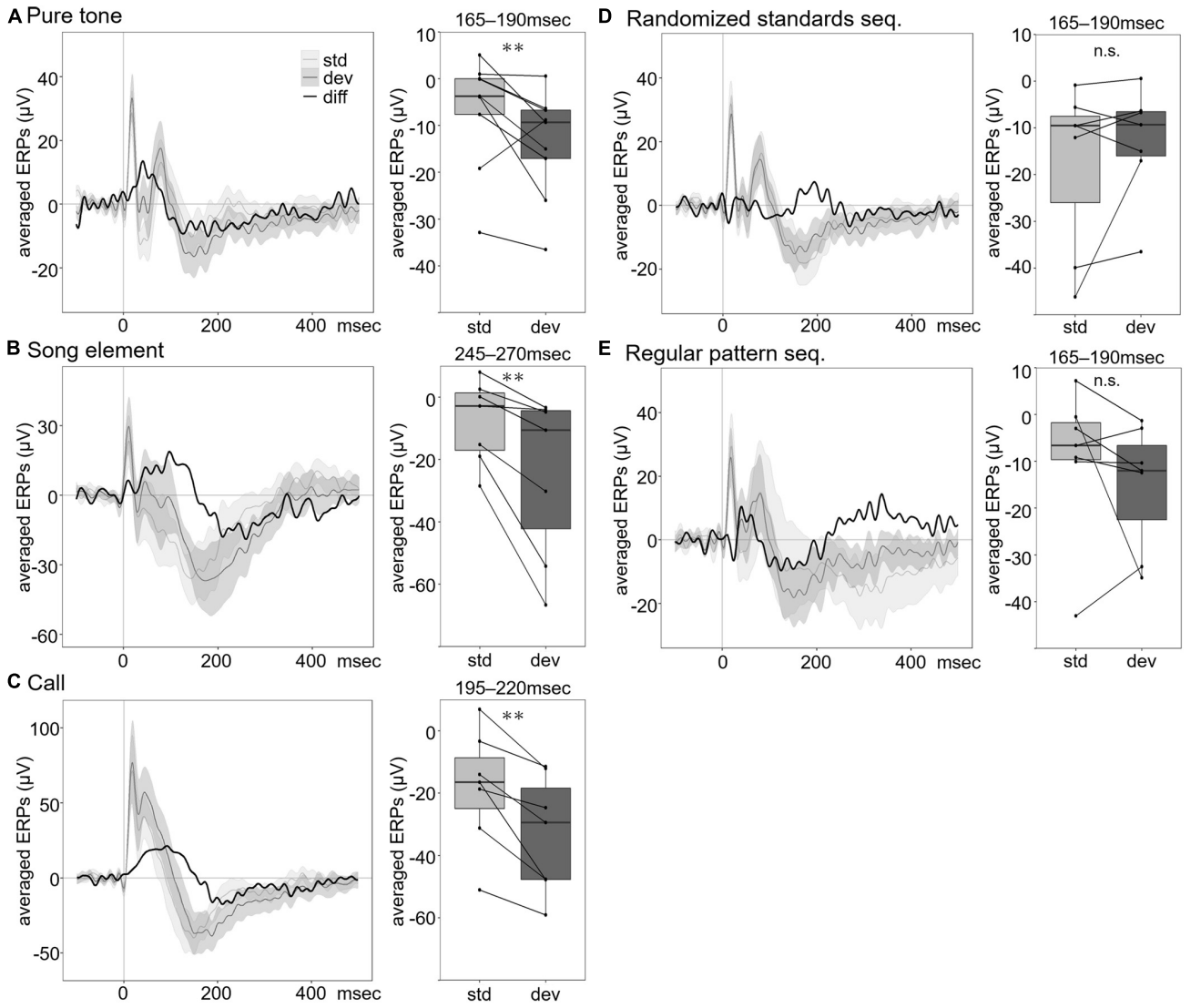


**FIGURE 2 |** Averaged ERPs to standard and deviant stimuli and averaged ERP amplitude at the specified time window. Group averaged ERPs to the oddball standard (light gray line) and deviant (dark gray line) stimuli for pure tones **(A)**, song elements **(B)**, and calls **(C)**. Comparison between group averaged ERPs of deviant stimuli in the oddball sequence and that of the standard stimuli in the randomized standard sequence **(D)** and the regular pattern sequence **(E)**. Black line shows the difference wave obtained by subtracting the ERP to standard stimuli from the ERP to deviant stimuli. The shading indicates the range of 95% CI of the waves. In the box plot on the right of the waveform, each box shows the quartiles of the ERPs to standard (light gray) and deviant (dark gray) stimuli at each time window (pure tone: 165–190 ms, song element: 245–270 ms, and call: 195–220 ms, after stimulus onset) in each sound sequence. Each dot represents the averaged ERPs for each individual (pure tone: $n = 9$, song element: $n = 7$, and call: $n = 7$). Averaged ERPs to deviant stimuli negatively shifted, compared with that of standard stimuli in the single oddball sequence, while no significant difference was found compared with averaged ERPs to standard stimuli in the randomized standard sequence ($n = 7$) and the regular pattern sequence ($n = 7$) (repeated measures ANOVA, $^{**}p < 0.01$). n.s., no significance.

not detected in NCM, and ERPs did not properly reflect stimulus predictability.

## The Event-Related Potentials in Caudomedial Nidopallium Were Sensitive to Differences in Triplet Temporal Pattern

To further examine sensitivity to the sequence order of sound patterns, we presented laboratory-created triplet sequences using natural calls. To evaluate the differences between ERPs to standard "AAB" and pattern-obeying deviants "AAB" (**Figure 3A**) or pattern-violating deviants "ABB" (**Figure 3B**), we focused on first and second negative components and compared with an averaged amplitude of LFP for each time window: 95–115 ms and 215–240 ms after stimulus onset (**Figure 3C**). These windows correspond to 95–115 ms after stimulus onset of the first and second sounds in the triplet sequence. Since the change in the pattern occurs in the second sound of the pattern-violating deviants, ERPs to second sounds are analyzed to investigate MMR and compared with the preceding ERPs to the first sound,

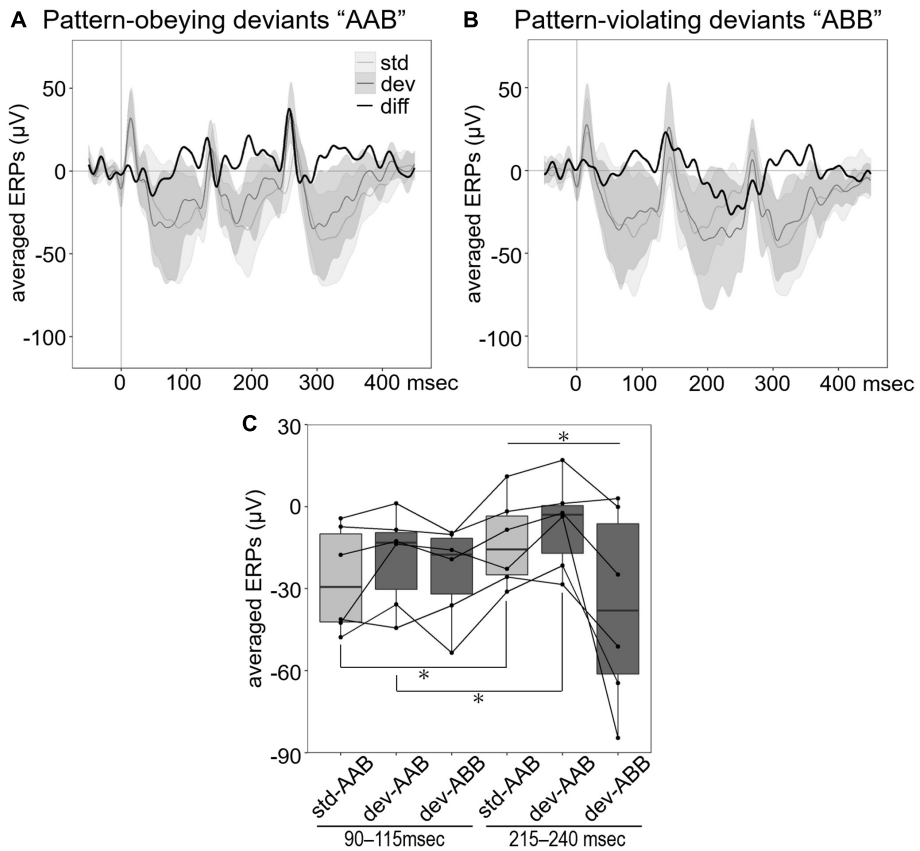


**FIGURE 3 |** Averaged ERPs to triplet sequences. **(A)** Group averaged ERPs to standard "AAB" (light gray line) and pattern-obeying deviants "AAB" (dark gray line). **(B)** Group averaged ERPs to standard "AAB" (light gray line) and pattern-violating deviants "ABB" (dark gray line). Black line in both **(A,B)** shows the difference wave obtained by subtracting the ERPs to standard stimuli from the ERPs to deviant stimuli. The shading indicates the range of 95% CI of the waves. **(C)** Each box shows the quartiles of the ERPs to standard (light gray) and deviant (dark gray) stimuli in each time window (90–115 ms for the first sound, 215–240 ms for the second sound in the triplet sequence). Each dot represents the averaged ERPs for each individual ($n$ = 6). The line connects data of the same individual. The negative waveform of ERPs to the second sound of triplet sequences reduced in standard "AAB" and pattern-obeying deviants "AAB" compared with pattern-violating deviants "ABB" (repeated measures ANOVA, $^*p < 0.05$).

which reflected most probable acoustical differences. Pattern-obeying deviants consisted of two different calls that physically differed from the standards but had the same pattern. Pattern-violating deviant differed from the standard both physically and in the pattern. The mean amplitude of the ERPs at each time window was analyzed by repeated-measures ANOVA. There was a significant effect of stimulus pattern ($F_{2,20} = 4.6968$, $p = 0.0213$) and an interaction between stimulus pattern and time window ($F_{2,20} = 5.1440$, $p = 0.0158$). The simple effect of the interaction showed that ERPs at 215–240 ms, corresponding to the negative peak, differed among stimulus patterns ($F_{2,20} = 5.4861$, $p = 0.0126$). Furthermore, the mean amplitude of the ERPs to standard "AAB" ($F_{1,10} = 7.5885$, $p = 0.0203$) and pattern-obeying deviants "AAB" ($F_{1,10} = 7.4773$, $p = 0.0210$) were significantly different between time windows, but those of pattern-violating deviants "ABB" were not ($F_{1,10} = 2.1542$, $p = 0.1729$). These results suggest that standard "AAB" and pattern-obeying deviants "AAB" showed a decreased negative shift of the ERPs to the second call in the triplet sequence compared with the first call, but the pattern-violating deviants "ABB" did not. This suggests a fundamental difference between the "AAB" deviants (standard and pattern-obeying) and the pattern-violating deviants "ABB."

## DISCUSSION

In this study, we demonstrated MMR in the NCM of Java sparrows in response to deviant pure tones and natural vocalizations in an oddball paradigm in which differences in the physical characteristics of the stimuli were controlled. However, in comparison, significant mismatch responses were not observed in the ERPs to the sequences controlled for probability and predictability of stimuli. These results indicate that the probability of stimulus frequency affected the shape of ERP waveforms. Furthermore, to investigate the sensitivity to the pattern of sequence, we compared the ERPs with three types of triplet sound sequences. The ERPs to pattern-violating deviants "ABB" differed significantly from ERPs to "AAB" triplet sequences. This

suggests that NCM has the sensitivity to differences in the elemental order.

In the oddball sequence, MMR appeared at 165–270 ms after the sound onset. This is consistent with previous studies in humans, primates, dogs, cats, rodents, and pigeons, in which a significant decrease in ERP amplitude is seen in response to deviant stimuli. The peak of human MMN usually appears at approximately 150–250 ms from the onset of stimuli (Näätänen et al., 2007). Non-human animal MMR sometimes exhibits shorter latencies than human MMN (Shiramatsu and Takahashi, 2021): 48–150 ms in macaque (Javitt et al., 1992; Javit et al., 1994; Molholm et al., 2005; Gil-Da-Costa et al., 2013), 37–131 ms in marmosets (Komatsu et al., 2015), 160–200 ms in dogs (Howell et al., 2011, 2012), 30–74 ms in cats (Csépe et al., 1987; Pincze et al., 2001), 50–150 ms in rodents (Umbricht et al., 2005; Ehrlichman et al., 2008; Shiramatsu et al., 2013; Christianson et al., 2014; Harms et al., 2014), and 50–250 ms in pigeons (Schall et al., 2015). The differences in MMR latency might result from differences in the network, structure, and size of the brain, although we cannot rule out the possibility that differences are due to the neural recording technique, experimental conditions (e.g., under anesthesia or freely behaving), or position of electrodes.

To separate MMN, reflecting a genuine deviance detecting property, from SSA, we performed recordings in which a randomized standard sequence and a regular pattern sequence were presented. Our results showed that using these two types of sequences in the oddball task did not elicit significant levels of deviance detection. At present, several studies have presented randomized standard sequences and successfully recorded MMN in human (Schröger and Wolff, 1996) and MMN-like responses in rats (Nakamura, 2011; Shiramatsu et al., 2013; Harms et al., 2014) and in pigeons (Schall et al., 2015), while some studies using macaques failed (Fishman and Steinschneider, 2012; Kaliukhovich and Vogels, 2014). Regarding the regular pattern sequence, some studies on human (Ruhnau et al., 2012) and rodents (Parras et al., 2017) demonstrated genuine deviance-detecting responses. However, Harms et al. could not find evidence of SSA-independent responses in the rat auditory cortex using this paradigm (Harms et al., 2014). Since the advantages and disadvantages of the control sequences are still controversial, further studies and considerations are needed for characterizing genuine deviance detection.

We used natural vocalizations, song elements, and calls of Java sparrows as stimuli for the oddball sequence and triplet sequence paradigm. Detection of deviants in natural auditory scenes is an important component of determining which ecological events are relevant to behavior and require an attention switch for further processing (Näätänen et al., 2001). This suggests that detecting deviance in natural complex sounds is critical for animal survival. Electrophysiological recording studies have reported that the auditory forebrain of songbirds shows larger responses to deviant calls than to standard calls using an oddball paradigm (Beckers and Gahr, 2012; Dong and Vicario, 2018). We demonstrated MMR elicited by deviant sounds using an oddball paradigm with song elements and calls. Song elements and calls for stimuli were sampled from a single and two individuals, which seemed to be a small sample size to generalize the result. However, MMR is also elicited by the changes in more complex triplet temporal patterns using the calls from five individuals. These suggest that MMRs reflect the deviance detection of natural communicative sounds. Song elements and calls elicited negative peaks that were larger in amplitude and longer in duration compared with those elicited by pure tones. In addition to containing more acoustic information than pure tones, call and song elements might attract more attention, which can modulate ERPs (Picton et al., 1971; Hromádka and Zador, 2007).

Previous studies indicated that human MMN can be elicited by categorical changes and violation of abstract rules of sequences (Tervaniemi et al., 2001; Pulvermu and Shtyrov, 2003). The detection of categorical changes is considered to reflect higher cognitive function rather than the effects of adaptation. Some studies using primates and rats demonstrated MMR elicited by the change in melodic contours (Ruusuvirta et al., 2007) and grammar-like sequences (Astikainen et al., 2014; Attaheri et al., 2015). In the triplet sequence paradigm, we detected MMR at 95–115 ms after the second sound onset (215–240 ms after the stimulus onset) specifically in pattern-violating sequences "ABB." Our result is consistent with a previous report of sequence sensitivity in NCM neurons using a triplet sequence oddball task (Ono et al., 2016), and it supports that MMR in this paradigm, such as MMN in humans, reflects genuine deviance detection rather than adaptation.

The current experiments showed that MMR elicited by oddball paradigm using pure tone could arise from SSA, but MMR elicited by violation of abstract rules of the sequences primarily reflect deviance detection in NCM. Songbirds including Java sparrows learn and produce songs composed of complex temporal sequences. They communicate by call interactions and maintain pair-bond and group. The acoustic structure and temporal pattern of successive calls reflect social relationships (Elie et al., 2011). Thus, they are sensitive to sound sequences. Even though more experiments have to be conducted, we believe our study shows that MMRs in Java sparrows share some characteristics with MMN and may provide insights into the electrophysiological properties of MMR and SSA, which represent sound sequence processing.

## CONCLUSION

We detected MMR in the songbird NCM to deviants with an oddball paradigm, whereas significant negative shifts were not detected using randomized standard sequences and regular pattern sequences. These results indicate that MMR demonstrated in the oddball paradigm reflects the adaptation to a repeated standard sound. Using a triplet sequence paradigm, we successfully detected MMR elicited by violation of the pattern of sound sequence, which is considered to reflect the ability to

extract structural information. Our findings can be used as a basis for understanding the neural mechanisms and functional role of MMR in songbirds and for comparing MMR with MMN in humans. However, it remains necessary to optimize the paradigm to investigate how neural adaptation and genuine deviance detecting properties contribute to ERP waveforms and further to combine recordings with pharmacological manipulations (e.g., antagonists of NMDA).

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Tokyo.

## AUTHOR CONTRIBUTIONS

CM and KO designed this study and wrote the manuscript. CM performed the experiments and data analysis. Both authors contributed to the article and approved the submitted version.

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# Imprintability of Newly Hatched Domestic Chicks on an Artificial Object: A Novel High Time-Resolution Apparatus Based on a Running Disc

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*Aoki N, Mori C, Fujita T, Serizawa S, Yamaguchi S, Matsushima T and Homma KJ (2022) Imprintability of Newly Hatched Domestic Chicks on an Artificial Object: A Novel High Time-Resolution Apparatus Based on a Running Disc. Front. Physiol. 13:822638. doi: 10.3389/fphys.2022.822638*

In filial imprinting, newly hatched chicks repeatedly approach a conspicuous object nearby and memorize it, even though it is an artificial object instead of their mother hen. Imprinting on an artificial object in a laboratory setting has a clear sensitive period from post hatch days 1–3 in the case of domestic chicks. However, the establishment of imprintability are difficult to investigate because of the limitations of the behavioral apparatus. In this study, we developed a novel behavioral apparatus, based on a running disc, to investigate the learning processes of imprinting in newly hatched domestic chicks. In the apparatus, the chick repeatedly approaches the imprinting object on the disc. The apparatus sends a transistor-transistor-logic signal every 1/10 turn of the disc to a personal computer through a data acquisition system following the chick's approach to the imprinting object on the monitor. The imprinting training and tests were designed to define the three learning processes in imprinting. The first process is the one in which chicks spontaneously approach the moving object. The second is an acquired process in which chicks approach an object even when it is static. In the third process, chicks discriminate between the differently colored imprinting object and the control object in the preference test. Using the apparatus, the difference in the chicks' behavior during or after the sensitive period was examined. During the sensitive period, the chicks at post hatch hour 12 and 18 developed the first imprinting training process. The chicks at post hatch hour 24 maintained learning until the second process. The chicks at post hatch hour 30 reached the discrimination process in the test. After the sensitive period, the chicks reared in darkness until post hatch day 4 exhibited poor first learning process in the training. Thus, this apparatus will be useful for the detection of behavioral changes during neuronal development and learning processes.

**Keywords: filial imprinting, learning process, *Gallus gallus domesticus*, domestic chicks, development, sensitive period, critical period**

**Abbreviations:** Phh, post hatch hour; Phd, post hatch day; TFT, thin film transistor.

## INTRODUCTION

Filial imprinting occurs in the early stages of precocial birds' lives (Spalding, 1873; Lorenz, 1935). Filial imprinting of domestic chicks is a useful model for early learning (Horn, 1986; Matsushima et al., 2003; McCabe, 2019). During imprinting, newly hatched chicks and ducklings repeatedly approach a conspicuous object nearby and then develop a preference toward it (Bateson, 1964, 1966). The learning processes of imprinting have been investigated in a laboratory setting since the 1960s (Bateson, 1966; Horn, 1998). In these studies, the neuronal and molecular mechanisms of imprinting have been investigated extensively (Horn, 1998, 2004; Yamaguchi et al., 2012; Solomonia and McCabe, 2015; Aoki et al., 2018, 2020). Additionally, several genes involved in memory formation during imprinting have been detected using comprehensive gene screening strategies (Yamaguchi et al., 2008, 2011a,b, 2012).

Although newly hatched chicks spontaneously explore static objects (Bolhuis, 1991; Dharmaretnam and Andrew, 1994; Vallortigara and Andrew, 1994; Versace et al., 2016), the chicks are strongly predisposed to approach conspicuous moving objects (Bateson, 1966; Bolhuis, 1991; Salvatierra et al., 1994; Maekawa et al., 2006; McCabe, 2013; Vallortigara and Versace, 2018). A runway-type apparatus was developed to investigate this innate predisposition. The chicks' motivation to approach the object depended on its characteristics; for example, color, size, shape, movement, and sound. Particularly, movement and sound were the most effective motivators (Collias and Joos, 1953; Smith and Hoyes, 1961; Hoffman, 1978). A wheel-type apparatus was developed to quantify the preference for the imprinting object by measuring the number of revolutions of the wheel (Johnson et al., 1985; McCabe and Horn, 1994). Recently established running wheel methods were able to automatically measure the approach distances of preference by the minute (Versace et al., 2017a,b). An automated tracking method had a time resolution of dozens of samples/second (Goldman and Wood, 2015; Lemaire et al., 2021). However, how the learning abilities for imprinting established after hatching have not been analyzed by the millisecond to investigate the relationship between the behavior and the neural activities.

Imprinting has a well-defined sensitive period (Ramsay and Hess, 1954; Hess, 1959; Bateson, 1966). In domestic chicks, the sensitive period peaks on post hatch day (Phd) 1 (Jaynes, 1957) and ends by Phd 4 (Yamaguchi et al., 2012). At Phd 4, even 2 h of training in which a chick was exposed and responded to the imprinting object did not make the chicks develop a preference for the imprinting object. The molecular mechanism of the sensitive period initiated by the inflow of the thyroid hormone into the brain has been investigated using a treadmill-type behavioral apparatus (Yamaguchi et al., 2012). In this apparatus, when chicks are close to the object on the treadmill in the training, they are forced backward to induce strong imprintability. A simultaneous choice test in the apparatus was effective in evaluating the ability to discriminate between the training and control objects because the chicks were able to observe and judge the differences between the objects with their feet firmly on the ground. Even in this treadmill-type apparatus, we could not analyze the precise timing of the chick's response to the appearance and movements of the objects in the training. Therefore, we still have not known how to deteriorate the imprintability after the end of the period.

By improving a devise of behavioral experiment further and by categorize the learning processes in detail, we hypothesized that the entire process of imprintability in the development and deterioration will be revealed. In this study, we developed a novel disc-based behavioral apparatus and designed the training and preference tests. This new apparatus made it possible to analyze the correlation between the precise timing of the chick's response and the appearance and movements of the objects by the millisecond. The significant improvement in the time resolution of the measurements using the disc-based behavioral apparatus allowed a clear discrimination of the different learning processes involved in the imprinting (i.e., the spontaneous approach, the acquired approach and the discrimination following approach behaviors). We examined how to develop the imprintability involved in the learning processes during the sensitive period and how to deteriorate it when the sensitive period is ending. We also examined whether the chicks trained by the disc-based apparatus showed preference to the imprinting object in the simultaneous choice test using the T-maze apparatus that we have used for many years to reveal the reliability of the novel apparatus.

## MATERIALS AND METHODS

### Animals

The experiments were conducted under the guidelines of the national regulations for animal welfare in Japan, with the approval of the Committee on Animal Experiments of Teikyo University (approval number: 18-015). In this study, 106 newly hatched domestic chicks (unknown sex) of the Cobb strain (*Gallus gallus domesticus*) were used. No subjects were excluded from the analyses. Fertilized eggs were obtained from a local supplier (3-M, Aichi, Japan) and incubated at 37°C for 21 days. Chicks were hatched in the incubator in the darkness. Three to six hours after hatching, the chicks were placed in dark plastic enclosures in a breeder at 32°C to prevent light exposure till the experiments (Izawa et al., 2001). After the behavioral experiments, the animals were euthanized with an overdose of isoflurane.

### Disc-Based Apparatus for Imprinting Training

We developed a new behavioral apparatus, in which the chick runs on a running disc 25 cm in diameter (**Figures 1A,B** and **Supplementary Video 1**). The chick was surrounded by the black-colored walls made from acrylic boards or thin paper which prevent a chick from dropping. The front wall was transparent to make the chick see the movie on the monitor. An imprinting object was displayed on a Thin Film Transistor (TFT) monitor (refresh rate: 56–75 Hz; size: 213 mm × 160 mm; type: LCM-T102AS, Logitec Corp., Tokyo, Japan). The distance between the apparatus and monitor was approximately 5.0 cm. Initial position of the chick was shown in **Figure 1B**. If the chick

approached the imprinting object on the monitor, the disc rotated clockwise. After the chick approached the monitor, it was brought back to its opposite side. This apparatus sends a transistor-transistor-logic signal every 1/10 turn of the disc to a personal computer through the OmniPlex data acquisition system (Plexon Inc., Dallas, TX, United States). The sampling frequency of the recording was 2,000 samples/second. The approach distance was calculated from the recorded data of the transistor-transistor-logic signals. Since the chicks walk approximately 6.5 cm away from the center of the disc, the estimated approach distance in one lap was about 40 cm. Therefore, one transistor-transistor-logic signal was 4.0 cm approach toward the monitor by the chick.

## Imprinting Training Using Disc-Based Apparatus

For imprinting training, two sessions (training 1 and 2) were conducted at 1-h intervals (**Figure 1C**). One session of training involved playing a movie, which was 20 s long, repeatedly on the monitor, 180 times for 1 h. The movie was created using the 3D creation software Blender (Blender Foundation, Amsterdam, Netherlands), which has been previously used for imprinting studies on domestic chicks (Batista et al., 2016; Lemaire et al., 2021). In the training movie, a yellow imprinting object appeared for 12 s. The object was static for the first 4 s, then turned clockwise and anti-clockwise repeatedly with artificial sounds from two speakers for 6 s. After the object stopped, the static object appeared again for the last 2 s to prevent the chicks from continuously approaching after the object disappeared. The interval between the object's appearances was 8 s. During this interval, the monitor presented a black screen. To analyze the chicks' performance during training, a trial of 20 s was divided into five periods (4 s per period) (**Figure 1C**). The approach distance in each period was calculated.

## Preference Test Using Disc-Based Apparatus

We examined whether the chicks trained by the disc-based apparatus showed preference to the imprinting object in the use of the test of disc apparatus. The preference test was conducted 1 h after training 2. For the preference test using the disc apparatus, two types of movies, 20 s each, were played pseudo-randomly for 1 h (**Figure 1C**). In one movie, the yellow imprinting object appeared for 12 s and did not move or make a sound. In the remaining 8 s, the monitor presented a black screen. In the other movie, a blue control object appeared for 12 s and did not move or make a sound. The approach distance during the object appearing (12 s) was measured and averaged in each trial type. The preference score was calculated by "approach distance to the imprinting object/(approach distance to the imprinting object + approach distance to the control object)." If the approach distance to the imprinting object is 15 cm/trial and that to the control object is 5 cm/trial, the preference score is 0.75. If the approach distance to the imprinting object and that to the control object are exactly same, the preference score is 0.50.

## Preference Test Using a T-Maze Apparatus

For the simultaneous choice test, we used a T-maze apparatus with a main arm of 20 cm and a side arm of 69 cm (Aoki et al., 2015). A yellow imprinting object and a control object were presented through a monitor placed at both ends of the side arm of the T-maze. The objects neither moved nor made a sound. After a chick started moving from the main arm, we counted the stay time of the approach area of each object for 120 s. The test was conducted four times and the stay time for each was averaged. We then calculated a preference score by subtracting the stay time for the control object from that of the imprinting object.

## Definition of Learning Processes in Filial Imprinting

The training and preference tests were designed to analyze the learning process of imprinting (**Figure 1C**). From the behavioral analysis, three learning processes were defined (i.e., spontaneous approach, the acquired approach, and the discrimination following approach behaviors) (**Figure 2A**). The representative behavior of a chick at Phh 30 is shown in **Figures 2B–E**. During the initial part of training 1, the chick responded to the object only when it was moving and made a sound (**Figure 2B**). These approaches to the object when it was moving and made sounds were considered to be a spontaneous approach behavior. This "spontaneous approach behavior" to the moving object was defined as the first learning process in imprinting. During training 2, the chick approached the static object more frequently than it did in training 1 (**Figure 2C**). These Approaches to the static object before it started to move as a result of exposure to repeated training trials were considered to be an acquired approach behavior. This "acquired approach behavior" was defined as the second learning process in imprinting. In the preference test, the yellow imprinting object or blue control object appeared on the monitor for 12 s, respectively. When the yellow imprinting object was presented, the chicks responded constantly to the static state of the object, as shown in the raster plots (**Figure 2D**). When the blue control object was presented, the chick responded to the object in some initial trials, but gradually stopped responding (**Figure 2E**). Accordingly, the approach distance to the imprinting object was greater than approach distance to the control object in the preference test. In this case, the preference score reached a high level (0.71). This status of the "discrimination following approach behavior" between the two objects in the test was defined as the third learning process in imprinting. The development of learning processes during the sensitive period was monitored using chicks at Phh 12, 18, 24, and 30. To examine which part of the learning processes the chicks at Phd 4 were unable to reach, the behavior of chicks at Phd 1 and 4 was compared.

## Statistical Analyses for Behavioral Data

For statistical analyses, R software (R Development Core Team) or MATLAB (Mathworks, Natick, MA, United States) for Windows was used. The number of animals used is shown in **Figures 3**, **5** and **Supplementary Figures 4–6**. The equalities of variance of behavioral data were checked by the Bartlett's

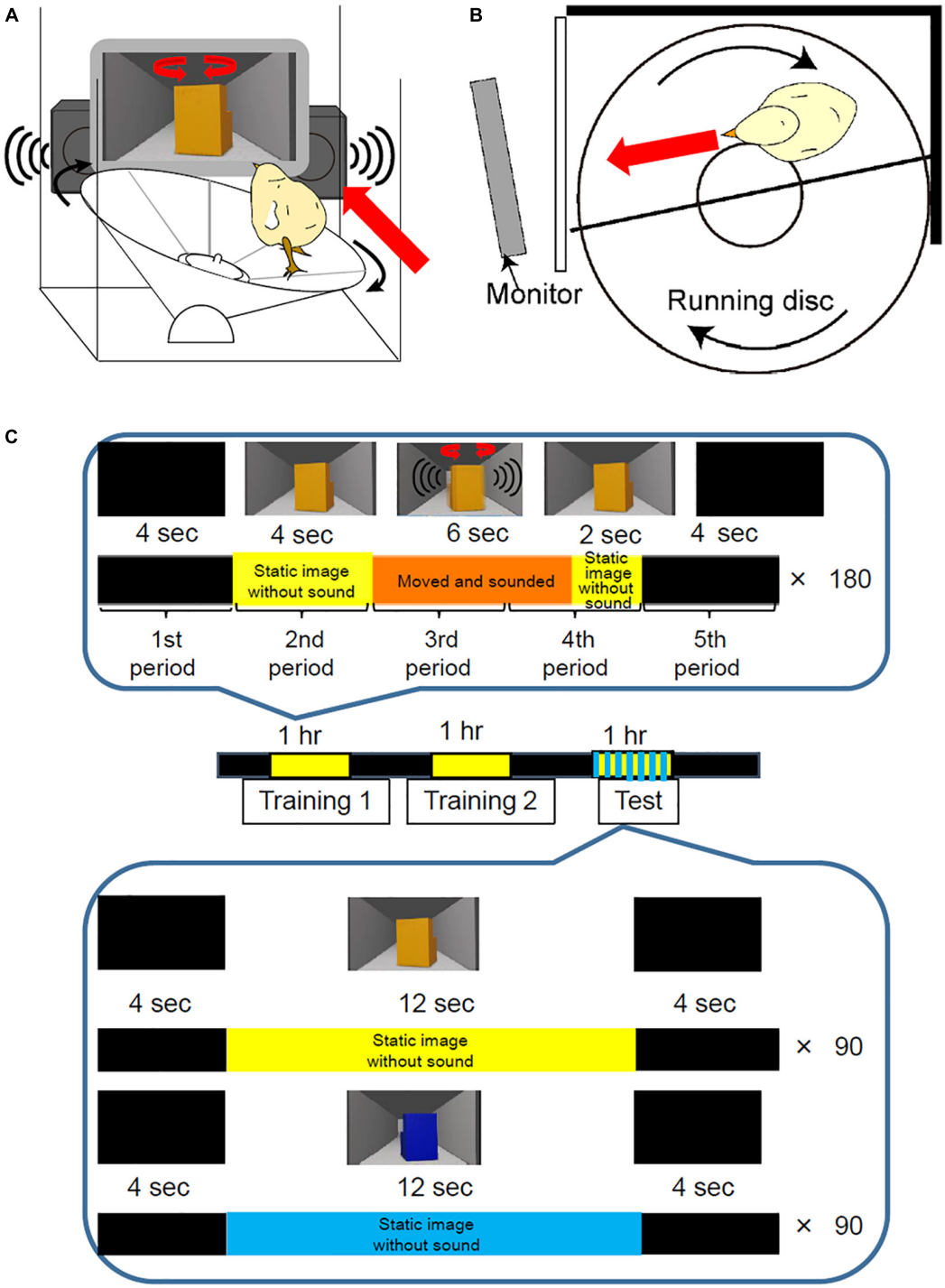


**FIGURE 1 |** Disc-based behavioral apparatus and procedure for imprinting training and test. **(A)** The running disc-based behavioral apparatus. An imprinting object was shown through a TFT monitor which was placed in front of the apparatus. Artificial sounds were played through the speakers. **(B)** A top view of the running disc-based behavioral apparatus. The chick is surrounded by the black-colored walls made from acrylic boards or thin paper. The front wall is transparent to make the chick see the movie on the monitor. The chick represented the initial position of the behavioral experiment. **(C)** Imprinting training and testing using the disc-based apparatus. During training, two sessions were conducted at 1 h intervals. In one session of training, a movie, 20 s long, was repeatedly played on the monitor 180 times for 1 h. In the training movie, a yellow imprinting object appeared for 12 s. The object was static for the first 4 s, then turned clockwise and anti-clockwise repeatedly with artificial sounds for 6 s. After the object stopped, it remained static again for the last 2 s. During the interval, the monitor presented a black screen. To analyze the chicks' performance during training, one trial of 20 s was divided into five periods (4 s each). In the preference test, two types of movies of 20 s were played pseudo-randomly for 1 h. In one movie, the yellow imprinting object appeared for 12 s and remained static without making a sound. In the remaining 8 s, the monitor presented a black screen. In another movie, a blue control object appeared for 12 s and remained static without sound.

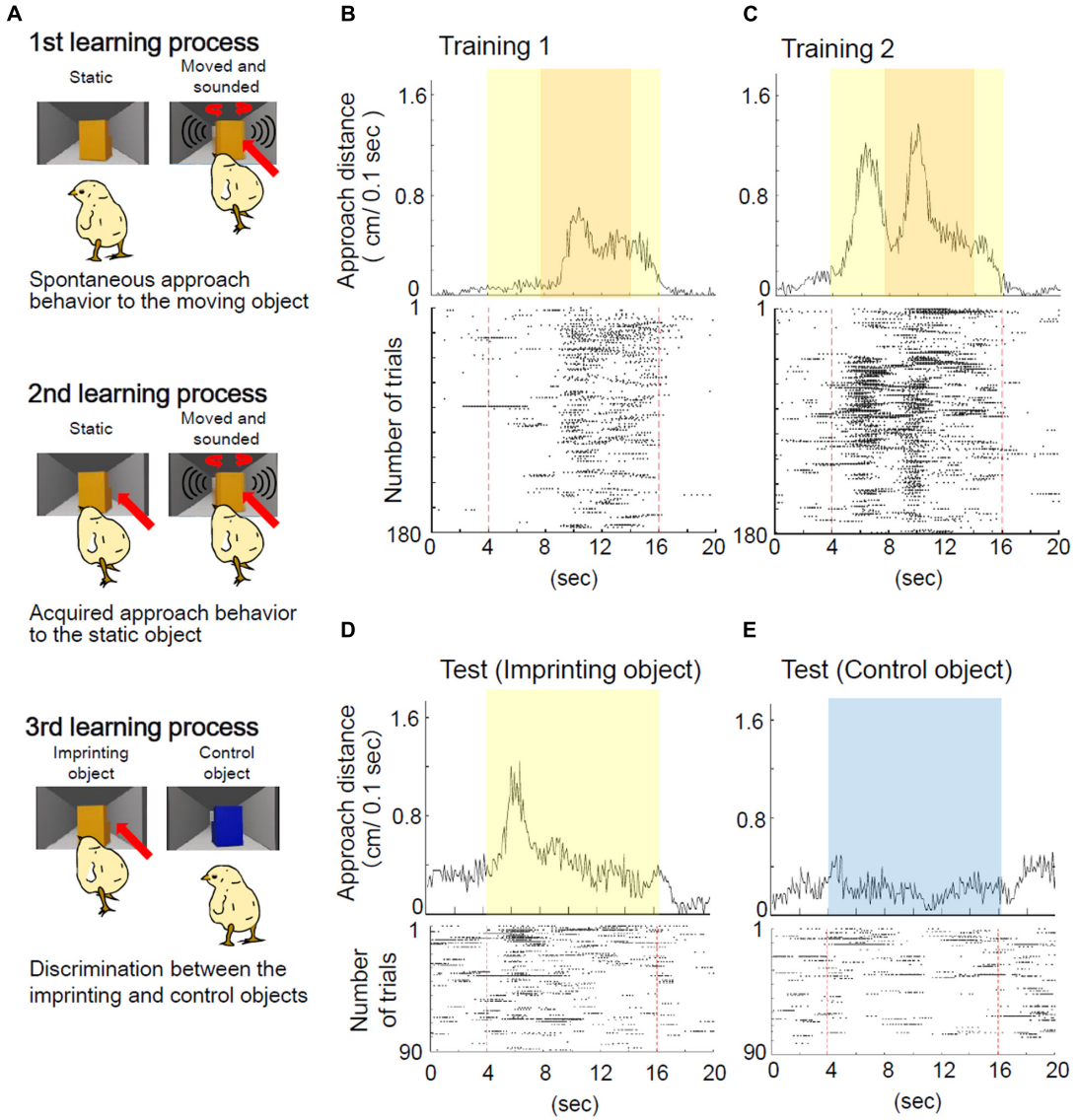


**FIGURE 2 |** Definition of learning processes in filial imprinting. **(A)** Three learning processes for the imprinting were defined. In the first process, the chick approaches spontaneously the moving object in the training, but does not approach the static object. In the second process, the chick becomes to approach the static object in the training. In the third process, the chick discriminates the imprinting object from the control object. Representative behavior of a chick at Phh 30 in the imprinting training and test are shown in panels **(B–E)**. **(B)** Averaged approach distances in training 1 are shown in the upper column. Raster plots of each trial are shown in the lower column. One plot indicates the timing of a 1/10 turn of the disc. Red broken lines indicate the onset and offset of the object presentation. In the initial part of training 1, a chick at Phh 30 responded to the object only when it was moving. In some trials of training 1, the chick responded to the object before the object started moving. **(C)** Averaged approach distances and raster plots of each trial in training 2 are shown. In training 2, the chick responded to the static object more frequently than the chick in training 1. **(D)** Averaged approach distances and raster plots in the imprinting object trials of the test are shown. The chicks responded constantly to the static state of the yellow object. **(E)** Averaged approach distances and raster plots in the control object trials of the test are shown. The chick responded to the static state of the blue object in several initial trials, but gradually stopped responding.

test. Since the results of the Bartlett's test showed that the variances were not equal, a Steel's multiple comparison test or the Welch's *t*-test was used as non-parametric tests. Using the Steel's multiple comparison test, the behavioral data between post hatch hour (Phh) 30 and other ages were compared. We used an R script developed by Dr. Shigenobu Aoki of Gunma University[1] to perform the Steel's multiple comparison test. The Welch's *t*-test was used to analyze the data and compare the behavioral data between chicks at Phd 1 and Phd 4. A paired *t*-test was used to compare the approach distance in the yellow

[1]http://aoki2.si.gunma-u.ac.jp/R/Steel.html

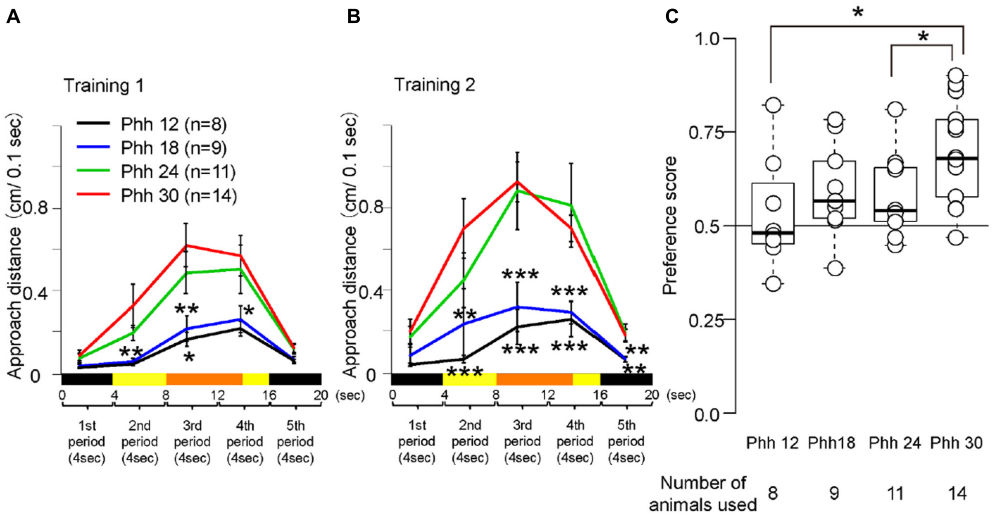


**FIGURE 3 |** Behavior of chicks at Phh 12, 18, 24, or 30 in the imprinting training and test. **(A)** Averaged approach distances in training 1 are shown. One trial of 20 s was divided into five periods (4 s each). The black line indicates the approach distances of chicks at Phh 12. The blue line indicates the approach distances of chicks at Phh 18. The green line indicates the approach distances of chicks at Phh 24. The red line indicates the approach distances of chicks at Phh 30. The approach distances of the chicks at Phh 24 were not significantly different from those of the chicks in any period at Phh 30. The approach distances of the chicks at Phh 18 from the second to fourth period were significantly less than those of the chicks at Phh 30. The approach distances of the chicks at Phh 12 in the second and third periods were significantly less than those of the chicks at Phh 30. **(B)** Averaged approach distances in training 2 are shown. The approach distances of the chicks at Phh 24 were not significantly different from those of the chicks in any period at Phh 30. The approach distances of the chicks at Phh 12 and 18 from the second to fifth period were significantly less than those of the chicks at Phh 30. **(C)** Preference scores in the test are shown. Preference scores of chicks at Phh 12 and 24 were significantly lower than those of chicks at Phh 30. The asterisks (*) indicate significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$).

trials with that in the blue trials in the preference test. To test the significance of the correlation between the preference score and behavioral data in the training test, Pearson's product-moment correlation was applied. Differences were considered statistically significant at $p$-values $< 0.05$. The $p$-values are shown in **Supplementary Tables 1–3**.

# RESULTS

## Establishment of Learning Processes During the Sensitive Period

The chicks at Phh 30 approached the imprinting object in the third and fourth periods when the object was moving in training 1 (**Figure 3A**). The chicks approached the object fewer times in the second period when the object was in a static state. In training 2, the approach distances of the chicks at Phh 30 increased from the second to the fourth period (**Figure 3B**). In the preference test, the chicks discriminated between the differences in the imprinting and control objects (**Figure 3C**). The approach distances and preference scores of the chicks at Phh 30 were compared with those of the chicks at other ages. The approach distances of the chicks at Phh 24 were not significantly different from those of the chicks in any period of training 1 and 2 at Phh 30 (**Figures 3A,B**). However, the preference score of the chicks at Phh 24 was significantly lower than that of the chicks at Phh 30 (**Figure 3C**). This means that the chicks at Phh 24 achieved up to the second learning process, but did not reach the third learning process. Subsequently, the approach distances of the chicks at Phh 12 and 18 from the second to the fifth period were significantly less than those of the chicks in training 2 at Phh 30 (**Figure 3B**). The preference scores of chicks at Phh 12 were significantly lower than those of chicks at Phh 30 (**Figure 3C**). The preference scores of the chicks at Phh 18 were not significantly different from those of chicks at Phh 30; however, they did not show clear discrimination (**Figure 3C**). This means that the chicks at Phh 12 and 18 achieved up to only the first learning process of imprinting.

Next, the significance of the correlations between the preference score and each period in the training trials was tested (**Supplementary Figure 1A**). The correlations were significant for the first to fourth periods. The results suggest that the approach to the imprinting object in training is related to the discrimination of the imprinting object in the test. The approach distances to the imprinting object will be a useful parameter of the learning process of imprinting. Furthermore, the total approach distances in training 2 were also significantly correlated with the preference score in the test (**Supplementary Figure 1B**). This indicates that total effort during training is also important for discrimination.

## Deterioration of Imprintability When the Sensitive Period Ended

The representative behavior of a chick at Phd 4 is shown in **Figure 4**. The chick at Phd 4 showed smaller responses from the second to the fourth period when the imprinting object appeared (**Figure 4A**). The responses continued to be smaller from the second to the fourth period even in training 2

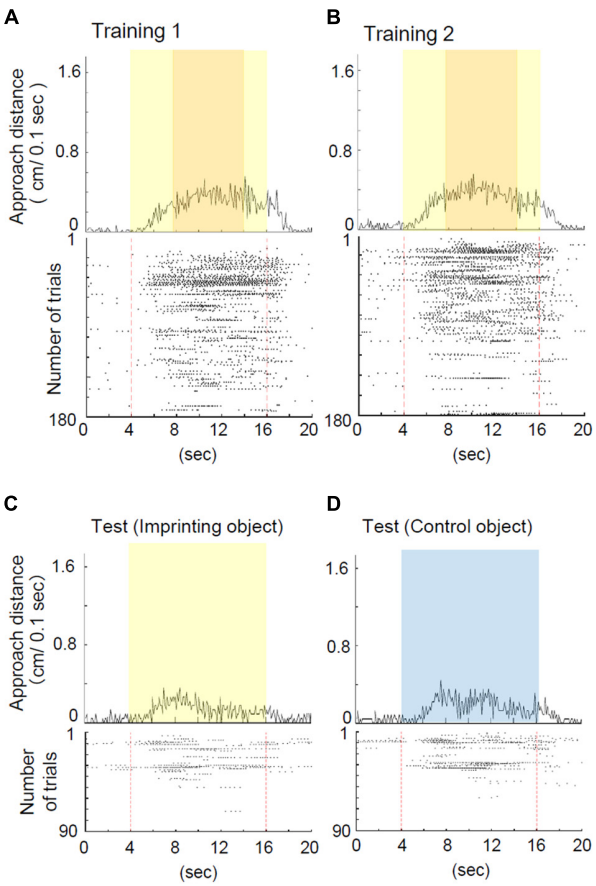


**FIGURE 4 |** Representative behavior of a chick at Phd 4 in the imprinting training and test. **(A)** Averaged approach distances and raster plots of each trial in training 1 are shown. In training 1, the chick responded to the moving object and, in some instances, responded to the object before it started moving. **(B)** Averaged approach distances and raster plots of each trial in training 2 are shown. In training 2, the chick responded to the object in a similar manner to training 1. **(C)** Averaged approach distances and raster plots in the imprinting object trials of the test are shown. Initially, the chicks responded to the static state of the yellow object, and gradually stopped responding. **(D)** Averaged approach distances and raster plots in the control object trials of the test are shown. Initially, the chicks responded to the blue object, and gradually stopped responding.

(**Figure 4B**). According to the raster plots, the chick gradually stopped responding to the object in both trainings 1 and 2. In the preference test, the chick responded to the yellow object as well as the blue control object and gradually stopped responding in both trials (**Figures 4C,D**). In this case, the preference score was negative (0.42).

Averaged behavioral data for Phd 1 and 4 is shown in **Figure 5**. The behavioral data of the chicks at Phh 30 are represented as Phd 1. In training 1, the approach distances of chicks at Phd 4 were significantly less than those of chicks at Phd 1 in the third and fourth periods when the object moved and made a sound (**Figure 5A**). In training 2, the approach distances of the chicks at Phd 4 were significantly less than those of chicks at Phd 1 in the second and third periods (**Figure 5B**). In the test, the preference scores of chicks at Phd 4 were significantly lower than those of chicks at Phd 1 (**Figure 5C**). This suggests that the learning ability of chicks from the first learning process gradually weakened until Phd 4, such that the chicks at Phd 4 did not reach the second process of learning.

The chicks at Phd 4 showed relatively smaller responses to the imprinting object and did not discriminate it from the control object (**Figure 5**). This indicates that the ability to respond to moving objects was weakened so that they lost the ability to transfer the innate approach behavior to the acquired approach behavior. The scatter plots between the preference score and approach distances of the data in **Figure 5** are shown in **Supplementary Figures 2A,B**. Interestingly, several chicks at Phd 4 still approached the imprinting object and discriminated it from the control object. This indicates that they maintained the ability of the learning process when reared in darkness until Phd 4. We also found differences in temporal changes in the behavior of the chicks at Phd 1 and 4 during the test (**Supplementary Figure 3**). In chicks at Phd 1, responses to the imprinting object gradually increased during the first hour of the test, whereas responses to the control object gradually decreased. In the chicks at Phd 4, both responses were less than those of chicks at Phd 1 and gradually decreased. These results suggest that both the decrease in responsiveness to the moving object and suppression of responses to the control object might cause a disability of imprinting when the sensitive period ends.

## The Simultaneous Choice Test Using the T-Maze Apparatus

In the simultaneous choice test using the T-maze apparatus, the preference scores of chicks trained by the disc-based apparatus at Phd 1 were high (**Supplementary Figure 4**). This indicates that chicks were able to be imprinted using the disc-based apparatus same as the previously used treadmill-type apparatus. The preference scores of chicks at Phd 1 were significantly greater than those of chicks at Phd 4 (**Supplementary Figure 4**), consistent to the preference score in the test using the disc-based apparatus. This suggests that the preference test using the disc-based apparatus was reliable same as the simultaneous choice test using the T-maze apparatus.

# DISCUSSION

In this study, a disc-based behavioral apparatus was found to be advantageous for the analysis of the learning process in imprinting. In previous studies, the runway-type (Hess, 1959), wheel-type (Horn, 1986), and treadmill-type apparatus have been used for the analysis of filial imprinting (Izawa et al., 2001; Yamaguchi et al., 2012; Aoki et al., 2020). The wheel-type apparatus is advantageous while analyzing approach distances to the object because approach distances are automatically measurable (Versace et al., 2017a,b). The treadmill-type apparatus is advantageous in the reliability of imprinting acquisition because the chicks have to walk by themselves in the

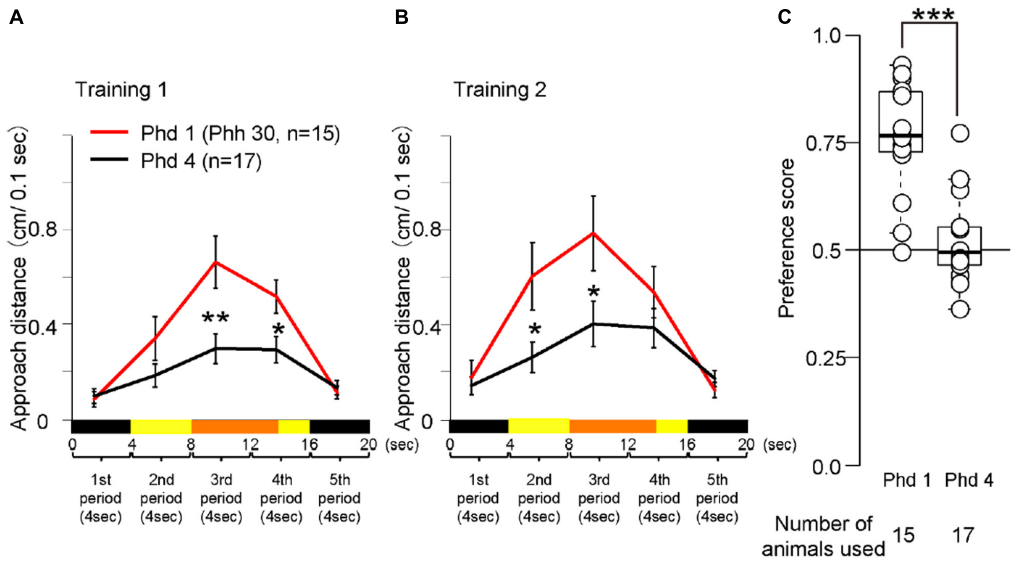


**FIGURE 5 |** Behavior of chicks at Phd 1 and 4 in the imprinting training and test. **(A)** Averaged approach distances in training 1 are shown. The red line indicates the approach distance of Phd 1 (Phh 30). The black line indicates the approach distance of chicks at Phd 4. In the third and fourth periods, the approach distances of chicks at Phd 4 were lower than those of chicks at Phd 1. **(B)** Averaged approach distances in training 2 are shown. In the second and third period, the approach distances of chicks at Phd 4 were lower than those of chicks at Phd 1. **(C)** Preference scores in the test are shown. Preference scores of chicks at Phd 4 were significantly lower than those of chicks at Phd 1. The asterisks (*) indicate significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$).

direction of the object through a binary choice test. In addition to those advancements above, we improved the time resolution in the disc-based behavioral apparatus. Furthermore, the training and tests were designed to define each learning process.

In previous studies using the wheel-type apparatus, the imprinting training has been performed with the physical red colored object (Johnson et al., 1985; Bolhuis et al., 1986; McCabe and Horn, 1994). In our experience, the strengths of the preference acquired by the imprinting training did not depend on the appearances of the object, e.g., physical or virtual, types of color. Using the treadmill-type apparatus, the chicks were able to be imprinted by the virtual stimulus on the monitor as well as the physical object (Takemura et al., 2018). In addition, the chicks trained with the blue-colored object showed a strong preference for the training object, similarly in the case of chicks trained with the yellow-colored object (**Supplementary Figure 5**). This suggests that the test was not likely to be affected by the differences in color. We think that an advantage of the virtual stimulus is that the timing of the visual and acoustic stimulus is able to be controlled exactly in every trial. That will be useful to define the learning processes in more detail. The preference scores using the novel apparatus had the same tendency of those using the T-maze apparatus (**Figure 5C** and **Supplementary Figure 4**). This suggested that the novel apparatus was available for the measurement of the preference score as well as the previous apparatus. Moreover, we are able to observe the chicks' behavior by millisecond in the test so that the processes of the decision making may be revealed by the combinational analysis of electrophysiology.

In the learning process of imprinting, it was important that chicks responded strongly to the moving object with sounds in the first process, and become responsive to the static object without movement and sound in the second process. Pearson's product-moment correlation between the approach distances in the second period (the static object) and those in the third period (moving object) was calculated using the data in **Figures 3B**, **5B**. The correlation was significant in both cases (**Supplementary Figures 1C, 2C**). These results suggest that strong responsiveness to a moving object is necessary for the acquisition of the approach behavior toward the object in a static state.

The chicks at Phh 24 approached the imprinting object eagerly, similar to the chicks at Phh 30. However, the approach distances of the chicks at Phh 24 between the imprinting and control object trials did not differ significantly (**Supplementary Figure 6**). This implies that a generalization of the image occurred in the brains of the chicks at Phh 24, by which a different-colored but same-shaped object was considered the same, suggesting an immaturity of discrimination of differences of the objects. Contrastingly, in chicks at Phh 30, the approach distances in the imprinting object trials were significantly greater than those in the control object trials. This suggests that the chicks at Phh 30 showed strong preferences and imprinted on the yellow object. Moreover, they suppressed the approach behavior to the control object, suggesting a maturity of discrimination.

In this study, we developed the high time-resolution apparatus based on a running disc for the filial imprinting. Using this apparatus, we defined three learning processes for the imprinting (i.e., spontaneous approach, the acquired approach, and the discrimination following approach behaviors). We showed that the chicks at Phh 30 reached the third learning process, but the chicks before that age remained first or second learning process, and that the chicks after the sensitive period exhibited

poor first learning process in the training. We propose that this apparatus is useful for the detection of the effects of infused drugs, deficits from local brain lesions, and developmental behavioral changes. In future, other statistical analyses such as generalized linear mixed models may reveal the unidentified factors which are important for the establishment of imprintability. We plan to investigate the regions of the brain involved in each learning process of imprinting by comparing gene expressions of immediate early genes among the brains at different stages of development. It is difficult to record the neuronal activity from a chick's brain during imprinting training using the previous apparatus, especially in the case of recording from a freely moving chick. In the new behavioral apparatus, the chick will be attached with a short cord for recording on the head and will be able to run in a limited area, 24 cm in diameter, of the disc. The relationship between neural activities and the learning process of imprinting in a living chick will be clarified using a disc-based behavioral apparatus.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experiments of Teikyo University.

## AUTHOR CONTRIBUTIONS

NA, CM, and KH were involved in the data analysis. NA and KH wrote drafts of the manuscript. All authors were involved in study design, data interpretation, critically commented and revised the drafts, and approved the final report.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.822638/full#supplementary-material

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BRIEF RESEARCH REPORT
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# Effect of Stressors on the mRNA Expressions of Neurosecretory Protein GL and Neurosecretory Protein GM in Chicks

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We recently discovered novel cDNAs encoding the precursors of two small secretory proteins, neurosecretory protein GL (NPGL) and neurosecretory protein GM (NPGM), in the mediobasal hypothalamus (MBH) of chickens. In addition, we found colocalization of NPGL, NPGM, and histidine decarboxylase (HDC; histamine-producing enzyme) in same neurons of the medial mammillary nucleus of the hypothalamus. In this study, we elucidated the effect of several stresses, including food deprivation, environmental heat, inflammation, and social isolation, on the mRNA expression of *NPGL*, *NPGM*, and *HDC* in chicks using real-time PCR. Food deprivation for 24 h increased *NPGM* mRNA expression in the MBH. On the other hand, an environmental temperature of 37°C for 24 h did not affect their mRNA expression. Six hours after intraperitoneal injection of lipopolysaccharide, an inducer of inflammation, the mRNA expression of *NPGM*, but not that of *NPGL* and *HDC* increased. Social isolation for 3 h induced an increase in the mRNA expression of *NPGL*, *NPGM*, and *HDC*. These results indicate that NPGM, but not NPGL or HDC, may participate in several physiological responses to stress in chicks.

**Keywords: neurosecretory protein, chicken, hypothalamus, stress, histamine**

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## INTRODUCTION

Biological stress responses are induced through the hypothalamus, which is known as the central region of the autonomic nervous system and endocrine system. In particular, the hypothalamic-pituitary-adrenal (HPA) axis is the most predominant pathway for the endocrine response to stress in vertebrates (Tsigos and Chrousos, 2002). In birds, exposure to stress induces the hypothalamus to secrete neuropeptides, corticotropin-releasing factor (CRF), and arginine vasotocin (AVT) in the paraventricular nuclei (PVN); and stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH; Barth and Grossmann, 2000; Jenkins and Porter, 2004). ACTH, secreted by the pituitary gland, stimulates the adrenal cortex through the bloodstream causing the synthesis and secretion of adrenal cortex hormones. Corticosterone is another biological marker of stress in birds (Dmitriev et al., 2001; Feltenstein et al., 2003; Pereyra and Wingfield, 2003). As chickens are one of the most important food resources, research on stress responses affecting egg and meat production is important (Tan et al., 2010;

Mignon-Grasteau et al., 2015; Nawaz et al., 2021). It is crucial to understand how stress response is regulated in the brain in order to minimize the effects of stress on the whole body. However, all pathways responsible for stress response in the chicken brain remain unknown. Therefore, novel factors involved in the stress response need to be investigated.

Recently, we discovered two novel cDNAs, paralogous genes, that encode the precursors of small secretory proteins in the mediobasal hypothalamus (MBH) of chickens (Ukena et al., 2014; Ukena, 2018). Because the predicted C-terminal amino acids of the small protein were Gly-Leu-$NH_2$ and Gly-Met-$NH_2$, the two small secretory proteins were named neurosecretory protein GL (NPGL) and neurosecretory protein GM (NPGM), respectively. Genomic database analysis showed that homologous genes of these novel cDNAs are highly conserved in vertebrates (Ukena, 2018). In chicks, NPGL and NPGM are produced in the medial mammillary nucleus (MM) and the infundibular nucleus (IN) of the hypothalamus (Shikano et al., 2018a). In particular, NPGL and NPGM are co-localized in the same neurons of the MM (Shikano et al., 2018a). We found that NPGL induces hyperphagia and fat deposition in rodents and chicks (Iwakoshi-Ukena et al., 2017; Shikano et al., 2018b, 2019; Fukumura et al., 2021). In contrast, NPGM induces fat deposition without hyperphagia in male chicks (Kato et al., 2021). Our previous studies suggest that NPGL and NPGM are related to energy homeostasis and/or growth in the hypothalamus of vertebrates.

Food deprivation is also a known naturally occurring stressor (Méndez and Wieser, 1993; Midwood et al., 2016). Our previous studies showed that *NPGL* mRNA expression levels were elevated by food deprivation in mice and rats (Iwakoshi-Ukena et al., 2017; Matsuura et al., 2017). In addition, we found the colocalization of NPGL, NPGM, and histidine decarboxylase (HDC; histamine-producing enzyme) in the MM of the chick hypothalamus (Bessho et al., 2014; Shikano et al., 2018a). HDC is a rate-limiting enzyme that synthesizes histamine from histidine. Neuronal histamine has a variety of physiological functions as a neurotransmitter or neuromodulator in the mammalian brain (Ito, 2000). In rodents, neuronal histamine is known to be involved in stress response. Histamine turnover is increased by various stressful situations in the brain, especially in the hypothalamus (Itoh et al., 1989; Ito, 2000). These results suggest that NPGL and NPGM play important roles in stress responses in rodents. There are many different types of stresses, such as energy deficiency, ambient temperature, inflammation, and social isolation. However, it is unclear whether NPGL, NPGM, and HDC respond to these stresses in birds. In this study, we analyzed the mRNA expression of *NPGL*, *NPGM*, and *HDC* in chickens exposed to different types of stressors.

# MATERIALS AND METHODS

## Animals

Male layer chicks of 1-day-old were purchased from a commercial company (Nihon layer, Gifu, Japan) and housed in groups in a windowless room at 28°C on a 20-h light (4:00–24:00)/4-h dark (0:00–4:00) cycle. Except for those in the food deprivation experiment, all chicks had *ad libitum* access to food and water.

## 24-h Food Deprivation Experiment

Male layer chicks ($n = 6$) of 17-day-old were fasted for 24 h. The chicks had *ad libitum* access to water. The control chicks ($n = 5$) had *ad libitum* access to food and water. Every 12 h, blood samples were collected from the ulnar veins. Blood glucose levels were measured using a GLUCOCARD G+ meter (Arkray, Kyoto, Japan). After fasting, the chicks were euthanized, and the MBH was collected. The MBH which includes the MM, IN, ventromedial hypothalamus, and periventricular stratum was dissected out using fine forceps and small scissors (Shikano et al., 2018b). The MBH was immediately snap-frozen in liquid nitrogen and stored at −80°C for real-time PCR analysis.

## Heat Stress Experiment

Male layer chicks ($n = 6$) of 14-day-old were housed at 37°C for 24 h. The control chicks ($n = 6$) were housed at 25°C. After the experiment, the chicks were euthanized, and the MBH was collected for real-time PCR analysis. Blood samples were collected in heparinized tubes at the endpoint.

## Lipopolysaccharide-Induced Inflammation Experiment

Male layer chicks ($n = 7$) of 6-day-old were administered intraperitoneal (i.p.) injection of 2 mg/kg (body mass) lipopolysaccharide (LPS) from *Escherichia coli* O127 (Wako Pure Chemical Industries, Osaka, Japan). LPS was dissolved in 0.9% NaCl. The control chicks ($n = 5$) were treated with an i.p. injection of 0.9% NaCl. After 6 h, the chicks were euthanized and the MBH was collected for real-time PCR analysis. Blood samples were collected at the endpoint.

## Social Isolation Stress Experiment

Male layer chicks were housed in groups until 7-day-old and had *ad libitum* access to food and water. The chicks ($n = 5$) were then isolated from the group breeding and housed individually for 3 h. The control chicks ($n = 6$) were housed in groups. After isolation, the chicks were euthanized and the MBH was collected for real-time PCR analysis. Blood samples were collected at the endpoint.

## Blood Corticosterone Test

Blood samples were centrifuged at $800 \times g$ for 15 min at 4°C to separate the blood plasma. Blood plasma samples were used to measure the corticosterone levels by a commercial ELISA kit (Assaypro, St. Charles, MO, United States).

## Real-Time PCR

RNA was extracted using RNAqueous-Micro Kit (Life Technologies, Carlsbad, CA, United States) following the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). PCR

amplification was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO): 95°C for 20 s followed by 40 cycles at 95°C for 3 s and 60°C for 30 s using a Step One Real-time Thermal Cycler (Applied Biosystems, Bedford, MA, United States). The ROX reference dye was added into each well to normalize the fluorescence intensity of SYBR green in real-time PCR. Relative quantification for each expression was determined by the $2^{-\Delta\Delta Ct}$ method using β-actin (*ACTB*) as an internal control. The primer sequences for *NPGL*, *NPGM*, *HDC*, and the gene of neuropeptide Y (*NPY*) are listed in **Supplementary Table 1**. *NPY* is frequently used as a biological marker for fasting experiments in chickens (Boswell et al., 1999).

## Statistical Analysis

Data were analyzed using the Student's *t*-test or one-way ANOVA for mRNA expression, blood glucose levels, and corticosterone levels in blood plasma. The significance level was set at $p < 0.05$. All results are expressed as the mean ± SEM.

# RESULTS

## Response to the 24-h Food Deprivation

To investigate the stress response of NPGL, NPGM, and HDC to 24 h food deprivation, we analyzed the mRNA expression of *NPGL*, *NPGM*, *HDC*, and *NPY*. The results showed that the mRNA expression of *NPGM* ($p < 0.005$, $F = 1.26$) and *NPY* ($p < 0.005$, $F = 2.87$) significantly increased after 24 h fasting (**Figures 1B,D**). However, *NPGL* ($p = 0.27$, $F = 1.86$) and *HDC* ($p = 0.16$, $F = 3.65$) did not change (**Figures 1A,C**). In this experiment, blood glucose levels ($p < 0.005$, $F = 12.99$) decreased after 24 h of food deprivation (**Supplementary Figure 1**).

## Response to Heat Stress

To investigate the effect of ambient temperature, we examined the mRNA expression of *NPGL*, *NPGM*, and *HDC* under heat stress. The chicks were kept at 37°C for 24 h, and then the mRNA expression levels of *NPGL*, *NPGM*, and *HDC* were quantified by real-time PCR. Corticosterone concentration was determined as a biological marker of stress in birds. The *NPGL* ($p = 0.37$, $F = 0.44$), *NPGM* ($p = 0.65$, $F = 3.17$), and *HDC* ($p = 0.53$, $F = 1.56$) mRNA expression levels (**Figures 2A–C**), and plasma corticosterone concentration ($p = 0.12$, $F = 0.43$; **Figure 2D**) were not affected by heat stress.

## Response to LPS-Induced Inflammation

LPS is an endotoxin derived from the outer membrane of gram-negative bacteria and is used to induce inflammatory stress. Thus, we administered i.p. injection of LPS to the chicks and quantified the mRNA expression levels of *NPGL*, *NPGM*, and *HDC* by real-time PCR. The mRNA expression of *NPGM* ($p < 0.05$, $F = 3.57$) significantly increased after i.p. injection of LPS (**Figure 3B**). However, the expression levels of *NPGL* ($p = 0.51$, $F = 2.90$) and *HDC* ($p = 0.95$, $F = 0.92$) mRNA did not change (**Figures 3A,C**). In addition, plasma corticosterone concentration ($p < 0.005$, $F = 0.00029$) significantly increased after the i.p. injection of LPS (**Figure 3D**).

## Response to Social Isolation Stress

Social isolation is a strong stressor, because chicks spend time in a group after hatching. To investigate the stress response to social isolation stress, 7-day-old chicks were isolated for 3 h, and the mRNA expression of *NPGL*, *NPGM*, and *HDC* was quantitated by real-time PCR. The results showed that the expression levels of *NPGL* ($p < 0.05$, $F = 1.33$), *NPGM* ($p < 0.005$, $F = 3.37$), and *HDC* ($p < 0.05$, $F = 2.67$) mRNA

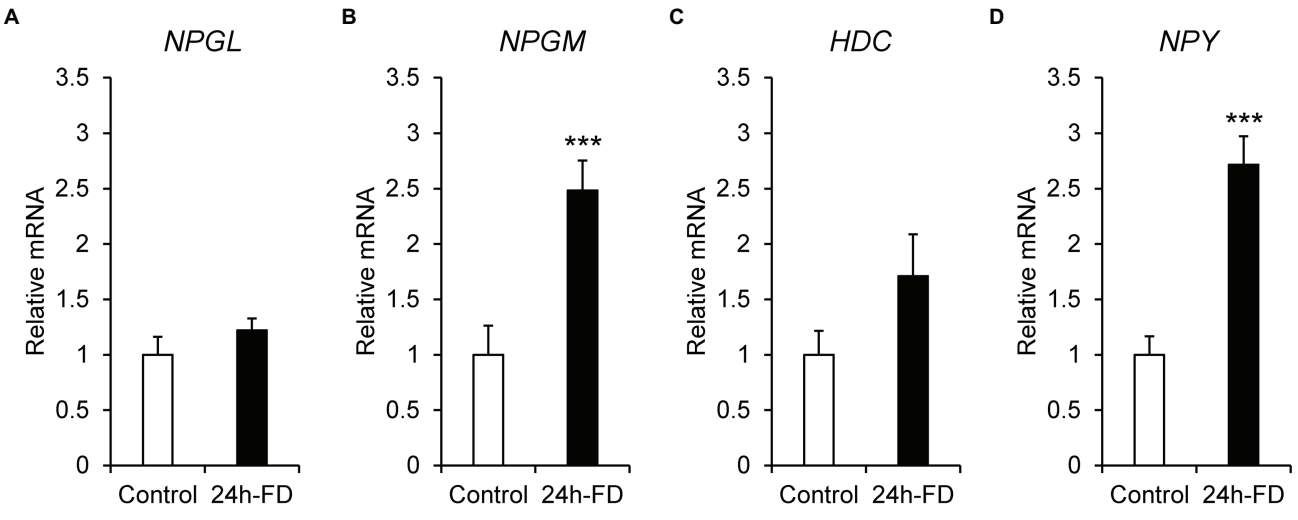


**FIGURE 1 |** mRNA expression levels, neurosecretory protein GL (*NPGL*; **A**), neurosecretory protein GM (*NPGM*; **B**), histidine decarboxylase (*HDC*; **C**), neuropeptide Y (*NPY*; **D**) in the mediobasal hypothalamus in the 24 h food deprivation experiment. Data are expressed as the mean ± SEM ($n = 5$–6). Data were analyzed by Student's *t*-test. An asterisk indicates a statistically significant difference (***$p < 0.005$).

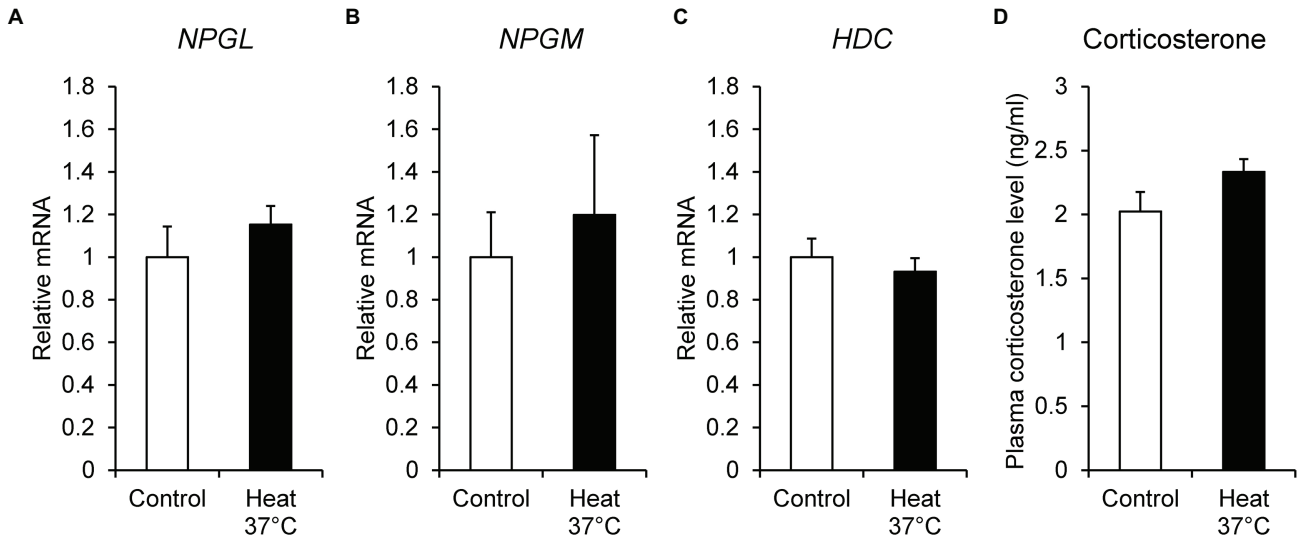


**FIGURE 2 |** mRNA expression levels, neurosecretory protein GL (*NPGL*; **A**), neurosecretory protein GM (*NPGM*; **B**), histidine decarboxylase (*HDC*; **C**) in the mediobasal hypothalamus in the heat stress experiment at 37°C for 24 h. Plasma corticosterone concentration level **(D)**. Data are expressed as the mean ± SEM ($n = 6$). Data were analyzed by Student's *t*-test.

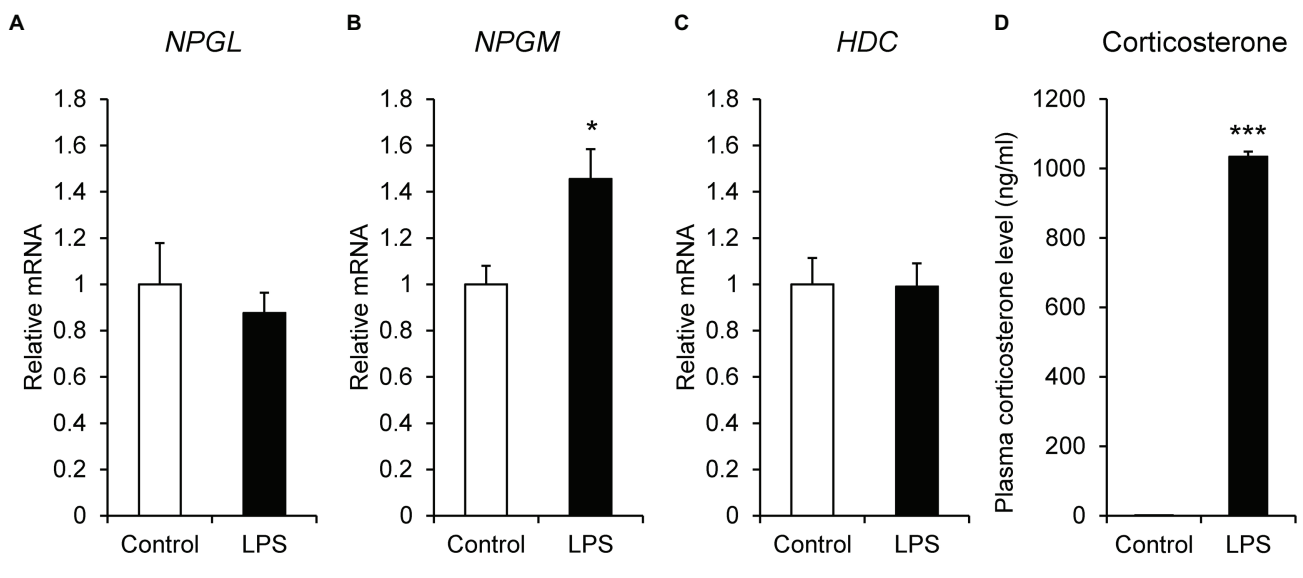


**FIGURE 3 |** mRNA expression levels, neurosecretory protein GL (*NPGL*; **A**), neurosecretory protein GM (*NPGM*; **B**), histidine decarboxylase (*HDC*; **C**) in the mediobasal hypothalamus in the lipopolysaccharide (LPS)-induced inflammation stress experiment. Plasma corticosterone concentration level **(D)**. Data are expressed as the mean ± SEM ($n = 5$–7). Data were analyzed by Student's *t*-test. An asterisk indicates a statistically significant difference (*$p < 0.05$, ***$p < 0.005$).

significantly increased after the isolation (**Figures 4A–C**). The isolated chicks were keeping to make distress calls for 3 h (data not shown), although plasma corticosterone concentrations ($p = 0.21$, $F = 0.33$) did not change after the isolation (**Figure 4D**).

## DISCUSSION

This study is the first to investigate the effect of various stress conditions on the mRNA expression of *NPGL*, *NPGM*, and *HDC* in the MBH. We selected four stressors: food deprivation, heat stress, inflammation stress, and isolation stress. The results of the stress experiments suggested that the changes in the mRNA expressions of *NPGL*, *NPGM*, and *HDC* depend on the type of stressor. *NPGM* mRNA expression was upregulated by food deprivation, inflammation stress, and social isolation stress. In contrast, the mRNA expression of *NPGL* and *HDC* was only increased by social isolation stress, although they were co-expressed with NPGM in the same neurons of the chicken MM (Shikano et al., 2018a). Heat stress at 37°C for

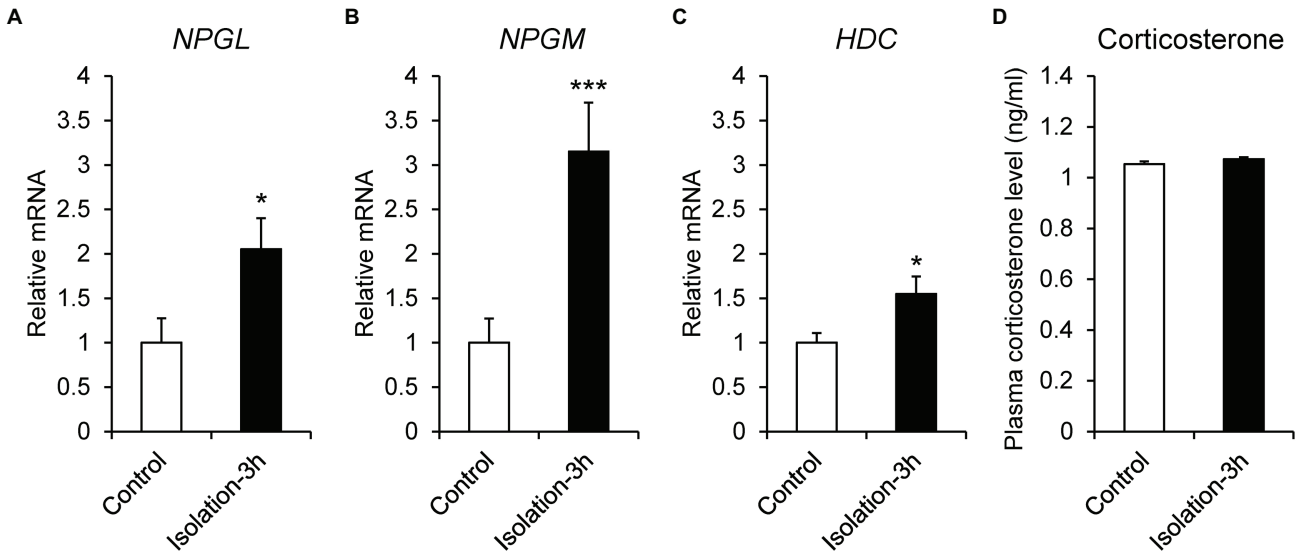


**FIGURE 4 |** mRNA expression levels, neurosecretory protein GL (*NPGL*; **A**), neurosecretory protein GM (*NPGM*; **B**), histidine decarboxylase (*HDC*; **C**) in the mediobasal hypothalamus in the isolation stress experiment for 3 h. Plasma corticosterone concentration level **(D)**. Data are expressed as the mean ± SEM ($n = 5$–6). Data were analyzed by Student's *t*-test. An asterisk indicates a statistically significant difference ($^{*}p < 0.05$, $^{***}p < 0.005$).

24 h had no effect on the mRNA expression of these genes. These results suggest that NPGM is more sensitive to energy deficiency, inflammatory response, and isolated states than NPGL or HDC.

Food deprivation experiments are widely used to understand the regulation and molecular mechanisms of feeding in many animals. Food deprivation for 24 h increased the mRNA expression of *NPGM*, whereas that of *NPGL* and *HDC* did not change. This result shows that hunger signaling regulates only the mRNA expression of *NPGM* in NPGL/NPGM/HDC-expressing neurons. The hypothalamus in chicks receives hunger signals, such as hypoglycemia, hormones from peripheral tissues, and other regions of the brain (Richards and Proszkowiec-Weglarz, 2007). For example, the central melanocortin system is one of the most characterized neuronal pathways in the regulation of feeding behavior and energy expenditure (Cone, 2005). In addition to melanocortin, when hunger signals *via* the blood or vagus nerve are transmitted from the peripheral tissues, NPY- and agouti-related peptide (AgRP)-expressing neurons are subsequently activated, which increases feeding behavior in chicks (Richards and Proszkowiec-Weglarz, 2007). In this study, *NPY* mRNA expression was increased by food deprivation for 24 h, and blood glucose levels gradually decreased during fasting. These responses indicated that the hunger signal was being generated adequately. Recently, a part of the NPY-related hunger signal pathway had been elucidated in rat brain (Nakamura et al., 2017). It has been reported that NPY, during hunger, activates GABAergic neurons in the reticular nuclei of the medulla oblongata, and GABAergic neurons inhibit energy expenditure (Nakamura et al., 2017). Our previous study showed that NPGM induces fat accumulation without increasing feeding behavior in chicks (Kato et al., 2021). It is possible that NPGM activated by hunger signaling may inhibit energy expenditure *via* GABAergic neurons. The relationship between NPGM-expressing neurons and GABAergic neurons in chicks is still unclear. Therefore, further studies are necessary to elucidate the biological significance of NPGM during the hunger period.

It is well known that heat stress is a particularly important environmental stressor, as it changes physiological responses, reduces the ability to digest nutrients, and decreases growth rate in chicks (Lara and Rostagno, 2013; Lu et al., 2017; Chowdhury, 2019). In addition, heat stress is often associated with high mortality in chicks. Exposure of chickens to high temperatures activates the HPA axis *via* the PVN from the preoptic area and leads to secretion of corticosterone (Bohler et al., 2021). Heat stress inhibits the mRNA expression of *NPY* and induces anorexia (Bohler et al., 2020). In mice, parts of CRF neurons from the PVN project into NPY neurons and inhibit these neurons (Stengel et al., 2009; Kuperman et al., 2016). The same phenomenon may occur in birds. However, the relationship between CRF neurons and NPGL/NPGM/HDC-expressing neurons remains unclear. In this study, heat stress at 37°C for 24 h did not change the mRNA expression of *NPGL*, *NPGM*, and *HDC* in the MBH. Additionally, the blood plasma corticosterone levels did not change, suggesting that it was stable on the HPA axis during this period. In chicks, corticosterone concentration is known to increase in response to acute (12 h) heat stress, but subsequently decreases to normal levels after heat stress for 24 h (Furukawa et al., 2016). The present data was coincident with the previous study (Furukawa et al., 2016). Therefore, it is possible that the mRNA expression of *NPGL*, *NPGM* and *HDC* may be affected by heat stress for 12 h. Future studies should investigate the effects

of short-time heat stress on the mRNA expression of *NPGL*, *NPGM*, and *HDC* in chickens.

Injection of LPS has often been employed to study systemic inflammatory reactions (Tonelli et al., 2008). LPS activates macrophagic response and induces oxidative stress in a dose-dependent manner (Koumakis et al., 2019). LPS-induced inflammation is also used in chicks, similar to that in mammals (Zhang et al., 2013; Liu et al., 2020). The i.p. injection of LPS induces hyperthermia and anorexia and causes inflammation stress and tissue damage in chicks (Johnson et al., 1993). LPS-induced inflammation is known to induce the mRNA expression of cytokines such as interleukin-1β (*IL-1β*) and interleukin-6 (*IL-6*) in chicken peripheral tissues and hypothalamus (Zhang et al., 2013; Grabbe et al., 2020). In particular, IL-6 induces calcium signaling in the hypothalamic neurons of chickens (Grabbe et al., 2020). Inflammation and immunoreactivity lead to energy wastage, thus, inhibiting the growth in chicks (Takahashi et al., 2008). In this study, plasma corticosterone levels were drastically increased by LPS-induced inflammation. Since other studies have shown that the same treatment increases corticosterone levels (Johnson et al., 1993), the inflammatory response is correctly induced. LPS-induced inflammation increased *NPGM* mRNA expression in the MBH. This result suggests that *NPGM* mRNA expression in NPGL/NPGM/HDC-expressing neurons may be activated by inflammatory cytokines. Although the pathway that promotes the transcription of *NPGM* has not been identified yet, the cytokine signaling pathway may promote the transcription of *NPGM*. In the future, it will be necessary to understand the relationship between NPGM and cytokines, such as IL-1β and IL-6.

It has been reported that social isolation stress can change the reactivity to stress and social behavior in both animals and humans, and it has been used as a biobehavioral assay to study attachment processes (Panksepp et al., 1980; Sufka et al., 1994). Social isolation stress activates various endocrinological changes, such as the HPA axis and secretion of glucocorticoids and catecholamines (Tsigos and Chrousos, 2002). In birds, this stress is thought to be related to the growth rate (John Savory and MacLeod, 1980). It has been reported that social isolation stress activates the HPA axis and stimulates the secretion of corticosterone after 10 min of isolation in layer chicks (Saito et al., 2005). In this study, there was no change in plasma corticosterone levels after 3 h of isolation, although chicks were making distress calls for 3 h. However, the mRNA expression of *NPGL*, *NPGM*, and *HDC* increased after 3 h of isolation. It is possible that the expression of *NPGL*, *NPGM*, and *HDC* is independent of the HPA axis in this experiment. The c-Fos expression in a variety of brain regions during isolation stress has been mapped in Japanese quails (Takeuchi et al., 1996). The density of c-Fos-immunoreactive cells is much higher in stressed groups than that of non-stressed groups in the dorsomedial mesencephalic areas (DMM; Takeuchi et al., 1996). We have identified that histamine neurons are localized in the MM and projected to these areas in chickens (unpublished data). We speculate that NPGL/NPGM/HDC-expressing neurons are activated by isolation stress and that NPGL, NPGM, and histamine are transmitted to a variety of brain regions. In the future, we need to analyze the projection of NPGL/NPGM/HDC-expressing neurons throughout the chick brain, and the regulatory mechanism of mRNA expression under isolation stress.

In conclusion, the present study revealed that the expression of *NPGL*, *NPGM*, and *HDC* depends on the type of stressor and may regulate stress responses to protect the whole body from stressors. This study shows that new factors contribute to stress mechanisms in chickens. Our previous study showed that the mRNA expression levels of *NPGL* increased, and *NPGM* decreased during post-hatching development in chicks (Shikano et al., 2018a). Thus, NPGL and NPGM have different physiological functions in addition to stress responses in chicks. However, the biological significance of the changes in mRNA expression under different stressors was not obvious in the present study. Future studies on the physiological functions of these brain factors and the gene-regulatory networks within the same neuron under stress are necessary. As the promoter regions of *NPGL*, *NPGM*, and *HDC* have not been studied yet, the presence of the stress response elements (STRE; Freitas et al., 2016; Iwata et al., 2017) in these regions remains unknown. On the other hand, this data was obtained using layer chicks. The layer is slower-growing and developed for egg production, while the broiler is fast-growing and selected for meat production. It is known that layers and broilers have different physiological and behavioral responses to stressors (Khan et al., 2015). Future studies need to analyze the mRNA expression of *NPGL*, *NPGM*, and *HDC* in broilers. Chickens must be reared under stress free conditions to prevent a decline in production and animal welfare. Therefore, elucidation of the molecular mechanisms of novel brain factors, NPGL and NPGM, may help to mitigate stresses in chickens.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by the experimental protocols were in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Hiroshima University (Higashi-Hiroshima, Japan).

## AUTHOR CONTRIBUTIONS

MK and KU: conceptualization. MK, EI-U, YN, MF, and KU: methodology and investigation. MK: writing—original draft

preparation and visualization. MK, YN, and KU: writing—review and editing. KU: project administration. EI-U and KU: funding acquisition. All authors contributed to the article and approved the published version of the manuscript.

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ORIGINAL RESEARCH
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# Chick Hippocampal Formation Displays Subdivision- and Layer-Selective Expression Patterns of Serotonin Receptor Subfamily Genes

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Hippocampal formation (HF) plays a key role in cognitive and emotional processing in mammals. In HF neural circuits, serotonin receptors (5-HTRs) modulate functions related to cognition and emotion. To understand the phylogenetic continuity of the neural basis for cognition and emotion, it is important to identify the neural circuits that regulate cognitive and emotional processing in animals. In birds, HF has been shown to be related to cognitive functions and emotion-related behaviors. However, details regarding the distribution of 5-HTRs in the avian brain are very sparse, and 5-HTRs, which are potentially involved in cognitive functions and emotion-related behaviors, are poorly understood. Previously, we showed that *5-HTR1B* and *5-HTR3A* were expressed in chick HF. To identify additional 5-HTRs that are potentially involved in cognitive and emotional functions in avian HF, we selected the chick orthologs of *5-HTR1D*, *5-HTR1E*, *5-HTR1F*, *5-HTR2B*, *5-HTR5A*, and *5-HTR7* and performed *in situ* hybridization in the chick telencephalon. We found that *5-HTR1D, 5-HTR1E, 5-HTR5A*, and *5-HTR7* were expressed in the chick HF, especially *5-HTR1D* and *5-HTR1E*, which showed subdivision- and layer-selective expression patterns, suggesting that the characteristic 5-HT regulation is involved in cognitive functions and emotion-related behaviors in these HF regions. These findings can facilitate the understanding of serotonin regulation in avian HF and the correspondence between the HF subdivisions of birds and mammals.

**Keywords: chick, hippocampal formation, serotonin receptor, subdivision, layer**

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## INTRODUCTION

The modulation of various neural functions by serotonin (5-hydroxytryptamine, 5-HT) is phylogenetically conserved (Marin et al., 2020). In particular, the association of the 5-HT system with cognition, behavior, and emotion is evolutionarily conserved in the animal kingdom (Kandel et al., 2000; Bacque-Cazenave et al., 2020). In mammals, processing of both cognition and emotion have been shown to involve the hippocampal formation (HF) (Fanselow and Dong, 2010; Anacker and Hen, 2017). The macrohistological features of HF are well-conserved in all mammals. More

specifically, HF consists of similarly convoluted and interlocked 3-layered subdivisions: the dentate gyrus (DG), Ammon's horns or Cornu ammonis (CA) fields 1 to 3 (CA1 to CA3), and the subiculum (Hevner, 2016; Medina et al., 2017b). Information flow in the mammalian HF was described as a "trisynaptic circuit", in which the entorhinal cortex (EC) projects to the DG, DG provides the projection to CA3 known as "mossy fiber", and CA3 relays it to CA1 (Sloviter and Lomo, 2012). To understand the phylogenetic continuity of the neural basis for cognition and emotion, it is essential to reveal the neural mechanisms in HF that process cognitive and emotional behaviors in nonmammalian animals. Birds are well-fitted model animals for understanding the evolutionary continuity of the neural basis of cognition and emotion (Rosa Salva et al., 2015; Papini et al., 2019). For instance, the polymorphism of the 5-HT transporter gene, which affects the levels of gene expression, has been suggested to modulate fear-related behavior in chickens (Krause et al., 2017; Phi Van et al., 2018; Krause et al., 2019). In addition, hippocampal formation in birds (HF, hippocampus proper (Hp), and area parahippocampalis (APH)) has been shown to be related to cognitive functions, such as spatial navigation (Colombo and Broadbent, 2000; Matsushima et al., 2003; Bingman, et al., 2005; Mayer, et al., 2016; Sherry, et al., 2017; Payne, et al., 2021), and controlling the stress response (Smulders, 2017; Smulders, 2021) and emotions, such as anxiety-like behavior (Mayer, et al., 2018; Morandi-Raikov and Mayer, 2020; Parada, et al., 2021). However, the neural circuits that control these behaviors and 5-HT regulation in the avian HF are largely unknown.

The avian HF is homologous to that of mammals (Reiner, et al., 2004; Herold, et al., 2015; Striedter, 2016). The ancient origin of HF in the amniote was supported by both recent large-scale and single-cell transcriptome studies (Belgard, et al., 2013; Tosches, et al., 2018). However, the avian HF is composed of a layered arrangement of densely packed neurons with poorly defined boundaries, whereas the mammalian HF has a clear laminar organization (Atoji and Wild, 2006; Herold, et al., 2015; Striedter, 2016). The existence of many subdivisions in the avian HF has been proposed to arise from multiple aspects, such as developmental origin, connectivity, histochemistry, and immunohistochemistry (Kuenzel and Masson, 1988; Atoji and Wild, 2004; Atoji and Wild, 2006; Suarez, et al., 2006; Puelles, et al., 2007; Gupta, et al., 2012; Herold, et al., 2014; Abellan, et al., 2014; Atoji, et al., 2016; Medinia, et al., 2017b). However, to date, a one-to-one correspondence of subdivisions between the avian and mammalian HF has not been established, especially owing to the controversy regarding its homology with the mammalian Hp (dentate gyrus and Ammon's horn) (Atoji and Wild, 2004; Atoji and Wild, 2006; Kempermann, 2012; Herold, et al., 2014; Abellan, et al., 2014; Streidter, 2016; Hevner, 2016; Atoji et al., 2016; Medina, et al., 2017b).

In mammals, various types of the 5-HT receptor (5-HTR) subfamily genes are expressed in the HF and are thought to play important roles in cognitive and emotional functions (Tanaka, et al., 2012; Strac et al., 2016; Zmudzka, et al., 2018; O'Leary, et al., 2020; Vilaro, et al., 2020). In our previous study, we pointed out that *5-HTR1B* and *5-HTR3A* were expressed in chick HF in a clear and characteristic manner (Fujita, et al., 2020). In the present study, we performed a detailed analysis and determined the subdivision of HF in which *5-HTR1B* and *5-HTR3A* are expressed. To comprehensively identify more 5-HTRs that are potentially involved in cognitive and emotional functions in avian HF, we investigated the expression of 5-HTR subfamily genes that were not analyzed in our previous study (Fujita, et al., 2020). We selected the chick orthologues of *5-HTR1D*, *5-HTR1E*, *5-HTR1F*, *5-HTR2B*, *5-HTR5A*, and *5-HTR7* and found that *5-HTR1D, 5-HTR1E, 5-HTR5A*, and *5-HTR7* were expressed in the chick HF. Among them, *5-HTR1D* and *5-HTR1E* showed subdivision- and layer-selective expression patterns, suggesting a characteristic 5-HT regulation in these regions. Our findings can be used as a basis for understanding 5-HT regulation in avian HF and the correspondence between the HF subdivisions of birds and mammals.

# MATERIALS AND METHODS

## Animals

Fertilized eggs of domestic chicks (*Gallus domesticus*, Cobb strain) were purchased from a local dealer (3-M, Aichi, Japan) and incubated at Teikyo University (Kaga, Itabashi-ku, Tokyo, Japan). Animal experiments were performed as previously described (Yamaguchi et al., 2008a; 2008b). Newly hatched chicks (P0) were transferred to dark plastic enclosures in a dark, warm cage at 30°C for 1 day (P1). We used seven chicks for *5-HTR1D* probes, nine for *5-HTR1E*, eight for 5-*HTR1F*, seven for 5-*HTR2B*, six for 5-*HTR5A*, six for 5-*HTR7*, three for 5-*HTR3A*, five for 5-*HTR1B*, and six for *lymphoid enhancer factor 1* (*LEF1*) (**Supplementary Table S1**). As for *5-HTR3A* and *5-HTR1B*, we have already investigated the expression of *5-HTR3A* and *5-HTR1B* throughout the entire brain in our previous study. In this study we focused on the expression of *5-HTR3A* and *5-HTR1B* in the HF. All procedures were reviewed and approved by the Committee on Animal Experiments of Teikyo University and were conducted in accordance with the guidelines of the national regulations for animal welfare in Japan.

## Tissue Preparation

P1 chicks were anesthetized by intraperitoneal injection (0.40 ml/individual) of a 1:1 solution of ketamine (10 mg/ml, Ketalar-10, Sankyo Co., Tokyo, Japan) and xylazine (2 mg/ml, Sigma, St. Louis, MO, United States). For brain fixation, the anesthetized chicks were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.5, PFA-PBS). Whole brain specimens were dissected and immediately immersed in PFA-PBS for 1 or 2 days at 4°C. For cryoprotection, fixed brain samples were placed in an 18% sucrose/PFA-PBS solution for 2 days at 4°C. Subsequently, brains with sucrose substitution were embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan), frozen immediately on dry ice, and stored at −80°C until sectioning. Frozen brain blocks were cut into 18 μm-thick sections using a cryostat (Leica CM3050S or Leica CM 1850, Leica Biosystems, Nußloch, Germany). Serial coronal sections were prepared at the level of A14.4–A4.4,

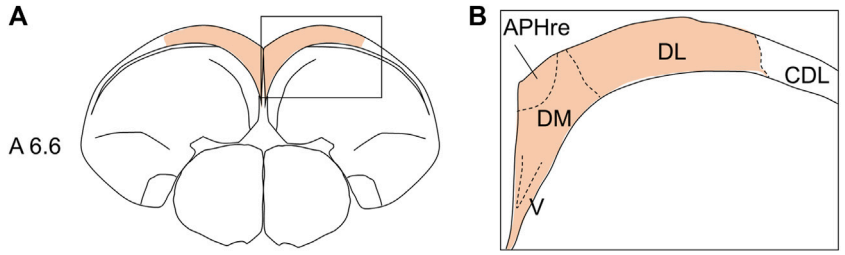


**FIGURE 1 |** Position of chick HF and its subdivisions **(A)** diagram of the coronal section of the chick telencephalon showing the HF position **(A)**. Subdivisions of the chick HF **(B)**. See **Table 1** for chick HF terminology. Regarding the HF range, to this day, no consensus or view for the inclusion of CDL exists (Puelles et al., 2007; Medina et al., 2017b). APHre, ectopic part of the rostral area parahippocampalis; CDL, corticoidea dorsolateralis; DL, dorsal lateral region of HF; DM, dorsal medial region of HF; V, V-shaped complex. Levels of sections were in accordance with those mentioned in Kuenzel and Masson's atlas (Kuenzel and Masson, 1988).

**TABLE 1 |** Comparison of terminology regarding avian HF.

| Chicken | | | Pigeon |
|---|---|---|---|
| This study, 2022 | Kuenzel and Masson, (1988) | Suarez et al. (2006); Puelles et al. (2007); Abellan et al. (2014) | Atoji and Wild, 2004; Atoji and Wild, 2006; Herold et al. (2014) |
| V | Hp | DGP | Vl |
| | | | Tr |
| DM | APH | APHm, APHi | DM |
| APHre | | APHre | Pa |
| — | | — | Po |
| — | | — | Ma |
| DL | | APHl | DL |

*APH, area parahippocampalis; APHi, intermediate APH; APHl, lateral APH; APHm, medial APH; APHre, ectopic part of rostral APH; DGP, dentate gyrus primordium; DL, dorsal lateral region of HF; DM, dorsal medial region of HF; Hp, hippocampus, Ma, magnocellular region of HF; Pa, parvocellular region of HF; Po, cell-poor region of HF; Tr, triangular region of HF; V, V-shaped complex; Vl, V-shaped layer region of HF.*

corresponding to those of the atlas by Kuenzel and Masson (1988).

## cDNA Cloning

Total RNA was extracted from chick brains using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and reverse-transcribed using the SuperScript III kit (Invitrogen) with an oligo (dT) primer, according to the manufacturer's protocol. Reverse transcription polymerase chain reaction (RT-PCR) for the amplification of *LEF1* was performed using gene-specific primers: forward, 5′-GATCCCCTTCAAGGACGAAG-3′; and reverse, 5′-GCCAAGAGGTGGTGTTATCTG-3′. PCR products were subcloned into the pGEM-T easy vector (Promega, Madison, WI, United States), the sequence of which was validated using Sanger sequencing. For 5-*HTR1B, 5-HTR1D, 5-HTR1E, 5-HTR1F, 5-HTR2B*, *5-HTR3A*, *5-HTR5A*, and *5-HTR7* probes, we used previously generated plasmids (Fujita et al., 2020; Fujita et al., 2022).

## RNA Probe Preparations

Plasmids containing cDNA fragments for *5-HTR1B, 5-HTR1D, 5-HTR1E, 5-HTR1F, 5-HTR2B, 5-HTR3A, 5-HTR5A*, *5-HTR7*, and *LEF1* were amplified by PCR using the M13 primer pair. Amplicons containing T7 and SP6 promoter sites were purified using a PCR purification kit (Qiagen, Valencia, CA, United States). Digoxigenin (DIG)-labelled sense and antisense RNA probes were prepared by *in vitro* transcription using a DIG RNA labelling kit (Roche, Basel, Switzerland) according to the manufacturer's protocol.

## *In situ* Hybridization

ISH experiments were performed as previously described in Fujita et al. (2019), with some modifications. Brain section specimens were refixed in 4% PFA-PBS, pretreated, and hybridized with DIG-labelled RNA probes at 70°C. After stringent washes with a series of saline-sodium citrate (SSC) buffers, hybridized probes were detected via immunohistochemical examination using an alkaline phosphatase-conjugated anti-DIG antibody (1:1,000; Roche). For signal visualization, a chromogenic reaction with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) was performed at 25°C for the following durations: *5-HTR1D, 5-HTR1E, 5-HTR1F*, and *5-HTR2B*, 18–42.5 h; *5-HTR5A*, 18.3–39.5 h; *5-HTR7*, 19.5–39.8 h; *5-HTR1B*, 18.2–39 h; *5-HTR3A*, 19.5 h; and *LEF1*, 18–38.8 h. Sense probes were used as negative controls in every experiment.

## Image Acquisition and Data Processing

Bright-field images of whole sections on each slide glass were semiautomatically taken using the NanoZoomer 2.0 HT or NanoZoomer XR systems (Hamamatsu Photonics, Shizuoka, Japan). Microscopic fields of interest were cropped using the

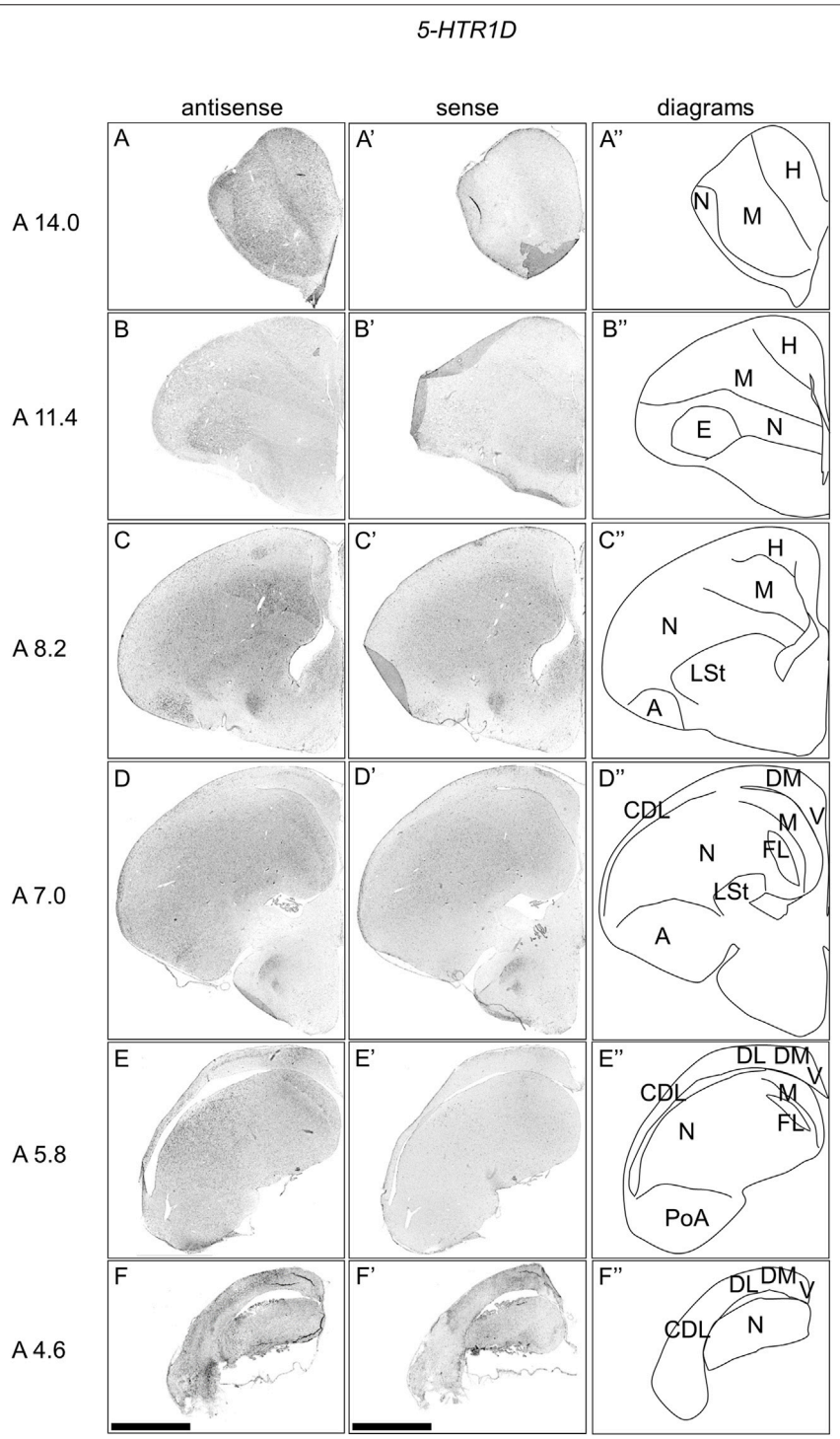


**FIGURE 2 |** *In situ* hybridization of *5-HTR1D* in P1 chick telencephalons. Digoxigenin-labelled RNA antisense **(A–F)** and sense **(A′–F′)** *5-HTR1D* probes were used for *in situ* hybridization in coronal sections of the P1 chick telencephalon. To evaluate the expression patterns of *5-HTR1D*, sections from seven chicks were analyzed, and representative images from four chick brain sections are shown. Diagrams of coronal sections are shown in the rightmost panels **(A–F∋)**. Levels of sections (A 14.0 to A 4.6) were in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). A, arcopallium; CDL, area corticoidea dorsolateralis; DL, dorsal lateral region of HF; DM, dorsal medial region of HF; E, entopallium; FL, field L; H, hyperpallium; LSt, lateral striatum; M, mesopallium; N, nidopallium; PoA, posterior pallial amygdala; V, V-shaped complex; P1: posthatch day 1. Scale bars = 2.5 mm.

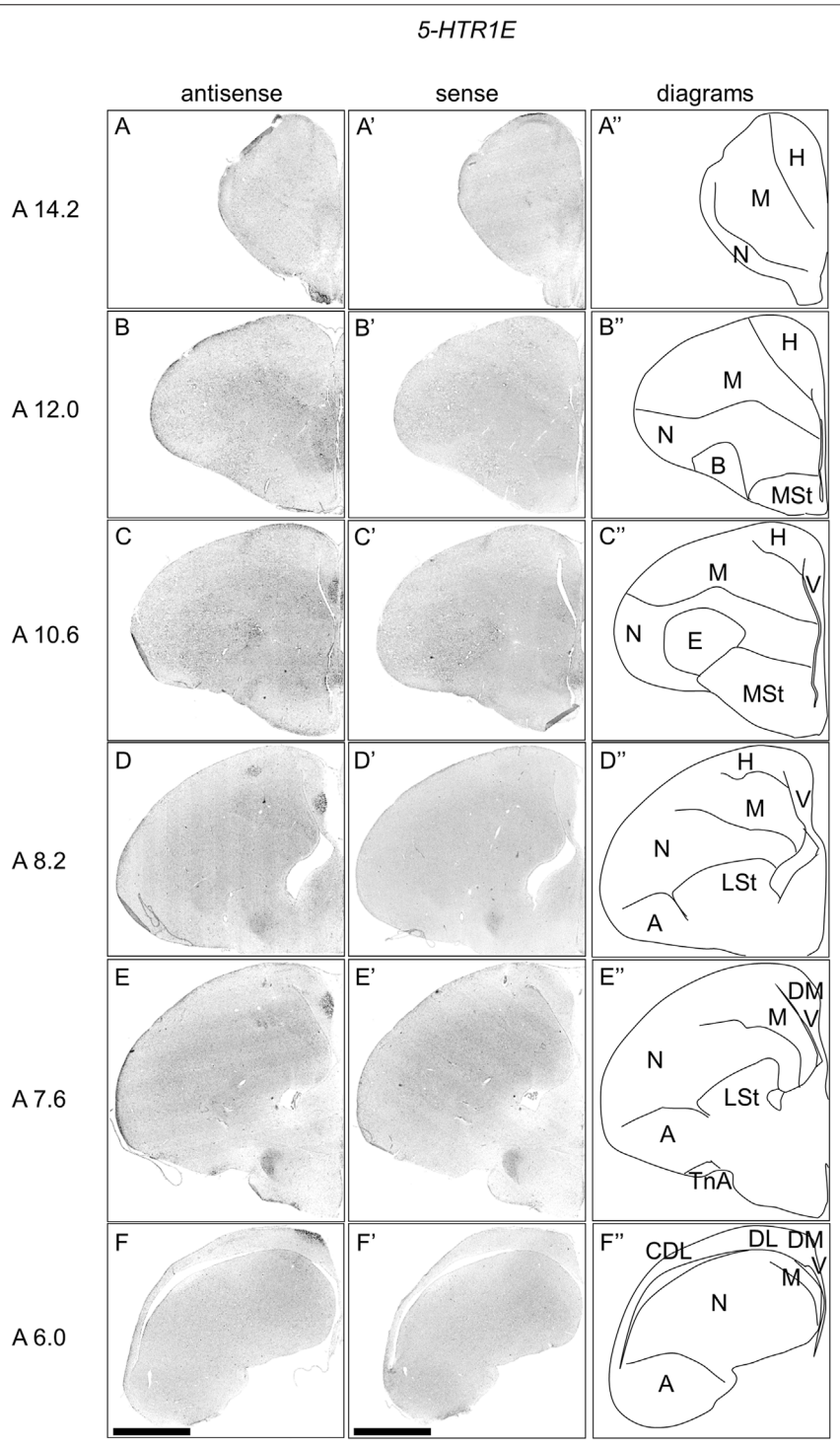


**FIGURE 3 |** *In situ* hybridization of *5-HTR1E* in P1 chick telencephalons. Digoxigenin-labelled RNA antisense **(A–F)** and sense **(A′–F′)** *5-HTR1E* probes were used for *in situ* hybridization in coronal sections of the P1 chick telencephalon. To evaluate the expression patterns of *5-HTR1E*, sections from nine chicks were analyzed, and representative images from three chick brain sections are shown. Diagrams of coronal sections are shown in the rightmost panels **(A–F∋)**. Levels of sections (A 14.2 to A 6.0) were in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). A: arcopallium; B: basorostralis; CDL: the area corticoidea dorsolateralis; DL: the dorsal lateral region of HF; DM: the dorsal medial region of HF; E: entopallium; H: hyperpallium; LSt lateral striatum; M, mesopallium; MSt, medial striatum; N, nidopallium; TnA, nucleus taeniae of the amygdala; V, V-shaped complex; P1: posthatch day 1. Scale bars = 2.5 mm.

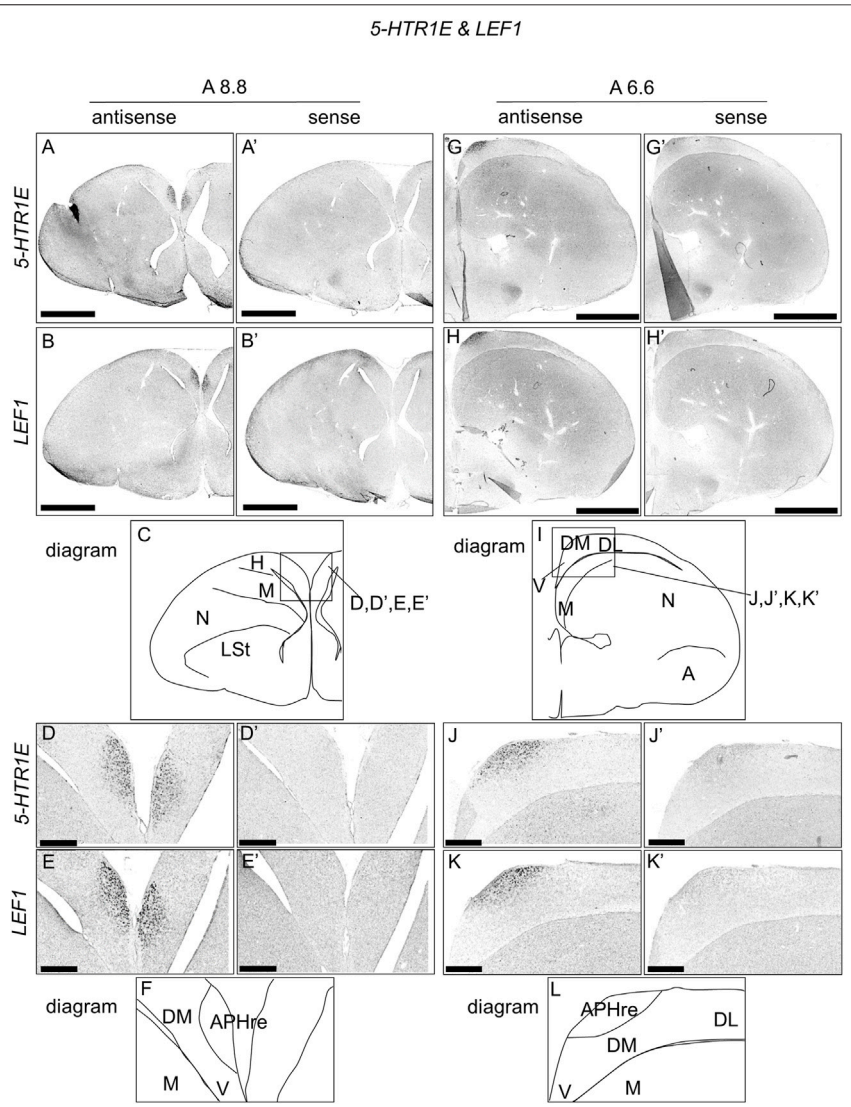


**FIGURE 4 |** Comparison of *5-HTR1E* and *LEF1* expression patterns in P1 chick telencephalon sections. *In situ* hybridization using DIG-labelled RNA antisense and sense *5-HTR1E* **(A,D,G,J**, **and A′,D′,G′,J′)**, respectively, and *LEF1* **(B,E,H,K**, **and B′,E′,H′,K′)**, respectively probes in P1 chick brain coronal sections are shown. Panels **(C) (F) (I),** and **(L)** show diagrams of **(A) (D) (G),** and **(J),** respectively. Levels of sections (A8.8 and A6.6) were in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). A: arcopallium; APHre: ectopic part of the rostral area hippocampalis; DL, dorsal lateral region of HF; DM, dorsal medial region of HF; H, hyperpallium; LSt, lateral striatum; M, mesopallium; N, nidopallium; V, V-shaped complex; P1: posthatch day 1. Scale bars = 2.5 mm **(A,B,G,H,A′,B′,G′,H′)** and 500 μm **(D,E,J,K,D′,E′,J′,K′)**.

NDP.view2 software (ver. 2.7.25; Hamamatsu Photonics, Shizuoka, Japan). Cropped images were converted to 8-bit images and their brightness and contrast were adjusted using ImageJ (ver. 1.52a, National Institute of Health, Bethesda, MD, United States).

## Terminology of Avian Hippocampal Formation

All histological terminologies used in this study were based on the atlas of Kuenzel and Masson (1988) and the descriptions of the avian brain nomenclature consortium (Reiner et al., 2004), except for HF and its subdivisions. The subdivision schemes of avian HF were based on various aspects and species, such as tract tracing and Nissl staining in pigeons (Atoji and Wild, 2004; Atoji and Wild, 2006), histochemistry of neurotransmitter radioligands in pigeons (Herold, et al., 2014), immunohistochemistry of several neurochemical markers in developing chickens (Suarez, et al., 2006), and combinatorial expression patterns of morphogenetic genes in developing chickens (Abellan, et al., 2014). No uniform nomenclature has been applied across species (Herold, et al., 2015). In this study, the histological position of HF in chicks was based on the nomenclature set by Puelles et al. (2007). Based on previous studies (Kuenzel and Masson, 1988; Atoji and Wild, 2004; Atoji and Wild, 2006; Suarez, et al., 2006; Puelles, et al., 2007; Abellan,

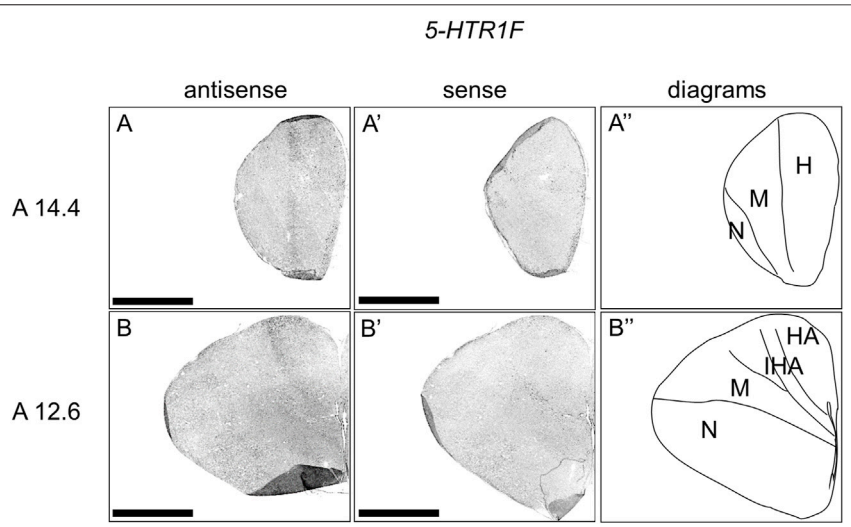


**FIGURE 5 |** *In situ* hybridization of *5-HTR1F* in P1 chick telencephalon. Digoxigenin-labelled RNA antisense **(A–B)** and sense **(A′–B′)** *5-HTR1F* probes were used for *in situ* hybridization in coronal sections of the P1 chick telencephalon. To evaluate the expression patterns of *5-HTR1F*, sections from eight chicks were analyzed, and representative images from two chick brain sections are shown **(A∋–B∋)** Diagrams of coronal sections are shown in the rightmost panels. Levels of sections (A14.4 and A12.6) were in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). H, hyperpallium; HA, hyperpallium apicale; IHA, interstitial part of the hyperpallium; M, mesopallium; N, nidopallium; P1: posthatch day 1. Scale bars = 2.5 mm.

et al., 2014; Herold, et al., 2014), we used the following subdivision terms: V-shaped complex (V), dorsal medial region (DM), ectopic part of the rostral APH (APHre), and dorsal lateral region (DL) (**Figure 1**). The relationships between the terminologies of avian HF used in this study and those in previous studies are summarized in **Table 1**.

# RESULTS

## Selection of Chick Orthologues of Mammalian 5-HTR Genes and LEF1 as APHre Marker Gene

We initially selected the chick orthologs of mammalian *5-HTR* genes, namely *5-HTR1B, 5-HTR1D, 5-HTR1E, 5-HTR1F, 5-HTR2B, 5-HTR3A, 5-HTR5A*, and *5-HTR7*. Among these, the expression patterns of *5-HTR1B* and *5-HTR3A* in the chick telencephalon have been previously reported (Fujita, et al., 2020). The chicken genome also contains *5-HTR6* (International Chicken Genome Sequencing Consortium, 2004); yet we could not obtain a subclone in our study. LEF1 is an important transcription factor for granule cell production in the dentate gyrus (Galceran, et al., 2000). We found that the chick *LEF1* ortholog exhibited sequence similarities with that of humans: 95% (protein) and 85.2% (DNA). In our previous studies, we searched for sequence similarities between chick *5-HTR1B, 5-HTR1D, 5-HTR1E, 5-HTR1F, 5- HTR2B, 5-HTR3A*, *5-HTR5A*, and *5-HTR7* with those from other animals (Fujita et al., 2020; Fujita et al., 2022). The accession numbers and molecular characteristics of ortholog gene probes are summarized in **Supplementary Table S2**. We accordingly designed probes to detect multiple transcript variants of orthologs registered in the database. Consecutively, we performed *in situ* hybridization (ISH) and analyzed the expression patterns of these orthologs in the telencephalon of chicks.

## Expression of *5-HTR1D* in Chick Telencephalon

To comprehensively examine the expression pattern of *5-HTR1D* in the chick telencephalon, we performed ISH using the *5-HTR1D* probe on coronal sections at approximately A14.4 to A 4.6 of naive chicks on posthatch day 1 (P1). We detected signals in a large part of the mesopallium (**Figure 2A–E, A′-E′**), entopallium (**Figure 2B, B′**), field L (**Figures 2D,E, D′-E′**), a part of the hyperpallium (**Figure 2C, C′**), a large part of the arcopallium (**Figure 2C,D, C′-D′**), and the lateral part of the nidopallium (**Figure 2C–F, C′-F′**). In addition, we detected signals in a part of the DM (**Figure 2C–F, C′-F′**), DL in a characteristic layered manner (**Figure 2C–F, C′-F′**), and the corticoidea dorsolateralis area (CDL) (**Figure 2D–F, D′-F′**).

## Expression of *5-HTR1E* in Chick Telencephalon

We examined the expression pattern of 5-*HTR1E* in sections A14.2 to A6.0 of P1 chick telencephalons (**Figure 3**). We detected strong signals in the nucleus taeniae of the amygdala (TnA) (**Figure 3E, E′**), and a part of the DM, in a cluster manner (**Figure 3C–F, C′-F′**). We also detected signals in a part of the mesopallium (**Figure 3A–F, A′-F′**), hyperpallium (**Figure 3D, D′**), DL in a layered manner (**Figure 3D–F, D′-F′**), and CDL (**Figure 3F, F′**).

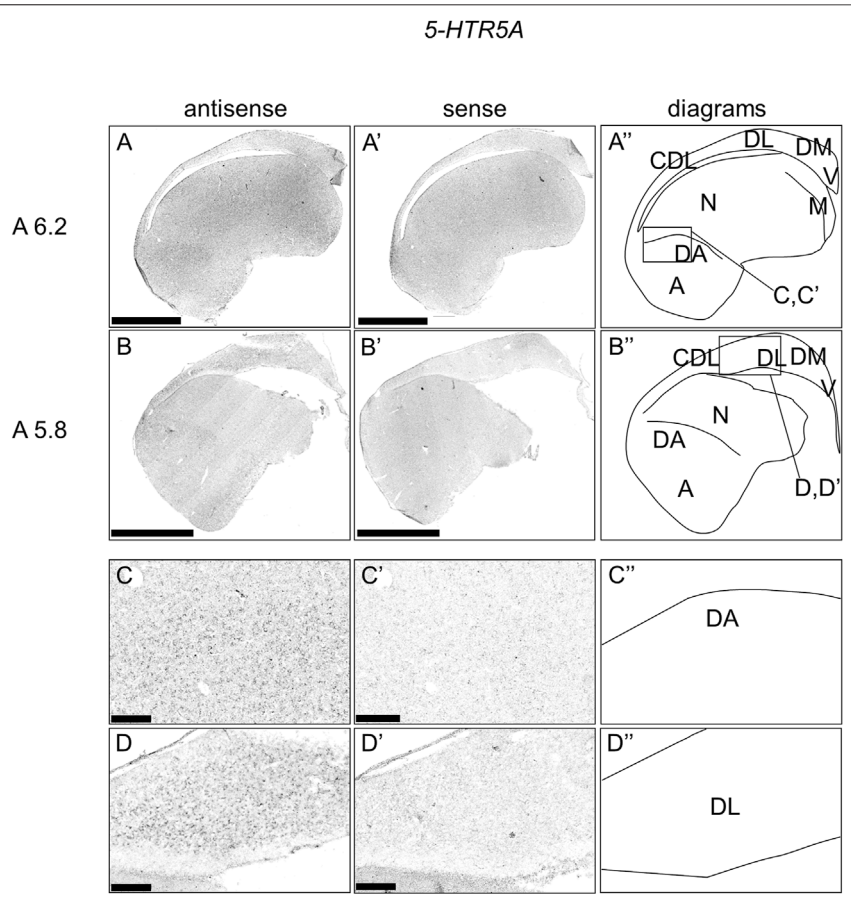


**FIGURE 6 |** *In situ* hybridization of *5-HTR5A* in P1 chick telencephalon. Digoxigenin-labelled RNA antisense **(A–D)** and sense **(A′–D′)** *5-HTR5A* probes were used for *in situ* hybridization in coronal sections of the P1 chick telencephalon. To evaluate the expression patterns of *5-HTR5A*, sections from six chicks were analyzed, and representative images from two chick brain sections are shown **(A∋–D∋)** Diagrams of coronal sections are shown in the rightmost panels. Levels of sections (A 6.2 and A 5.8) are in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988) **(C–D and C′–D′)** Magnified views of brain areas shown in the boxes in (A″ and B″). A: arcopallium; CDL, area corticoidea dorsolateralis; DA, dorsal arcopallium; DL, dorsal lateral region of HF; DM, dorsal medial region of HF; M, mesopallium; N, nidopallium; V, V-shaped complex; P1: posthatch day 1. Scale bars = 2.5 mm **(A–B and A′–B′)** and 250 μm **(C–D and C′–D′)**.

## Comparison of *5-HTR1E* and *LEF1* Expression Patterns in Chick APH

To compare the expression patterns between *5-HTR1E* and *LEF1*, which is the APHre region marker (Abellan et al., 2014), we performed ISH using *5-HTR1E* and *LEF1* probes in neighboring sections of A8.8 and A6.6, respectively (**Figure 4**). Interestingly, we found that the regions of expression of *5-HTR1E* and *LEF1* in the APHre matched well at both sections (**Figure 4D,E, J-K, D′-E′, J′-K′**).

## Expression of *5-HTR1F* in Chick Telencephalon

We then examined the expression patterns of *5-HTR1F* in sections A14.4 to A5.0 of P1 chick telencephalons (**Figure 5**). Our analysis revealed the presence of signals in the interstitial part of the hyperpallium (**Figure 5A,B, A′-B′**).

## Expression of *5-HTR5A* in Chick Telencephalon

We also examined the expression patterns of 5-*HTR5A* in sections A13.8 to A4.4 of P1 chick telencephalons (**Figure 6**). We accordingly detected the expression of 5-*HTR5A* in the dorsal arcopallium, lateral nidopallium, DL, and CDL (**Figure 6A,B, A′-B′**).

## Expression of *5-HTR7* in Chick Telencephalon

We further examined the expression patterns of *5-HTR7* in sections A13.6 to A4.4 of P1 chick telencephalons (**Figure 7**) and found that *5-HTR7* was mainly expressed in a large part of the arcopallium, lateral nidopallium, DM, DL (**Figure 7A–C, A′-C′**), and CDL (**Figure 7B,C, B′-C′**).

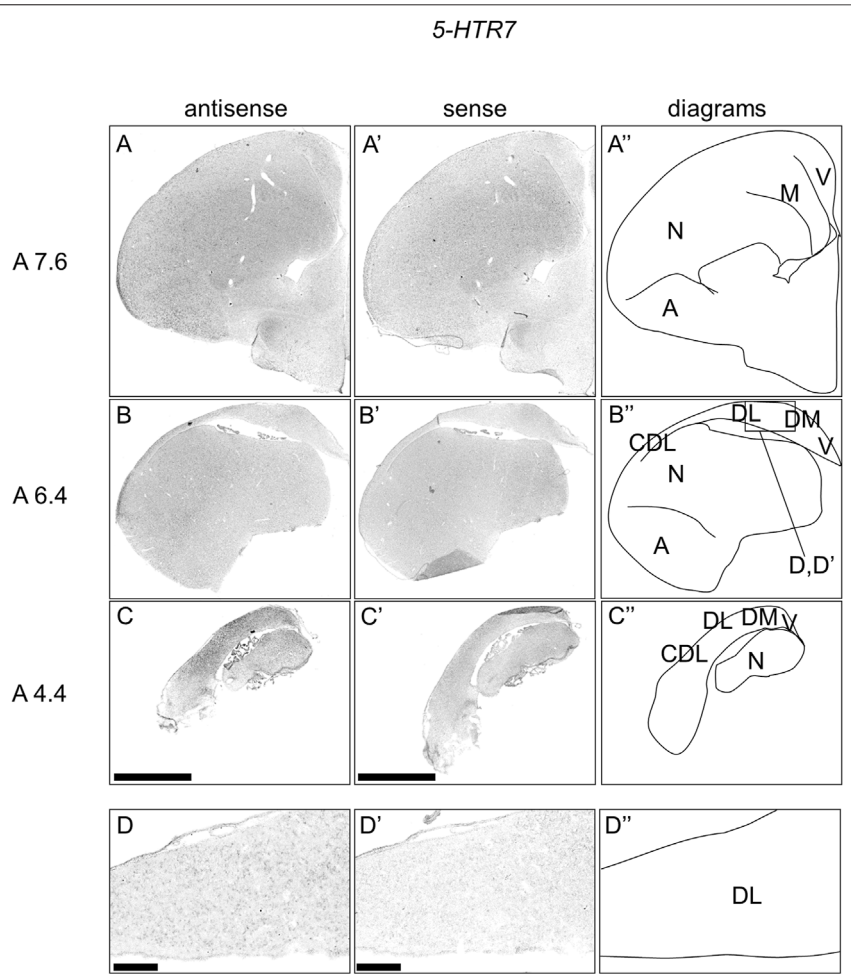


**FIGURE 7 |** *In situ* hybridization of *5-HTR7* in P1 chick telencephalons. Digoxigenin-labelled RNA antisense **(A–D)** and sense **(A′–D′)** *5-HTR7* probes were used for *in situ* hybridization in coronal sections of the P1 chick telencephalon. To evaluate the expression patterns of *5-HTR7*, sections from six chicks were analyzed, and representative images from three chick brain sections are shown **(A∋–D∋)** Diagrams of coronal sections are shown in the rightmost panels. Levels of sections (A 7.6, A 6.4, and A 4.4) were in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988) **(D and D′)** Magnified views of brain areas shown in the boxes in **(B∋)**. A, arcopallium; CDL, area corticoidea dorsolateralis; DL, dorsal lateral region of HF; DM, dorsal medial region of HF; M, mesopallium; N, nidopallium; V, V-shaped complex; P1: posthatch day 1. Scale bars = 2.5 mm **(A–C and A′–C′)** and 250 μm **(D and D′)**.

## Expression of *5-HTR2B* in Chick Telencephalon

Finally, we examined the expression patterns of *5-HTR2B* in sections A13.2 to A5.0, but did not detect any signal (data not shown), suggesting that the levels of expression of *5-HTR2B* were either very low or cells expressing *5-HTR2B* were very rare.

## Comparison of *5-HTR1B, 5-HTR1D, 5-HTR1E,* and *5-HTR3A* Expression Patterns in Chick Hippocampal Formation

We found that *5-HTR1D* and *5-HTR1E* were clearly expressed in chick HF. In our previous study, we revealed the characteristic expression patterns of *5-HTR1B* and *5-HTR3A* (Fujita, et al., 2020). Subsequently, to understand the relationship between the regions of expression of these *5-HTR*s in chick HF, we performed ISH using *5-HTR1B, 5-HTR1D, 5-HTR1E,* and *5-HTR3A* probes on neighboring sections of A8.8 to A8.6 (**Figures 8**, **9**A6.6 to A6.4 (**Figures 10**–**13**). We found that *5-HTR1B* was expressed in the whole DL (**Figure 8A, A′**, **Figure 9**, **Figure 10A, A′**, **Figure 13**), whereas sparsely in the DM (**Figure 8A, A′**, **Figure 9**, **Figure 10A, A′**, **Figure 12**). We also detected the expression of *5-HTR1D* in a part of DM (**Figure 8B, B′**, **Figure 9**, **Figure 10B, B′**, **Figure 12**), and in DL in a layered manner (**Figure 8B, B′**, **Figure 9**, **Figure 10B, B′**, **Figure 13**). We found that *5-HTR1E* was expressed in APHre (**Figure 8C, C′**, **Figure 9**, **Figure 10C, C′**, **Figure 12**), and in DL in a layered manner (**Figure 8C, C′**, **Figure 9**, **Figure 10C, C′**, **Figure 13**). We detected sparse signals of expression of *5-HTR3A* in both the DM and DL (**Figure 8D, D′**, **Figure 9**, **Figure 10D, D'**, **Figure 12**, **Figure 13**). Finally, we observed that *5-HTR1B* and *5-HTR3A* were sparsely expressed in V, whereas we detected a faint

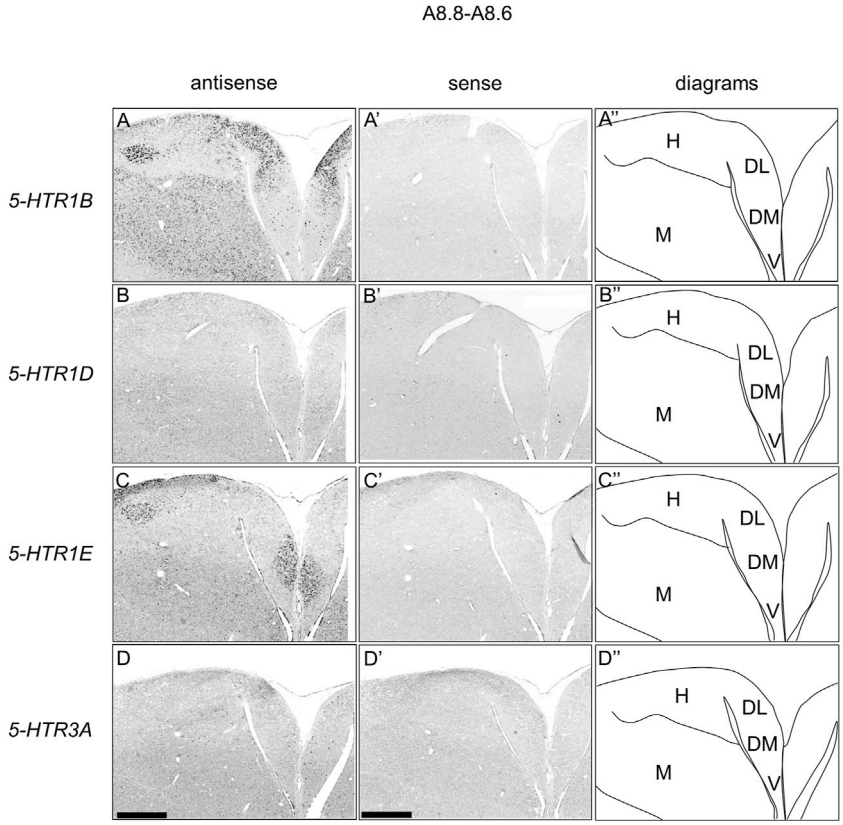


**FIGURE 8 |** Comparison of *5-HTR*s expression patterns in HF in P1 chick telencephalons using neighboring sections at approximately A 8.8 level. *In situ* hybridization using DIG-labelled RNA antisense and sense *5-HTR1B* **(A and A')**, *5-HTR1D* **(B and B')**, *5-HTR1E* **(C and C')**, and *5-HTR3A* **(D and D')** probes in coronal sections of P1 chick telencephalons **(A∋–D∋)** Diagrams of coronal sections are shown in the rightmost panels. DL, dorsal lateral region of HF; DM, dorsal medial region of HF; H, hyperpallium; M, mesopallium; V, V-shaped complex; P1: posthatch day 1. Scale bars = 1 mm.

expression of 5-*HTR1E* and no expression of *5-HTR1D* in this region (**Figure 11**).

# DISCUSSION

## Expression Patterns of *5-HTR* Subfamily Genes in Chick HF

In the present study, we revealed that 4 *5-HTR*s, *5-HTR1B, 5-HTR1D, 5-HTR1E*, and *5-HTR3A*, were expressed in a subdivision- and layer-selective manner in chick HF (**Figure 14**). We also found that *5-HTR5A* and *5-HTR7,* which were faintly expressed, did not clearly show subdivision- or layer-selective expression patterns in the chick HF.

We found that *5-HTR1B* was highly expressed in the DL, whereas sparsely in V, DM, APHre, while *5-HTR3A* was expressed in all the V, DM, DL, APHre, sparsely. In comparison, in mammals, *5-Htr1b* is expressed in Cornu Ammonis 1 (CA1) and CA3 pyramidal cells, GABAergic interneurons of the hilus, granule cells of dentate gyrus (DG), the subgranular zone, the layer II pyramidal cells in the entorhinal cortex (EC) (Tanaka, et al., 2012), while *5-Htr3A* is expressed in GABAergic interneurons of the hilus, in the subgranular zone, in scattered EC cells, CA1, CA3, and DG (Tecott, et al., 1993; Fonseca et al., 2001; Morales and Wang 2002; Tanaka, et al., 2012; Koyama, et al., 2017). Considering the expression patterns of *5-HTR1B* and *5-HTR3A*, our results support the correspondence of V and DM in the avian HF to the mammalian hippocampal proper (DG and CA subfields) (Montagnese et al., 1996; Szekely, 1999; Atoji and Wild, 2004; Atoji and Wild, 2006; Suarez, et al., 2006; Puelles et al., 2007; Herold et al., 2014; Abellan et al., 2014; Atoji et al., 2016; Medina et al., 2017b). To date, a one-to-one correspondence of subdivisions between the avian and mammalian HF has not been established, while various studies have postulated on possible areas in avian HF corresponding to mammalian DG. One suggestion is that the mammalian DG corresponds to V in avian HF (Atoji and Wild, 2004; Suarez, et al., 2006; Puelles et al., 2007; Gupta, et al., 2012; Herold et al., 2014; Atoji et al., 2016). Another theory is that the mammalian DG corresponds to DM in avian HF (Montagnese et al., 1996; Szekely, 1999). However, another possibility is that DG might be a novel acquisition in mammals, which would imply that there is no homolog in birds. The avian HF has undergone divergence over hundreds of millions of years of evolution, thus making it difficult to compare the subdivisions of HF between birds and mammals

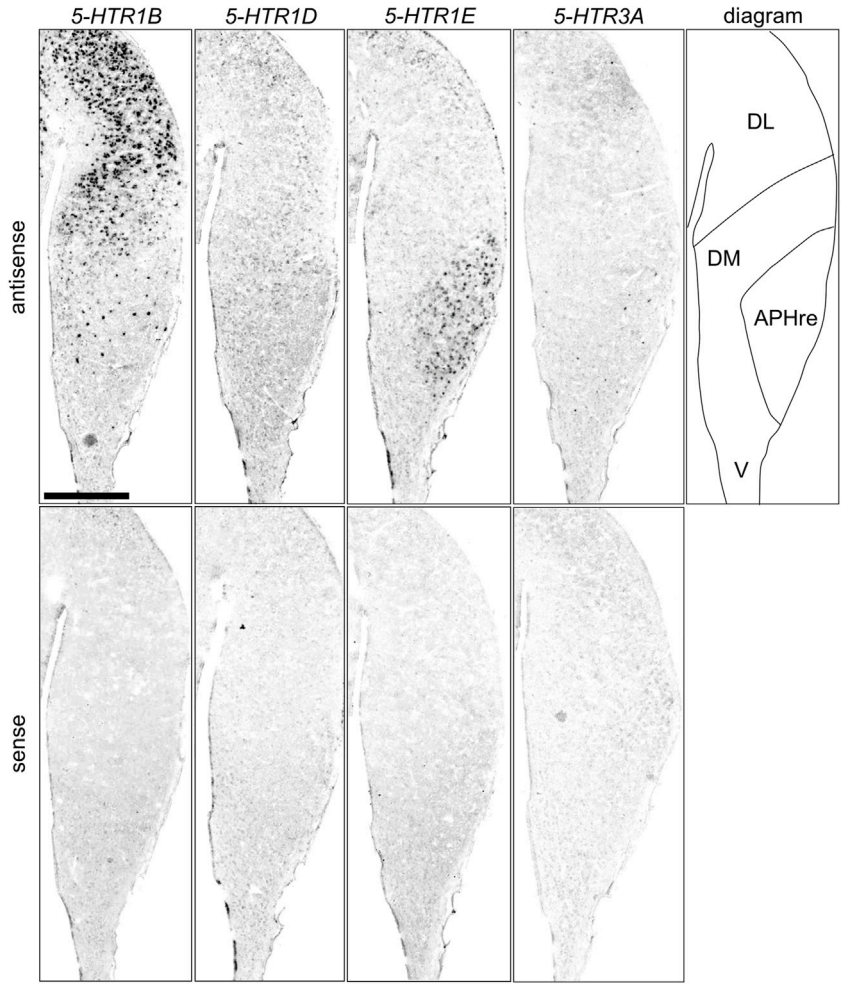


**FIGURE 9 |** Comparison of *5-HTR*s expression patterns in HF in P1 chick telencephalons using neighboring sections at approximately A 8.8 level focused on HF. Magnified views of HF surrounding regions are shown in **Figure 8**. APHre: ectopic part of the rostral area hippocampalis; DL: dorsal lateral region of HF; DM: dorsal medial region of HF; V: V-shaped complex; P1: posthatch day 1. Scale bars = 1 mm.

(Hevner, 2016; Striedter, 2016). In fact, although no macrostructure corresponding to mossy fibers in the mammalian DG has been observed in avian HF (Faber et al., 1989; Montagnese et al., 1993, 1996; Tombol et al., 2000; Herold, et al., 2014), this does not exclude the existence of a neuron population in the avian HF corresponding to granule cells in the mammalian DG. This candidate neuronal cell population corresponding to granule cells might be unevenly distributed in the avian HF. We assumed that using chick orthologs of another marker gene to distinguish pyramidal from granule cells in mammalian HF could provide new clues for the correspondence between avian and mammalian HF.

In chicks, *5-HTR1E* was highly expressed in APHre, and weakly in the V and DL in a layer-selective manner, suggesting that the APHre cell population that preferentially expresses *5-HTR1E* has novel characteristics. In contrast, in mammals, *5-Htr1e* was shown to be expressed in the CA fields and DG (Bruinvels et al., 1994a; Mengod, et al., 2006; Vilaro et al., 2020). However, because the relationship between APHre in avian HF and other HF subdivisions and the function of APHre are completely unknown (Abellan et al., 2014), it was difficult to determine the correspondence of APHre to the mammalian subdivision of HF. In the future, a better understanding of the features of APHre in chicks with respect to multiple aspects, such as connectivity, electrophysiological properties, and behavioral function, is expected to help determine the correspondence of APHre subdivisions.

Patch RNA-sequencing analysis in mammals showed that *5-Htr1d* was expressed in the GABAergic interneurons of CA1 (Luo, et al., 2019). We here found that *5-HTR1D* was expressed in DM, APHre, and DL in a layer-selective manner. Based on this,

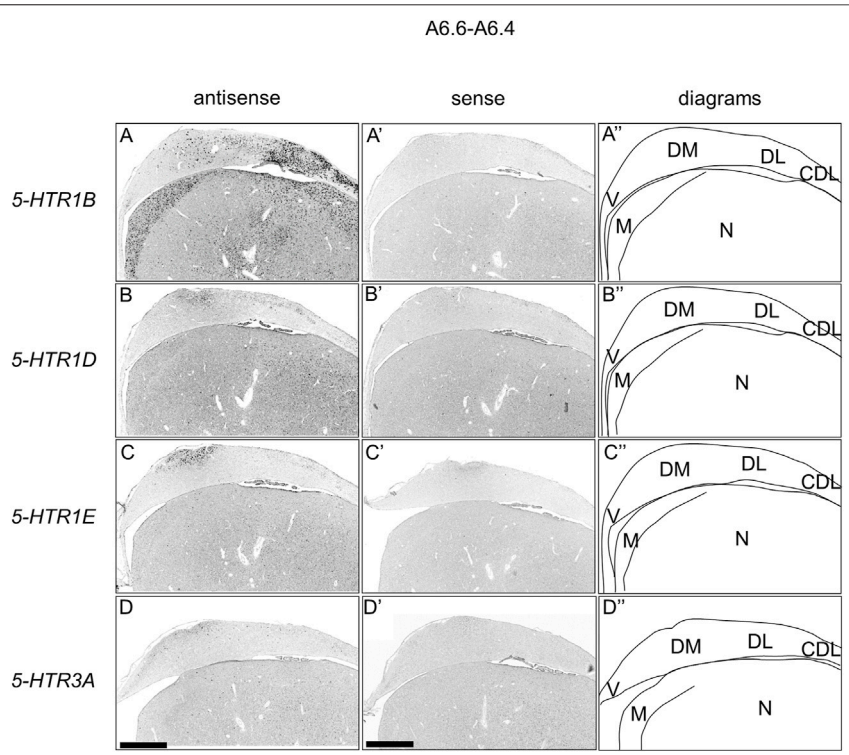


**FIGURE 10 |** Comparison of *5-HTR*s expression patterns in HF in P1 chick telencephalons using neighboring sections at approximately A 6.6. *In situ* hybridization using DIG-labelled RNA antisense and sense *5-HTR1B* **(A and A′)**, *5-HTR1D* (B and B′), *5-HTR1E* **(C and C′)**, and *5-HTR3A* **(D and D′)** probes in coronal sections of P1 chick telencephalons **(A∋–D∋)** Diagrams of coronal sections are shown in the rightmost panels. CDL: area corticoidea dorsolateralis; DL: dorsal lateral region of HF; DM: dorsal medial region of HF; M: mesopallium; N: nidopallium; V: V-shaped complex; P1: posthatch day 1. Scale bars = 1 mm.

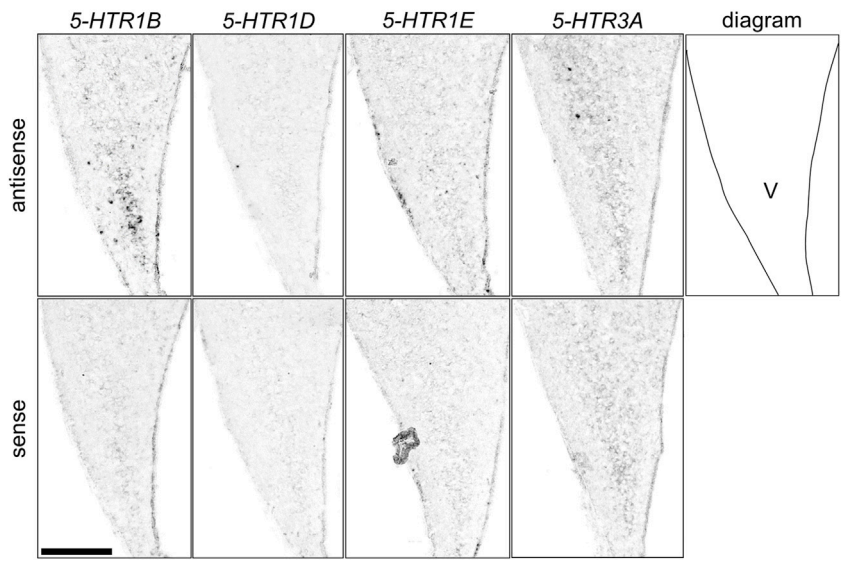


**FIGURE 11 |** Comparison of *5-HTR*s expression patterns in the HF in the P1 chick telencephalons using neighboring sections around A 6.6 focused on V. Magnified views of HF surrounding regions shown in **Figure 10**. V: V-shaped complex; P1: posthatch day 1. Scale bars = 250 μm.

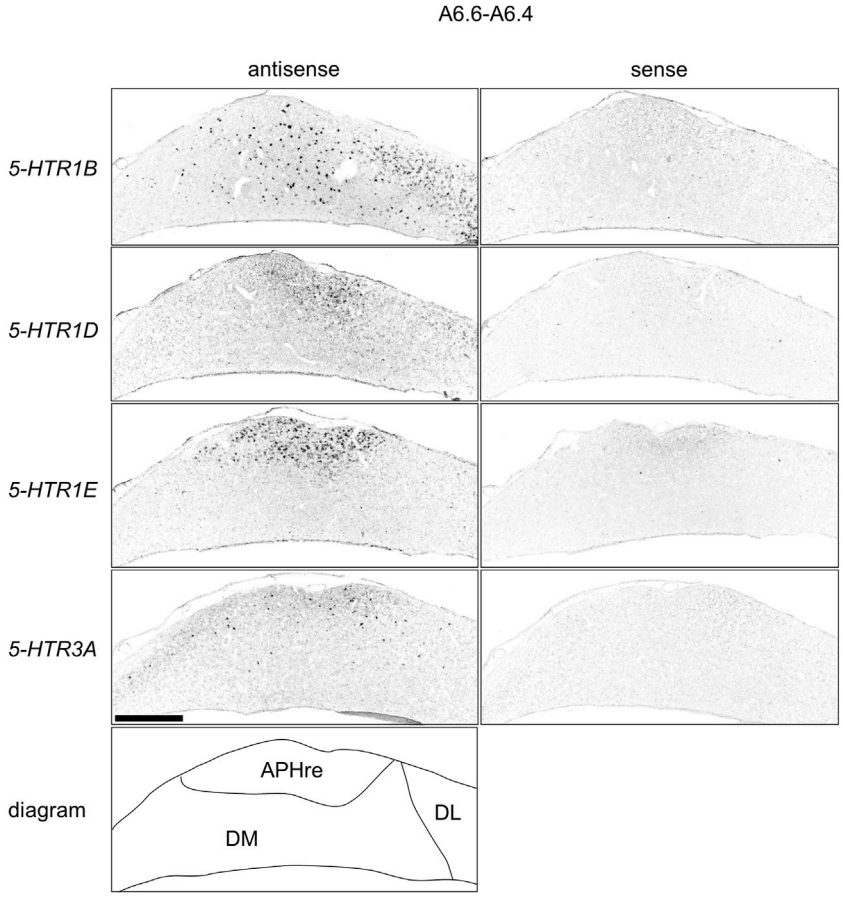


**FIGURE 12 |** Comparison of *5-HTR*s expression patterns in HF in P1 chick telencephalons using neighboring sections at approximately A 6.6 focused on DM. Magnified views of HF surrounding regions shown in **Figure 10**. APHre, ectopic part of the rostral area parahippocampalis; DL, dorsal lateral region of HF; DM, dorsal medial region of HF; P1: posthatch day 1. Scale bars = 500 μm.

we assumed that *5-HTR1D* might also be expressed in GABAergic interneurons in chick HF.

In the past, the cytoarchitecture of avian HF was considered quite different from that of the mammalian 3-layered HF, as it appeared to have completely lost its layered structure. Three layers are clearly present in lizards but less visible in crocodiles (closest to birds) as for HF homologue. (Hevner, 2016; Striedter, 2016). However, detailed immunohistochemical analysis and combinatorial expression analysis using developmental regulatory genes revealed the existence of a layered cytoarchitecture orthogonal to radial glial fibers in the chick HF during its developmental stages (Redies et al., 2001; Abellan et al., 2014). Such a layered structure has also been confirmed in the HF of adult pigeons (Suarez et al., 2006; Herold et al., 2019). In our study, we showed that the expression patterns of *5-HTR1D* and *5-HTR1E* in DL were layered, suggesting a selective regulation of the DL layer by these receptors. Our data indicated that the neuronal population in this layer might have functional roles in cognition and emotion.

We also examined the expression of *5-HTR2B* and failed to detect it in chick telencephalons, suggesting either a low level of expression of *5-HTR2B* or the rarity of expressing cells. Interestingly, the expression level of *5-Htr2b* in the telencephalon of mammals was found to be low (Bonaventure et al., 2002; Vilaro, et al., 2020). This finding was consistent with our obtained results for the expression of *5-HTR2B*.

## Possible Functions of Chick 5-HTRs in Chick Telencephalon Other Than HF

Regarding its the expression pattern, we noticed that *5-HTR1D* was expressed in a large part of the mesopallium, arcopallium, and a part of the hyperpallium, nidopallium, HF, CDL, and major part of the intercalated nidopallium (entopallium and field L (Jarvis et al., 2013)). Of note, the intercalated nidopallium receives sensory projections from the thalamus (Reiner et al., 2004; Jarvis et al., 2013). We previously showed that *5-HTR2C* was preferentially expressed in intercalated nidopallium in chicks (Fujita et al., 2020). Considering the expression combination of *5-HTR1D* and *5-HTR2C*, it is possible that these 2 5-HTRs might work together in sensory input information processing in the intercalated nidopallium in birds. In mammals, 5-HTR1D was demonstrated to be distributed in the frontoparietal cortex, primary olfactory cortex, accumbens nucleus, caudate-

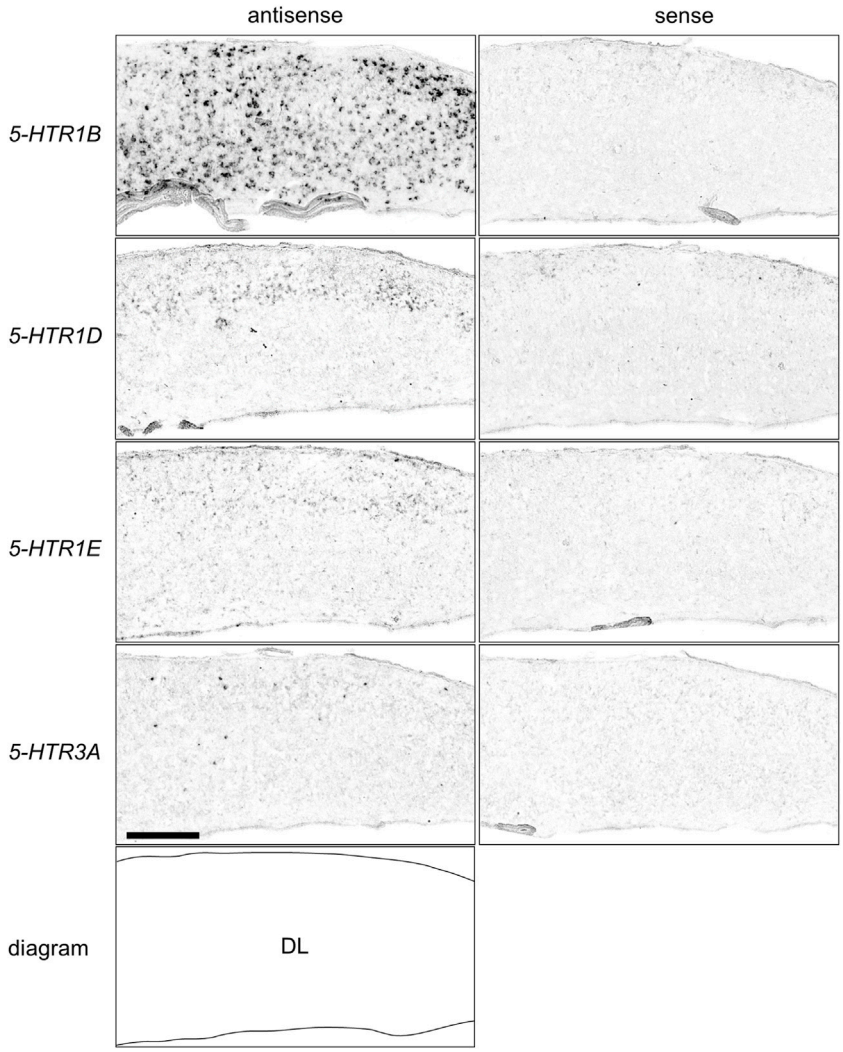


**FIGURE 13 |** Comparison of *5-HTR*s expression patterns in HF in P1 chick telencephalons using neighboring sections at approximately A 6.6 focused on DL. Magnified views of HF surrounding regions shown in **Figure 10**. DL: dorsal lateral region of HF; P1: posthatch day 1. Scale bars = 250 μm.

putamen, and lateral mammillary nucleus (Bruinvels, et al., 1994a, 1994b; Vilaro et al., 2020). However, the regional expression of *5-HTR1D* in the chick hyperpallium appeared to be limited (**Figure 2C, C'**), suggesting a serotonergic modulation in the neuronal population of the hyperpallium via 5-HTR1D.

In the case of the expression pattern of *5-HTR1E*, we found that the major regions of expression were the APHre, TnA, and a part of the hyperpallium. We previously showed that *5-HTR2C* and *5-HTR4* were preferentially expressed in the TnA in chicks (Fujita, et al., 2020), whereas in mammals, *5-HTR2C* and *5-HTR4* are expressed in the amygdala (Huang and Kandel, 2007; Bombardi 2014; Bocchio et al., 2016). TnA is considered to be the counterpart of the mammalian medial amygdala (Reiner et al., 2004; Yamamoto et al., 2005; Yamamoto and Reiner, 2005; Hanics et al., 2017), which is functionally associated with social behaviors, including sexual behavior and social interactions (Ikebuchi, et al., 2009; Mayer et al., 2017; Mayer, et al., 2019). These findings indicated that 5-HT might play a key role in shaping social responses in birds and mammals. In this study, we found another *5-HTR*, *5-HTR1E*, which was preferentially expressed in TnA. Whereas, in mammals, 5-*Htr1e* is expressed in the amygdala (Lowther et al., 1992; Bruinvels et al., 1994a; Mengod et al., 2006). Our findings regarding *5-HTR1E* also support the potentially conserved roles of *5-HTR*s in the mammalian medial amygdala and avian TnA. Furthermore, the expression patterns of *5-HTR1E* and *5-HTR1D* in the hyperpallium, appeared to be similar (**Figure 8A,C, A′, C'**), suggesting a serotonergic modulation in the neuronal population of the hyperpallium via these receptors.

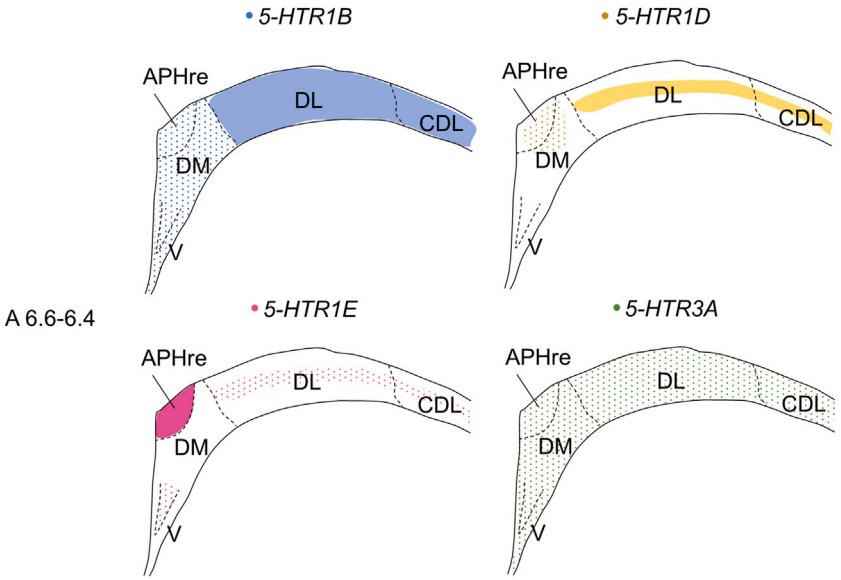


**FIGURE 14 |** Schematic summary of expression patterns of *5-HTR1B, 5-HTR1D, 5-HTR1E,* and *5-HTR3A* in subdivisions in the P1 chick HF. Representative expression patterns in sections at approximately A 6.6 to A 6.4 are shown in colored areas (blue, *5-HTR1B*; yellow, *5-HTR1D*; magenta, *5-HTR1E*; green, *5-HTR3A*). A dotted pattern indicates the sparse distribution of expressing cells. Levels of sections were in accordance with those mentioned in Kuenzel and Masson's chick atlas (Kuenzel and Masson, 1988). APHre: ectopic part of the rostral area parahippocampalis; CDL, corticoidea dorsolateralis; DL, dorsal lateral region of HF; DM, dorsal medial region of HF; V, V-shaped complex; P1: posthatch day 1.

Regarding the expression pattern of *5-HTR1F*, we observed that it was selectively expressed in the interstitial part of the hyperpallium (IHA). According to developmental studies, the hyperpallium of birds is a region homologous to the mammalian neocortex (Fernandez, et al., 1998; Puelles et al., 2000), and is composed of four pseudolayers: the apical part of the hyperpallium, IHA, the intercalated part of the hyperpallium, and the densocellular part of the hyperpallium (Medina and Reiner 2000; Reiner et al., 2004). Among them, the IHA has projection terminals of sensory information from the thalamus, which is considered equivalent to layer IV of the mammalian neocortex (Bangnoli and Burkhalter, 1983; Miceli and Reperant, 1985; Miceli et al., 1990; Atoji et al., 2018; Atoji and Wild, 2019). Despite some species differences, *5-Htr1F* is expressed in the intermediate cortical layers (layers IV and V) in mammals (Waeber and Moskowitz 1995; Mengod et al., 1996; Pascual et al., 1996; Lucaites et al., 2005). Taken together, both *5-HTR1F*-expressing neurons in chick IHA and *5-Htr1F*-expressing neurons in layer IV of the mammalian neocortex might have conserved functions in processing sensory input under serotonergic modulation.

We also detected that *5-HTR5A* was expressed in the dorsal arcopallium, lateral nidopallium, HF, and CDL. In mammalian brains, *5-HTR5A* is distributed in the piriform cortex, habenula, and HF, suggesting its involvement in the regulation of cognition, anxiety, and sensory perception (Plassat et al., 1992; Erlander et al., 1993; Matthes et al., 1993; Kinsey et al., 2001; Mengod et al., 2006; Vilaro et al., 2020). The dorsal arcopallium is the proposed region homologous to the basolateral amygdala in terms of embryonic origin and expression combinations of conserved morphogenetic genes (Martinez-Garcia et al., 2009; Medina et al., 2017a; Martinez-Garcia and Lanuza, 2018). The similarity in the regional expression of *5-HTR5A* between mammalian and chick telencephalons suggested its conserved function in the serotonergic modulation in telencephalons.

Finally, we observed that *5-HTR7* was expressed in the arcopallium, lateral nidopallium, HF, and CDL. In the mammalian telencephalon, *5-Htr7* is distributed in some brain regions, including the HF and amygdala (Gustafson et al., 1996; Mengod et al., 1996; Martin-Cora and Pazos, 2004; Mengod et al., 2006; Tanaka et al., 2012). This finding suggested the conserved 5-HTR7-mediated serotonergic modulation in the amygdala and HF between birds and mammals.

## CONCLUSION

We comprehensively revealed the expression patterns of 5-HTR subfamily genes in the chick telencephalon and specifically found that *5-HTR1D*, *5-HTR1E*, *5-HTR5A*, and *5-HTR7* were expressed in chick HF. These receptors might be involved in the regulation of HF neural circuits that control cognitive and emotion-related functions in birds. In addition, we found that *5-HTR1B*, *5-HTR1D*, *5-HTR1E*, and *5-HTR3A* were expressed in HF in a subdivision- and layer-selective manner. Our findings can facilitate the improved understanding of the correspondence between the avian and mammalian HF.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Teikyo University.

## AUTHOR CONTRIBUTIONS

TF and SY designed the study and performed the experiments; TF, NA, CM, EF, KH and SY analyzed the data; TF, NA, CM, TM, KH and SY wrote the paper. All authors have reviewed the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.882633/full#supplementary-material

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BRIEF RESEARCH REPORT
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# Contribution of Endocannabinoids to Intrinsic Motivation for Undirected Singing in Adult Zebra Finches

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Songbirds, such as zebra finches, spontaneously produce many song renditions for vocal practice even in the absence of apparent recipients throughout their lives. Such "undirected singing" is driven by intrinsic motivation, which arises within individuals for internal satisfaction without immediate external rewards. Intrinsic motivation for undirected singing in adult zebra finches was previously demonstrated to be critically regulated by dopamine through D2 receptors. Here, we further investigate the mechanisms of intrinsic motivation for undirected singing by focusing on endocannabinoids, which modulate dopamine signaling and contribute to motivation and reward in mammals. In songbirds, endocannabinoids have been shown to be involved in the production of undirected songs, but whether they are involved in the intrinsic motivation for undirected singing remains unknown. Using latencies of the first song production following temporary singing suppression as a measure of intrinsic motivation for undirected singing, we demonstrate that systemic administration of the direct cannabinoid agonist WIN55212-2 decreases intrinsic motivation for singing and that those effects are largely reversed by the cannabinoid antagonist SR141716A co-administered with WIN55212-2. Administration of SR141716A alone or that of two indirect cannabinoid agonists did not significantly affect intrinsic singing motivation. These results suggest that endocannabinoids are critically involved in regulating intrinsic motivation for undirected singing and provide new insights into the neural mechanisms of intrinsically motivated motor behaviors.

**Keywords: endocannabinoid, motivation, songbird, dopamine, reward, vocalization, voluntary behavior, vocal learning**

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## INTRODUCTION

Animals, including humans, spontaneously exhibit various behaviors, even without receiving any immediate external reward such as food or money. Such voluntary behaviors are driven by intrinsic motivation and are critical for the development and optimization of cognitive, social, and physical functions throughout life (Parisi et al., 2019; Ryan and Deci, 2000). Songbirds, such as zebra finches, offer a unique opportunity to study the neural substrates of intrinsic motivation because they spontaneously produce hundreds of renditions of stereotyped songs every day, even in the absence of apparent recipients ("undirected singing") (**Figure 1A**) (Dunn and Zann, 1996; Sossinka and Böhner, 1980). Undirected singing is thought to serve, at least in part, as a vocal practice by which birds develop and optimize song structure to prepare for future courtship activity (Brainard and Doupe, 2001; Konishi, 1965; Lombardino and Nottebohm, 2000; Tumer and Brainard, 2007);

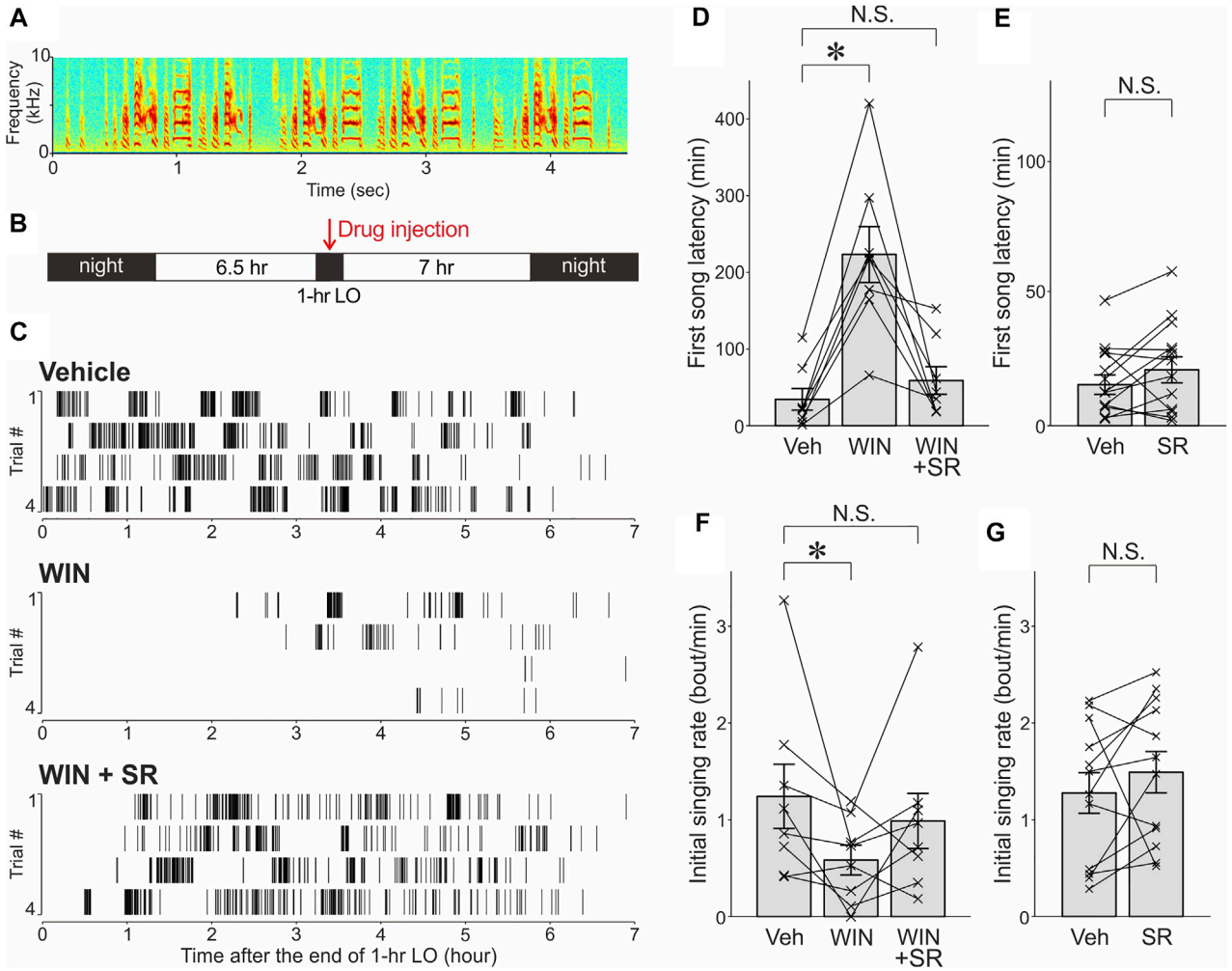


**FIGURE 1 |** The cannabinoid agonist WIN significantly decreased intrinsic motivation for undirected singing and this effect was reversed by the cannabinoid antagonist SR. **(A)**. Spectrogram of an undirected song bout in a representative bird. **(B)**. Schedule of lights-out (LO) and drug administration. Drugs or their vehicles were injected 30 min prior to the offset of 1-h LO periods. **(C)**. Raster plot of song bouts produced after LO periods with vehicle (*top*), WIN (*middle*), or WIN & SR (*bottom*) administrations in a representative bird. **(D)**. The effects of WIN and SR administrations on first song latencies. First song latencies were significantly increased after WIN administrations compared with those after vehicle administrations ("Veh"), and these effects were largely reversed by co-administration of SR and WIN (mean ± SEM, $^*p < 0.01$, significance level α was corrected from 0.05 to 0.0167). Each line indicates a single bird. N.S., not significant. **(E)**. Effects of SR administration alone on first song latencies. **(F)**. Effects of WIN and SR administrations on initial singing rates. $^*p < 0.05$. Conventions are as in *D*. **(G)**. Effects of SR administration alone on initial singing rates.

other functions of undirected singing have also been suggested (EensHausberger et al., 1995; EensHausberger et al., 1997; Jesse and Riebel, 2012).

We have previously demonstrated that the intrinsic motivation for undirected singing in adult zebra finches is critically regulated by dopamine through D2 receptors (Kim et al., 2021). In mammals, dopamine signaling is modulated by endocannabinoids (El Khoury et al., 2012; Wenzel and Cheer, 2018) and they play a critical role in motivation for voluntary behaviors, such as social play and physical exercise (Muguruza et al., 2019; Parsons and Hurd, 2015; Vanderschuren et al., 2016). In songbirds, endocannabinoids are involved in undirected singing: the amount of undirected singing is dramatically decreased by systemic administration of the direct $CB_1$ cannabinoid agonist WIN55212-2 (WIN), and such effects are partially reversed by the $CB_1$ cannabinoid antagonist SR141716A (SR) (Soderstrom and Johnson, 2001). Although these results suggest that endocannabinoids play a role in the production of undirected songs, they do not necessarily indicate that endocannabinoids are directly involved in the intrinsic motivation for undirected singing. There is evidence that undirected singing acts as internal reward and is associated with a positive affective state (a state of "liking") (Riters and Stevenson, 2012). Because such singing-associated reward may facilitate subsequent song production, the inhibitory effect of WIN on undirected song production could result from a reduction in singing-associated reward rather than a direct influence of the drug on intrinsic singing motivation. In

support of this view, significant correlations between singing-associated reward and endocannabinoid-related gene expression have been found in songbirds (Hahn et al., 2017). Thus, it remains to be determined whether the endocannabinoid system is involved in intrinsic singing motivation (a state of "wanting") and/or singing-associated reward (a state of "liking").

To better understand the role of endocannabinoids in undirected singing, we examined whether they are critically involved in intrinsic singing motivation. To quantify the motivation for undirected singing, we used the latency to the first song following the temporary suppression of singing (referred to as "first song latency"), a measure recently developed and described in our previous work (Kim et al., 2021). Unlike the singing rate and song amount, the first song latency is not directly influenced by any process following the act of singing, allowing for examination of intrinsic singing motivation independent of singing-associated reward. We systemically administered WIN and SR as in a previous study that examined their effects on undirected song production (Soderstrom and Johnson, 2001), and examined their effects on the first song latency to determine the role of the endocannabinoid system in intrinsic singing motivation. We additionally examined the effects of these drugs on the mean singing rates measured over a 30-min period starting at the first song after singing suppression ("initial singing rate"), which can reflect both intrinsic singing motivation and singing-associated reward (Kim et al., 2021). Moreover, we assessed the effects of two indirect cannabinoid agonists, URB597 and VDM11, on undirected singing, both of which have been shown to enhance intrinsically motivated social play in adolescent rats (Manduca et al., 2014; Trezza and Vanderschuren, 2009, 2008a, 2008b).

# MATERIALS AND METHODS

## Subjects

All the subjects were adult male zebra finches (*Taeniopygia guttata*, 90–146 days post hatching). Birds were raised in our colony with their parents and siblings until ~60 days old and then housed with their siblings and/or other male conspecifics until the experiments started. The care and treatment of animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Brain Research Institute.

## Song Recording and Measuring Singing Motivation

Birds were housed individually in sound-attenuating chambers (MC-050, Muromachi Kikai) under a 14:10 h light:dark cycle throughout the experiments. Undirected songs, which the birds spontaneously produced in a solo context, were recorded as previously reported (Kim et al., 2021). Briefly, the output from a microphone (PRO35, Audio-Technica) positioned above the cage was amplified using a mixer (402-VLZ4, Mackie) and digitized via an audio interface (Octa-Capture UA-1010, Roland) at 44.1 kHz (16-bit). Recording was controlled by a custom-written song recording program (R. O. Tachibana at the University of Tokyo), which triggered recording if it detected four or five consecutive sound notes, each of which was defined based on the sound magnitude, duration, and intervening gap duration. Each recording ended when the silent period lasted longer than 0.5 s. Birds with sufficient singing rates (>300 song bouts per day) were used in further experiments.

Intrinsic motivation for undirected singing was quantified by measuring the latency of the first song produced following the temporal suppression of singing ("first song latency") (Kim et al., 2021). Singing was suppressed by turning off the light in the sound-attenuating chambers for 1 or 2 h depending on the drugs administered (see below), and the time interval from the offset of the lights-out (LO) period to the onset of the first song was measured. Furthermore, the mean singing rates over a 30 min period starting at the first song following LO periods ("initial singing rates") were measured. To obtain all songs produced during those periods, all sound files recorded during those periods were screened to exclude non-song files (which include calls and/or noise) using a previously reported semi-automated method (Kim et al., 2021).

## Drug Administrations

For pharmacological manipulation of cannabinoid signaling, the following drugs or the corresponding vehicle were injected into the pectoral muscle: the direct $CB_1$ cannabinoid agonist WIN (Sigma-Aldrich, W102; 1 mg/kg); selective $CB_1$ cannabinoid inverse agonist/antagonist SR (Sigma-Aldrich, SML0800; 5 mg/kg); and the indirect endocannabinoid agonists URB597 (Sigma-Aldrich, U4133; 0.2 and 1 mg/kg) and VDM11 (Sigma-Aldrich, V3264; 1 and 5 mg/kg). The doses were selected based on previous studies (Achterberg et al., 2016; Gilbert and Soderstrom, 2013; Muguruza et al., 2019; Trezza and Vanderschuren, 2009, 2008a). All drugs were stored as stock solutions in DMSO at −20 °C; WIN and VDM11 were dissolved in saline before use; SR and URB597 were dissolved in 5% Tween-80/5% polyethylene glycol/saline. All birds received a single injection of a drug or vehicle every 1–2 days with a fixed LO schedule: For all drugs except URB597, birds received 1 h LO in the middle of the day (**Figure 1B**) and the drug or vehicle was injected 30 min before the end of the LO periods; for URB597, birds received 2 h LO period and the drug or vehicle was injected at the beginning of those LO periods, following a previous study (Achterberg et al., 2016). Different sets of birds were used for different experiments: 8 birds were injected with WIN, the mixture of WIN and SR, and the vehicle; 13 birds were injected with SR and the vehicle; 12 birds were injected with 0.2 and 1 mg/kg of URB597 and the vehicle; 12 birds were injected with 1 and 5 mg/kg of VDM11 and the vehicle. The drugs and corresponding vehicle were injected sequentially (only a single injection per day), and those injections were repeated 2–5 times; the results (first song latency and initial singing rate) for the same drug/vehicle were averaged across injections. The temporal order of drug and vehicle injections was as follows: For the WIN and WIN + SR experiment, vehicle, WIN, and WIN + SR were injected in order but the order of WIN and WIN + SR was

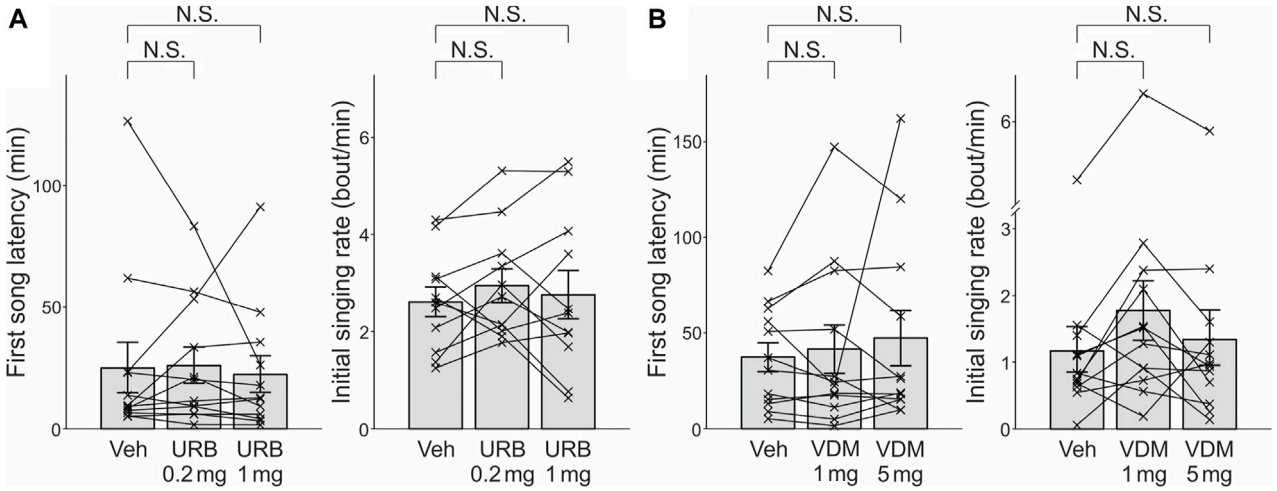


**FIGURE 2 |** The effects of the indirect cannabinoid agonists URB597 and VDM11 on first song latencies and initial singing rates. **(A)**. URB597 administered at 0.2 and 1 mg/kg did not have significant effects either on first song latency (*left*) or initial singing rate (*right*). Conventions are as in **Figure 1D**. **(B)**. VDM11 administered at 1 and 5 mg/kg did not have significant effects either on first song latency (*left*) or initial singing rate (*right*).

switched after every round of injections. For the SR alone experiment, vehicle and SR were injected alternately. For the URB597 and VDM11 experiments, vehicle, lower dose and higher dose of the drug were injected in order. We confirmed that all birds produced substantial amounts (>50 renditions) of undirected songs during the light period before each drug injection to ensure that the drug injection on the preceding day did not severely affect singing behavior on the next day.

## Statistical Analysis

To examine the effects of drug administration, bird behaviors (first song latencies and initial singing rates) were compared between those occurring after drug administration and those after vehicle administration, using a Wilcoxon signed-rank test. For multiple comparisons, the significance threshold (alpha) for rejecting the null hypothesis was adjusted using the Bonferroni correction. All statistical analyses were performed using the MATLAB software.

# RESULTS

## The Effects of WIN and SR

Systemic administration of the cannabinoid agonist WIN at a dose that has been shown to effectively suppress undirected song production (1 mg/kg) (Soderstrom and Johnson, 2001) markedly prolonged first song latencies following temporal suppression of spontaneous undirected singing: First song latencies observed after WIN administrations were significantly longer than those after vehicle administration (**Figures 1C,D**; n = 8 birds, $p$ = 0.0078, Wilcoxon signed-rank test with a Bonferroni correction for multiple comparisons, significance level α was corrected from 0.05 to 0.0167). Moreover, this prolongation was largely reversed by the cannabinoid antagonist SR (5 mg/kg), which was co-administered with WIN (**Figures 1C,D**; n = 8 birds, $p$ = 0.20 vs. vehicle). The effect of SR administration alone was assessed on first song latencies in a separate experiment, and no significant effects were observed (**Figure 1E**; n = 13 birds, $p$ = 0.17, Wilcoxon signed-rank test).

The effects of these drugs on initial singing rates were also examined. Consistent with the results of the first song latencies, the initial singing rates were significantly (but marginally) reduced by WIN administration (**Figure 1F**; n = 8 birds, $p$ = 0.0156), and these effects were substantially reversed by SR co-administered with WIN (**Figures 1C,F**, n = 8 birds, $p$ = 0.5 vs. vehicle). In addition, no significant effects were observed after SR administration alone (**Figure 1G**; n = 13 birds, $p$ = 0.15).

## The Effects of URB597 and VDM11

We also examined the effects of the indirect cannabinoid agonists URB597 and VDM11 on both first song latencies and initial singing rates. For each drug, relatively low and high doses were administered, based on similar experiments in previous studies (0.2 and 1 mg/kg for URB597 and 1 and 5 mg/kg for VDM11) (Manduca et al., 2014; Trezza and Vanderschuren, 2009, 2008a, 2008b). No significant effects of URB597 were found on either first song latencies (**Figure 2A** *left*; n = 12 birds, $p$ = 1 and 0.47 for 0.2 mg/kg and 1 mg/kg, respectively) or initial singing rates (**Figure 2A** *right*; n = 12 birds, $p$ = 0.21 and 0.76 for 0.2 mg/kg and 1 mg/kg, respectively). Similarly, no significant effects of VDM11 were observed for either first song latencies (**Figure 2B** *left*; n = 12 birds, $p$ = 0.97 and 0.47 for 1 mg/kg and 5 mg/kg, respectively) or initial singing rates (**Figure 2B** *right*; n = 12 birds, $p$ = 0.044 and 0.52 for 1 mg/kg and 5 mg/kg, respectively, significance level α was corrected from 0.05 to 0.0167).

# DISCUSSION

Our results showing the marked increases in first song latencies by WIN administration and substantial reversal of those effects

by SR administration suggest that endocannabinoid signaling through $CB_1$ receptors is critically involved in the initiation of the first song after temporary singing suppression. Given that behavioral latencies have generally been used to quantify the levels of motivation for various behaviors including birdsong (Salamone and Correa, 2012; Volkow et al., 2017; Berke, 2018; Mohebi et al., 2019; Haakenson, Balthazart, and Ball 2020), these results demonstrate that endocannabinoids critically contribute to the regulation of intrinsic motivation for undirected singing. These findings greatly advance the understanding of the role of endocannabinoids in undirected singing. A previous study using the same drugs and a similar method examined the effects of these drugs on the amount of singing (the number of song bouts over a 90-min period) and demonstrated that the singing amount was decreased by WIN and partially reversed by SR (Soderstrom and Johnson, 2001). Since undirected song production has been suggested to act as internal reward (Riters and Stevenson, 2012), which may facilitate subsequent song production, the singing amount could reflect not only singing motivation, but also singing-associated reward. Thus, the previous study measuring only singing amounts does not demonstrate whether endocannabinoids are involved in intrinsic singing motivation, singing-associated reward, or both. Because the first song latency directly reflects intrinsic singing motivation independent of singing-associated reward (Kim et al., 2021), our results provide the first evidence, to our knowledge, of the significant role of endocannabinoids in intrinsic motivation for undirected singing in songbirds. The findings are consistent with those of previous studies in mammals showing that direct cannabinoid agonists, including WIN, affect the motivational aspects of both food- and drug-seeking behaviors (for review, see Parsons and Hurd, 2015).

The results of reduced initial singing rates following WIN administration also support the involvement of endocannabinoids in intrinsic singing motivation because reduced motivation for singing should result in reduced singing rates. However, these results do not eliminate the possibility that endocannabinoids are involved in singing-associated reward. As in the case of the singing amount described above, it is possible that WIN indirectly decreases initial singing rates by affecting singing-associated reward, in addition to directly affecting intrinsic singing motivation. Thus, endocannabinoids could play a role in singing-associated reward as well as in intrinsic singing motivation. In accordance with this idea, significant correlations between singing-associated reward and endocannabinoid-related gene expression have been found in songbirds (Hahn et al., 2017). This idea is also consistent with the roles of endocannabinoids in both motivational and pleasurable aspects of various behaviors in mammals (for review, see Sagheddu et al., 2015; Solinas et al., 2008).

We previously demonstrated that dopamine plays a critical role in regulating intrinsic motivation for undirected singing though D2 receptors (Kim et al., 2021). Because endocannabinoids modulate the dopamine system in mammals (for review, see Wenzel and Cheer, 2018), it is likely that endocannabinoids regulate intrinsic singing motivation by interacting with the dopamine system. In mammals, the interactions between endocannabinoids and the dopamine system vary across different brain areas (Wenzel and Cheer, 2018; Lovinger et al., 2022). For example, endocannabinoids disinhibit dopaminergic neurons in the midbrain by suppressing GABA releases from GABAergic interneurons, resulting in enhanced dopaminergic neuron activity and enhanced dopamine release from their axon terminals (Wang and Lupica, 2014). In contrast, endocannabinoids also have a function to decrease dopamine release via local actions in the striatum: dopamine release enhanced by glutamatergic inputs to the striatum is inhibited by the activation of $CB_1$ cannabinoid receptors expressed on cortical glutamatergic terminals (Covey et al., 2017). Moreover, endocannabinoids differentially interact with dopamine D1 and D2 receptors (El Khoury et al., 2012), and these receptors are differentially involved in motivational processes (Olivetti et al., 2019; Volkow et al., 2017). In songbirds, it remains unclear how endocannabinoids interact with dopamine signaling to regulate singing motivation, although both dopamine-related signals and endocannabinoid-related signals are associated with the production of undirected song in many brain areas such as VTA, the medial preoptic area, and the periaqueductal gray, and the songbird basal ganglia nucleus Area X (Haakenson et al., 2020; Hahn et al., 2017; Heimovics et al., 2009, 2011; Kubikova et al., 2010; Merullo et al., 2016; Yanagihara and Hessler, 2006). Identifying the neural circuits and detailed mechanisms underlying the interaction between endocannabinoids and dopamine signaling will advance our understanding of how those neuromodulators regulate singing motivation.

In contrast with the direct cannabinoid agonist WIN, which binds to $CB_1$ cannabinoid receptors, the indirect cannabinoid agonists URB597 and VDM11 increase cannabinoid binding to receptors by preventing the breakdown or reuptake of cannabinoids, respectively. These indirect cannabinoid agonists enhance social play in rats, an intrinsically motivated rewarding behavior (Manduca et al., 2014; Trezza and Vanderschuren, 2009, 2008a, 2008b). Such enhancing effects on social play are not consistent with our results of no significant effects of these drugs on undirected singing. This discrepancy could result from different mechanisms between birdsong and social play, and/or between animal species. In rodents, the effects of URB597 on social play depend on the age, strain, and behavioral context, and such effect patterns vary across different behaviors (Manduca et al., 2014). Given that songbirds have discrete neural circuits specialized for song learning and production (for review, see Mooney, 2009), it is likely that the mechanisms by which endocannabinoids regulate intrinsic singing motivation differ in many aspects from mechanisms of other voluntary behaviors in other animals. Nevertheless, the tractable nature of zebra finch songs and song control circuits will enable us to understand the detailed neurophysiological mechanisms underlying intrinsic motivation for complex learned motor behaviors. Moreover, endocannabinoids in songbirds are implicated, not only in singing motivation, but also in song development (Soderstrom and Gilbert, 2013; Soderstrom and Johnson, 2003), song recognition (Whitney et al., 2003; Hahn et al., 2019), and stress responses (Dickens et al., 2015),

illustrating the importance of songbirds as a useful model system to study the roles of endocannabinoids in various biological functions.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the Korea Brain Research Institute.

## AUTHOR CONTRIBUTIONS

YK and SK conducted the experiments and data analyses; SK designed the experiments; YK and SK wrote the article.

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# Song Preference in Female and Juvenile Songbirds: Proximate and Ultimate Questions

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Birdsong has long been a subject of extensive research in the fields of ethology as well as neuroscience. Neural and behavioral mechanisms underlying song acquisition and production in male songbirds are particularly well studied, mainly because birdsong shares some important features with human speech such as critical dependence on vocal learning. However, birdsong, like human speech, primarily functions as communication signals. The mechanisms of song perception and recognition should also be investigated to attain a deeper understanding of the nature of complex vocal signals. Although relatively less attention has been paid to song receivers compared to signalers, recent studies on female songbirds have begun to reveal the neural basis of song preference. Moreover, there are other studies of song preference in juvenile birds which suggest possible functions of preference in social context including the sensory phase of song learning. Understanding the behavioral and neural mechanisms underlying the formation, maintenance, expression, and alteration of such song preference in birds will potentially give insight into the mechanisms of speech communication in humans. To pursue this line of research, however, it is necessary to understand current methodological challenges in defining and measuring song preference. In addition, consideration of ultimate questions can also be important for laboratory researchers in designing experiments and interpreting results. Here we summarize the current understanding of song preference in female and juvenile songbirds in the context of Tinbergen's four questions, incorporating results ranging from ethological field research to the latest neuroscience findings. We also discuss problems and remaining questions in this field and suggest some possible solutions and future directions.

**Keywords: birdsong, song preference, mate choice, song tutor choice, vocal learning, auditory learning, neural circuit**

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## 1 INTRODUCTION

Birdsong has been extensively studied in multiple disciplines that address animal behavior. A large body of field and laboratory work in songbirds (order Passeriformes, suborder Passeri) has revealed that songs are typically used for courtship and territorial defense (Catchpole and Slater, 2008). Because of historical and geographical biases, function of birdsong is particularly well studied in species where only males sing to repel male rivals or to attract female mates, although song is not a

male-specific trait in a substantial number of species (Odom et al., 2014; Riebel, 2016). Since the finding of learned aspects of vocal behavior such as song dialects and cultural transmission (Marler and Tamura, 1964), great effort has been devoted to elucidating the mechanisms of song learning in males (Brainard and Doupe, 2002; Bolhuis and Gahr, 2006; Mooney, 2009; Ikeda et al., 2020). Now, growing evidence shows that the process of song development in songbirds is quite similar to that of speech acquisition in humans, and these similarities are evident at behavioral, neural, and genetic levels (Doupe and Kuhl, 1999; Bolhuis et al., 2010; Prather et al., 2017). Partly due to these shared features with human speech, neuroethology of birdsong has primarily focused on the signalers. As a result, neural mechanisms of song learning and production in males have been more intensively studied than those of song perception and recognition in either sex (Hernandez et al., 2008; Riebel, 2009).

Birdsong, like human speech, is used to convey information. Although relatively less attention has been paid to song receivers compared to signalers, investigation into the mechanisms of song perception and recognition should also be an important focus as we seek to attain a deeper understanding of vocal behavior. It has been demonstrated in several species that adult and juvenile birds of both sexes can discriminate songs of different categories or acoustic and temporal features (Searcy and Yasukawa, 1996; Riebel, 2009; Rodríguez-Saltos, 2017). Their sensitivity to differences between song stimuli has been measured by behavioral, physiological, and molecular methods, and their acuity suggests the ability to recognize species, local populations, and individuals based on song features (Miller, 1979; Dooling and Searcy, 1980; Clayton, 1988; Gentner and Hulse, 2000; Maney et al., 2003; Woolley and Doupe, 2008). Especially in case of females, such song discrimination abilities have been explored in the theme of mate choice. In a series of studies both in the field and laboratory, selectivity of behavioral responses to one song stimulus over others is often called "song preference." For instance, if female birds show more frequent responses to conspecific songs than to heterospecific songs, such selective responses are described as a preference for songs of their own species, and those preferences are interpreted as evidence that the females are more likely to mate with conspecific males based on their songs (Wagner, 1998).

Female song preference has traditionally been studied in the field of behavioral ecology, but recent studies on female songbirds have also begun to reveal the neural basis of song preference, as detailed in subsequent sections. Moreover, there are other new studies of song preference in juvenile birds which suggest possible functions of preference in learning associated with song production. Neuroethological studies on these topics have just begun to emerge, and there is still much to explore, including how song preference is formed, maintained, expressed, and altered. To pursue this line of research, however, it is important to grasp current methodological challenges in defining and measuring song preference. In addition, consideration of ultimate questions (i.e. functions in reproductive success and evolution of song preferences) should also be helpful for laboratory researchers in designing experiments and interpreting results. To clarify the problems and discuss future directions, here we summarize the current understanding of song preference in female and juvenile songbirds in the context of Tinbergen's four questions, incorporating results ranging from ethological field research to the latest neurobiological findings.

# 2 WHAT IS SONG PREFERENCE

## 2.1 Terminology and Definition

"Preference" is a term that is used very commonly and in many contexts. Its flexibility of use is evident in several different research contexts even within the field of animal behavior. The definition of song preference based on the act of measurement sometimes differs from one study to another. To better understand how the term "song preference" is defined either conceptually or operationally, here we briefly summarize the usage and definition of the term.

"Song preference" has been used in studies of female mate choice where it is postulated that female birds select a suitable mate among multiple males they encounter. In addition to a range of features that provide insight into a suitor's fitness, such as plumage, song is an especially important element in how females evaluate the quality of male birds. Thus, investigating how females respond to within- or between-species song variation is fundamental to understanding of songbird mating preference and mate choice. Here, mating preference denotes a disposition or propensity that a female possesses, while mate choice is the manifestation of preference which is also affected by other conditions including external factors such as social environment and habitat structure (Jennions and Petrie, 1997; Widemo and Sæther, 1999; Cotton et al., 2006; Kirkpatrick et al., 2006). Song preference can be regarded as one aspect of mating preference, because morphological and behavioral traits other than song are also available for mate evaluation (Collins and Ten Cate, 1996; Grant and Grant, 1997; Brazas and Shimizu, 2002; Gomes et al., 2017). The term "song preference" is also used in studies of song tutor choice in juvenile males who are about to memorize and imitate songs from conspecific adults. Song preference in juveniles potentially enable them to selectively attend to an appropriate song model among multiple options, but the preference for a tutor may also be influenced by other behaviors and traits of the adults or environmental factors (Clayton, 1987; Soma et al., 2009). More background will be reviewed in another section, but the basic idea regarding juvenile song preference is similar to that of females in that both focus on how birds respond to different songs and use those experiences to shape their subsequent behavior. In summary, song preference is a tendency or likelihood for an individual to respond to one song among multiple song samples that the bird may hear, and preference for a song has considerable relevance to the preference for the associated singer. These preferences predict future behavior, but they do not prevent departure from those preferences. Thus, one must keep in mind that preferences bias behavior, but there can be a difference between perception of a preference and expression of the associated choice.

Whatever the underlying concept of the term, such as females choosing a mate or males selecting a tutor to imitate, researchers need a means to operationally define song preference in scientific studies. To operationally define song preference, we need observable motor responses to song stimuli; and we need ways to measure and compare those responses to different stimuli. In the field of animal behavior, an animal's response to a stimulus (e.g., approaching food or avoiding predators) is generally interpreted as an expression of the animal's motivation (Berridge, 2004). Accordingly, preference denotes a difference between the strength of motivation to obtain or avoid one stimulus over others (Fraser and Matthews, 1997; Kirkden and Pajor, 2006). Thus, an experimenter can quantify the animal's preference by playing alternative sound stimuli with a suitable presentation (Wagner, 1998) and comparing the frequency, duration, or intensity of a given behavior that is produced in response. Songbird researchers have found several types of behaviors that are available to quantify preferences in laboratory playback studies (Searcy, 1992).

Taken together, song preference in birdsong ethology and neuroscience is operationally regarded as the behavioral response selectivity for one song over others, and this preference is assumed to underlie the process of mate choice or tutor choice at a conceptual level. While accepting the operational definition in practice, it is also important to consider what the bird may actually be perceiving and indicating in their behavioral indicators of preference that are measured in experiments. Because it is important to be aware of strengths and weaknesses of each type of behavioral measurements, we consider several examples in the following subsection.

## 2.2 How to Measure Song Preference

In attempts to quantify and understand cognitive behaviors such as mate preference, it can be challenging to assess an organism's performance. It can be challenging to develop tests that reveal a female songbird's preference for the features of individual songs and their intention to choose the associated singer as their mate. In such studies, researchers must be careful to control for other features of a male bird's behavior or other characteristics, such as the quality of a male's plumage or a male's expression of other courtship behaviors. This exclusion of facets of communication other than song can be accomplished by recording the songs produced by male birds and playing them through a speaker when no male is physically present. Song is an especially impactful stimulus in female evaluation of the quality of the male suitor, and song is sufficient to drive female courtship and copulatory behaviors even when no male is present (Woolley and Doupe, 2008; Caro et al., 2010; Dunning et al., 2014, Dunning et al., 2020; Heinig et al., 2014; Perkes et al., 2019, Perkes et al., 2021; Fujii and Okanoya, 2022). Thus, song is a unimodal stimulus that facilitates investigations of how the qualities of that stimulus affect female evaluation of the quality of those signals. The remaining challenges center on how to assess female responses and interpret the relation between those behaviors and a female's preferences. Researchers have sought to develop ways to detect evidence of a bird's cognitive behaviors such as song perception and evaluation.

Many paradigms have been employed to assess female song preference, and these can be broadly categorized as operant versus naturalistic response to song stimuli. In both paradigms, a variety of songs are typically presented to a female bird while observing her immediate responses to those stimuli. To avoid pseudoreplication, many songs should be recorded from each male so that each is represented by many exemplars of his song performances. The difference between operant and naturalistic approaches lies in the nature of the responses that the female displays. In operant conditioning, rewards or punishments are used to train a bird to express a specific behavior in response to songs that they perceive as having a specific characteristic. For example, a bird may be taught to express one behavior when she hears a song that she recognizes as familiar or finds very attractive, and she may be taught to express another behavior in response to a song that is novel or that she finds less attractive (Burt et al., 2000). These sorts of operant behavioral responses can include things such as hopping onto one perch or another, pulling a string, or pecking a target. Often, some form of reward or punishment is used to motivate the behavior, such as food or brief periods of darkness, respectively. A distinct advantage of studying female songbirds is that song appears to be inherently rewarding, such that the ability to induce song playback is sufficient to motivate the females to engage in behavior (e.g., Riebel and Slater, 1998). This facilitates experimentation by making it relatively easy to induce the birds to engage in the associated behavior, and it removes a possible confound to interpreting the nature of the preference that the female is revealing in her responses.

The advantages of operant paradigms are significant, but an operant approach commonly requires subjects to engage in some behavior that is different than they would do in response to songs heard in the natural environment. This has led some researchers to measure female preference by observing naturalistic behaviors that females perform in response to song stimuli. These behaviors include things such as adopting the unusual posture that defines a courtship solicitation display (CSD), calling in different amounts in response to different stimuli, approaching one speaker or another, or orienting their body toward the source of sound in a behavior called phonotaxis (e.g., Caro et al., 2010) As in the case of operant conditioning, there are both advantages and disadvantages to these more naturalistic approaches. For example, the fact that females express behaviors naturally means that researchers can avoid concerns inherent to the training and reward processes used in operant conditioning. However, naturalistic studies are not immune to difficulties in interpreting the meaning of a female's behavior. For example, a female may approach a speaker for a number of different reasons, such as seeking to hear the song more clearly, seeking to associate with something they recognize as simply familiar, or seeking to investigate the source of a song that they find attractive as a courtship signal. In each case, the observed behavior (approach) is the same, but the underlying motivation could be quite different. The female could be seeking to gain more information in their assessment, the safety of associating with a particular individual, or a reproductive opportunity in response particularly attractive song. These potential confounds emerge in

any condition in which researchers are seeking to interpret the actions of an experimental subject that can't clearly communicate its intent. One idea is that some combination of operant and naturalistic behaviors could be the best way to measure female song preference, but it remains unknown what method or combination of methods may be the most revealing and least confounding way to assess that perception.

Some researchers have used the expression of CSD's as the ultimate indicator that a female finds a signal attractive (e.g., Anderson, 2009; Dunning et al., 2014). Reports have revealed congruence between the expression of CSDs and tests performed on the same birds using either operant approaches or measures of naturalistic behaviors (e.g., Anderson, 2009; Dunning et al., 2014). These results support the idea that each of these approaches is a valid behavioral measure of song preference; however, the robust expression of CSD's often requires an altered state, such as subcutaneous implants to induce elevated levels of hormones such as estradiol (reviewed in Maney, 2010; see also Maney et al., 2008). This is especially important for researchers seeking to understand the neural mechanisms that underlie song perception and evaluation, as the presence or absence of estradiol can have a profound impact on auditory responses of neurons that are thought to play essential roles in those cognitive processes (Maney et al., 2003, Maney et al., 2008; Krentzel et al., 2018, Krentzel et al., 2020; Lee et al., 2018). A state-dependent difference in patterns of neural activity gives rise to the possibility that the mechanisms and patterns of activity that are at work in one state could be different than those at work in another state (i.e., artificially elevated levels of estradiol versus physiological levels). An additional complication is that the effects of estradiol are not constant over time, with implants commonly inducing robust expression of CSDs for only a brief window of time (discussed in Anderson, 2009). These complications are largely unavoidable in the experimental process, and they point to the value of multiple approaches to measure female song preference and mate choice. An important goal of future studies should be to reconcile data from these multiple approaches and physiological states as they seek to discern general principles that underlie the processes of song perception and decision-making in the natural condition.

# 3 FEMALE SONG PREFERENCE

## 3.1 Ultimate Questions

### 3.1.1 Function

To understand the mechanisms and evolutionary development of song preferences, it is important to understand how they may provide benefit for songbirds. Song performances not only provide the listeners insight into the quality of the associated singer but serve many purposes, both within and outside of a courting pair. One of the primary functions of song preference in songbirds is to identify the singer. Song evaluation and preference allow the listener to determine whether the singer is a member of their own species (conspecific) or another species (heterospecific). When given the choice, females across species prefer the songs of conspecifics over those of heterospecifics (Clayton and Pröve, 1989; Hernandez and MacDougall-Shackleton, 2004; Diez et al., 2019). This preference for conspecifics helps to facilitate social interactions and courtship behaviors with members of their own group.

Male behavior also facilitates those social interactions, with males engaging in courtship behaviors to different degrees around different females (Heinig et al., 2014). For example, males can perform songs in association with overt bodily orientation and hopping back and forth as a signal to the female that the song performance is being directing toward her (Frith and Frith, 1988). These "directed songs" are acoustically more precise than other "undirected songs" performed either in isolation or in groups without any overt orientation toward a specific receiver (Riters and Stevenson, 2012). Female songbirds prefer those directed song performances more than undirected songs from the same individual (Woolley and Doupe, 2008; Riters and Stevenson, 2012; Dunning et al., 2014; Heinig et al., 2014; Schubloom and Woolley, 2016). Exposure to songs that the female finds attractive is thought to facilitate the physiological processes associated with successful mating, allowing her preference to directly impact reproductive success (Slater and Mann, 2004). This is especially clear in the case to birds that live in tropical climates, where courtship behaviors are thought to play an even greater role than photoperiod or other seasonal shifts in affecting reproductive status (e.g., Hoffmann et al., 2019). During successful courtship by a male, the female engages in a duet by singing along with him in remarkable temporal precision (discussed in Elie et al., 2019). Together, these studies from tropical climates, where reproductive status is most strongly influenced by behavioral cues, and temperate climates, where reproductive status is shaped by both environmental and behavioral cues, offer opportunities to understand the mechanisms that underlie the expression of song preferences in mate choice.

In addition to recognition of species identity, songs provide sufficiently high-resolution information that birds can identify individuals from the same local area and even the same family. Females of many species prefer songs that are performed by members of their local population over songs performed by males from other populations (Spitler-Nabors and Baker, 1983; O'Loghlen and Rothstein, 1995; Le Maguer et al., 2021). Such preferences are thought to confer benefit to the females by enabling them to mate assortatively, permitting social, geographic, and genetic stability within their population (Spitler-Nabors and Baker, 1983). Studies from many species have shown that females prefer their father's song over unfamiliar songs from males of the same species (Miller, 1979; Clayton, 1988; Fujii et al., 2021; Le Maguer et al., 2021). The nature of that preference has remained unclear, as females will approach or otherwise choose to interact with the source of that song, but it is not clear what sort of intent might be associated with that. For example, the female could be seeking familiarity, or seeking the protection of their family group. A recent study showed that the father's song can be a strong stimulus in eliciting CSDs in female Bengalese finches. (Fujii and Okanoya, 2022). If the father's song is consistently the female's most preferred song, that could have

detrimental effects on fitness. Many additional aspects of a female's developmental history also affect their song preference. The female hears her father's song many times throughout development, and a relation between preference and the father's song is consistent with the idea that the juvenile's experience of their father's song could possibly help them form a mental model of what a high-quality song sounds like (Miller, 1979; Clayton, 1988; Fujii et al., 2021).

Song preferences are also thought to confer advantage by allowing female to evaluate the quality of male suitors as potential mates (reviewed in Catchpole and Slater, 2008). Two key challenges in research involving those preferences have been identifying which features of song are most salient in communicating quality and determining whether quality of song performance may be associated with other more general aspects of cognitive ability. Although this latter idea has been alluring and persistent, many studies have found no consistent relationships between song ability and other forms of cognitive ability (Templeton et al., 2014; Anderson et al., 2017). Nonetheless, song may provide insight into the life history of the singer and thus provide insight into his potential quality as a mate. If a male experiences nutritional stress during juvenile development, that negative experience can have a similarly negative impact on many facets of development, including learned song performance (O'Loghlen and Rothstein, 1995; Nowicki et al., 2002; Lauay et al., 2004; Chen et al., 2017; Bircher and Naguib, 2020). Females are able to detect those subtle impacts, and they express greater preference for songs performed by normally reared birds than for their nutritionally stressed counterparts (reviewed in Nowicki et al., 1998, Nowicki et al., 2002). This is thought to provide benefit by helping females avoid nutritionally compromised and developmentally challenged mates. This idea is also reflected in the reduced female preference for songs performed by males that are experiencing infection or other ailments that engage the immune system (Garamszegi et al., 2004; Spencer et al., 2005; Dreiss et al., 2008; Munoz et al., 2010).

Just as female preference for song can be affected by the status of the singer, the female's preference can also be affected by her own status. For example, one study raised broods of songbirds under a variety of conditions to create what they termed high-quality (good nutrition and parental care) and low-quality (poor nutrition and parental care) offspring (Burley and Foster, 2006). When queried about their mate preferences, high-quality females tended to prefer songs of high-quality males. When the females' status was negatively impacted by trimming the most distal portion of their feathers, their preferences tended to change to now prefer the songs of low-quality males (Burley and Foster, 2006; Holveck and Riebel, 2010; Holveck et al., 2011). Thus, care must be taken when interpreting data regarding mate preferences, as they can vary not only across individuals but also within an individual tested at different in different conditions.

The impact of female mate preferences goes beyond the initial mating ritual, affecting many aspects of a pair's mate bond. This is evident in the greater likelihood of success for pairings in which females select the partner whose song she prefers. When this is not the case, such as instances when mate pairs are selected arbitrarily by breeders, the number and health of the offspring are less than when the female's choice is the prime determinant of the pairing (Schubloom and Woolley, 2016). The role of female preference is also evident in pair maintenance long after the initial pairing. For example, females that have mated with males that perform high-quality songs are less likely to seek extra-pair copulations, while females that have mated with low-quality males are more likely to seek extra-pair copulations (Garamszegi et al., 2004; Chiver et al.,2008; Byers and Kroodsma, 2009). Together, these data make clear the role of mate preferences of both female and male birds in what is ultimately a mutual mate choice, and they lead to questions about how those preferences have emerged through evolution and how they are expressed throughout ontogeny.

### 3.1.2 Evolution

Questions about the evolution of female song preference is of great scientific significance for neurobiologists who study the mechanisms underlying song preference. Explanations for the evolution of mating preference are mostly based on direct or indirect benefit to individuals as a result of their mate choices, or sensory or perceptual biases that affect the receivers in general contexts (Jennions and Petrie, 1997; Kokko et al., 2003; Ryan, 2021). Both these are general and well-established theoretical frameworks, but in case of birdsong research, particularly the former one has been the target of intensive discussion and empirical examination. Reconstructing the evolutionary process of a certain preference is a challenging task, but it can be addressed by estimating the fitness costs and benefits of possessing the preference and/or by comparing the preference among multiple extant species with analysis of the phylogenetic relationship. Here, we take up some examples where the evolutionary background of the preference has been investigated through these approaches.

Just as there is variation between species in what song features they prefer, there is also variation even within species such that different females may have different profiles of song preference. Nonetheless, years of research have revealed some song features that are broadly impactful across members of a species, and even across different species, and are thus significant influences on female birds' responses. Typically, females prefer songs of higher output, such as longer duration, higher frequency, or larger amplitude (Nowicki and Searcy, 2004; Catchpole and Slater, 2008). Because longer or louder singing requires more time and energy and should also be conspicuous to predators, song output is thought to be a reliable indicator of male signaler (Nowicki and Searcy, 2005; MacDougall-Shackleton and Spencer, 2012). There is empirical data showing correlation between song output and male reproductive success or other indices of male quality (Gil and Gahr, 2002; Catchpole and Slater, 2008), which supports the assumption of this signal honesty.

In addition, preference for song complexity has been commonly demonstrated in several species (Searcy and Yasukawa, 1996; Catchpole and Slater, 2008). Complexity in this context can refer to the number of song types in a male bird's repertoire, or the size of song note repertoire. In

contrast to song output, it is not apparent whether and how the song complexity can be a reliable indicator of male quality. However, an idea proposed as the "developmental stress hypothesis" (Nowicki et al., 1998, Nowicki et al., 2002) provides a clue to solve this puzzle. A key point of this hypothesis is that male birds learn their songs as juveniles, and they are particularly susceptible to nutritional or environmental stress during that time, though the degree of stress varies across species (c.f., Russell et al., 2004). It is thought that individuals who are resistant to such stress or suffer less stress can allocate metabolic resources to the development of neural structures for motor or cognitive abilities including singing (Crino and Breuner, 2015). In this way, song quality of an adult male is thought to be correlated with other traits that are also affected by stress during one's developmental history. Substantial number of studies support the predictions of the hypothesis, such as the effect of stress on the behavior or neural structure in males, for example in song sparrows, starlings, and canaries (reviewed in MacDougall-Shackleton, 2015; MacDougall-Shackleton and Spencer, 2012). Nonetheless, other reports indicate little correlation between aspects of song performance and features of general cognition, so understanding what benefit may be gleaned from selecting a mate with a complex song remains a topic of ongoing research (Anderson et al., 2017; DuBois et al., 2018).

Another specific aspect of song complexity that varies across and within species is the transitional pattern of song notes (Okanoya, 2004a; Soma et al., 2006a). It was previously found that Bengalese finches' songs contain complex transition patterns (Honda and Okanoya, 1999; Katahira et al., 2013), and that a certain population of females prefer more complex songs than simpler songs (Okanoya and Takashima, 1997; Morisaka et al., 2008; Kato et al., 2010). In addition, early developmental condition affects the sequential complexity of the song and the body size of males (Soma et al., 2006b), supporting the possibility that the preference for sequential complexity is also explained by the developmental stress hypothesis.

There are some caveats when considering possible explanations for potential benefits from selecting a mate based on specific aspects of learned song performance. First, the manipulation of developmental conditions in a laboratory may not exactly mimic the stresses that birds encounter in the natural environment. Therefore, balance between experimental control and external validity of methodology should be considered (MacDougall-Shackleton, 2015). Second, male song complexity may not only be influenced by female preferences but also advantages of signal modulation in managing social interactions with diverse individuals through song, including between-males song matching (Byers and Kroodsma, 2009). Also, some people indicate that the cost of having mating preferences is much less explored than the benefit, despite its importance (Jennions and Petrie, 1997). In this regard, investigation into the neural mechanisms that enable evaluation of song complexity may shed light on the cognitive cost, and eventually the evolutionary process of the song preference.

## 3.2 Proximate Questions

### 3.2.1 Development

Similar to how male birds learn their songs from the sounds they hear during development, female song preference is also shaped by juvenile experience. For example, female songbirds commonly prefer songs performed by members of their local population more than songs performed by males from a different population (Spitler-Nabors and Baker, 1983; O'Loghlen and Rothstein, 1995; Le Maguer et al., 2021). This suggests that females may prefer songs that they heard during development, but it leaves open the question of whether that preference is learned or related to some genetic predisposition. A study of female swamp sparrows addressed this by collecting hatchling birds from the wild and rearing them in the laboratory under carefully controlled acoustic conditions. When the females reached adulthood, they expressed a preference for the songs that they had heard during development (Anderson et al., 2014). Importantly, the females' preference for familiar songs from their local population was much greater than their responses to songs that were also recorded from their local population but were not played to them during juvenile development (Anderson et al., 2014). Another study in the same species further revealed the importance of juvenile experience. In that study, females were collected from the wild as adults and tested in the laboratory for their preference for songs recorded from either their local breeding population or from another population over 500 km away (Anderson, 2009). Initial tests revealed that females expressed a strong preference for songs from their local population. The same females were then provided with extensive exposure to songs from the distant population, and then the females were tested again. Even after extensive and exclusive exposure to songs from the more distant population, the females' preference for songs from their local breeding population remained stable (Anderson, 2009). Together, these results indicate that female preference in this species is strongly influenced by developmental auditory experience and is much less vulnerable to the influence of adult auditory experience.

In other species, females can modify their song preferences to include songs that they heard only later in life. For example, reports have documented that females of many species prefer songs that they heard during juvenile development over songs performed by males that they encountered only in adulthood and are therefore novel to them at the time of testing (King et al., 1980; Baker et al., 1987; Clayton, 1990; Searcy, 1990; Searcy et al., 1997, Searcy et al., 2002; Freeberg et al., 2001). However, when adult zebra finch females are provided with extensive exposure to songs that they had previously never heard, they can expand their preferences to include those novel songs (Clayton, 1988). This pattern is also evident in female canaries and cowbirds, as females typically show a preference for songs that they heard during juvenile development, but their preferences can expand to include songs of unfamiliar males with which they have formed pair bonds and raised successful broods of offspring (Nagle and Kreutzer, 1997; West et al., 2006). When we consider results obtained from many species, a common theme is that females prefer songs from males in their local population and those they experienced during juvenile

development. The ability of adult females to modify their preferences seems to vary within a range.

Acquisition of song preferences during juvenile development and possible plasticity of song preference throughout adult life afford many possible advantages and disadvantages. For example, a static preference could facilitate recognition of not only members of the female's own species, but also members of her home group. This could lend stability to populations even if home ranges become disrupted or otherwise displaced. Such a pattern could also confer disadvantage in that it could restrict dispersion into new areas and integration with other populations. This could place constraints on genetic diversity and the ability of a group to engage in adaptive behaviors such as hybridization. In contrast to preferences that are static throughout life, dynamic preferences could enable adaptation to emergent conditions such as dispersal by unusual influences, such as storms or errors in migratory navigation. Preferences that can be enlarged through adult auditory and social experience could provide a greater potential for reproductive success and thereby provide a potential mechanism to facilitate genetic diversity and population success.

Many researchers have been intrigued by the ability of different species of songbird to modify their behavior to various degrees in response to adult experience. This is evident in female birds, and male birds may also continue to modify their songs throughout adulthood. For example, males of some species are very sensitive to the influence of auditory experience during juvenile development but are much less vulnerable to the influence of songs that they encounter during adulthood (reviewed in Mooney et al., 2008). These species are said to be "closed learners" and are commonly studied to identify neural mechanisms that enable birds to be so pliable during development and yet so persistent in their skill throughout adulthood. Males of other species are sensitive to the influence of auditory experience during both juvenile development and adulthood. For example, mockingbirds are able to imitate sounds that they hear at any point throughout their lives. These species are called "open ended learners" and offer opportunities to study the neural mechanisms of learning as it occurs in both the juvenile and the adult physiological conditions (reviewed in Mooney et al., 2008). Taking a broad perspective and incorporating results from both types of learners provides an opportunity to investigate the neural mechanisms at work in both conditions (reviewed in Murphy et al., 2017; Schmidt, 2010). Such experiments can address many fascinating questions such as whether adult learning recapitulates the phenomena of juvenile learning, or whether adult learning relies on a different set of mechanisms so that skills and experiences acquired in juvenile learning are preserved while also permitting other forms of behavioral plasticity.

As researchers have looked more closely and more expansively across species, a realization has emerged that learning is more evident across species than was previously appreciated. Even in species in which song was thought to be an innate behavior and the male did not rely on auditory experience to learn the sounds that compose their songs, closer inspection revealed that songs performed by males of those species contain small elements that vary as a function of experience (Saranathan et al., 2007; Kroodsma et al., 2013). Thus, the ability to learn throughout ontogenetic experience appears to be more akin to differences along a spectrum rather than any categorical distinctions between learning versus non-learning species. Incorporating a comparative approach into future studies of how males acquire their songs and how females acquire their song preferences will afford many opportunities to study the possible contributions of a wide range of factors such as experience, genetics, endocrine influences, and especially the neural mechanisms that underlie that behavior (Murphy et al., 2017, Murphy et al., 2020).

Learning in males is closely associated with a network of neural structures called the "song system." The acquisition of female preferences during juvenile development is reminiscent of song learning that occurs in males, but structures that compose the song system are typically quite atrophied or entirely absent in female birds (reviewed in Mooney et al., 2008). For many years, this led to the impression that song was relatively uncommon among female birds, but more recent studies have indicated that females having the ability to sing either solo or as part of a duet is much more common that was previously appreciated (e.g., Odom et al., 2014; Elie et al., 2019). These new insights into the neurobiology of song perception in female birds have heightened interest in the question of how females acquire their preferences, store those perceptions and preferences in long-term memory, and then recall those experiences in adulthood and use those memories to modify ongoing behaviors. The link between learning and preferences helps elevate the importance of preference research to understand learning. Many researchers focus on male songbirds' song-learning process to help understand the neurological building blocks of learning. Because female songbirds' song preferences and mate choice are also influenced by acoustic and social experience, they offer a unique model to study how learning can affect the process of decision making. Researchers have focused on that question much more in recent years. As elaborated in the following section, results have begun to reveal brain sites and pathways that underlie female learning, memory, and behavioral expression of preference for specific songs and the associated singers.

### 3.2.2 Neural Mechanisms

Behavioral experiments have made it clear that experience plays an important role in shaping those preferences. Here, we turn to consideration of the neural mechanisms that are also shaped by that experience and that underlie the perception and expression of mate preferences. Electrophysiological recordings and pathway tracing studies from many research groups have revealed pathways through which auditory experience is relayed from the ears to the brain (reviewed in Dunning et al., 2018; Bloomston et al., 2022). Activity originating in hair cells of the inner ear is propagated through a network of auditory neurons in the brainstem and thalamus, and it eventually reaches a forebrain area which is the avian analog of the mammalian primary auditory cortex (Field L) (Lewicki and Arthur, 1996; Grace et al., 2003; Amin et al., 2004; Meliza and Margoliash, 2012;

Prather, 2013). This first post-thalamic processing region is thought to contribute to the neural basis of natural sound recognition. Electrophysiological recordings in female zebra finches have revealed that Field L neurons respond more robustly to unfamiliar conspecific songs as compared to white noise or synthetic sound stimuli (Hauber et al., 2007). These findings are consistent results from studies of male zebra finches and other songbird species (Leppelsack and Vogt, 1976; Grace et al., 2003). Together these results indicate that at this stage of the auditory processing pathway there may be similar mechanisms that are present in both sexes.

Field L is typically split into three subsections: L1, L2 and L3. L2 is the thalamorecipient portion of Field L, and L1 and L3 relay information to higher auditory areas such as the caudal mesopallium (CM; specifically the medial portion of CM called CMM) and the caudal nidopallium (NC; specifically the medial portion of NC called NCM) (Gobes et al., 2010). Because of these patterns of connectivity, portions L1 and L3 are sometimes considered hierarchically more advanced in the sound processing pathway. Ascending through that pathway, auditory responses become progressively more selective to specific elements of songs and calls (Terleph et al., 2007; Meliza and Margoliash, 2012). CM and NC are secondary auditory processing regions analogous to layers II and III of the mammalian auditory cortex (Karten, 1991). These two forebrain regions are sometimes collectively called the auditory lobule (Cheng and Clayton, 2004; London and Clayton, 2008) and their medial portions (CMM and NCM) have been implicated as potential contributors to formation and storage of auditory memories in male songbirds. Studies of female birds have extended that idea by also implicating them in the expression of learned song preference in females (Riebel, 2003). These and other studies have implicated several brain sites in affecting female song preference, giving rise to additional questions about the neural circuits through which females perceive and evaluate the quality of male song.

Investigations into female song evaluation originated as lesion studies performed using female canaries (*Serinus canaria domestica*). Lesions placed in an auditory-vocal forebrain region known as HVC resulted in a change in female song preference (Brenowitz, 1991). The lesions in that study were quite robust and covered not only most of HVC but also parts of other adjacent auditory-responsive areas including CM and NC. At the time of those experiments, those areas were not appreciated as potentially important in female song perception, and those authors did not control for the possibility that impact of their lesions on sites other than HVC may have also contributed to the observed change in female song perception. A subsequent study of song preference in female zebra finches addressed that limitation by placing smaller and more focal lesions in either HVC or CM. Those authors found that lesions in HVC did not alter song preference. Instead, it was damage to CM that resulted in altered song preferences (MacDougall-Shackleton et al., 1998). Despite those authors' increased focus on placing small lesions, some of their lesions were so large that they extended beyond the border of CM, thus also impacting regions outside of CM. Moreover, those lesions were generated electrolytically, thus damaging not only somas located within CM but also fibers of passage that course through CM and may need to be intact in order to establish females' mate preferences. Although these studies have their caveats, they collectively pointed to CM as likely playing an important role in processing auditory signals=.

Additional investigations of CM have revealed pathways through which neurons in that site may communicate with downstream areas to exert their influence on song preference and mate choice. Dunning and colleagues (Dunning et al., 2018) used anterograde tracers to identify the pathways through which CM projects to downstream targets in female Bengalese finches. They found broad agreement between pathways present in females and those previously reported for males (Vates et al., 1996; Mandelblat-Cerf et al., 2014). CM projects robustly to other portions of the auditory lobule (NC). CM also receives small amounts of reciprocal input from NC, as evident in some reports that reveal weak projections from NC to CM and others that detected no connection between those areas (Vates et al., 1996; Dunning et al., 2018; Bloomston et al., 2022). Dunning et al. (2018) also found that CM projects into pathways that provide dopaminergic input back to cortical areas (ventral portion of the intermediate arcopallium, AIV). AIV in male songbirds has been shown to play a role in vocal-motor learning, and is likely a key region for establishing motivational state, which is important for reinforcement learning (Mandelblat-Cerf et al., 2014). In males, AIV provides a connection to midbrain dopaminergic regions, namely the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNpc) (Mandelblat-Cerf et al., 2014). This projection may also play a key role in learning of song and mate preferences in females. Together, these pathways provide mechanisms through which information in CM could be integrated with activity in other auditory processing sites as well as pathways implicated in behavioral motivation and reward.

Dunning et al. (2018) also described two novel pathways emerging from CM in female birds. The first of these is a projection from CM to a site implicated in production of behavioral responses (robust nucleus of the arcopallium, RA). A projection from CM to RA provides a link between a site implicated in auditory perception and downstream sites implicated in production of behavioral indicators of a female's degree of song preference. Specifically, RA is implicated in the production of calls, which are a way that females indicate their preference for specific songs (Dunning et al., 2014). RA also projects to the midbrain dorsomedial nucleus of the intercollicular complex (DM) (Wild, 1993; Tobari et al., 2006), where activity is both necessary and sufficient for the production of calls (Simpson and Vicario, 1990; Fukushima and Aoki, 2000, Fukushima and Aoki, 2002). DM also projects to the shell of the auditory thalamic nucleus (ovoidalis; $Ov_{shell}$), which then projects to the ventromedial nucleus of the hypothalamus and the mediobasal hypothalamus (MBH) (Durand et al., 1992; Cheng and Peng, 1997). Investigations using measures of immediate early gene expression in female canaries reveal a relationship between levels of activity in CM and MBH in response to songs females find attractive (Cheng and Zuo, 1994; Monbureau et al., 2015). Finally, DM also projects to a medullary respiratory center

(nucleus retroambigualis, Ram) which then projects to motor neurons in the spinal cord that control muscles in the cloaca through which females engage in copulation (Wild and Botelho, 2015). This pathway from CM to RA and beyond provides a route through which auditory stimuli may drive expression of behavioral indicators of song preference and mate choice.

The second novel pathway described by Dunning et al. (2018) was a connection between CM and the caudal striatum (CSt). Reiner and colleagues reported that CSt shares characteristics with an auditory region of the mammalian striatum which receives auditory input from Layers 2 and 3 of the auditory cortex (Reiner et al., 2004). This finding points to a role for the auditory areas CM and NC in higher order processing of complex auditory stimuli. The striatum has also been implicated in behavioral selection in mammals (Jiang and Kim, 2018; Cox and Witten, 2019), suggesting another possible route through which activity in auditory cortical areas may influence expression of selective behavioral responses to one suitor among many. This possibility is supported by data showing that optogenetic stimulation of CM can induce dramatic changes in female expression of song preference (Elie et al., 2019). Thus, CM and its projections play a key role in regulating the expression of those behaviors. Future studies will seek to discern the respective contributions of each of the many pathways that emerge from CM.

The reciprocal connections between CM and NC suggest that both sites may contribute to female song preference and mate choice. In support of a role for NC in auditory perception, previous studies of NC have revealed that following lesions to that area there is a reduction in preference for familiar (tutor) songs (Gobes and Bolhuis, 2007). Consistent with this possible role for NC activity in coding familiarity, activity of individual NC neurons habituates rapidly in response to repeated song playback, especially when those song stimuli are conspecific songs as opposed to heterospecific songs (Chew et al., 1995). Additional studies using female zebra finches have shown that when NCM is temporarily inactivated using the sodium channel blocker lidocaine, females show decreased affiliative behavior with conspecific males (Tomaszycki and Blaine, 2014). Taken together, these results indicate that NC may provide a site through which activity in CM may influence perception of song stimuli and the associated expression of affiliative social behaviors. Further insight into these circuits and the cellular mechanisms that compose them are the focus of ongoing research.

# 4 JUVENILE SONG PREFERENCE

## 4.1 Ultimate Questions (Functions)

### 4.1.1 Song Production Learning

Songbirds acquire their song through vocal learning. The process of song learning has been well documented in some species such as white-crowned sparrows, zebra finches, Bengalese finches, and canaries (Brainard and Doupe, 2002; Okanoya, 2004b; Prather et al., 2017). Although there is diversity in this process (Brenowitz and Beecher, 2005), song learning is commonly characterized by 2 phases: sensory learning and sensorimotor learning (Brainard and Doupe, 2002). Young birds hear and memorize songs of adult conspecifics (song tutor) in the sensory learning phase, and practice singing to match their own vocalization to the memorized song model in the sensorimotor learning phase. Because song is an important courtship signal, choosing an appropriate conspecific song model should be critical for reproductive success. If a juvenile has the opportunity to hear more than one song during sensory learning, how does the bird choose which song to imitate? One possibility is that song preference in juveniles plays a role to guide song tutor choice.

Some species of birds share habitat with phylogenetically close heterospecifics or live in a large flock of conspecifics. In such cases, young birds may hear multiple songs from conspecific and heterospecific adults, but they usually do not learn them all (Mann and Slater, 1995; Takahasi et al., 2010; Peters and Nowicki, 2017). A series of pioneering studies in chaffinches and white-crowned sparrows demonstrated that juveniles selectively learn songs of their own species (Thorpe, 1958; Marler and Peters, 1977; Soha and Peters, 2015; Soha, 2017). When hand-reared juveniles were tutored with playbacks of both conspecific and heterospecific songs, they typically produced species-specific sound features as adults (Marler, 1970). Based on these findings, it was hypothesized that juvenile birds have an auditory template that guides them to pay attention to conspecific song in the sensory learning (Marler, 1970, Marler, 1999). At that time, however, the auditory template was assumed to be some set of mechanisms in the juvenile's brain but not directly observed either as neuronal or behavioral responses to songs. More recent studies have revealed neuronal responses to juvenile experience that persist even in the adult brain, but they leave open the question of how the template may exert its influence on learning (Prather et al., 2010; Moseley et al., 2017).

In the classical studies, where a disposition in song learning was measured by the consequent song output, there was a methodological problem that one cannot tell if the selective learning is a consequence of perceptual preference of the learner (Rodríguez-Saltos, 2017). Indeed, species-specific physical properties define the range of sounds that juveniles learn to produce, and there should also be interaction between such motor constraints and perceptual preference. However, there is empirical evidence suggesting that juveniles do have preferences in a sense of selective behavioral responses to song stimuli, and such preferences are mostly consistent with the previously found selectivity of song learning. For example, juvenile white-crowned sparrows emit more calls in response to playbacks of conspecific song than to heterospecific song (Nelson and Marler, 1993). Moreover, they acquire memories of specific tutor songs and vocalize more to the familiar songs over unfamiliar conspecific songs (Nelson et al., 1997). Similarly, song-naive zebra finches juveniles prefer their own species song compared to starling songs, when measured by operant behavior associated with song playbacks (Braaten and Reynolds, 1999). In zebra finches, preference for a tutor song (typically father's song) is also demonstrated with phonotaxis and vocal response assay (Clayton, 1988) as well as key pecking operant conditioning (Rodríguez-saltos et al., 2021). Selective approaches and vocal

responses to father's song was also reported in juvenile Bengalese finches (Fujii et al., 2021).

These findings collectively indicate that male juveniles can discriminate and probably be attracted to the song that they are more likely to imitate. However, in the studies above, the scope was limited to the song preference itself, so the relationship between preference and song learning was not examined. Strict demonstration of the causal relationship between song preference and song imitation learning may be challenging, but we can at least make the following prediction. If juveniles selectively learn the song that they prefer, there should be a positive correlation between the behaviorally measured song preference and the performance of song imitation learning. By testing this prediction, researchers can evaluate the validity of this hypothetical function of song preference. As such, in relatively early years, two studies in zebra finches recorded both the preference and song learning performance (Houx and Ten Cate, 1999; Terpstra et al., 2004). In these studies, preference was tested for the tutor song compared to unfamiliar conspecific song using operant conditioning, but the preference tests were conducted in adulthood, where the birds already finished song learning. Both studies found that the males preferred their tutor's song over unfamiliar song on average. However, when they examined the relationship of the degree of preference and the tutor-tutee song similarity, one study reported no significant correlation (Terpstra et al., 2004), and the other had a mixed results depending on the condition of song tutoring (Houx and Ten Cate, 1999).

In more recent years, some researchers investigated how social interaction with the song tutor enhance song learning in juveniles (Chen et al., 2016; Baran, 2017). In these studies, the authors did not measure the song preference itself but showed interesting relationships between the behavior of juvenile learners during song tutoring and the result of song production leaning. For example, juveniles generally pay closer attention to song when performed by a live tutor than when played through a speaker, and the degree of attention towards the song during tutoring predicts the accuracy of song imitation (Chen et al., 2016). In this study, attentiveness was defined by the lack of engagement in other behaviors such as feeding, flying, or vocalizing. Another study pointed out a correlation between social attachment to parents and song copying from the father in juvenile zebra finches (Baran et al., 2016, Baran et al., 2017). Although the relationship among the preference, attention, and social interaction is still unclear, this line of research may shed light on the possible function of song preference in sensory learning.

We also note here that preference for tutor song may help not only sensory learning but also sensorimotor learning. In a recent study using zebra finches, the authors quantified juveniles' approach to their singing tutor and found that individual variability of approach behavior correlated with the precision of tutor song imitation (Liu et al., 2021). They proposed a two-step auditory learning process; juveniles first acquire a crude memory of their tutor song, which drives repeated interaction with the singing tutor, leading to ensure the selective and precise vocal imitation. As they did not compare the juveniles' response among different songs or song tutors, the degree of attentive listening behavior may not be directly equivalent to song preference as measured in other studies. However, it suggests a possibility that early acquisition of preference for the song of an individual tutor may function to further reinforce accurate imitation.

Regardless of which phase of learning the conspecific or tutor song preference may serve, crucial empirical evidence for the causality between preference and song production learning is still lacking, either at behavioral or neural levels. Yet, a breakthrough may be provided by cutting-edge studies. By using a combination of carefully controlled live tutoring and well-designed operant conditioning preference tests, it was recently reported that song preference of juveniles in song development predicts the quality of future song imitation (Rodríguez-saltos et al., 2021). Another study from the same group using the same experimental system attempts to reveal the neural mechanisms underlying the tutor song preference and vocal learning (Pilgeram et al., 2021).

### 4.1.2 Other Possibilities

There are also other possible functions of song preference in young birds. One example is parental recognition. Because of the entire nutritional dependence on parental care in young altricial songbirds, correct recognition of the species or identity of adult birds should be critical to the survival of birds at a very early developmental stage. Some studies have shown that nestlings (Shizuka, 2014; Wheatcroft and Qvarnström, 2017; Hudson et al., 2020) and young fledglings (Nelson and Marler, 1993; Whaling et al., 1997; Soha and Marler, 2001) respond to conspecific songs with more calls than to heterospecific songs. These results support the idea that the preference may help very young birds correctly recognize their parents and beg for food or care. However, it should be noted that parental recognition can also be accomplished through other sensory domains. For example, nestling zebra finches can use odor to detect the presence of their parents and initiate begging behavior (Caspers et al., 2017). In addition, it is likely that young birds use vocalizations other than song in species where only males sing, as biparental care is a typical characteristic of songbirds in either group of female song present or absent (Langmore, 1998; Cockburn, 2006). Thus, future studies are awaited to figure out whether young birds use their parents' (or only father's) song as a cue for parental recognition.

Finally, we add some notes for fair estimation of the utility of juvenile song preference. First, the fact that young birds respond differently to song stimuli in experimental settings does not necessarily indicate that the birds do utilize such discrimination ability in natural social settings (Nelson and Marler, 1990). In addition, it is also possible that birds acquire song preference when young, but the preference comes to have its utility only after the birds become adult. In such a case, even if the song preference is not in effect, it can be observed in young birds if researchers attempt to do so. Conversely, juvenile song preference can also have multifaceted functions, thus different functions described above are not mutually exclusive. Lastly, as we currently know very little about the phylogeny of juvenile

song preference, more comparative studies are expected in the future.

## 4.2 Proximate Questions—Mechanisms and Development

Although we currently know less about the neural mechanisms underlying song preference of juveniles compared to that of females, there are a substantial number of neurobiological studies suggesting the relevance of higher auditory forebrain areas including NCM. This auditory region originally attracted attention because evidence indicated that it is the locus of tutor song memory as a template of imitative song learning (Adret, 2004; Bolhuis and Gahr, 2006; Hahnloser and Kotowicz, 2010; Bolhuis and Moorman, 2015, but see also; Ikeda et al., 2020). It is still one of the hottest topics in the songbird neuroscience, and the role of forebrain auditory areas in song production learning is well discussed in the reviews listed above. Here, we focus on the literature that is particularly related to the preference for conspecific songs or the tutor song.

It was first found in adult songbirds that NCM exhibits stimulus-specific neural responses (Mello et al., 1992; Chew et al., 1996; Bolhuis et al., 2000; Phan et al., 2006). Later, mainly in zebra finches, the neural responses were compared across ages to investigate how such neural selectivity develops. Consistent with the sensitivity to own-species song at a behavioral level, electrophysiological recordings (Stripling et al., 2001; Schroeder and Remage-Healey, 2021) and immunohistochemical assay (Stripling et al., 2001; Bailey and Wade, 2003, Bailey and Wade, 2005) showed that some neurons in the NCM selectively respond to conspecific song over heterospecific song either at a single-cell level or at a population level. These results indicated that the selectivity is already present at the age of 20 days post hatch, which is said to be the beginning of the sensory learning in this species (Roper and Zann, 2006). Similarly, it was also found that a population of NCM neurons selectively respond to tutor song among other unfamiliar conspecific songs. The hypothesis that the NCM neurons code the tutor song memory was originally suggested by a series of findings in adult birds (Bolhuis et al., 2000, 2001; Terpstra et al., 2004; Phan et al., 2006), but recent electrophysiological studies showed that tutor song selective neurons are present at an early stage of sensory learning but may be small in number (Miller-Sims and Bottjer, 2014), and that such selectivity is actually shaped through auditory experience of tutor song (Yanagihara and Yazaki-Sugiyama, 2016; Moore and Woolley, 2019). It is not clear, however, whether these neural activities are the basis of tutor song preference. To our knowledge, there has been no study that impaired the neural activity of NCM in juveniles and examined the effect of impairment on the song preference, although one study reported that NCM lesion in adult male zebra finches impaired the tutor-song selective approaching behavior (Gobes and Bolhuis, 2007).

Another line of studies suggests the importance of midbrain catecholaminergic systems during song tutoring. For example, in male juvenile zebra finches, noradrenergic neurons in the locus coeruleus as well as dopaminergic neurons in VTA show activities correlated with attention to tutor song. Behaviorally measured attentional state and the neuronal responses in these midbrain areas both increased during live tutoring compared to playback tutoring (Chen et al., 2016). Also, a recent study from the same group showed that noradrenaline release in NCM during social tutoring is important for the formation of tutor song memory (Chen and Sakata, 2021). Another study reported that pharmacological manipulation of the dopaminergic system in juvenile zebra finches changed the approaching behavior towards tutor songs, and that activation of immediate early gene in the NCM in response to song playbacks was associated with the listening approaching behavior of the juveniles (Liu et al., 2021). Thus, the noradrenergic and dopaminergic systems seem to be involved in the mechanism of juvenile song preference, though the specific contributions of these systems in the expression or plasticity of preference are currently unknown.

# 5 DISCUSSION

The results of studies focusing on birdsong preference have accumulated and now researchers have a basis to develop a more precise investigation into its neural mechanisms. In this section, we describe some problems that should be kept in mind when further exploring both the proximate and ultimate explanations for song preference and discuss possible future directions.

## 5.1 Choice and Validation of Behavioral Index of Preference

When choosing a method, it is important to balance the practicality and ecological validity. Indeed, if a bird consistently shows different degrees of a given behavioral response to different songs, it means that the birds can discriminate between stimuli. However, whether the discrimination is interpreted as the preference in the context of mate choice or song tutor choice is not clear until additional tests can verify the ecological validity of the behavior observed in the tests. We believe this is a critical issue for any behavioral and neurobiological laboratory study. Without the firm understanding of the focal behavior, one can lose sight of the mapping between neural activity and natural behaviors (Krakauer et al., 2017).

Here we take call back assay as an example. Although the method has been used to test preferences in females and juveniles of some species (Clayton, 1988; Nelson et al., 1997; Chen et al., 2017), it was not a standard procedure to differentiate the categories of calls with consideration of the function of each call type (but see Nagle et al., 2002). Of course, such a fact does not necessarily deny the reliability of the assay. Nonetheless, birds usually communicate with others in various behavioral contexts through different categories of calls (Marler, 2004; Elie and Theunissen, 2016), and calls are sometimes regarded as an indicator of the more general attentional state such as familiarization to stimuli (Dong and Clayton, 2009; Ono et al., 2015; Dai et al., 2018). Probably, the best way to verify the calls as a measure of preference would be to carefully observe the animals' behavior in natural social settings. As this would not always be very practical, an alternative way is to examine the correlation

between behavioral indices in laboratory experiments. As such, in a series of studies of female Bengalese finches, the response rate of a specific call type was positively correlated with CSDs as well as with phonotaxis at an individual or a population level (Kato et al., 2010; Dunning et al., 2014; Fujii et al., 2021; Fujii and Okanoya, 2022). Because using multiple behavioral tests to measure subjects within a single study can be logistically challenging, the most practical approach may be to gradually accumulate the results from an entire research community over time. This would also enable researchers to examine the reproducibility of results obtained from the same experimental system, as well as the consistency of results between different types of experiments. This cycle of careful reconsideration and application of methods should also be vital to evaluate the impact of neural manipulation on song preferences.

The choice of behavioral index may be critical in figuring out the causal relationship of juvenile song preference and vocal learning. When investigating the correlation between behaviorally expressed preference and the results of song learning, do differences in the choice of behaviors lead to different results? To date, some studies utilized operant conditioning to quantify the tutor song preference (Terpstra et al., 2004; Pilgeram et al., 2021; Rodríguez-saltos et al., 2021), while others measured birds' activities including approaching behavior as an indicator of stimulus engagement or attentional state (Chen et al., 2016; Liu et al., 2021). Currently, we do not know whether these behaviors capture the same aspect of song preference or whether they share the neural basis. In future studies, identifying the neural substrate responsible for juvenile song preference and testing the effect of manipulation of the preference-related neural activity on the performance of vocal imitation would be one of the most powerful approaches to examine the function of preference in learning.

## 5.2 Inter- and Intra-Species Variation of Song Preference

Another important perspective is the variation of song preferences. As seen in the diversity of song characteristics across species, there is great species-diversity in the function, evolutionary background, and the developmental process of songs (Brenowitz and Beecher, 2005; Catchpole and Slater, 2008). This diversity means that we cannot necessarily generalize the knowledge of one species to another, and thus a comparative viewpoint is needed for a comprehensive understanding of any aspects of birdsong, including how and why females or juveniles prefer a certain song over others. In a case where many different species of birds possess a similar profile of preference, either by convergent evolution or inheritance from a common ancestor, studying one representative model species helps construct a general understanding. However, the postulation itself is the very subject of scientific examination, and there has been a bias in the choice of subject species particularly in the neurobiological laboratory experiments; zebra finches are the most studied species, and other birds such as Bengalese finches and cowbirds follow. Even the few species listed here show great diversity in their habitat, life history, or evolutionary background (Okanoya, 2004b; Perkes et al., 2019; Griffith et al., 2021), further advancing the existing research topics in these species and others is quite worthwhile. However, direct comparison of a limited number of species of great phylogenetic distance is rather difficult and may not be relevant. Thus, extending the scope to more diverse taxa of songbirds as well as comparing multiple species of close phylogenetic relationship would be meaningful in future studies.

Individual difference of preference within a species should also be considered as a direction to extend research efforts. The importance of individual variation has already been pointed out particularly for the study of mating preference; investigation into the basis of variation in mating preferences has the potential to reveal new insights into the evolutionary history of sexually selected signals and the signal preference itself (Jennions and Petrie, 1997). We believe this is also a context in which Tinbergen's four questions can interact in a fruitful way. For instance, in the studies on Bengalese finches from our laboratories, we found that while some song features are preferred by majority of females, there are also substantial individual differences in the song preference (Morisaka et al., 2008; Kato et al., 2010; Dunning et al., 2014, 2020). Other studies provide a possibility that the source of individual variation lies in the early-life experience of father's song (Fujii et al., 2021; Fujii and Okanoya, 2022). As the Bengalese finch song is particularly interesting for the sequential complexity and its evolutionary change through domestication process (Okanoya, 2004a; Okanoya, 2004b), scrutinizing the universality and individual differences of female song preference from the perspectives of neural mechanisms and development potentially help investigate the function and evolution of the sequential complexity, and vice versa. This is also the case for song preference of juveniles, as they have a potential to filter the song features transmitted to the next generation by selective imitation (Soma et al., 2009; Peters and Nowicki, 2017).

## 5.3 Neural Basis of Song Preference

The neuroscience of female song preference began with the exploration of brain areas involved in the expression of preference using electrolytic or pharmacological lesions as well as correlation analysis between the neural and behavioral response selectivity to song stimuli. The results from recent neural tracing and imaging studies (Dunning et al., 2018; Van Ruijssevelt et al., 2018; Perkes et al., 2019), together with the techniques of precise manipulation of neural activities (Elie et al., 2019; Barr et al., 2021) now provide a prospect of investigation into the mechanisms of song preference at a circuitry level. To elucidate the functional roles of each component in the proposed neural circuits for the expression of song preferences (Dunning et al., 2018; Van Ruijssevelt et al., 2018; Perkes et al., 2019), optogenetic manipulation of specific neuronal populations with a fine anatomical and temporal resolution would be a powerful tool (Elie et al., 2019).

The neural mechanisms underlying plasticity of song preference is also worth studying. Strong influence of postnatal experience on the signal preference is one of the most interesting features in songbirds compared to other vertebrate or

invertebrate taxa (Riebel, 2009; Verzijden et al., 2012), and experience-dependent changes in song preferences can occur throughout life (Riebel, 2009). Recent studies in adult female zebra finches showed the involvement of the dopaminergic system in the acquisition of song preference using pharmacological manipulation (Day et al., 2019; Barr et al., 2021), but the neural mechanisms by which song preference is shaped early in development is still unclear. It would be interesting to examine the degree to which the neural circuitry composed of higher auditory forebrains and dopaminergic neuromodulation also plays a role in the brain of juveniles similarly to that of adults.

We also note that the inter-species comparison of auditory neural response properties may shed light on the evolutionary process that have shaped song preferences. Although the evolutionary mechanisms of bird song preference other than fitness benefits remain poorly understood (but see Collins, 1999; Eda-Fujiwara et al., 2006), such a comparative approach succeeded in anurans to find the significant role of sensory bias in the preference of acoustic signals (Ryan and Cummings, 2013).

In a case of male juvenile songbirds, the neuronal activities in the forebrain auditory area NCM have been explored in search of the neural substrate of tutor song memory used for vocal imitation (Bolhuis and Gahr, 2006; Bolhuis and Moorman, 2015). It is known from classical studies that male juveniles have a disposition to selectively learn conspecific songs. More recent behavioral and neurobiological studies suggest the relevance of juveniles' perceptual preference among conspecific songs (Fujii et al., 2021; Liu et al., 2021; Pilgeram et al., 2021; Rodríguez-saltos et al., 2021) as well as the importance of juveniles' physiological or arousal state during the exposure to the song they are about to learn (Chen et al., 2016; Yanagihara and Yazaki-Sugiyama, 2019; Chen and Sakata, 2021; Liu et al., 2021). These researchers focus on NCM, while also indicating the involvement of the midbrain catecholaminergic system in the processes of memory formation or increased attention to the tutor song. To date, we do not know if there are distinct neural substrates of song preference and song template within NCM. Attempts to experimentally dissociate the two kinds of neural representation will be challenging, but this direction of neurobiological research should also be helpful to reveal the relationship of preference and song learning at a behavioral level. In addition, it is not known if the midbrain catecholamine neurons differentially respond to more preferred or less preferred songs. Moreover, it needs to be examined if the catecholaminergic signaling is relevant not only to the formation or plastic changes of song preference but also to the expression of preference. Future studies might ask to what extent the neural activity correlated with behavioral song preference depends on previous auditory experience. It would be important to figure out whether certain neural response properties are actually the basis of behavioral preference for a song with specific features, and whether they guide song tutor choice.

## 5.4 Summary and Conclusion

Song preference of female and juvenile songbirds has been studied as an important factor in mate choice and song tutor choice, respectively. Based on the previous ethological findings, researchers are now investigating the neural mechanisms underlying the representation, expression, and plasticity of song preferences. The higher auditory forebrain areas and the midbrain catecholaminergic systems are of particular interest in both cases of females and juveniles. For quantification of the preference in the neurobiological laboratory research, experimenters need to carefully choose a method of behavioral test, balancing the practical use and ecological validity. We suggest that incorporating the knowledge about ultimate questions of songs and song preferences is helpful in doing so. Conversely, the future exploration into the proximate questions of song preference would shed light on the function and evolution of the preference, leading to more comprehensive and general understanding of a complex nature of vocal behavior. The multifaceted approach to the questions of birdsong preference will potentially give insight into the study of speech communication in humans.

# AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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# Suppressive Modulation of the Chick Forebrain Network for Imprinting by Thyroid Hormone: An *in Vitro* Study

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The thyroid hormone 3,5,3′-triiodothyronine ($T_3$) is considered to act acutely in the chick forebrain because focal infusion of $T_3$ to the intermediate medial mesopallium (IMM) causes 4 to 6-day-old hatchlings to become imprintable approximately 30 min after the infusion. To understand the mechanism of this acute $T_3$ action, we examined synaptic responses of IMM neurons in slice preparations *in vitro*. Extracellular field potential responses to local electrical stimulation were pharmacologically dissociated to synaptic components mediated by AMPA and NMDA receptors, as well as GABA-A and -B receptors. Bath-applied $T_3$ (20–40 μM) enhanced the positive peak amplitude of the field potential, which represented the GABA-A component. Bicuculline induced spontaneous epileptic bursts by NMDA receptor activation, and subsequent application of $T_3$ suppressed the bursting frequency. Pretreatment of slices with $T_3$ failed to influence the synaptic potentiation caused by tetanic stimulation. Intracellular whole-cell recording using a patch electrode confirmed the $T_3$ actions on the GABA-A and NMDA components. $T_3$ enhanced the GABA-A response and suppressed the NMDA plateau potential without changes in the resting membrane potential or the threshold of action potentials. Contrary to our initial expectation, $T_3$ suppressed the synaptic drives of IMM neurons, and did not influence activity-dependent synaptic potentiation. Imprinting-associated $T_3$ influx may act as an acute suppressor of the IMM network.

**Keywords: imprinting, thyroid hormone, sensitive period, NMDA receptor, GABA-A receptor**

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## 1 INTRODUCTION

Since being coined by Lorenz (1937), imprinting has amassed a long history of ethological and neuroscientific investigation as a unique form of behavioral development toward formation of social attachment in precocial animals such as domestic chicks and ducklings (Bolhuis et al., 1985; Bolhoius 1999). Beside the cellular and molecular bases of imprinting memory formation (Horn, 1998, Horn, 2004; Horn et al., 2001), considerable efforts have addressed the sensitive (or critical) period, one of the core characteristics of imprinting (Hess, 1958, Hess, 1959; Bateson, 1978). However, the mechanisms underlying this aspect of imprinting remained vague until recently.

Thyroid hormone (3,5,3′-triiodothyronine, $T_3$) is the critical molecule determining the sensitive period (Yamaguchi et al., 2012). Imprinting enhances expression of the converting enzyme (type2 iodothyronine deiodinase, Dio2) in the vascular endothelial cells of the dorsal pallium (including the intermediate medial mesopallium, IMM), leading to a rapid influx of $T_3$ to the neuronal network involved in imprinting. Two distinct modes of $T_3$ action occur, an acute and a chronic effect. Usually,

4 to 6-day-old hatchlings cannot be imprinted, but injection of exogenous $T_3$ (either systemically or focally into the IMM that is responsible for imprinting) makes them imprintable approximately 30 min after the injection; $T_3$ acutely re-opens the previously-closed sensitive period. Systemic $T_3$ application also increases the imprinting score in 1-day-old chicks, indicating that thyroid hormone is functional at hatching or earlier. Furthermore, imprinting on day 1, or a single injection of exogenous $T_3$, makes the treated chicks imprintable to an object of novel color at 4–6-days post-hatching, when the sensitive period is normally closed; $T_3$ elongates the sensitive period so that the imprinted chicks remain imprintable.

Recently, Batista et al. (2018) revealed that the mechanistic target of rapamycin complex 1 (mTORC1) is critically involved in the acute effect of thyroid hormone; its blockade by rapamycin injection impaired imprinting, and its activation re-opened the sensitive period at 4-days post-hatching, similar to thyroxine injection. Furthermore, we have found that development of GABA receptor-mediated transmission in the IMM is also critical in the control of the sensitive period (Aoki et al., 2018); systemic intravenous injection of GABA-B agonist (baclofen) made 4-day-old hatchlings imprintable, whereas its selective blockade impaired imprinting in 1-day-old hatchlings. Thyroid hormone can act on the mTORC1 kinase cascade and GABA receptors to affect behavior.

Despite these clear-cut pharmacological findings, the assumed biochemical cascades have not been causally linked to the network properties of the IMM. It is important to determine how thyroid hormone affects in the IMM neurons and synapses, and how these cellular/synaptic events change the neural network dynamics. Furthermore, which of these changes lead to the behavioral expression of social attachment in hatchlings needs to be determined.

In addition to detailed Golgi cytoarchitecture (Tömböl et al., 1988), ample histochemical and connectivity data are available for the IMM (Bradley et al., 1985; Csillag, 1999). A neurophysiological study reported activity-dependent synaptic potentiation in the IMM (Bradley et al., 1991), although the synaptic transmission responsible was not specified. We (Yanagihara et al., 1998) then showed that a DNQX-sensitive excitatory component (namely AMPA receptor mediated) is potentiated by locally applied tetanic electrical stimulation, but the plastic change was not linked to immediate early gene (*c-fos*) expression, which was clearly associated with the imprinting score at the individual level (McCabe and Horn, 1994). Since the single-unit characterization of visual objects in the IMM (Horn et al., 2001), little progress has been made and our understanding of imprinting mechanisms remains elusive at the neuron level.

In this study, inspired by the recent findings concerning thyroid hormone, we investigated the neuronal/synaptic target of the hormonal action using IMM slice preparation *in vitro*. If $T_3$ activated the IMM network, exogenous bath application of $T_3$ should enhance the neuronal excitability, excitatory synaptic drive or facilitate synaptic potentiation. Alternatively, $T_3$ might not modulate IMM excitability, but might act via other aspects of the neural network. As reported for cultured mammalian hippocampal neurons (Losi et al., 2008; Puia and Losi 2011), $T_3$ can suppress the synaptic transmission mediated by GABA and glutamate receptors. This also seems to be the case in chicks, as we show here.

# 2 MATERIALS AND METHODS

## 2.1 Slice Preparation

Domestic chicks of a white leghorn strain (called "Julia" by the local name of hatchery; 2–5 days post-hatching, mostly 3 days old) were used. Fertilized eggs were purchased from a local supplier (Sankyo Labo Service Co., Sapporo, Japan; Iwamura Poultry Co., Yubari, Japan; Nippon White Farm Co., Shiretoko Farm, Abashiri, Japan) and incubated in the laboratory. To avoid possible complication due to uncontrolled learning experiences, hatchlings were individually housed in cages placed in a dark incubator at 37°C until used in experiments. Chicks were decapitated under a deep anesthesia by i. m. injection of 0.8 ml ketamine-xylazine cocktail; a 1:1 mixture of 10 mg/ml ketamine hydrochloride (Daiichi-Sankyo Propharma Co., Tokyo, Japan) and 2 mg/ml xylazine (Sigma-Aldrich Co., St Louis, Missouri, United States ). The left hemisphere of telencephalon was dissected out, and quickly immersed in ice-chilled Krebs solution composed of (in mM): NaCl, 113; $NaHCO_3$, 25.0; KCl, 4.7; $KH_2PO_4$, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.2, glucose, 11.1; pH was adjusted to 7.2–7.4 by bubbling the solution with 95% $O_2$—5% $CO_2$. Using a vibrating microslicer (DTK-1000, Dosaka EM Co., Kyoto, Japan), two to three 400 μm-thick coronal slices were cut from the brain and stored for recovery (>1 h) in an interface-type slice chamber at room temperature (ca. 26–28 °C) supplied with a continuous flow of humid $O_2/CO_2$ gas. Thereafter, slices were placed in a submersion-type recording chamber (ca. 2 ml in volume), which was continuously perfused with Krebs solution held at 30 °C at a flow rate of 1.5 ml/min. The following drugs were applied to the perfusing solution: 3,5,3′-triiodo-L-thyronine ($T_3$, Sigma Co.); 6,7-dinitroquinoxaline-2,3-dion (DNQX, Tocris Bioscience Co.); 1(S),9(R)-(-)-bicuculline methiodide (bicuculline, Sigma Co.); D (-)-2-amino-5-phosphonovaleric acid (D-AP5, Wako Co.) [3-[[(3,4-dichlorophenyl)methyl] amino]propyl](diethoxymethyl)-phosphinic acid (CGP52432, Tocris Bioscience Co.). Concentration of receptor blockers in the perfusing bath solution was fixed at: DNQX (10 μM), bicuculline (10 μM), D-AP5 (50 μM), and CGP52432 (1 μM). When $Ca^{2+}$ was removed, no divalent cation was substituted.

## 2.2 Electrical Stimulation and Recording of Extracellular Field Potential

Electrical stimulation was delivered via a concentric metal electrode (o.d. = 300 μm; UB-9007, Unique Medical Co., Tokyo Japan) placed on the slice surface. The center (or sleeve) of the electrode was connected to the cathode (or

anode) of the isolating unit (SS-203 J) and pulse generator (SEN-3301, Nihon Koden Co., Tokyo, Japan) in the constant current mode. Field potential was recorded using a pair of Krebs-solution-filled glass micropipettes (o.d. = 1.2 mm, i. d. = 0.69 mm, borosilicate glass; #BF120-69-10, Sutter Instruments Co., Novato, California, United States ) with fine tips made by pulling and cutting (o.d. = ca. 50 μm). One pipette (plus pole) was placed in the slice tissue, and another (minus pole) was submerged in the perfusing Krebs solution as the reference electrode. These pipettes were connected through inserted silver wires to a differential amplifier (DAM-50, World Precision Instruments Inc. Sarasota, Florida, United States ) set at frequency range of 1 to 1,000 Hz and gain of x1,000. The amplified signals were stored in a computer via an A/D interface and sampling software (Power 1401 and Signal ver 5, Cambridge Electronic Design Ltd. Cambridge, United Kingdom) after digitizing at 5 kHz.

## 2.3 Whole-Cell Intracellular Recording by Nystatin-Perforation Patch Recording

To characterize components of the recorded field potential responses, intracellular voltage recordings were obtained using Nystatin-perforation patch recording. For details of the methods, see Matsushima and Aoki (1995). Briefly, patch glass pipettes (tip size: 1–2 μm) were filled with a solution containing (in mM: K-gluconate, 123; KCl, 18; NaCl, 9; $MgCl_2$, 1; EGTA, 0.2; HEPES, 10; pH adjusted to 7.3 with KOH) with a DC-resistance of 3–6 MΩ. Nystatin (Sigma-Aldrich) was added to the solution at 100 μg/ml just before recording. To load chloride ($Cl^-$) intracellularly, K gluconate was substituted by equimolar KCl, 141 mM. Membrane potential was recorded with a single-electrode voltage-clamp amplifier (CEZ-3100, Nihon Kohden Co., with bridge balance mode or single electrode current clamp mode, sampling clock rate = 30 kHz, high-cut filter = 2 kHz) and stored in a computer. Data were discarded when resting membrane potential was above −50 mV or stable responses lasted for less than 30 min.

## 2.4 Histological Verification of Stimulating and Recording Sites

After recording, slices were fixated in 4% paraformaldehyde in phosphate buffer (0.1 M PB, pH = 7.2) for ca. 30 min at room temperature, embedded in gelatin and post-fixed in the same fixative for 1 day at 4°C. Sections, 50 μm thick, were cut from the slices using a microslicer (DTK-3000, Dosaka EM Co.), and then mounted on slide glasses, air dried, and stained with cresyl violet. Data were discarded when holes made by the recording electrodes were found outside of the mesopallium. The recording sites were superimposed on a representative intact section after adjusting the location of the ventricle and the boundary between the mesopallium and the nidopallium. Histological judgements were based on the chick brain atlas by Kuenzel and Masson (1988) and terminology followed nomenclature reform (Reiner et al., 2004).

# 3 RESULTS

## 3.1 Local Synaptic Transmission and Network Dynamics of the Pallial Neural Network

### 3.1.1 Excitatory and Inhibitory Synaptic Transmission in the IMM

As reported previously (Matsushima and Aoki 1995), field potential responses to local electrical stimulation are composed of an initial negative spike-like component (S-waves) followed by slower positive waves (P-waves); **Figure 1Aa** shows an averaged trace (~30 successive responses at 10 s intervals). Histological examination confirmed that stimulation electrodes were located at ca. 500 μm dorsal to the recording sites in the IMM (**Figure 1C**). The S-wave contained pre-synaptic action potentials that were unmasked in $Ca^{2+}$-free Krebs solution (**Figure 1Ab**), whereas the later component of the S-wave (indicated by *) was of post-synaptic origin because this and the following P-waves (indicated by #) were almost completely diminished in $Ca^{2+}$-free conditions. In other words, the post-synaptic response was composed of the initial negative (*) and the late positive wave (#) components. Subsequent successive addition of blockers to the bath (DNQX for AMPA receptor blockade, **Figure 1Bb**; bicuculline for GABA-A receptor blockade, Bb; CGP52432 for GABA-B receptor blockade, 1Bc; D-AP5 for NMDA receptor blockade, 1Bd) confirmed that the P-waves represents a compound of early excitatory (glutamatergic) and late inhibitory (GABA) transmission; all traces in Ba-d are non-averaged single traces. The blue trace represents a typical single response trace in normal Krebs; inlet traces show the time expansion. DNQX blocked the initial P-waves component (blue arrow in **Figure 1Ba**) and the late component (brown arrow). The GABA-A blocker unmasked a lasting barrage of unsynchronized spikes (green arrow in **Figure 1Bb**, late P-waves). Blockade of GABA-B transmission augmented the spiking barrages (black arrow in Bc), which were abolished by D-AP5 (Bd) leaving the pre-synaptic action potentials as in **Figure 1Ab**.

### 3.1.2 Epileptiform Excitatory Bursts After GABA-A Blockade

In normal Krebs, without electrical stimulation, field potential recording did not show any spontaneous activities. Bath-applied bicuculline induced DNQX-sensitive epileptiform bursts (indicated by %) that emerged spontaneously at varying intervals of ca. 5–15 s (**Figure 1Db**). The activities were not localized but spread ventrally from the dorsomedial pallium. Field potentials were simultaneously recorded dorso-ventrally in medial forebrain regions, with the reference recording site set in the IMM (**Figure 1F**); experiments were repeated in eight slices. The intermediate medial hyperpallium apicale (IMHA) was 500 μm dorsal to the IMM, and the medial nidopallium (MN) was 500–1,000 μm ventral. **Figure 1E** shows three occasions, in which simultaneous IMHA (thick line traces, onset indicated by downward arrow) and IMM (thin line

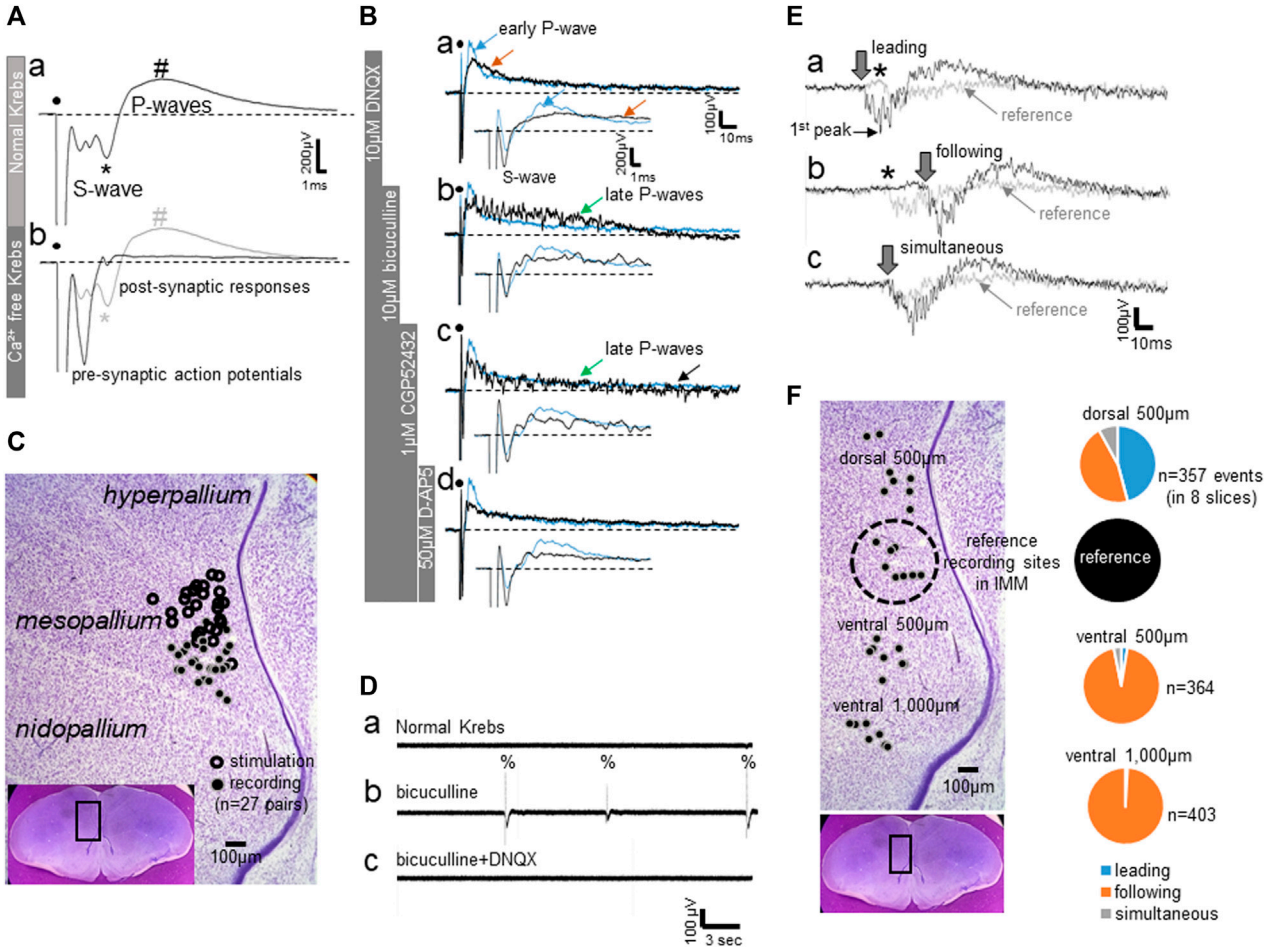


**FIGURE 1 | (A–C)** Field potential responses to local electrical stimulation of the IMM. **(A)** Averaged field potentials recorded in normal Krebs and in $Ca^{2+}$-free Krebs; ~30 successive responses at 10 s intervals were averaged. Superimposed traces reveal a pre-synaptic component (S-wave) and post-synaptic responses (P-waves). **(B)** Bath-applied blockers dissociated components of the P-waves; single traces (not averaged). The trace shown in blue indicates the control recorded in normal Krebs. DNQX-sensitive fast excitatory transmission is represented by the early rising phase of the P-wave, whereas the late component (late P-waves) is NMDA-receptor-mediated (D-AP5-sensitive) slow excitation that was unmasked by GABA-A and–B blockers (bicuculine and CGP52432, respectively). **(C)** Sites of electrical stimulation and field potential recording plotted on a representative photomicrograph of a frontal section (see inlet). **(D–F)** Field potential recording of spontaneous epileptiform activities induced by bath-applied bicuculline. **(D)** Spontaneous epileptiform discharges (%) with bicuculline, which disappeared after DNQX was added to the bath. **(E)** Epileptiform activities simultaneously recorded from two sites, one from a point in the IMM (shown by grey traces) and another from a point 500 μm dorsal to the reference site (shown by black traces). Three examples are shown where the dorsal activity led **(Ea)**, followed **(Eb)** or occurred simultaneously **(Ec)**. Downward arrows indicate onset of the negative component, and asterisks denote the initial positive component recorded in the IMM, or the reference point. **(F)** Proportion of the leading (blue), following (orange) and simultaneous (grey) activities. Recording sites in eight slices are plotted on the same photomicrograph as C; number of events (*n*) indicates the total events obtained from these eight slices.

reference traces, onset indicated by asterisk) recordings were superimposed. The IMHA bursts led (**Figure 1Ea**) or followed (Eb) the IMM bursts in almost equal frequency, indicating that the bursts could emerge from epileptic focal spots scattered around IMHA-IMM regions. However, bursts in the MN almost always followed the IMM bursts. Within the frontal plane of the pallial network, IMHA-IMM could be the most excitable hotspot, from which bursts spread ventrally to the MN.

As described below, we then examined the effects of exogenously applied $T_3$ on local synaptic transmissions and epileptiform bursts in the IMM.

## 3.2 Acute Neurophysiological Effects of $T_3$

### 3.2.1 $T_3$ Enhanced the Bicuculline-Sensitive Synaptic Transmissions, Field Potential Analysis

Bath-applied $T_3$ gradually enhanced the peak amplitude of the P-wave (**Figure 2A**); averaged traces (30 successive records at 10-s intervals) obtained from one control slice with vehicle (Aa) and another slice with $T_3$ (40 μM) (Ab) are compared. Responses recorded at 25–30 min after the onset of drug application (black traces) were superimposed on those during a 5-min period just before the application (grey traces with red upward arrows). Effects were not found on the onset slope of the initial S-wave (indicated by dashed blue lines), indicating that the neuronal

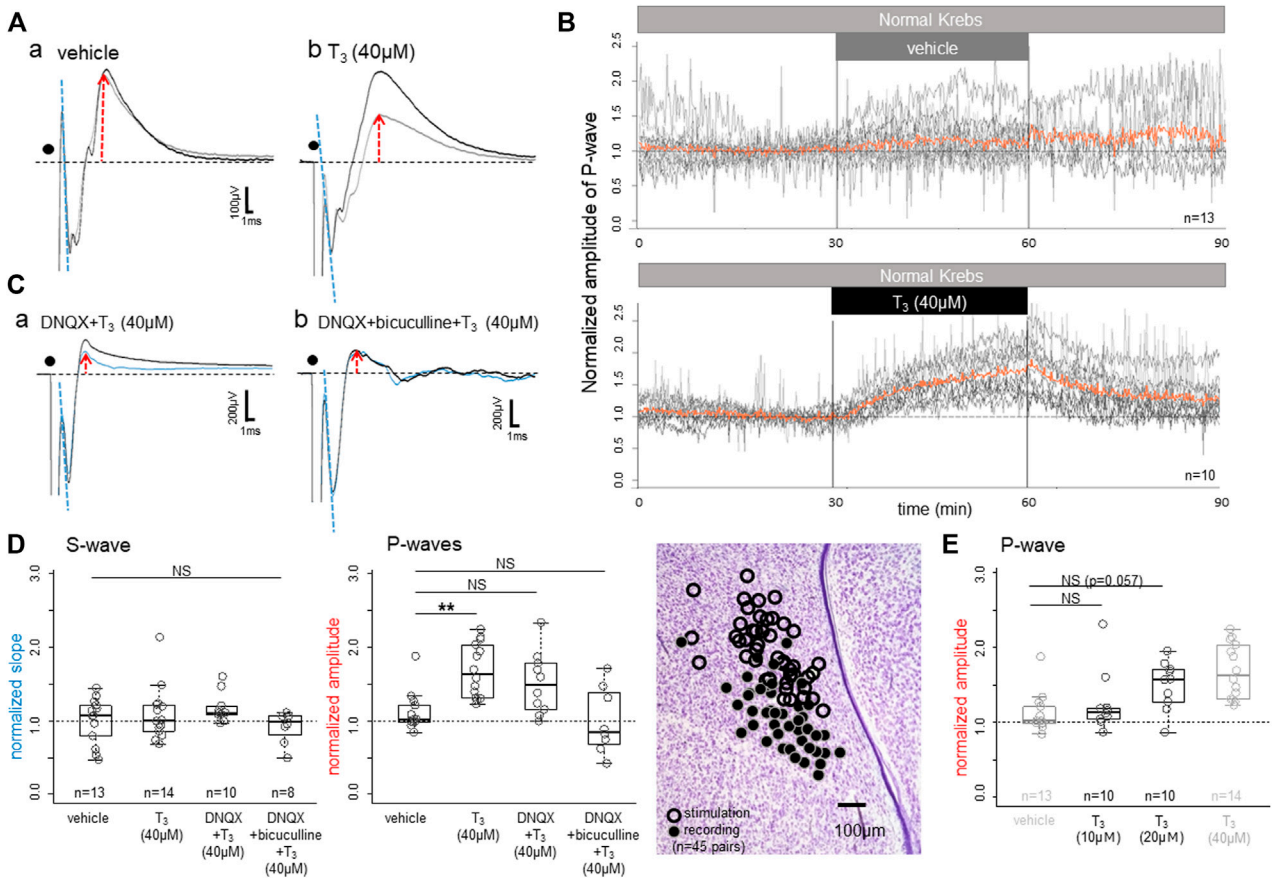


**FIGURE 2 |** Effects of bath-applied $T_3$ on the field potential responses to local electrical stimulation in the IMM. **(A)** Superimposed averaged traces (~30 successive responses at 10 s intervals in **(A,C)** before (thin grey traces) and after (thick black traces) application of vehicle **(Aa)** and $T_3$ (40 μM; **(Ab)**). **(B)** Time course of the $T_3$ effect. Superimposed plots are shown for the vehicle control (top) and $T_3$ (bottom); orange lines indicate the population average. **(C)** $T_3$ was bath applied after the slice had been treated with DNQX **(Ca)** or DNQX + bicuculline **(Cb)**: the thin blue traces denote the pre-$T_3$ responses. **(D)** $T_3$ effects on the normalized slope of the S-wave (left; initial negative slope) and P-wave amplitudes (middle and right; peak amplitude). Note that the $T_3$ effect survived even in DNQX, but not in DNQX + bicuculline (middle). Sites of stimulation and recording ($n$ = 45 slices) are plotted. **(E)** Dose dependence of the $T_3$ effects on the early P-wave; the vehicle control and the $T_3$ (40 μM) data are duplicated (shown in grey) for comparison. Normalization of the slope/amplitude was done against those control records during the last 5 min (i.e., 30 trials) of the control condition before $T_3$ was applied. NS denotes not significant. Asterisks denote the level of significance as; * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

excitability to the applied electrical stimulation remained unchanged.

**Figure 2B** shows the time course of changes in the vehicle control (upper) and the $T_3$ experimental (lower) slices. Recording was composed of pre-application (0–30 min), application (30–60 min) and post-application wash-out (60–90 min). Peak amplitude of the P-wave was plotted every 10 s after normalization against the responses during the last 5 min of the pre-application. Red traces indicate the population average of the superimposed recordings (13 slices for vehicle and 10 slices for $T_3$). The P-wave amplitude started to rise as soon as $T_3$ was introduced to the bath and rose continuously during the application period. After $T_3$ was removed from the bath, the P-wave dropped immediately, but the amplitude stayed higher than 1.0 at 90 min, at 30 min after washout.

The $T_3$ effects survived after AMPA receptor-mediated excitatory transmission was blocked by DNQX (**Figure 2Ca**) but disappeared when GABA-A receptor was also blocked (Cb). Also note that DNQX strongly suppressed the P-wave; when compared with the control traces (gray traces in Aa,b), P-wave was much smaller in DNQX (blue traces in Ca). Population data are shown in **Figure 2D** for the normalized slope of the S-wave (the initial drop phase; dashed blue lines) and the peak amplitude of the early P-wave (normalized against the pre-$T_3$ perfusion; red arrows). One-way ANOVA revealed no significant difference in the S-wave ($p$ = 0.28), but the differences were significant in the early P-wave ($p$ = 0.0016); further post-hoc multiple comparisons were made by Tukey's method and revealed a significant difference between vehicle and $T_3$ (40 μM). The dose response relationship of $T_3$ effects was examined in an additional 20 slice preparations as shown in **Figure 2E**. Comparisons with the duplicated data of **Figure 2D** revealed a significant effect of dose (ANOVA, $p < 0.001$), although post-hoc multiple comparisons showed only a suggestive level of difference ($p$ = 0.057) for $T_3$ (20 μM) over vehicle.

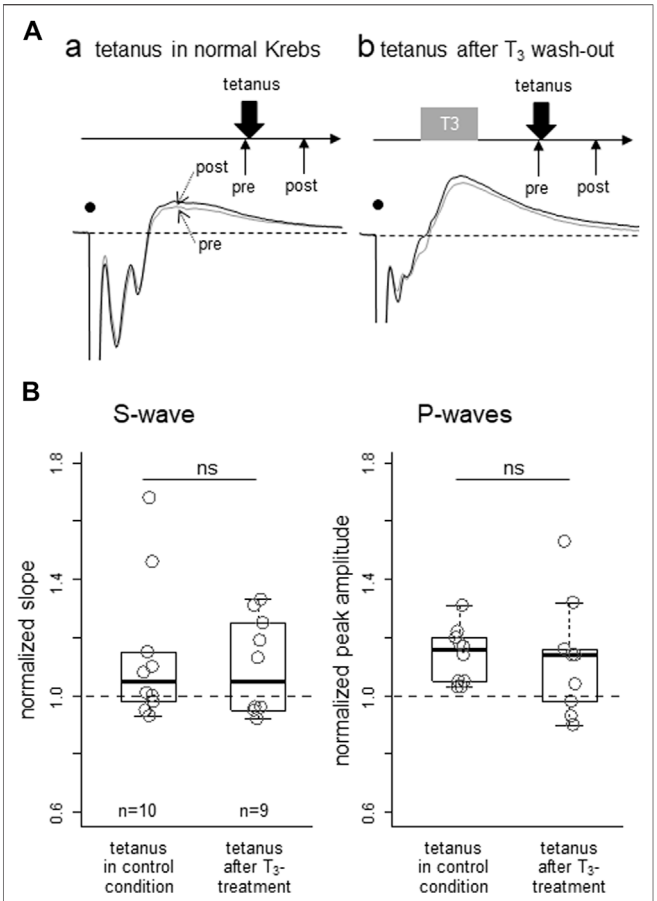


**FIGURE 3 |** Lack of $T_3$ effect on lasting potentiation of synaptic responses after low-frequency tetanic stimulation applied locally in IMM. Effect of $T_3$ pre-treatment on the subsequent induction of potentiation was examined. **(A)** Two examples of pre- (thin) and post-tetanus (thick) traces, one with $T_3$ pretreatment **(Aa)** and one without **(Ab)**; ~30 successive responses at 10 s intervals **(B)** Normalized initial negative slope of the S1-wave (left) and normalized peak amplitude of the early P-wave were compared between two groups, but no significant difference was apparent.

These findings indicated that $T_3$ enhances GABA-A-ergic inhibitory synaptic transmission without changes in the pre-synaptic action potentials, namely the neuronal excitability. The early P-waves are considered to be composed of GABA-A-ergic synaptic inhibition. At this post-hatch stage, GABA-A receptor mediated synaptic current is hyperpolarizing by $Cl^-$ influx (see below and **Figure 5Bb** for the reversal potential of the $Cl^-$ ion), giving rise to positive waves when recorded extracellularly as field potential response. However, these results do not reject the possibility that the AMPA receptor-mediated fast synaptic excitation is also modulated by $T_3$.

### 3.2.2 $T_3$ Failed to Facilitate Synaptic Potentiation by Low-Frequency Tetanic Stimulation, Field Potential Analysis

To examine if $T_3$ could also modulate activity-dependent synaptic plasticity, effects of low frequency tetanic stimulation were compared between two groups of slice preparations, one with $T_3$ bath application for 30min (with an additional interval of 30min before tetanus) and another without $T_3$ (control). In both groups, slice preparations were maintained in the recording chamber with test stimulation applied at 10-s intervals. As shown in **Figure 3A** (averaged traces for 5 min from two examples) and **Figure 3B** (population data), pre-treatment by $T_3$ yielded no significant differences; S-wave ($p$ = 0.71; Mann-Whitney's $U$-test) and early P-wave ($p$ = 0.49), respectively. As the slice preparations were obtained from naïve inexperienced chicks (see 2.1. in Methods), we assume that non-specific saturation of synaptic plasticity did not occur as reported by Bradley et al. (1999).

### 3.2.3 $T_3$ Suppressed the Frequency of the Epileptiform Bursts Induced by Bicuculline, Field Potential Analysis

The spontaneous epileptic bursts induced by bicuculline are composed of two negative components. The first peak (**Figure 4Aa** and **Figure 4b**; also see **Figure 1E**) represents the highly synchronized compound action potentials due to fast excitatory transmission, and the second peak coincides with the lasting strong depolarization of the NMDA receptor mediated plateau potential (Yanagihara et al., 1998). The bursts repeated for several hours, and the amplitude (both 1st and 2nd peaks) gradually became smaller (**Figure 4Aa**); superimposed traces recorded at 30 min (grey trace) and 60 min (black trace). The gradual decrease in amplitude was accompanied by gradual increase in frequency (number of bursts in 5 min, **Figure 4Ca**). Further blockade of GABA-B-ergic transmission by CGP52432 (**Figure 4Ac**) strongly enhanced the 2nd peak, on which barrages of epileptiform bursts were superimposed for several seconds. These barrages were suppressed by D-AP5 (data not shown), indicating that GABA-B transmission potently masked the NMDA receptor mediated long-lasting excitation of the network. However, CGP52432 failed to change the first component (1st peak in **Figure 4B**), which is the fast compound action potentials.

$T_3$ increased the amplitude and decreased the frequency of the epileptic bursts. Population data (**Figure 4B**) revealed that both components were enhanced by $T_3$; one-way ANOVA revealed significant differences among the three groups ($p$ = 0.015 for the 1st peak, $p$ = 0.038 for the 2nd peak), with both peaks showing significant pair-wise differences between control and $T_3$ (post-hoc Tukey's multiple comparisons). In the control condition in which only bicuculline was applied, the frequency gradually increased (**Figure 4Ca**). When $T_3$ (40 μM) was added, the frequency increase was arrested (Cb), and the slope of the frequency change during the latter half of the recording session (30 min) was significantly lower (**Figure 4D**) ($p$ = 0.003; Tukey's multiple comparison). CGP52432 caused a much stronger suppression ($p$ < 0.001) compared with the control and $T_3$.

These $T_3$ effects on epileptiform bursts can be accounted for either by 1) enhanced NMDA receptor mediated depolarization or 2) reduced GABA-B receptor mediated inhibition. Furthermore, $T_3$ could modulate the resting membrane

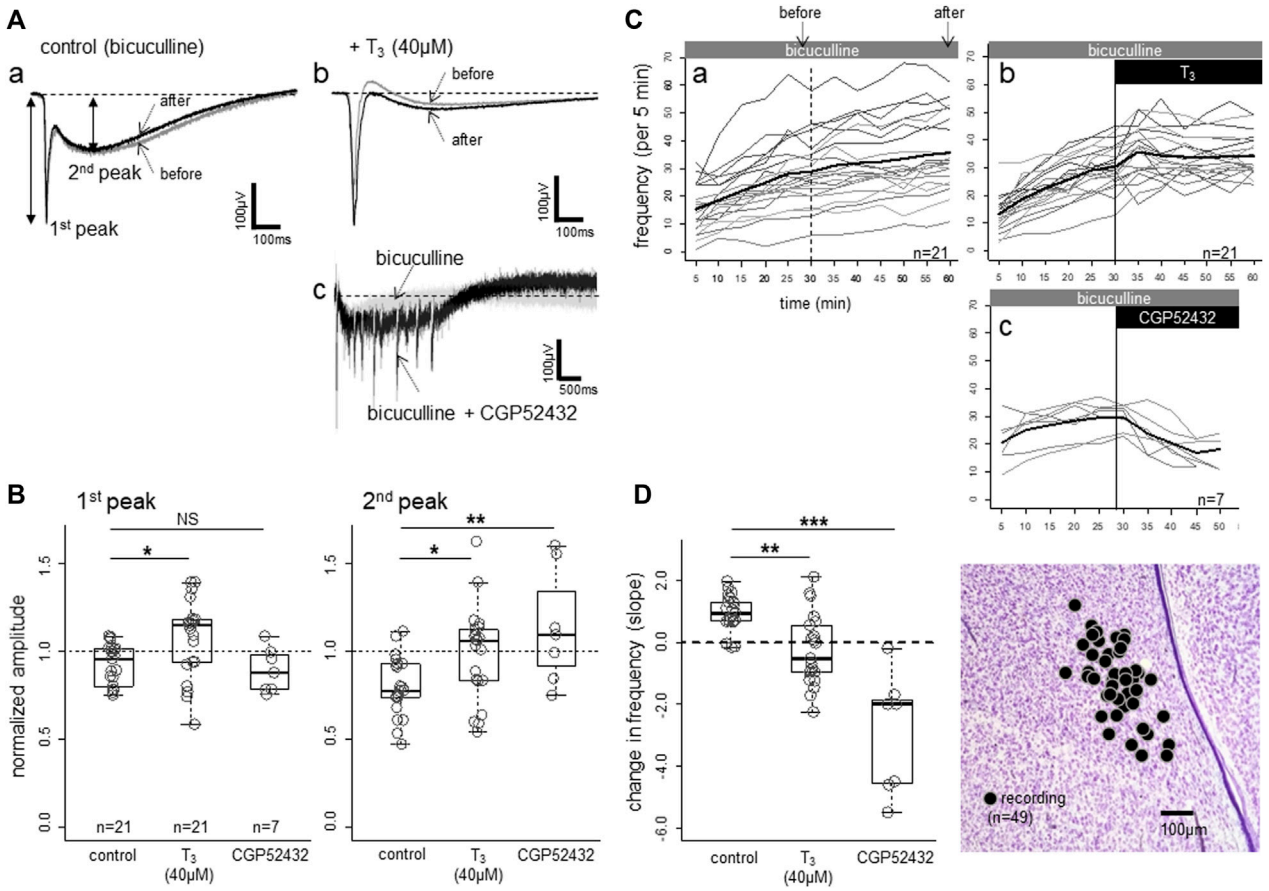


**FIGURE 4 |** Effects of bath-applied $T_3$ (40 μM) on bicuculline-induced spontaneous epileptiform activities. **(A,B)** $T_3$ effect on the waveform. The activities were composed of a sharp negative component (1st peak) followed by a slower negative component (2nd peak), both of which were larger in $T_3$. CGP52432 (GABA-B blocker) in the bath caused a lasting barrage of sharp negative components. **(C,D)** $T_3$ effects on frequency (number of events per 5-min bin; thin lines indicate individual slices and thick line the average. Note a gradual increase in the control slices. Change in frequency of events (per 5 min bin) was fitted to linear lines for the data of each slice, and the estimated slope of the change was plotted and compared. Sites of recording ($n = 49$) plotted on the same photomicrograph as **Figures 1**, **2**. Asterisks denote the level of significance as; * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

potentials, spiking threshold and other factors involved in spike generation, rather than synaptic transmissions. We have therefore examined $T_3$ effects by intracellular recordings from IMM neurons.

### 3.2.4 $T_3$ Suppressed the NMDA Plateau Potential and Enhanced GABA-A Responses, Intracellular Whole-Cell Recording Analysis

NMDA receptor activation produces a bistabile property in the membrane potential by negative resistance because of depolarization-induced release of the $Mg^{2+}$ blockade. When disinhibited by bicuculline, local electrical stimulation reliably elicited a plateau potential for a considerable period without any additional synaptic drive. **Figure 5A** shows an example recorded in single-electrode current clamp mode using a normal (low $Cl^-$ concentration) pipette solution. After a depolarizing current injection through the recording electrode (duration = 100 msec, indicated by a rectangle above the traces), four repetitive electric shocks were applied at 40 msec intervals (dots); six consecutive responses recorded at 10 s intervals were superimposed. The depolarizing current intensity was adjusted so that one action potential was induced at the peak of the depolarization. In control conditions (normal Krebs in the bath), each shock generated quickly decaying excitatory post-synaptic potentials (EPSPs) with action potentials on the peak of the first and/or the second shocks. When bicuculline was added (Ab), a depolarizing plateau (indicated by * at the shoulder) appeared and lasted for ca. 400 msec after the electric shocks; notice that the depolarization muted the neuron and action potentials failed to occur during the plateau. Bath applied $T_3$ (40 μM, Ac and Ad) gradually reduced the duration of the plateau (3 and 10 min after $T_3$ reached the chamber) without suppressing the initial component (indicated by #); fast EPSPs were thus supposed to be insensitive to $T_3$. D-AP5 completely suppressed the plateau (Ae), confirming its NMDA-receptor origin. Notice also that the resting membrane potential, the depolarization by the current injection, and the threshold membrane potential for generating spikes remained unchanged.

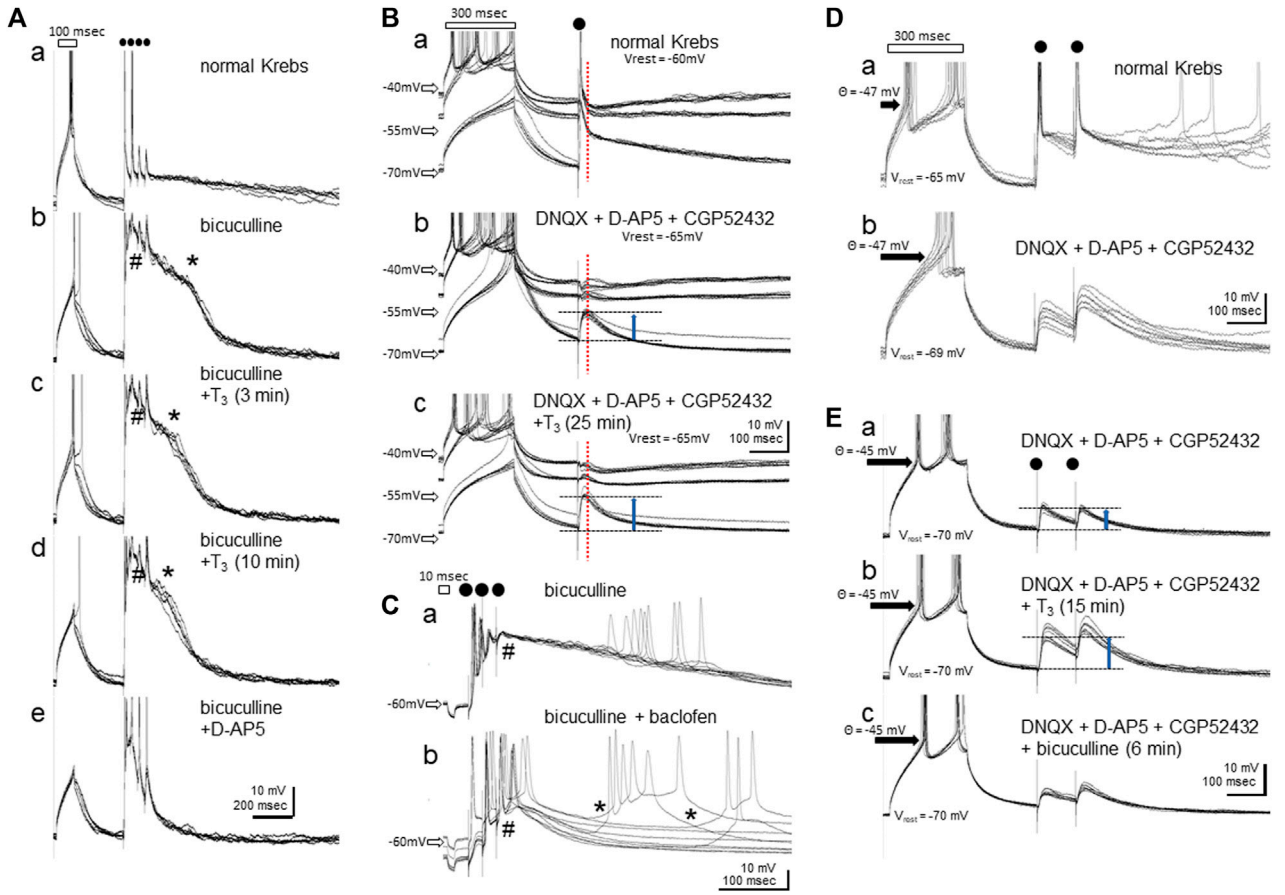


**FIGURE 5 |** Effects of bath-applied $T_3$ (40 μM) on the membrane potentials in IMM neurons. **(A–C)** show recordings using normal $Cl^-$ pipette solution. **(D,E)** show recordings with high-$Cl^-$ electrode and GABA-A responses revealed as depolarization. **(Aa-e)** $T_3$ suppressed the NMDA-plateau. Initial depolarizing current injection (indicated by a rectangle, 100 msec) was followed by four repetitive local electric shocks (dots); # indicates the initial onset phase of the NMDA plateau potential by bicuculline, and * the shoulder (onset of the decaying phase) of it. Six consecutive traces recorded at 10 s intervals are shown for each. **(Ba-c)** Responses recorded at three steps of tonic current injection through the recording electrode. Vertical dashed red line indicates the peak GABA-A response that is reversed at ca. −50 mV. Vrest means the resting membrane potential without current injection. **(Ca-b)** Baclofen induced sporadic NMDA-plateau depolarization (*). **(Da-b)** Blockade of AMPA, NMDA and GABA-B receptors unmasked GABA-A receptor mediated depolarization. **(Ea-c)** Further addition of $T_3$ enhanced the GABA-A depolarization (upward blue arrows). No effects were found in the resting membrane potential ($V_{rest}$ = −70 mV), membrane resistance, or threshold for spike generation (θ = −45 mV).

The NMDA-plateau potential was induced by bicuculline application in all 15 neurons recorded from eight slice preparations, with resting membrane potentials ranging from -50 to -67 mV (mean ± s. e.m. = -59.5 ± 1.6 mV) and stable recording maintained for 40–84 min. $T_3$ effects (40 μM, >15 min bath application) were successfully examined in 11 neurons, and the NMDA plateau was suppressed by $T_3$ in 10 out of these 11 neurons; no changes were detected in the remaining one neuron. Vehicle control was tested in other four neurons, and none of them showed changes in the plateau.

The GABA-A receptor-mediated inhibitory synaptic response was examined after glutamatergic (AMPA- and NMDA-R mediated) and GABA-B ergic transmission was blocked (**Figure 5B**, bridge balance mode). In normal Krebs (Ba), tonic current injections at three levels (two depolarizing and one hyperpolarizing) set the membrane potential at ca. −45 mV, −50 mV and −70 mV. Single shocks of local electrical stimulation elicited fast EPSPs, and single action potentials followed by a hyperpolarization at −45 mV (indicated by vertical dashed red line). The hyperpolarization phase turned to depolarization if held at −70 mV, indicating the reversal of the GABA-A response at approximately −50 mV. After glutamatergic and GABA-B-ergic transmission were blocked by DNQX, D-AP5 and CGP52432 (Bb), the resting membrane potential was hyperpolarized by 5 mV. Synaptic responses were examined by adjusting the tonic current injection levels. GABA-A ergic depolarization was clear at −70 mV (upward blue arrow). When $T_3$ was added (40 μM, Bc), the depolarizing GABA-A response increased without changes in the reversal potential, resting potential, or threshold of spike generation. The bicuculline-induced lasting depolarization was further augmented by baclofen (20 μM) (**Figure 5Ca,b**); note that the synaptically driven initial depolarization (indicated by #) was suppressed, thus allowing slow depolarization of varying

latency and amplitude (indicated by *). The GABA-B receptor activation paradoxically facilitated the spontaneous bursting caused by NMDA receptor activation. Actually, the delayed depolarization was not accompanied by synaptic drives if measured in voltage clamp mode and disappeared with the addition of D-AP5 (data not shown). Most probably, the hyperpolarizing effect of baclofen via GABA-B receptor activation set the membrane potential within an appropriate range for the NMDA-induced plateau to spontaneously arise.

The GABA-A responses were examined in nine neurons from four slices, with resting membrane potentials ranging from −55 to −69 mV (mean ± s. e.m. = -63.9 ± 1.5 mV), and $T_3$ was tested in eight neurons and vehicle in 1 neuron. Of these eight neurons, enhancement by $T_3$ occurred in six, suppression in one and no detectable effect was found in another. The effect of baclofen on the NMDA plateau was reproduced in all of the other three neurons tested. Because the reversal potential of the GABA-A response was close to the resting potential (usually ~5mV above than the resting level), a small accidental change in the resting level had a big influence on the GABA-A response amplitude. We therefore re-examined the effects of $T_3$ using high-$Cl^-$ pipette solution.

Increase in the GABA-A response by $T_3$ was confirmed (**Figures 5D,E**). At the resting membrane potential, the GABA-A response quickly turned depolarizing after poking the neuron, and reached equilibrium within ~10 min. In normal Krebs, two pulse electric shocks (100 msec interval) induced initial spiking followed by lasting depolarization (Da). The depolarizing response remained after DNQX, D-AP5 and CGP52432 were added (Db). The unclamped resting membrane potential was slightly hyperpolarized (from −65 to −69 mV) but the spiking threshold potential (−47mV) remained unchanged. In another neuron (Ea), $T_3$ enhanced the depolarizing response (Eb). The depolarization was suppressed by bicuculline (Ec) although not completely. We therefore argue that GABA-A receptor mediated responses was enhanced by $T_3$. $T_3$ application caused no changes in the spiking threshold ($\theta$ = −45 mV) in response to a rectangular depolarizing current injection through the recording electrode.

The reversed GABA-A potential was reliably recorded from six neurons from five slice preparations, with resting membrane potentials ranging from −59 to −77 mV (mean ± s. e.m. = −68.7 ± 3.1 mV), and $T_3$ effects (>15 min of bath application) successfully examined in all six neurons. Enhancement by $T_3$ occurred in five out of these six neurons, whereas suppression occurred in the other neuron.

The possibility that $T_3$ enhances the NMDA-receptor mediated response (see **Section 3.2.3**) should therefore be rejected, and an alternative hypothesis must be considered such that GABA-B mediated inhibition was suppressed. Taken together, $T_3$ could have opposite effects on the GABA-mediated responses, namely, enhancement of the fast GABA-A-mediated inhibition and suppression of the slow GABA-B-mediated inhibition of the IMM network.

# 4 DISCUSSIONS

## 4.1 Thyroid Hormone, the Issue of the Effective Dose

Our results showed that bath application of $T_3$ at 20–40 μM reliably modified synaptic transmission, namely it enhanced GABA-A and suppressed NMDA responses. In our previous behavioral pharmacology study (Yamaguchi et al., 2012), we reported that single injection of a small amount of $T_3$ (10 μM x 100 μl, intravenous injection) effectively re-opened the sensitive period. The low dose effects of thyroid hormones have been confirmed by Batista et al. (2018) for the $T_4$ effect on imprinting, Miura et al. (2018) for the $T_3$ effect on biological motion preference, and Lorenzi et al. (2021) for the $T_3$ effect on animacy preference. We must consider two possible explanations for the discrepancy. Systemically injected $T_3$ could be diluted to sub-micromolar level in the circulation and brain tissue, and the high dose effects found in this study are not supposed to be physiological. Alternatively, the thyroid hormone transporter system in the dorsal telencephalon could accumulate $T_3$ in the peri-vascular neural tissue, and the concentration could reach a supra-micromolar level in the peri-neuronal space.

In favor of the latter possibility, localized infusion of the monocarboxylate transporter inhibitor (bromsulphthalein) to IMM effectively nullified the effects of systemically injected $T_3$ (Yamaguchi et al., 2012). The transport and accumulation mechanism may not function in the slice preparations and the actual effective dose may be supra-micromolar. Accordingly, in rat primary hippocampal neuron culture (Losi et al., 2008), $T_3$ inhibited the NMDA current at $IC_{50}$ ~ 15 μM. Although the opposite $T_3$ action to that in our present study, these authors reported that $T_3$ inhibited GABA current at $IC_{50}$ ~ 13 μM (Puia and Losi, 2011). When systemically applied *in vivo*, however, repeated i. p. injection of levothyroxine was effective at a low dose of 20 μg (~26 nano mole)/kg/day in rescuing the hypothyroidism-induced cognitive impairment (Alzoubi et al., 2009). This dosage was several magnitudes lower than that applied to cultured neurons. Changes in any of the following processes could contribute to the effectiveness of thyroid hormone action: synthesis and release of thyroxine ($T_4$) by the thyroid gland, conversion of $T_4$ to $T_3$ at the target organ (vascular endothelial cells) and transportation to the peri-neuronal space.

It may also be interesting to examine the $T_4$ action *in vitro*, even though $T_3$ is supposed to be critical player as argued above. Because we do not yet fully understand the mechanism of the $T_3$ action, several interesting possibilities arise. $T_4$ may act as $T_3$ at similar dose *in vitro* but has no functional significance because the brain content of $T_4$ is nearly at the detectable level (Yamaguchi et al., 2012, **Figure 3A**). Alternatively, we may assume that $T_4$ acts differently, so that the $T_3$ action is modulated by $T_4$ at the extremely low concentration. Further studies are needed to address these possibilities, but only after we are quite sure about the $T_3$ action.

## 4.2 Opposing Roles of GABA-A and B Receptors

Our recent qPCR analysis revealed that GABA-A receptor expression increases from day1–5, whereas GABA-B expression gradually decreases during this early post-hatching period (Aoki et al., 2018). Therefore, the ratio of GABA-A to -B receptor (referred to as the A/B ratio below) gradually increases. We have also found that direct infusion of CGP-52432 (GABA-B antagonist, thus increasing the A/B ratio) into the IMM suppressed imprinting on day 1. However, infusion of baclofen (GABA-B agonist) or bicuculline (GABA-A antagonist) decreased the A/B ratio and made 4-day old chicks imprintable as on day 1. Based on these effects, we hypothesized that a low A/B ratio could be one of the neurophysiological phenotypes of the sensitive period of imprinting. Importantly, the GABA-A and B receptors could have opposing roles in the regulation of the sensitive period. One possible explanation is that activation of GABA-B receptors on pre-synaptic terminals leads to suppression of GABA release from inhibitory interneurons (Gassmann and Bettler, 2012; Aoki et al., 2018), thus excitability of the IMM network is enhanced through GABA-B-mediated suppression of the $Ca^{2+}$ influx into presynaptic terminals, which is responsible for GABA release (Deisz and Prince, 1989).

The A/B ratio increase during the days 1–5 post-hatching means a monotonic decrease in network excitability. The present finding that the GABA-B agonist, baclofen, induced the NMDA-plateau (**Figure 5Cb**) supports this possibility. An appropriate decreased in the A/B ratio seems to be necessary for spontaneous NMDA bursting to occur in the IMM network. GABA-A blockade alone (Ca) strongly depolarizes neurons, thereby muting action potentials, whereas additional activation of GABA-B lowers the membrane potential to around the NMDA plateau, so that short barrages of synaptic inputs can cause lasting spiking responses after transient synaptic drives. Alternatively, as GABA also acts on somatic receptors extra-synaptically via volume transmission (Matsushima et al., 1993; Tegnér et al., 1993), activation of GABA-B receptors and the associated G-proteins may have directly enhanced the NMDA-plateau of the recorded neurons. Further analyses are needed to fully understand how GABA controls the dynamics of the IMM network.

Contrary to our initial expectation, $T_3$ was a potent enhancer of the GABA-A response (**Figures 5B,E**), thereby increasing the A/B ratio. As $T_3$ partially mimicked the effect of CGP52432 on the bicuculline-induced spontaneous bursts ($2^{nd}$ peak amplitude and frequency; **Figure 4Ab** and **Figure 4B**), $T_3$ may suppress GABA-B so that the A/B ratio is further decreased. Blockade of GABA-B receptors on pre-synaptic terminals of inhibitory interneurons would disinhibit GABA release, so that the post-synaptic GABA-A response would be enhanced. However, the neuronal cascades through which $T_3$ facilitates imprinting remain to be clarified.

Why $T_3$ target on GABA? We have yet no clear idea about the functional link between the central thyroid hormone effect and the imprinting memory formation. Beside the link discussed above, we may assume that immediate early genes (such as *c-fos*) could be linked, as imprinting is reported learning-related increase in Fos-immunoreactivity specifically in GABA neurons (Ambalavanar et al., 1999). The thyroid hormone influx to IMM might lead to the enhanced GABA release through expression of those genes selectively in the GABA-ergic inhibitory interneurons, and the GABA release mechanism is downstream to the c-fos.

## 4.3 NMDA-Plateau Potential and Activity-dependent Synaptic Potentiation

McCabe et al. (1992) reported that blockade of NMDA receptors in the dorsomedial pallium impairs imprinting, while imprinting upregulates the glutamatergic receptors in the IMM (McCabe and Horn, 1988), subsequently leading to morphological and functional changes in IMM synapses (Horn, 1998). Accordingly, DNQX-sensitive fast excitatory transmission was potentiated by a low-frequency tetanic stimulation (Matsushima and Aoki, 1995), which turned out not to be linked to the immediate early gene expression associated with memory (Yanagihara et al., 1998). Two decades later, our understanding of imprinting remains limited, mostly because we do not know what activities occur in the IMM network during imprinting *in vivo*, nor which activities are critical for imprinting-related memory formation.

Dynamic behaviors of the IMM neurons *in vitro* could give us a hint about their *in vivo* behaviors. During the low-frequency tetanic stimulation for 1 min (5 Hz × 300 pulses), GABA-A mediated synaptic inhibition quickly fades away, and is replaced by NMDA-receptor mediated slow depolarization (Matsushima and Aoki, 1995). If the bicuculline-induced bursts observed in slice preparations paralleled the activities assumed *in vivo*, facilitatory modulation of the 1st and 2nd spike by $T_3$ (**Figure 4Ab**, **Figure 4B**) would underlie the initial phase of memory formation, rather than the subsequent potentiation of the post-synaptic responses (**Figure 3**).

In this study, we could not specify whether $T_3$ action was genomic or nongenomic. At the behavioral level, $T_3$ facilitated imprinting within a few hours after injection. In slices, bath applied $T_3$ changed the synaptic transmission immediately (within a few min; **Figure 2B**), in favor of non-genomic effects. Naturally, the late phase of the $T_3$ actions could be genomically caused. At present, we are unable to dissociate these two processes.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Experiments were conducted under the guidelines and approval of the

Committee on Animal Experiments of Hokkaido University (approval number 18-0167).

## AUTHOR CONTRIBUTIONS

TM and KH conceived the study. YS and TM designed the experiments. YS, NA and TM carried out the experiments and analyzed the data. TM wrote the manuscript. All the authors gave final approval for publication.

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# GSK-3β Inhibition in Birds Affects Social Behavior and Increases Motor Activity

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Glycogen synthase kinase-3 (GSK-3) is a highly conserved serine/threonine protein kinase that plays a central role in a wide variety of cellular processes, cognition and behaviour. In a previous study we showed that its α and β isozymes are highly conserved in vertebrates, however the α gene is missing in birds. This selective loss offers a unique opportunity to study the role of GSK-3β independently. Accordingly, in the present study we aimed to investigate the role of GSK-3β in social behaviour, motivation, and motor activity in zebra finches (*Taeniopygia guttata*). We did that by selective inhibition of GSK-3β and by using tests that were specifically designed in our laboratory. Our results show that GSK-3β inhibition: 1) Affected social recognition, because the treated birds tended to move closer towards a stranger, unlike the control birds that stood closer to a familiar bird. 2) Caused the treated birds to spend more time in the more middle parts of the cage compared to controls, a behaviour that might indicate anxiety. 3) As the experiment progressed, the treated birds took less time to make a decision where to stand in the cage compared to controls, suggesting an effect on decision-making. 4) Increased in the motor activity of the treated birds compared to the controls, which can be regarded as hyperactivity. 5) Caused the treated birds to pass through a barrier in order to join their flock members faster compared to controls, and regardless of the increase in the level of difficulty, possibly suggesting increased motivation. Our study calls for further investigation, because GSK-3 is well acknowledged as a central player in regulating mood behaviour, cognitive functions, and neuronal viability. Therefore, studying its impact on normal behaviour as we did in the current study, unlike most studies that were done in diseases models, can advance our understanding regarding GSK-3 various roles and can contribute to the discovery and development of effective treatments to repair cognition and behaviour.

**Keywords: GSK-3, birds, zebra finches, cognition, motor activity, sociability**

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## INTRODUCTION

Glycogen synthase kinase-3 (GSK-3) is a highly conserved serine/threonine protein kinase that plays a central role in a wide variety of cellular processes, including embryonic development, cellular growth, and metabolism (Eldar-Finkelman, 2002; Doble and Woodgett, 2003; Beurel et al., 2015). The versatility of GSK-3 is also based on its broad range of substrates, including a predicted number over 500 substrates and about 100 "physiological substrates" that are related to diverse cellular

functions (Linding et al., 2007). While GSK-3 is constitutively active under basal conditions, in order to phosphorylate its targets, most GSK-3 substrates demand pre-phosphorylation within GSK-3's recognition site catalyzed by other protein kinase (termed "priming" kinase) (Woodgett and Cohen, 1984; Fiol et al., 1987). Phosphorylation by GSK-3 typically inhibits its targeted substrate, which attenuates the downstream signaling pathway (Eldar-Finkelman, 2002; Doble and Woodgett, 2003; Beurel et al., 2015).

GSK-3 exists as two isozymes that are encoded by two separate genes, GSK-3α and β (Woodgett, 1990). A splice variant of GSK-3β with a 13-residue insert in the catalytic domain has also been described (Mukai et al., 2002). The GSK-3 isozymes share 98% identity in the catalytic domains, but there are significantly differences in the N- and C-terminal domains (Woodgett, 1990; Ali et al., 2001). The two GSK-3 isozymes exhibit both similar and distinct functions. In some cases, the isozymes fulfil non-redundant physiological functions, but in others, there is a possibility of compensation (Doble et al., 2007; Force and Woodgett, 2009; Rippin and Eldar-Finkelman, 2021). GSK-3 is of medical importance, with its high activity having been determined in several human pathogenesis including in Alzheimer's and Parkinson's diseases, psychiatric disorders such as bipolar disorder and schizophrenia, and metabolic diseases including diabetes (Zhou and Snider, 2005; Kim et al., 2009; Llorens-Martin et al., 2014; Beurel et al., 2015; Ruiz and Eldar-Finkelman, 2021). It has therefore been considered a therapeutic target and the use of GSK-3 inhibitors has indeed demonstrated beneficial outcomes in respective diseases models. Hyperactivity of GSK-3 in Alzheimer's disease is linked to the formation of amyloid-β plaques and neurofibrillary tangles (NTF) (Leroy et al., 2007; Lauretti et al., 2020), while its inhibition has been shown to reverse disease pathology and improved cognitive and social skills in Alzheimer's disease mice model (Ly et al., 2013; Licht-Murava et al., 2016). There is also evidence that inhibition of GSK3 activity is therapeutic for mood disorders (Jope, 2011) and stress-induced depression-like behaviors (Polter et al., 2010).

In a previous study we investigated the presence of GSK-3 isozymes across evolution. We showed the α and β isozymes diverged from a common precursor around the time vertebrates emerged, and both genes are highly conserved in fish, amphibians, reptiles, and mammals (Alon et al., 2011). Interestingly, we also found that the α gene is missing in birds. Our findings were initially based on the available draft genome of chickens, domestic turkeys, and zebra-finches, however a search of the updated genomic data confirmed the general selective loss of GSK-3α in the avian species (Alon et al., 2011, and unpublished results from our laboratory). We suggested that the selective loss of GSK-3α in birds offers a unique opportunity to study the role of GSK-3β independently, which could contribute to the intensive studies aimed at deciphering the function of the two isozymes in mammals.

The role of GSK-3 activity in regulating cognitive functions and mental behavior has been mostly explored in mammals. These studies showed that GSK-3 can impact mood behavior, increase motor activity, and cognitive capabilities (Ackermann et al., 2010; and reviews by Beaulieu et al., 2007; Rippin and Eldar-Finkelman, 2021 respectively), as well as fear, anxiety, and social behavior (Beurel et al., 2015; Jung et al., 2016; Mines et al., 2010). However, since mammals express the two isozymes, it was not possible to distinguish between the two isozymes. Furthermore, while GSK-3β knockout model resulted in lethality, GSK-3α knockout animals did not show robust changes as compared to wild-type (MacAulayet et al., 2007; Kaidanovich-Beilin et al., 2009). On the other hand, birds offer a "natural" GSK-3α knockout model that may reflect the necessity of GSK-3b and enable to study the role of GSK-3β in a non-genetic manipulation system. In the CNS arena, birds also present an advantageous model to study brain functions due to their robust adult neurogenesis and neuronal architecture (Barnea and Nottebohm, 1994; Doetsch and Shcarff, 2001); and are particularly suitable for studies that integrate genomes, brain, and behavior (Clayton et al., 2009). In addition, their long life-span (compared to rodents for example), makes them a good alternative model to study age-related diseases (Austad, 2011). Therefore, in a previous study we used zebra finches (*Taeniopygia guttata*), as our working model to study the role of GSK-3β in neuronal proliferation and singing behavior. We showed that inhibition of GSK-3β enhanced cellular proliferation in the ventricular zone, where new neurons are born, and altered singing behavior patterns (Aloni et al., 2015). As zebra finches are highly social birds with a wide variety of behavioral types (Zann, 1996; Schuett and Dall, 2009), it is possible that our findings pointed toward a broader impact of GSK-3 in affecting social and cognitive abilities of the bird. Zebra finches are known to maintain strong monogamous bonds, and they are also gregarious, exhibit a high degree of social tolerance, and maintain multiple social bonds, including bonds with same-sex conspecifics (reviewed in Prior et al., 2020). Moreover, in the wild, individuals from the same colony that are synchronized in their reproductive timing, keep stable social ties across years (Brandl et al., 2019). Recently, it has also been shown that familiarity enhances behavioral coordination in zebra finches' dyads, and that overall females were more active than males (Prior et al., 2020). Therefore, in the current study we choose to focus on female zebra finches as a suitable model to examine the ability of GSK-3β in manipulating social recognition and motivation, two important characteristics for individuals that tend to be part of a flock. To achieve this, we evaluated the impact of selective GSK-3β inhibition in birds on their social recognition and motivation under isolation stress conditions, by using tests that were specifically designed and developed in our laboratory. In addition, because GSK-3β inhibition in rodents was found to increase motor activity (see citations above), we also measured this behavior in our birds.

# MATERIALS AND METHODS

## Experimental Design

This study was approved by the Tel-Aviv University Institutional Animal Care and Use Committee (permit No. 04-18-014) and

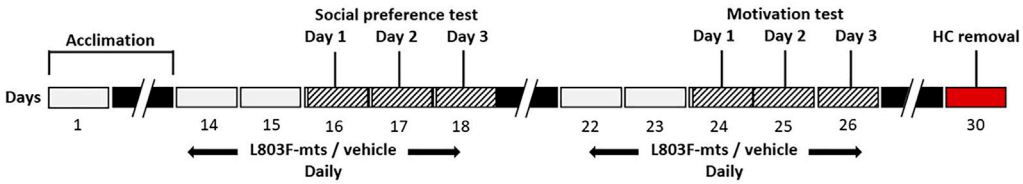


**FIGURE 1 |** A schematic depiction of the study. Black rectangles represent time gaps. See text for details.

was carried out in accordance with its regulations and guidelines regarding the care and use of animals in experimental procedures. The experimental group comprised of 20 adult female zebra finches (*Taeniopygia guttata*), that had been raised in our outdoor breeding colonies at the I. Meier Segals Garden for Zoological Research at Tel-Aviv University, Israel. The birds were taken from several colonies, in order to lower the chance of kinship, since it has been shown that zebra finches can recognize their kin (Krause et al., 2012). We choose to use females in our study because they have less distinct visual characteristics on their faces and bodies than males, and because they do not sing as males. These features make the social tasks harder and decrease the possibility of a bias in the behavioral tests that we performed. At the age of 100–300-days-old these 20 females were moved to an indoor cage (140 × 33 × 33 cm), where they were kept together throughout the entire study, which lasted one-month. These 20 birds included experimental and control birds, as well as familiar flock members, as described below.

A schematic depiction of the timeline of the experiment is presented in **Figure 1**. Following transfer of the birds from the breeding colonies to the indoor cage, they underwent an acclimation period of 14 days, under a light regime of 14:10 L: D and temperature of 24°C. The birds were then assigned to three groups (but still kept in one cage) as follows: six females in the experimental group, six as controls, and eight as flock members in the behavioral tests (see below). The control and experimental birds were equalized according to body mass, to create two balanced groups. Following the acclimation period, for two additional weeks the six experimental birds were administered with the GSK-3 inhibitor L803Fmts (as explained below), while the six control birds received only the vehicle. During these two weeks we conducted two behavioral tests: a social preference test (days 16–18 in Figure 1, and a motivation test (days 24–26 in **Figure 1**). The birds were then euthanized and their hippocampus was dissected out of their brains for immunoblot analysis.

## Nasal Administration

We had previously demonstrated (Aloni et al., 2015) that avian GSK-3β, which has a similar sequence alignment of the catalytic domain to human GSK-3β, was inhibited by the inhibitor L803mts in the brains of adult zebra finches. L803-mts has since been refined, generating a new peptide inhibitor - L803Fmts - with improved inhibitory capacity (Licht-Murava et al., 2011). Here we used this newer, refined GSK-3β inhibitor—L803Fmts - and tested its effect on the behavior of adult female zebra finches compared to controls. The L803Fmts peptide (Myr-GKEAPPAPPQS(p)PF) was synthesized by Geneme Synthesis Inc. L803Fmts (60 μg total in vehicle solution, 128 mM NaCl, 8 mM citric acid monohydrate, 17 mM disodium phosphate dehydrate and 0.0005% benzalkonium chloride), was administered to the experimental birds intra-nasally using a narrow pipette tip (5 μL of solution was introduced to each nostril), while the control birds received only the vehicle. Nasal administration was chosen to deliver the peptide since it has proven to be an effective route for such small peptides (Born et al., 2002). Following the acclimation period, the inhibitor was administered each morning for five consecutive days a week, starting two days prior to the first day of the behavioral test (**Figure 1**). To avoid stress effects on the birds from the handling during administration, which might have affected the behavioral tests, there was a 2-h interval between administration and the tests, which are described below.

## The Social Recognition Test

This test was designed to determine whether GSK-3β inhibition affects the social preferences of the birds, and was conducted in a cage (70 × 33 × 33 cm), (as shown in **Figure 2A**), with camera was placed above the cage. The walls of the central chamber of the cage allowed the tested bird that was placed in it to see and hear the birds in the side chambers. A flock member was placed in the left chamber and a stranger bird in the right one. We choose live birds as a social stimulus because we wanted to test the effect of the GSK-3 inhibition under more natural and complex conditions. The stranger birds were females that hatched and then kept together throughout the study in a separate and far away cage, with no vocal or visual contact with the other birds, and therefore were completely unfamiliar to the tested birds. The flock members had lived together with the tested birds (control and GSK-3β inhibited ones) from hatching until adulthood, and during the whole duration of the study.

The social recognition test lasted three days (days 16–18 in **Figure 1**), with all the tested birds, six control and six GSK-3β -inhibited, being released daily into the main chamber, one at a time in a randomized order, each one for 10 min. The release was performed through a double door, designed to avoid human interference. Each day during the three test days, different flock members and different strangers were used, to avoid any bias that might have resulted toward specific birds due to specific inter-individual relationships. The floor of the main chamber was lined with a grid (4 cm × 4 cm) with numbered squares, and each

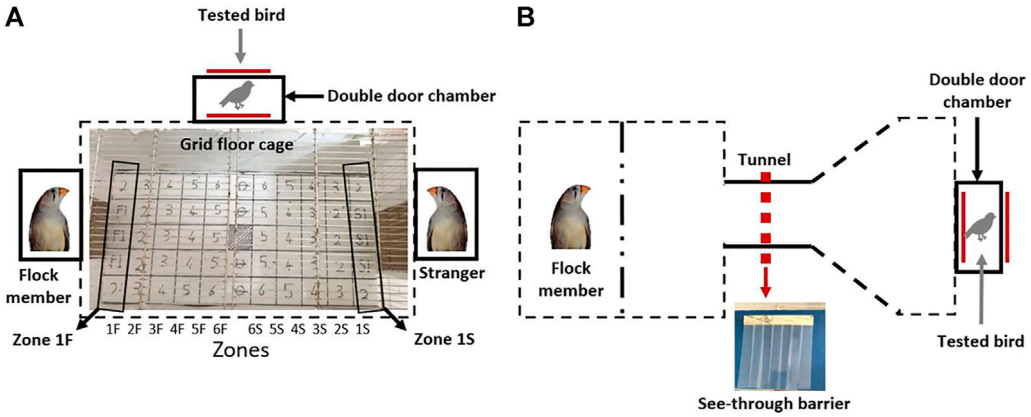


**FIGURE 2 |** A schematic depiction of the cages designed for the **(A)** social preference and **(B)** motivation tests. The grid with numbered squares that was printed on the main chamber floor in the cage of the social preference test is shown in **(A)**. Each vertical line of squares was defined as a "zone". Zones 1F and 1S are indicated as examples. See text for details.

vertical line of squares was defined as a "zone" (see **Figure 2A** and **Figures 5B–D**). For example, zone 1F represents all squares that were closest to the flock member, zone 2F represents the squares that were next closest to the flock member, etc. Similarly, zone 1S represents the squares that were closest to the stranger, zone 1S the one next to it, etc. During the 10 minute-test of each bird we recorded the time that it spent in each square, as well as the time it spent moving, as an indication of its motor activity. A bird was recorded as standing in a square if it did not move for 2 or more seconds from that square; if the time spent in a square was less than 2 s, the bird was recorded as moving. Throughout the entire duration of the 10-min test the birds were undisturbed by human presence.

## The Motivation Test

This test was designed to determine whether GSK-3β inhibition affects social motivation (**Figure 2B**). The tested bird was introduced into the right hand chamber (15 × 15 × 15 cm), which was connected to a tunnel (15 cm in length), through which it could see and hear its flock member located in a chamber at the other end of the tunnel. The tunnel was initially blocked by a plastic barrier comprising a 1 cm thick plastic sheet, attached only to the ceiling of the cage, not to the floor. In order to pass, the bird had to push its way through the barrier. The barrier also served as a visible obstacle, partially blocking the tested bird's view of its flock members. Each tested bird was released from the right side of the cage through a double door, to avoid human interference. Upon entering the cage, the bird faced the tunnel with the barrier, but could see partially through to the other side, where the flock member bird was located. Each tested bird was allowed a total of 10 min, and the latency to pass the barrier was recorded. As most of the birds passed the barrier within the first 25 s, and only a few did not pass in the time allowed, we artificially represented the maximum latency to pass as 30 s (**Figure 7**). The experiment was run for three consecutive days (days 24–26 in **Figure 1**), with an additional plastic barrier being added each day, adjacent to the previous one (i.e., 1 barrier on day 1, 2—on day 2, 3—on day 3). The addition of the barriers not only required the bird to use more physical force in order to pass through, but also the visibility of its flock members on the other side decreased, thus making it harder each day to cross the barrier. Therefore, the birds needed to increase their motivation each day, in order to fulfil the task.

## Immunoblot Analysis

The birds were euthanized with $CO_2$ and their brains were dissected. The hippocampus, which in birds is located in the dorsal part of both hemispheres, was cut out with 2 mm margins on each side (**Figure 3**). The hippocampal tissue was immediately frozen in liquid nitrogen until homogenization with Polytrone in ice-cold buffer H (10 mM β-glycerophosphate, 10% glycerol, 1–7.5 mM EGTA, 1–5 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 0.5 mM orthovanadate, 1 mM benzamidine, 5 μg/ml leupeptin, 25 μg/ml aprotinin, 5 μg/ml pepstatin and 0.5% Triton x-100). The homogenates were then centrifuged at 14,000 × g for 20 min and supernatants were collected. Protein concentrations were determined by Bradford analysis. Equal amounts of protein were subjected to gel electrophoresis followed by immunoblot analysis using the following antibodies: β-catenin (Transduction Laboratories, Lexington, Ky., United States) and GAPDH (Cell Signaling LTD.), followed by incubation with a secondary HRP-conjugated antibody. Signal was developed by enhanced chemiluminescence solution (ECS) (Roche, Basel, Switzerland). Detected bands were quantified by ImageJ software (http://imagej.nih.gov/ij/) using "area under the curve" procedure. The levels of GAPDH were used to demonstrate equal protein loading.

## Statistical Analysis

SPSS Statistics for Windows, version 27.0 (SPSS Inc., Chicago, Ill., United States) was used for all analyses. The Shapiro-Wilk normality test was performed before each statistical test to confirm normal distribution. The motivation and movement

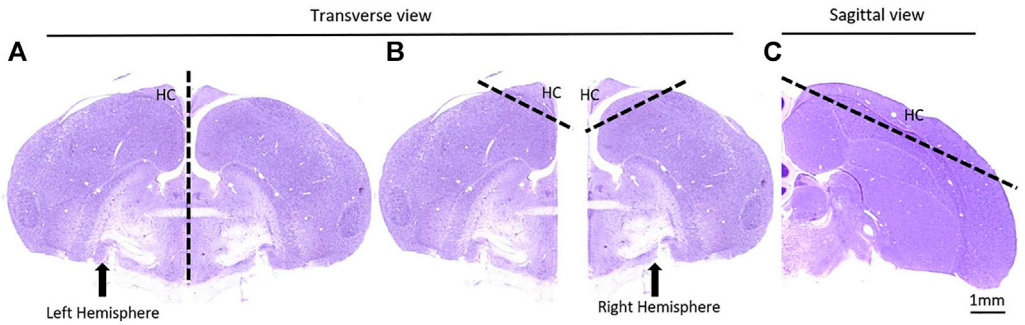


**FIGURE 3 |** A schematic depiction of the removal of the hippocampus (HC). **(A)** The brain was separated into the two hemispheres; **(B)** A generous 60° cut was made in both hemispheres to encapsulate all of the HC; **(C)** The same cut is shown in a sagittal view. Images adapted from Karten et al., 2008.

data were found to be normal whereas the social preferences data was not. For overall motion and motivation tests we used Repeated Measures ANOVA with between subject factor as treatment and within subject factors as days of the test, with post-hoc Tukey HSD (Honestly Significant Difference) tests to determine differences of means between control and experimental groups and for each day. Non parametric repeated measures Friedman's test was performed for the social preferences data followed by Kruskal-Wallis test to check for the difference between control and experimental groups. For biochemistry analyses, we used a *t*-test between treated and control birds. Alpha was set to 0.05.

# RESULTS

## L803Fmts Inhibits GSK-3β in the Zebra Finch Hippocampus.

We used an improved version of the previously described GSK-3 peptides inhibitor L803mts, termed L803Fmts (Licht-Murava et al., 2011). To validate inhibition of GSK-3b by L803Fmts, brains were collected at the end of the behavioral experiments and brain extracts were subjected to immunoblot analysis to detect the GSK-3 downstream target β-catenin in the hippocampus. This brain region was chosen for our analysis because of its homology to the mammalian hippocampus (e.g. Striedter, 2015), and evidence suggest that, as in mammals, also in birds, it plays a role in stress response (Smulders, 2017; Gualtieri Iders, 2017). This homology is further supported by the importance of avian hippocampus in spatial memory in birds in general (reviewed in Barnea and Pravosudov, 2011), and also specifically in zebra finches (Watanabe et al., 2008; Mayer and Bischof, 2012). In addition, recent evidence indicates, similar to mammals, the existence of place-cells in the avian hippocampus, including that of zebra finches (Payne et al., 2021). As inhibition of GSK-3 stabilizes β-catenin (Yost et al., 1996; Ikeda et al., 1998), we expected to find elevation in β-catenin expression levels in the treated animals. Indeed, β-catenin levels were elevated in the hippocampi of the L803Fmts treated birds as compared to controls by about 2.5 fold ($F_{(3,17)} = 45.875$, $p = 0.00007$; **Figure 4**). This provided a strong evidence that L803Fmts inhibited brain GSK-3β.

## GSK-3β Inhibition Affects Social Recognition

The average percentage time spent in zones S1-S6 during days 1–3 (which is complimentary to F1-F6) is presented in **Figure 5A**. The detailed percentage time spent in the cage in each of the days of the experiment is presented in **Figures 5B–D**. Overall, the distribution of the residuals was not normal. A Friedman's test showed no difference between

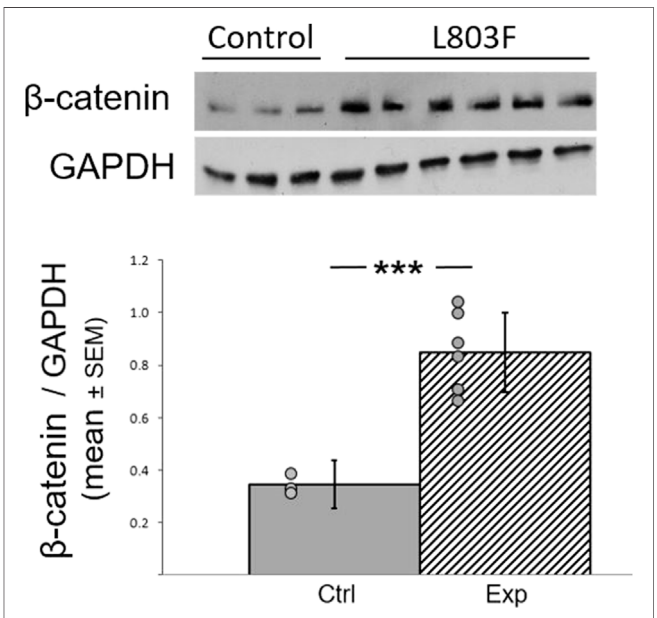


**FIGURE 4 |** L803Fmts inhibits GSK-3β in the adult avian hippocampus. Female zebra finches were treated with L803Fmts and compared to untreated controls. Brain tissue from the hippocampus was subjected to western blot analyses using GAPDH and β-catenin antibodies. Ratios of β-catenin to GAPDH from densitometry analyses are shown below the gel image. Results are means (±SEM) for control ($n = 3$) and L803Fmts treated birds ($n = 6$). Individual dots indicate the value of each sample. ***$p < 0.0001$.

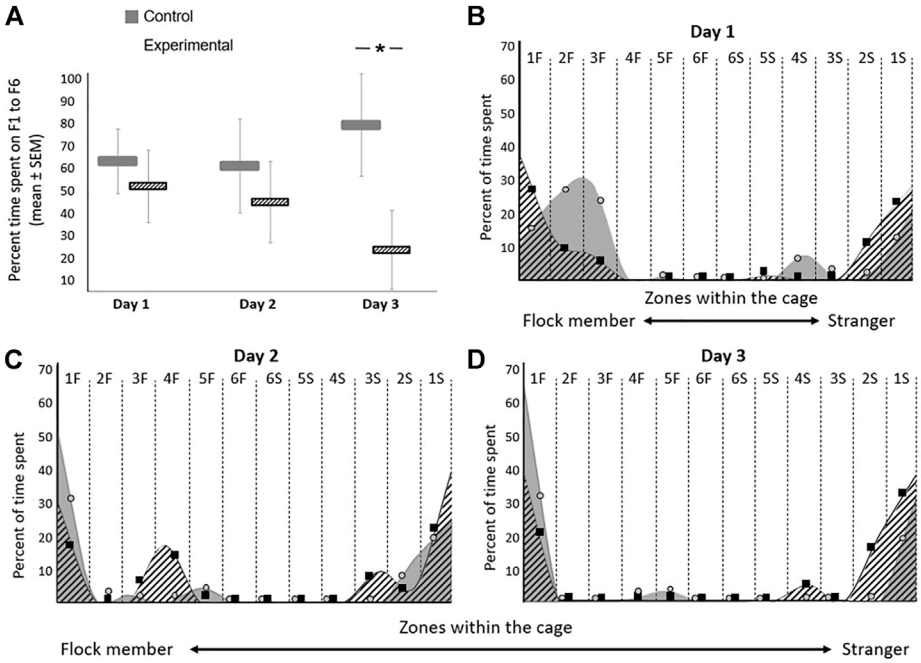


**FIGURE 5 |** Effect of GSK-3β inhibition on social recognition in zebra finches during the three days of the test. The average time spent on each day for both groups are presented. **(A)** Average percentage time spent (seconds ± SEM) in zones F1-F6 on days 1–3 of the test. **(B–D)** The percentage of time spent in each zone within the cage on each day of the experiment is represented with circles (control) and squares (experimental). A smoothing algorithm was used to present the data in a more uniform way overlapping the circles and squares. N = 6 birds/group; $^{*}p < 0.01$.

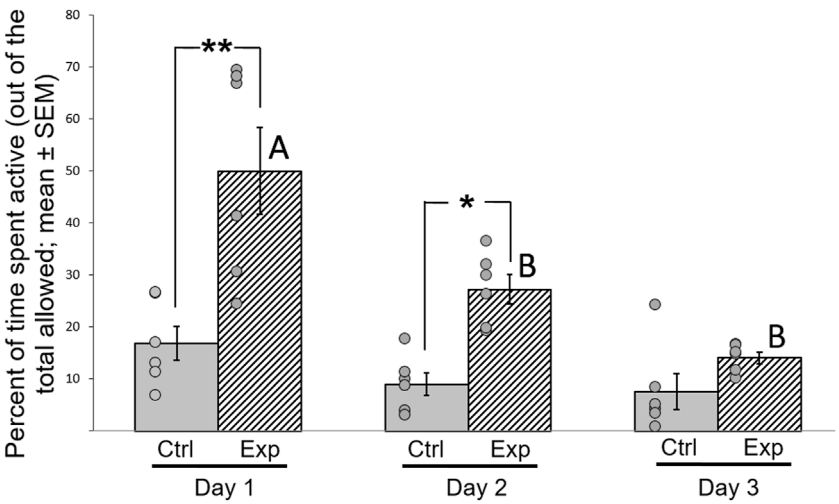


**FIGURE 6 |** Effect of GSK-3β inhibition on motor activity of zebra finches during the 3 days of the test. Activity was calculated as percentage (mean ± SEM) of time spent moving in the cage, out of the total time allowed. N = 6 birds/group; $^{*}p < 0.01$; $^{**}p < 0.001$. Ctrl = control birds; Exp = GSK-3β-inhibited birds. Letters represent the significance within the experimental group between days. Dots represent data points of individual birds.

days ($p = 0.08$). However, the difference between the groups was significant in Kruskal-Wallis test ($p = 0.01$), as can be seen in **Figure 5A**. During the first two days of the tests, inhibition of GSK-3β did not affect the birds' location compared to controls, when introduced into a cage with a flock member on one side and a stranger on the other side (**Figure 5A**). However, on day 3, the GSK-3-inhibited birds showed a significant inclination to move towards the stranger compared to towards the flock member ($F_{(1,11)} = 12.367$, $p = 0.0245$; **Figure 5A**). In both groups and on all days of the tests, the birds avoided standing in the middle part of the cage and preferred to move to one of the two sides (**Figures 5B–D**).

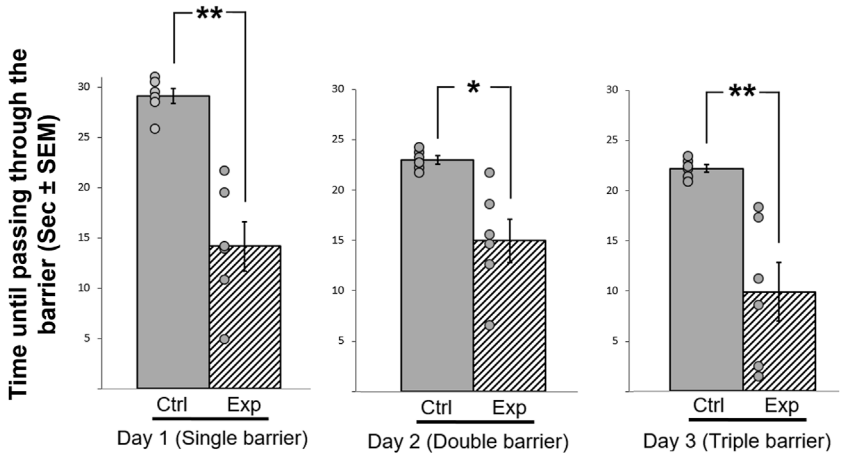


**FIGURE 7 |** Effect of GSK-3β inhibition on the time (sec± SEM) taken to pass a barrier with increasing difficulty every day, during three consecutive days. The time taken to pass through the barrier is represented relative to the maximum of 10 min allowed, which is shown as maximum of 30 s. N = 6 birds/group; *$p < 0.01$; **$p < 0.001$. Ctrl = control birds; Exp = GSK-3β -inhibited birds. Dots represent individual birds.

## GSK-3β Inhibition Increases Motor Activity

In the social recognition test we also found that motor activity of the GSK-3β-inhibited birds significantly increased compared to the controls, measured by repeated measures ANOVA ($F_{(1,11)} = 7.658$, $p = 0.001$; **Figure 6**). There were also interactions between activity time*day ($F_{(1,33)} = 11.219$, $p = 0.003$), therefore post-hoc analyses was performed. On day 1 ($F_{(1,11)} = 4.317$, $p = 0.049$) and day 2 ($F_{(1,11)} = 5.518$, $p = 0.041$) there were significant differences in motor activity between the groups, whereas on day 3 no such difference was observed. In addition, the motor activity of the GSK-3β-inhibited birds significantly decreased between days 1 and 2 ($F_{(1,5)} = 4.436$, $p = 0.018$) and between day 1 and 3 ($F_{(1,5)} = 7.001$, $p = 0.001$), while during days 2 and 3 their activity levels did not differ significantly. Overall, the activity of the control birds was similar throughout the three days of the test (**Figure 6**).

## A Possible Effect of GSK-3β Inhibition on Motivation

In this motivation test, we exploited the natural tendency of zebra finches, which are highly social birds, to join their flock members and to be a part of the flock. The results are presented in **Figure 7**. Overall repeated measures ANOVA revealed a significant difference between the groups were observed ($F_{(1,11)} = 5.118$, $p = 0.001$; **Figure 7**). There were also significant interactions between treatment*day ($F_{(1,33)} = 8.595$, $p = 0.005$), therefore post-hoc analyses was performed. During the first day of the test, four out of the six control birds did not pass through the barrier and could not complete the task. However, during the two consecutive days, all of the control birds completed the task successfully, and overcame the increasing level of difficulty (e.g., more barriers) that were added each day. In contrast, the GSK-3β inhibited birds passed through the barrier from day one, a process that was significantly faster as compared to controls ($F_{(1,11)} = 18.019$, $p = 0.0132$), and regardless of the daily increase in the level of difficultly. Hence, the experimental birds always took significantly less time to pass through the obstacle (day 1 ($F_{(1,11)} = 7.161$, $p = 0.001$), day 2 ($F_{(1,11)} = 4.341$, $p = 0.048$) and day 3 ($F_{(1,11)} = 6.625$, $p = 0.001$). These results might suggest that inhibition of GSK-3β increased motivation of the bird to join the flock.

# DISCUSSION

We have previously shown that, unlike other vertebrates that harbor both GSK-3 genes, birds have only one isozyme - GSK-3β (Alon et al., 2011). In a recent study (Aloni et al., 2015) we found that inhibition of brain GK3-3β affected the capability and singing behavior patterns of zebra finches, reflecting its potential impact on the social interactions of the birds. This prompted us to further deepen our investigation on the role of GSK-3β in controlling lower and higher brain functions, with a particular focus on sociability and a self-motivation to be part of the flock.

## GSK-3β Inhibition Affects Social Recognition

For many species of birds and mammals, sociability is a key component of communal living and is a crucial need for reproduction and survival. Our experimental birds, the zebra finches, are known for their intensive social interactions (Zann, 1996), and hence may serve as a good model system to study mechanisms controlling social behavior.

Our uniquely designed behavioral tests provided us with a platform to study the effects of GSK-3β inhibition on social recognition in zebra finches. However, before we discuss this issue, it is important to understand this species' normal social behavior, as reflected in the control birds. Firstly, as shown in **Figure 5A**, the control birds preferred to stand next to the familiar flock member during all three days of the test. This is an expected behavior for social birds that benefit from group living for

various reasons, such as reduced predation risk or improved foraging success, and which therefore generate non-random social networks and maintain cohesion with familiar individuals (reviewed in Silk et al., 2014). Such maintenance of physical proximity between flock members is known also in our study model, the zebra finch (Zann, 1996; Prior et al., 2020). Furthermore, one of our previous studies (Fleischman et al., 2016) supports this observation, by demonstrating that female zebra finches possess a very good ability to recognize their mates and other socially closely-related individuals that are members of their flock. Moreover, in that study we found that the individual integrity of the flock and that the relationships between flock members are maintained over a long period of time. Another characteristic behavior of the control birds was that on all three days of testing they mostly avoided standing in the middle parts of the cage (**Figures 5B,D**), a pattern that has been found also in rodents (e.g., Jensen et al., 2003), which avoid wide-open spaces. A third observation regarding the control birds was that during the first day of the experiment they explored the test cage relatively more compared to on the following days, during which they tended to spend more time in more restricted areas at the sides of the cage (**Figure 5B** vs. **Figure 5C, D**). Such pattern, of decreased activity on repeated tests is well known for a long time, and has been described in various species (e.g., by Warren and Callaghan, 1976 in fish, and Jones et al., 1977 in rats). The general interpretation is that the gradual decrease in ambulation after a repeated exposure to a situation is a result of habituation of the animal to that situation, which is considered to be a learning process (Thorpe, 1956). Similarly, this outcome in our control birds can indicate that they had learned the situation as the experiment progressed and were more relaxed after the first day of testing.

Inhibition of GSK-3β affected the social recognition of the experimental birds, because over the course of the three days of the test they tended to move closer towards the stranger, unlike the control birds that stood closer to the familiar bird. This finding is in agreement with studies in mice, where GSK-3 influenced social preference and social interaction (e.g., Mines et al., 2010). In our study, we can not determine whether inhibition of GSK-3β affected actual social preference of the birds, or that social preference remained intact, but the treatment caused other changes (such as memory loss, hearing, vision, or olfaction), which in turn affected behavior. Further investigation in required to answer this question, for example by presenting a more controlled stimulus to the birds, such as a pair-wise presentation of two images, or vocalizations. On the other hand, if the inhibition of GSK-3β impaired aspects such as those that are mentioned above, then one could expect a negative effect on the activity/ability of the birds, and in addition that they will stay equally closer to strangers and familiar birds. However, we the treatment actually caused an increase in motor activity, and a tendency to move closer towards the strangers. Therefore, these findings, combined with previous evidence from mammals, that inhibition of GSK-3 affected sociability in mice (Mines et al., 2010), lead us to suggest that this might be the case also in birds.

In addition, overall, our experimental birds stood more in central parts of the cage compared to the control birds (**Figures 5B–D**), reflected on day 1 of the test (**Figure 5B**), in which the experimental birds stood both in zones 1F–3F (closer to the familiar bird), and in zones 1S-3S (closer to the stranger). In mammals, such behavior, of spending more time in the more middle parts of the cage, can be interpreted as anxiety, as already suggested for mice (e.g., Ramboz et al., 1998). Specifically, in relation to GSK-3, it has been shown that silencing GSK-3β in mice did not affect anxiety behavior (Chew et al., 2015), although other studies (e.g., Jung et al., 2016) have shown that GSK-3 knockout mice revealed aberrant anxiety. If spending more time in a central place reflects anxiety also in birds, then it could be suggested that GSK-3β inhibition caused anxiety-like behavior in our experimental birds. However, to the best of our knowledge, there are no supporting evidence for such interpretation from other studies in birds. The only study that we found, which compared the proportion of time spent in the center of an arena as opposed to its corners in house sparrows (*Passer domesticus*), used this measure to quantify neophobic behavior, not specifically anxiety (Ben Cohen and Dor, 2018). Therefore, at this stage, without additional physiological measurements, we are aware that our single behavioral parameter is not sufficient to determine that inhibition of GSK3β causes anxiety in birds. In any case, as the experiment progressed, the experimental birds stood in fewer zones in the cage also reduced the time that they spent in motion. These observations could indicate that it took them less time to make a decision where to stand compared to controls, and hence might suggest that inhibition of GSK-3β affects decision-making, which is a higher brain function and cognition.

## GSK-3β Inhibition Increases Motor Activity and Possibly Motivation

During the three consecutive days of the social preference test, the control birds spent a similar amount of time being active during the full period of the test on each day (**Figure 6**). This finding indicates that even if the new situation on the first day had caused any stress to the birds, it did not affect their motor activity. In contrast, the GSK-3 inhibited birds showed a significant increase in motor activity during the first two days compared to the controls, but this faded on the third day. The increased motor activity can be regarded as hyperactivity, and was most obvious during the first day of the test. This interpretation is supported by previous findings in mice, indicating the role of GSK-3 in regulation of hyperactivity (Ackermann et al., 2010). In addition, GSK-3 inhibition in mice was found to affect dopamine mechanisms in neurons and the expression of dopamine-associates behaviors such as hyperactivity (Beaulieu et al., 2007).

The increased hyperactivity of the GSK-3β-inhibited birds is also in line with the results of the motivation test, in which the control birds showed a gradual learning process reflected in a progressive improvement of passing the barrier in order to join the flock members, in contrast to the GSK-3β inhibited birds, which passed the barrier faster during all days of the test, and regardless of the increase in the level of difficulty. However, since motivation is a very complex intrinsic state that can be interpret by a combination of various reasons, such as the need to join a flock or to find a safe place, further investigation is needed in order to better understand the role that GSK-3 might have in its regulation. It could be that there

was no effect on motivation, and the different latencies between groups to fly to join the flock members are due to the increased activity that we observed in the GSK-3β inhibited birds. Other alternative explanations are also possible at this stage, for example that the difference between the control and the experimental birds in this test results from different degrees of neophobia (the tendency to avoid novel objects and foods), a phenomenon that has been discussed and described in birds (e.g., Greenberg, 1990), and could apply in our case. When facing a novel situation, as getting through barriers, a tension can exist between the attraction to join flock members and the fear from the barriers, and the response can be avoidance or slowing the passage through the barriers. Our data showed that the GSK-3β inhibited birds passed the barriers faster then the controls, and from Day 1 of the experiment, which might suggest that the treatment decreased their neophobia. In any case, it is obvious that in order to resolve this issue further investigation is needed, also because most of the studies regarding the effects of GSK-3 were done in diseases models, for the search of adequate therapy for neurodegenerative disorders, which are spreading worldwide and are one of the greatest threats to public health. GSK-3 is now acknowledged as a central player in regulating mood behavior, cognitive functions, and neuron viability (reviewed in Rippin and Eldar-Finkelman, 2021). Therefore, studying its impact on normal behavior, as we did in the current study, can advance our understating regarding its various roles and can contribute to the discovery and development of effective treatments to repair cognition.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Tel-Aviv University Institutional Animal Care and Use Committee (permit No. 04-18-014).

## AUTHOR CONTRIBUTIONS

SM: conceptualization, animal care, experimentation, experimental design, data analysis, writing—original draft. IR: experimentation, experimental design, and data analysis, writing. JT: conceptualization, experimentation, experimental design. HE-F: conceptualization, experimental design, funding acquisition, supervision, and writing. AB: conceptualization, experimental design, funding acquisition, supervision, and writing. All authors reviewed, edited, contributed to the article, and approved the submitted version.

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# "Prefrontal" Neuronal Foundations of Visual Asymmetries in Pigeons

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This study was conducted in order to reveal the possibly lateralized processes in the avian nidopallium caudolaterale (NCL), a functional analogue to the mammalian prefrontal cortex, during a color discrimination task. Pigeons are known to be visually lateralized with a superiority of the left hemisphere/right eye for visual feature discriminations. While animals were working on a color discrimination task, we recorded single visuomotor neurons in left and right NCL. As expected, pigeons learned faster and responded more quickly when seeing the stimuli with their right eyes. Our electrophysiological recordings discovered several neuronal properties of NCL neurons that possibly contributed to this behavioral asymmetry. We found that the speed of stimulus encoding was identical between left and right NCL but action generation was different. Here, most left hemispheric NCL neurons reached their peak activities shortly before response execution. In contrast, the majority of right hemispheric neurons lagged behind and came too late to control the response. Thus, the left NCL dominated the animals' behavior not by a higher efficacy of encoding, but by being faster in monopolizing the operant response. A further asymmetry concerned the hemisphere-specific integration of input from the contra- and ipsilateral eye. The left NCL was able to integrate and process visual input from the ipsilateral eye to a higher degree and thus achieved a more bilateral representation of two visual fields. We combine these novel findings with those from previous publications to come up with a working hypothesis that could explain how hemispheric asymmetries for visual feature discrimination in birds are realized by a sequential buildup of lateralized neuronal response properties in the avian forebrain.

**Keywords: birds, tectofugal system, lateralization, single unit recording, nidopallium caudolaterale**

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## INTRODUCTION

Asymmetries of brain and behavior are not only widespread among vertebrates, but also extend to bilaterians and thus presumably to the majority of animals (Vallortigara and Rogers, 2005; Ocklenburg et al., 2013; Güntürkün et al., 2020). This ubiquity of non-human animals with cerebral asymmetries provides a great opportunity to develop animal models in order to reveal the mechanisms with which genetic (Norris, 2012; Brandler and Paracchini, 2014), neural (Moorman et al., 2012; Chou et al., 2016; Washington et al., 2021), and cognitive systems (Regolin et al., 2005; Della Chiesa et al., 2006; Yamazaki et al., 2007) govern lateralized behavior. Avian models like chicks and pigeons proved especially suitable to discern the neural foundations of such asymmetries. Both adult pigeons and chicken hatchlings show left-right differences of behavior in various visual tasks that involve discrimination of object details (Güntürkün, 1985; Rogers, 2014), spatial locations (Prior

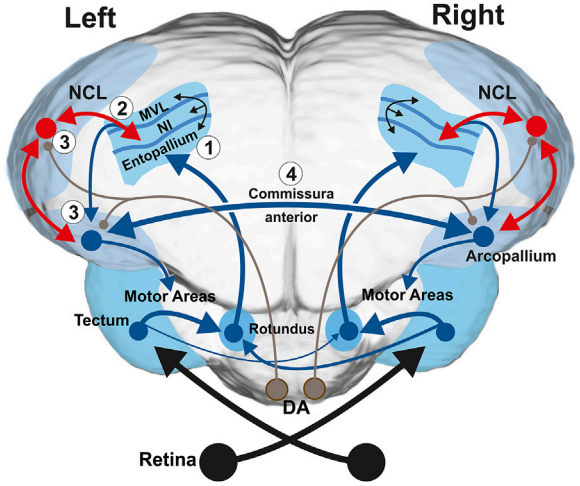


**FIGURE 1 |** Schematic frontal depiction of the tectofugal visual system along with the anterior commissure, the projection of the tectofugal system to the prefrontal-like nidopallium caudolaterale (NCL) and the projection of NCL onto the motor arcopallium (A). The subsystems that play key roles for the present study are depicted in red. The tectorotundal axons have a larger number of fibers that cross from right to left, thereby constituting a more bilateral representation of visual information in the left hemisphere. In the current study, we recorded the activity patterns of single NCL neurons. The NCL receives input from the tectofugal system via the nidopallium intermediale (NI) and feedback projections from the arcopallium. In addition, dopaminergic (DA) brainstem projections (depicted in brown color) can modify visuo-associative and visuo-motor connections in experience dependent manner. Numbers 1–4 refer to the four steps of the working hypothesis on lateralized visual discrimination learning and task execution at pallial level in birds. Further abbreviation: mesopallium ventrolaterale (MVL).

et al., 2004; Tommasi et al., 2012), or social companions (Daisley et al., 2009). It is relevant to emphasize that both the left and right hemisphere of birds provide specific contributions to visual behavior. While pigeons and chicks reach higher accuracy and speed during pattern or color discrimination tasks when using their left hemisphere/right eye (Vallortigara, 1989; Rogers, 2014; Xiao and Güntürkün, 2018), the right hemisphere/left eye excels when social or spatial stimuli are to be distinguished (Rosa-Salva et al., 2012; Tommasi et al., 2012). Thus, avian brain asymmetries are not about an overall hemispheric dominance, but implies the existence of task- and hemisphere-specific circuits.

These asymmetries can be traced down to underlying neural networks. In birds, visual information is processed in two parallel ascending pathways, the thalamofugal and the tectofugal system (Mouritsen et al., 2016). The thalamofugal system transfers input from the retina *via* the contralateral n. geniculatus lateralis, pars dorsalis (GLd) bilaterally to the visual hyperpallium (Güntürkün and Karten, 1991). In chicks, this system is asymmetrically organized with more bilateral inputs to the right hyperpallium (Rogers, 2018) that consequently displays lateralized activity patterns (Costalunga et al., 2021). The second ascending visual pathway is the tectofugal system that projects *via* the contralateral midbrain tectum opticum and the thalamic n. rotundus (Rt) to the entopallium. In pigeons, this pathway evinces various anatomical left-right differences (Güntürkün et al., 2020). Most of tectum axons ascend to the ipsilateral Rt, while a smaller contingent crosses the midline and reaches the contralateral Rt (**Figure 1**). These contralaterally projecting fibres are more numerous from the right tectum to the left Rt than vice versa (Güntürkün et al., 1998; Letzner et al., 2020). Therefore, a larger bilateral input arrives through the left Rt into the left entopallium (Güntürkün et al., 1998; Güntürkün and Hahmann, 1999; Skiba et al., 2002; Letzner et al., 2020). Verhaal et al. (2012) recorded multi-unit responses from right and left entopallium while pigeons were discriminating colors. Similar to the observations of Colombo et al. (2001), entopallial neurons started to respond with an initial phasic burst when processing the rewarded stimulus. This initial activity peak, however, was only present in left entopallial neurons, making an initial recruitment of a larger population of left entopallial neurons. Such an abrupt unilateral avalanche of entopallial activity could activate a larger number of downstream left-hemispheric associative and motor structures of the left hemisphere, thereby resulting in visually controlled behavior that is mostly governed by the left hemisphere.

The entopallium is part of a cortex-like avian sensory pallium that is constituted by radially and tangentially organized neurites that create an orthogonally organized fiber pattern (Stacho et al., 2020). Local communities of neurons that are embedded in this structure create iteratively repeated columnar canonical circuits (Güntürkün et al., 2021). These circuits are tangentially intersected by axons that cross-connect visual tectofugal columns with the (pre)motor arcopallium and the prefrontal-like nidopallium caudolaterale (NCL) (**Figure 1**).

The arcopallium projects to various brainstem areas to control visually guided behavior (Wild et al., 1985; Davies et al., 1997). However, the arcopallium also gives rise to the anterior commissure that is the main source for interhemispheric crosstalk in birds (Letzner et al., 2016). Xiao and Güntürkün (2018), Xiao and Güntürkün (2021) recorded from single arcopallial neurons while the pigeons were discriminating colors and discovered two key mechanisms. First, the neurons of the left arcopallium were faster in triggering the conditioned response, thereby providing the left hemisphere a significant time-advantage that translates into a hemisphere-specific control of behavior during visual object discriminations (Xiao and Güntürkün, 2018). Second, visual information was asymmetrically exchanged *via* the commissure with the left arcopallium providing the right side more information about ipsilateral stimuli than vice versa (Xiao and Güntürkün, 2021).

The second major target of tectofugal columns is the NCL (Stacho et al., 2020) (**Figure 1**). The NCL receives massive dopaminergic input (von Eugen et al., 2020) and constitutes a large avian pallial hub that is situated between all ascending sensory and descending motor systems (Kröner and Güntürkün, 1999). Based on this connectivity pattern, the neurochemical architecture (Herold et al., 2011) and prefrontal-like executive functions (Güntürkün, 1997a; Hartmann and Güntürkün, 1998; Diekamp et al., 2002a), it is likely that the NCL is a functional avian analogue to the mammalian prefrontal cortex (Güntürkün, 2012). This analogy was importantly substantiated by single unit recordings in pigeons and crows. The avian NCL neurons showed

identical neuronal properties as monkey prefrontal neurons during working memory performance (Diekamp et al., 2002b; Johnston et al., 2017; Hahn et al., 2021), stimulus value judging (Kalenscher et al., 2005; Dykes et al., 2018), serial order behavior (Johnston et al., 2020), decision making (Lengersdorf et al., 2014; Veit et al., 2015), prediction error signaling (Packheiser et al., 2021), abstract rules (Veit and Nieder, 2013), as well as coding for numerical information (Nieder, 2017), and conscious experiences (Nieder et al., 2020). The bulk of these evidences indicate that mammals and birds evolved strikingly similar "prefrontal" pallial areas in convergent manner (Güntürkün et al., 2021).

Taken together, the avian NCL is the key area for storing, gating and weighing incoming sensory formation and subsequent planning of appropriate actions. Since visual asymmetries in birds affect perceptual, cognitive, and action-related tasks, it is likely that NCL neurons might show left-right differences during visual discrimination. We therefore set out to analyze single NCL neurons during a color discrimination task. The current paper derives from a comprehensive study of which the arcopallial results have been published (Xiao and Güntürkün, 2018; Xiao and Güntürkün, 2021). The data from NCL were up to now not analyzed and reported.

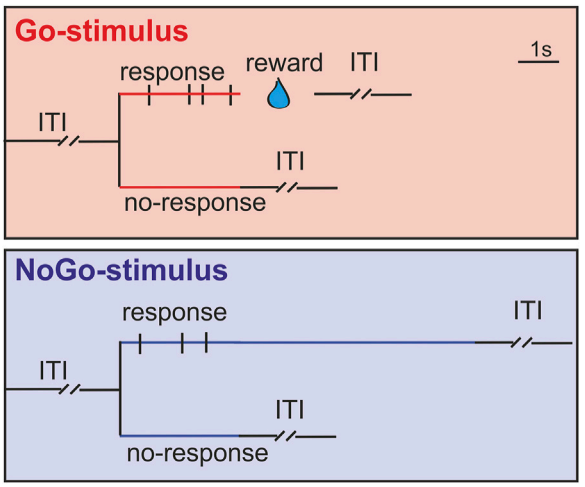


**FIGURE 2 |** Schematic drawing of the training paradigm. Stimuli were presented to each eye in pseudorandomly interleaved trials with 15 s inter-trial intervals (ITI). Animals were trained to either respond to Go-stimuli (red line) or withhold responses to NoGo-stimuli (blue line) during a 3 s response period after the stimulus onset. Correct responses in Go-trials were rewarded with water at the end of response period and the stimuli were switched off during the reward time. Correct responses (no jaw movements) to NoGo-stimuli were not rewarded, whereas jaw movements during the 3 s response period prolonged stimulus presentation time from 3 s to 9 s. Black vertical bars during the 3 s response period indicate jaw movements of animals (mandibulations).

# METHODS

## Bird Training

Six adult homing pigeons (*Columba livia*) from local breeders and of unknown sex were employed and maintained on a 12 h day/night cycle. Food was available at all times. On non-training and non-test days, the birds were allowed to drink as much as they wanted. The day before training or testing, the water bowl in the cage was removed in the afternoon to motivate the animals to receive a water reward during the experiment in the next day. On average, the animals received approximately 4 ml of water during a single session. After training or testing, the birds were allowed to drink ad libitum in the cages for several hours. The weight and health of the animals were monitored on a daily basis. All procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by a state committee (North Rhine-Westphalia, Germany).

We used a procedure that was first introduced by Mallin and Delius (1983). Before the training started, a metal head-fixation pedestal (1 × 0.7 × 0.4 cm, 1.6 g) was glued onto the animals' skull with dental cement to prevent head movements. Isoflurane (~2% by volume in $O_2$, Medical Developments International) was used for anesthesia during surgery. Body temperature was maintained at 40°C by an electric warming pad. A recording trough of 5 × 5 mm was built with dental acrylic on the skull to access the target area of each hemisphere. After surgery, animals were allowed to recover for at least 7 days. The experiments were run in a dark room. The color stimuli were provided by light-emitting diodes (LED) located at each side with a distance of 5 cm away from each eye. LEDs were inserted into a tube with a diameter of 11 mm that pointed closely towards one eye to avoid the diffusion of another eye. This arrangement made sure that the light stimulation was limited and only reached one eye. LEDs were controlled by a custom software written in Matlab (Mathworks, R2009a, United States). Pigeons were trained to discriminate four colors (blue, green, yellow and red) each with the luminance of 0.6 cd/m$^2$. The colors were 2 × 2 paired and each pair of colors (Go- and NoGo-stimulus) was exclusively learned by one eye/hemisphere. Color pairs were balanced between animals. Using the custom-made water container (1 × 0.6 × 0.4 cm) and the beak monitoring system, each beak movement (mandibulation) was detected by an infrared light barrier (Zweers, 1982) and synchronically recorded with task events including the onset and offset of stimulus and reward delivery. The tip of the beak was placed in the middle of the water container. The water pump needed ~0.5 s to fill or empty the container. After the water container was full, water remained for 0.5 s.

During experiments, we characterized visual responses of NCL neurons of both hemispheres. A training session consisted of 80 trials with 20 trials for each color. Only one eye was stimulated in each trial. The stimuli were presented in pseudorandomly interleaved trials with 15 s inter-trial intervals (ITIs). Once an LED that pointed to one eye was switched on (stimulus onset), the animals had a 3 s response period to either answer to the Go-stimulus or withhold responses to the NoGo-stimulus. Correct responses in Go-trials were rewarded at the end of the response period while the stimulus was switched off during reward. Correct NoGo responses ("rejections") were not rewarded, but mandibulations during NoGo trials ("false alarms") prolonged stimulus presentation time from 3 s to 9 s (**Figure 2**). After the

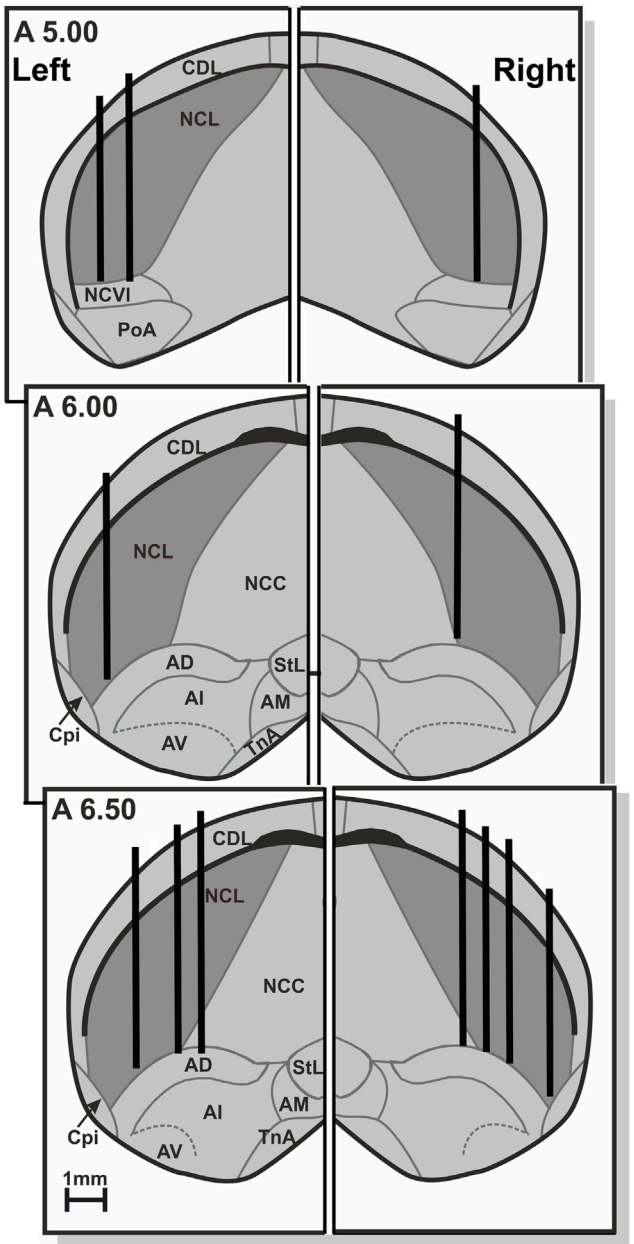


**FIGURE 3 |** Schematic frontal depiction of the electrode positions in the left and right NCL, based on lesions and electrode tracks. Only the lateral 2/3 of the telencephalon of each hemisphere is shown, and omitting the medial 1/3. Abbreviations: AD, arcopallium dorsale; Al, arcopallium intermediale; AM, arcopallium mediale; AV, arcopallium ventral; CDL, area corticoidea dorsolateralis; Cpi, cortex piriformis; NCC, nidopallium caudale pars centralis; NCL, nidopallium caudolaterale; NCVl, nidopallium caudoventrale pars lateralis; N. PoA, posterioris amygdalopallii; StL, striatum laterale; N. TnA, taeniae amygdalae.

signal for reward delivery was given, the pump needed 0.33 s to fill half of the water-container and 0.5 s to fill it completely. When the animals' correct responses to Go- and NoGo-stimuli for each eye both reached 85% on three continuous days, electrophysiological recordings started. The animals' behavioral responses detected by laser detector and task events were recorded synchronically.

## Extracellular Recording

Before recording, a small craniotomy was made at the location of the targeted area under isoflurane anesthesia as described above. Animals were allowed to recover for 1 week and were retrained again until reaching criteria. At the end of each recording day, the recording trough was filled with dental silicone (0.1%, Sigma). There were 2–4 training days between two continuous recording days for each animal.

Neuronal responses from NCL (A: 5.0–7.0; L: 6.0–8.0; D: 1.0–3.0) (Karten and Hodos, 1967; Kröner and Güntürkün, 1999) of two hemispheres were recorded (Thomas recording, 1–2 MΩ, 7-channels Eckhorn System, Germany) while animals were engaged in discriminating colors. Spikes were amplified (×1,000–2,500), filtered (500–5,000 Hz, single-unit activity filter/amplifier system, Thomas Recording, Germany) and displayed on the oscilloscope. Signals were continuously acquired at 20.8 kHz on a 16-channels Spike2 system (CED, Micro1401-mk2, Cambridge Electronic Design Ltd, United Kingdom). Task events and beak movements were digitized at a sampling rate of 1 kHz.

After experiments were finalized, birds were anesthetized with ketamine hydrochloride (initial dose of 40 mg/kg followed by supplements of 20 mg/kg/h) and xylazine hydrochloride (5 mg/kg followed by 2 mg/kg/h) into the pectoral muscle. An electrolytic lesion was placed by a 50 μA positive current (20–30 s). Then, equithesin (0.45 ml/100 g body weight) was added, the animals were perfused and brains were removed. Using the classic nissl histology, the exact positions of lesion sites and electrode tracks were determined, and were all confined in NCL (Karten and Hodos, 1967; Kröner and Güntürkün, 1999) (**Figure 3**).

## Data Analyses

Using a one-way analysis of variance (ANOVA), neurons were defined as task-related when their firing rates during the response or reward phase across all correct response trials were significantly different with spontaneous activities at a same length of time period during ITI before each trial. The data were quantitatively analyzed off-line by Spike2 software (CED) and custom-made MATLAB routines. Single units were classified based on full wave templates and clustered by principle component analysis and direct waveform feature measures. Only well isolated units were included in this study.

To calculate response onset times of each neuron to the stimulus onset or the animals' first response, all spikes were trial-to-trial aligned to either stimulus onset or first mandibulation. We applied the trial-to-trial Poisson spike train analysis to calculate the response onset time of each neuron (Hanes et al., 1995). Response onset times of excited neurons were calculated as the mean time of the first burst of this neuron relative to stimulus onset across all correct response trials. The response onset times of inhibited neurons were the mean value of inhibitory onset time relative to the Go-stimulus onset across all correct response trials. The inhibitory onset time in each

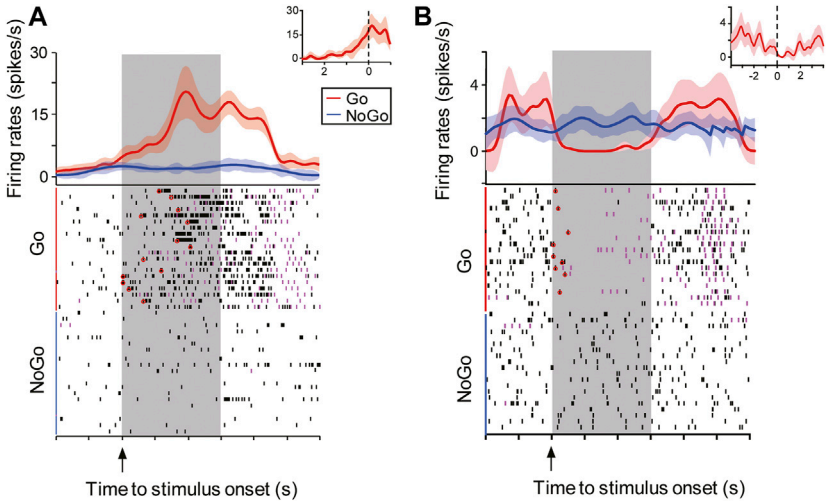


**FIGURE 4 |** Representative example of an excited and an inhibited visuomotor neuron in the NCL. Animals had 3 s (gray shading) to either respond (magenta ticks: mandibulations) to the Go-stimulus (red line) or refrain from responding to the NoGo-stimulus (blue line). The visuomotor neurons were either excited **(A)** or inhibited **(B)** by Go-stimuli (mean ± SEM), but they did not respond to the NoGo-stimuli. The neuronal onset time for each trial was determined by Poisson spike train analyses and are marked with hollow red circles in the raster plots shown in the lower half. After trial-to-trial aligning all spikes of each neuron to the animal's first mandibulation (zero) during the 3 s response period, these neurons showed preceding excitatory or inhibitory activity before the animal's first response (insets, mean ± SEM).

trial was the time at which the firing rate of this neuron was lower ($p < 0.05$) than the predicted one according to spike trains during ITI.

Responses of each NCL neuron were calculated based on their spike density function, which was determined after trial-to-trial aligning all spikes either to the stimulus onset or the first mandibulation during the 3 s response period. This spike density function of each neuron was estimated with kernel density estimations (Shimazaki and Shinomoto, 2010). After subtracting the mean value of spontaneous activity (10 s of ITI), the spike density function across all successful response trials was the temporally pure firing rate change. The peak response times and peak responses were calculated based on the spike density functions of each excited neuron. For inhibited neurons, the inhibitory duration was the time period between the inhibitory start and end time, which was the intersection of the spike density function of each neuron after stimulus onset with the mean activity minus standard deviation of spontaneous responses during ITI.

To quantify the response selectivity of each neuron to Go-/NoGo-stimuli, the spike density function of each neuron to Go- and NoGo-stimuli during 3 s of response period was compared with a receiver-operating characteristic (ROC) analysis. Each point of ROC curve depicted the proportion of bins (20 ms) on which the NoGo-responses exceeded a criterion level against the proportion of bins on which the Go-responses exceeded the same criterion. The criterion level was increased from minimum spikes per bin to the maximum one in one-spike increments. The area under the ROC curve (AUROC) gives the strength of firing that varies in a range from 0 to 1. A value of 0.5 indicates a complete overlap of neuronal responses to Go- and NoGo-stimuli, whereas a value of 0 or 1 indicates a perfect separation. If the AUROC-value of the excited neuron is closer to 1, it implies a higher selectivity of this neuron to the Go-than to the NoGo-stimulus. The AUROC-value of inhibited neuron close to 0 implies a higher discrimination ability.

# RESULTS

## Pigeons Learn and Respond Faster to Stimuli Presented to the Right Eye

All six pigeons used in the current experiment reached the learning criterion faster when the stimuli were presented to the right eye (left-eye: 48.5 ± 14.9 sessions; right-eye: 25.7 ± 8.4 sessions; mean ± SEM; two-tailed Wilcoxon signed-rank test, $p = 0.03$). Once animals mastered the task, the subsequent single-unit recordings in NCL were started. Across all recording sessions, all animals maintained a high correct response rate to Go- and NoGo-stimuli in two monocular viewing conditions (left-eye: 86 ± 0.41%; right-eye: 85.5 ± 0.45%; n = 115 sessions). On average, pigeons responded faster when the stimuli were presented to the right eye (left-eye: 1.53 ± 0.1 s; right-eye: 1.21 ± 0.11 s; mean ± SEM; two-tailed paired $t$-test: $p = 0.04$).

## Go-Stimuli Drive NCL Visuomotor Neurons Which Then Remain Active Until the Animals' Behavioral Onset

In total, we separated 627 NCL neurons in the experiment. In order to link the neuronal activities with the animals' behavior, we trial-to-trial aligned all spikes of each neuron either to the stimulus onset or the animal's first jaw movement (mandibulation) during the 3 s response period. We then calculated the peristimulus time histogram (PSTH) of each neuron by averaging all spikes across aligned trials. From PSTHs aligned with the stimulus onset, we found that 309

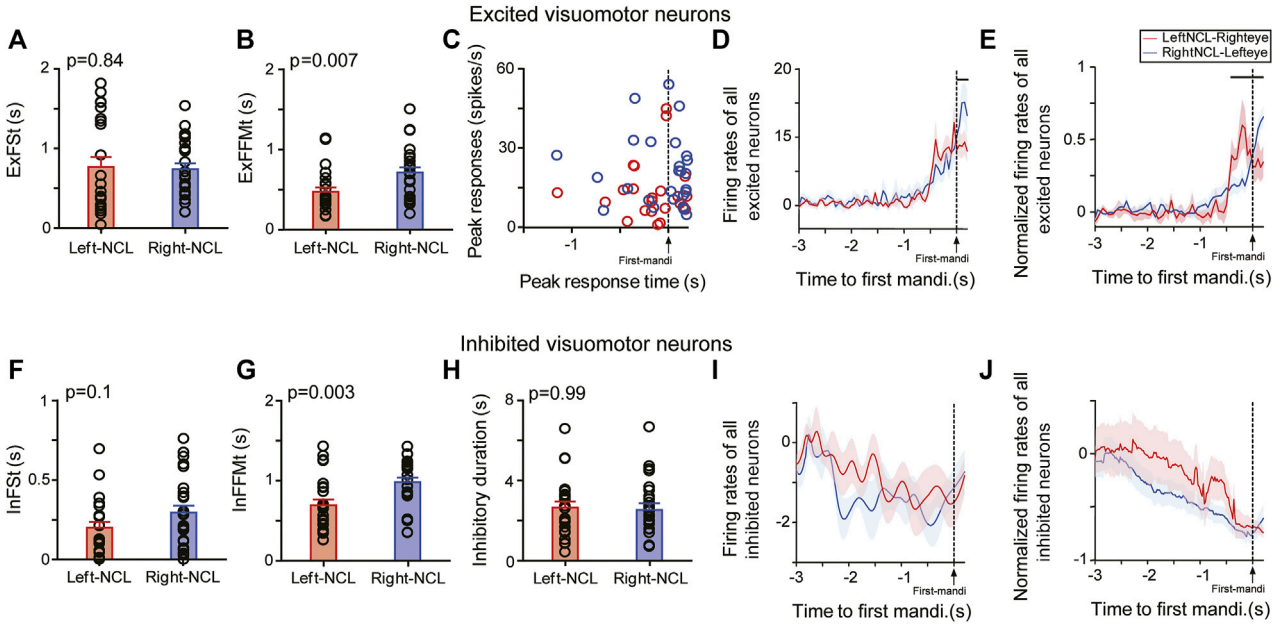


**FIGURE 5 |** Responses of excited (upper row) and inhibited (lower row) visuomotor neurons to Go-stimuli presented to the contralateral eye. Mean response onset time (± SEM) of excited **(A)** and inhibited neurons **(F)** to Go-stimuli. Mean latency (± SEM) between neuronal and behavioral onset of excited **(B)** and inhibited neurons **(G)**. **(C)** Peak responses and peak-firing times of excited neurons. Mean firing rates (± SEM) of left and right excited **(D)** or inhibited visuomotor neurons **(I)** relative to the first mandibulation of animals. Normalized mean responses (± SEM) of all visuomotor neurons relative to the first mandibulation of animals, in which firing rates of each neuron were normalized to its peak responses of excited neurons **(E)** or lowest responses of inhibited neurons **(J)**. Horizontal lines indicate time points of significant firing rate differences between left and right NCL excited neurons ($p < 0.05$). **(H)** Duration of inhibitory responses of inhibited neurons.

neurons (150 left, 159 right) were stimulus-driven. Each animal contributed 25 ± 9 neurons and 26 ± 7 neurons (mean ± SD, n = 6 animals) from the left and right NCL, respectively. By comparing the activity of these neurons during task execution with their spontaneous activity during ITI, we could separate neurons into two groups. They were either excited or inhibited by Go-stimuli, but were not affected by NoGo-stimuli. Depending on their response patterns to Go-stimuli, 233 neurons were classified as excitatory (113 left, 120 right) and 76 neurons as inhibitory (37 left, 39 right). Based on PSTHs aligned with the animal's first mandibulation, some task-related neurons showed preceding responses before the animals' first mandibulation to Go-stimuli (**Figure 4**). Since these neurons were driven by visual inputs and started their excitatory or inhibitory responses prior to the motor output, we dubbed these neurons excited or inhibited visuomotor neurons. These visuomotor neurons responded either to input from one eye (contralateral or ipsilateral) or from both eyes (excited neurons: 23 left, 26 right; inhibited neurons: 24 left, 26 right). Since error rates were very low, our results did not contain a separate analysis of trials with misses or false alarms.

## Left and Right Visuomotor Neurons Respond Equally Fast to Go-Stimuli

Remember that pigeons were faster to respond when seeing the Go-stimuli with the right eye (left hemisphere). In order to investigate neuronal correlates of speed differences of left-right responses, we analyzed the spike latencies to Go-stimuli. To this end, we applied trial-to-trial Poisson spike train analyses and calculated response onset times of excited (ExFSt) and inhibited visuomotor neurons (InFSt) to Go-stimulus onsets. ExFSt-values of excited neurons give the mean time of the first burst relative to Go-stimulus onset across all correct response trials. Correspondingly, InFSt-values of inhibited neurons give mean values of inhibitory onset times to Go-stimulus onsets which were calculated as the time at which firing rates started to be lower ($p < 0.05$) than the predicted one according to ITI spike trains. Since visuomotor neurons did not respond to NoGo-stimuli, such values were not available for this stimulus class.

Excited and inhibited visuomotor neurons of both hemispheres revealed no differences in response onset times to contralateral Go-stimuli. For excited neurons, ExFSt-values were 0.77 ± 0.13 s for left (mean ± SEM, n = 23) and 0.74 ± 0.07 s for right NCL neurons (n = 26) (two-tailed *t*-test: $p = 0.84$; **Figure 5A**). Similarly, inhibited NCL neurons of both hemispheres had comparable inhibitory onset times relative to Go-stimuli (InFSt: left-NCL: 0.2 ± 0.04 s, n = 24; right-NCL: 0.29 ± 0.04 s, n = 26; mean ± SEM, two-tailed *t*-test: $p = 0.1$; **Figure 5F**). Thus, neuronal spike onset times could not explain hemispheric differences in response speed of the birds. However, these analyses showed that inhibited neurons of both hemispheres responded faster to Go-stimuli than excited ones (left and right NCL: $p$'s < 0.0002; **Figures 5A,F**).

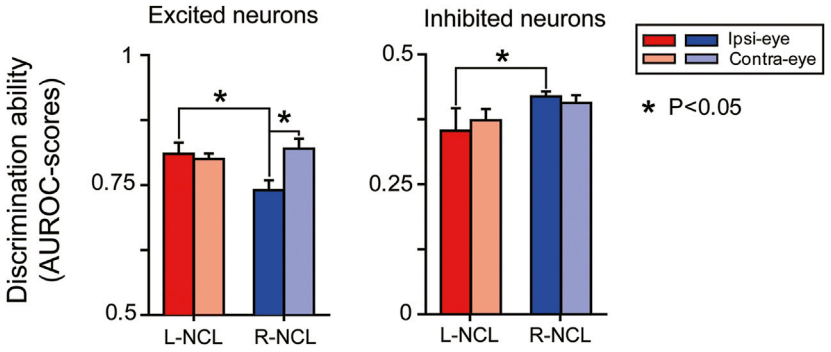


**FIGURE 6 |** Discrimination scores (AUROC-value: mean ± SEM) of NCL visuomotor neurons in left (L) or right NCL (R).

## The Left Hemisphere is More Efficient in Activating the Motor Output

To evaluate the latency between the neuronal response and the animal's movement onset, we calculated the ExFFMt-value for excited and InFFMt for inhibited neurons. The ExFFMt-value of each excited neuron is the mean latency between the first burst and the animals' first mandibulation across all correct response trials. Similarly, InFFMt-values are the latencies between inhibitory onset and the animals' first mandibulation across all correct response trials.

Our results showed that after Go-stimulus onset, the excited visuomotor neurons in left NCL had shorter ExFFMt-values than those in right NCL (left: 0.47 ± 0.05 s, n = 23; right: 0.71 ± 0.06 s, n = 26; mean ± SEM $p = 0.007$) (**Figure 5B**). Thus, NCL neurons of the left hemisphere were faster in turning their activation into an appropriate motor output. But which mechanism produced this left-right difference? To find an answer, we looked at the neuronal dynamics of NCL neurons. First, we compared Go-stimulus driven peak-firing times of excited neurons. As shown in **Figure 5C**, the majority of left NCL neurons reached their peak activities before the animals' first mandibulation (left: 74%, 17/23; right: 35%, 9/26; two-tailed chi-square test: $p < 0.01$). To analyze the relation between the timing of the motor output and the neuronal activity of NCL neurons, we compared the activities of these neurons 200 ms before and during the animal's first mandibulation. The mean firing rates of left NCL neurons were comparable shortly before and after the behavioral motor output (before: 6.55 ± 1.48 spikes/s; during: 4.48 ± 1.49 spikes/s; $p = 0.22$). On the contrary, the neuronal activities of right NCL neurons continuously increased after the onset of the animal's first mandibulation (before: 9.17 ± 1.93 spikes/s; during: 16.66 ± 2.85 spikes/s; $p < 0.0003$). No inter-hemispheric activity differences were observed before the animal's first mandibulation ($p = 0.35$). However, right NCL neurons reached higher peak firing rates than those on the left but this peak came after mandibulation ($p = 0.004$) (**Figure 5D**). We then normalized the firing rates of each neuron to the peak value during 3 s before the animal's response. As shown in **Figure 5E**, normalized firing rates sharply increased in left NCL before the animals' first mandibulation, while those in right NCL did so after first mandibulation. The left and right NCL neurons were significantly more active before and after the animals' response onset, respectively (each $p < 0.05$). Thus, the left NCL was able to determine the animals' responses by its early rise in activity. As a result, the right NCL reached its peak activity when the animals' response had already started and consequently came too late.

We then turned our attention to the NCL neurons that were inhibited by Go-stimulus onset. These neurons displayed higher spontaneous activities during ITI than the excited ones (both left and right NCL, $p$'s = 0.03). After the appearance of Go-stimulus, inhibited neurons of the left NCL had shorter InFMMt-values than those in the right NCL (InFMMt: left: 0.69 ± 0.07 s, n = 24; right: 0.98 ± 0.06 s, n = 26; mean ± SEM, two-tailed $t$-test: $p = 0.003$) (**Figure 5G**). The durations of inhibitory activities were comparable between left and right NCL neurons (left: 2.65 ± 0.3 s, right: 2.64 ± 0.27 s, $p = 0.99$; **Figure 5H**). In each hemisphere, the activities of inhibited neurons declined slowly after Go-stimulus onset and reached its lowest level before the animals' first mandibulation. The mean activities before and during the animal's first mandibulation (200 ms) were comparable for neurons in the left (before: 1.27 ± 0.58 spikes/s; during: 0.99 ± 0.71 spikes/s; $p = 0.19$) and right NCL (before: 1.58 ± 0.52 spikes/s; during: 1.26 ± 0.43 spikes/s; $p = 0.59$). No significant inter-hemispheric activity differences were found before ($p = 0.73$) and during the first response of animals ($p = 0.73$) (**Figure 5I**). This result was also valid for normalized data patterns (**Figure 5J**).

## Integrating Inputs From Both Eyes

The optic nerves of birds with their laterally placed eyes cross virtually completely in the optic chiasm. Thus, the optic fibers of each eye project nearly only to the contralateral half brain (Güntürkün and Karten, 1991). At the subpallial level, the re-crossing of visual information is enabled by mesencephalic and meso-diencephalic commissural projections (Stacho et al., 2016). As shown in **Figure 1**, the tectorotundal commissural crossover is asymmetrically organized such that the left hemisphere integrates visual inputs from two hemispheres to a larger extent than the right hemisphere (Güntürkün et al., 1998; Letzner et al., 2020; Rogers, 2020). As a result, the left visual entopallium receives more information from both visual fields (Verhaal et al., 2012).

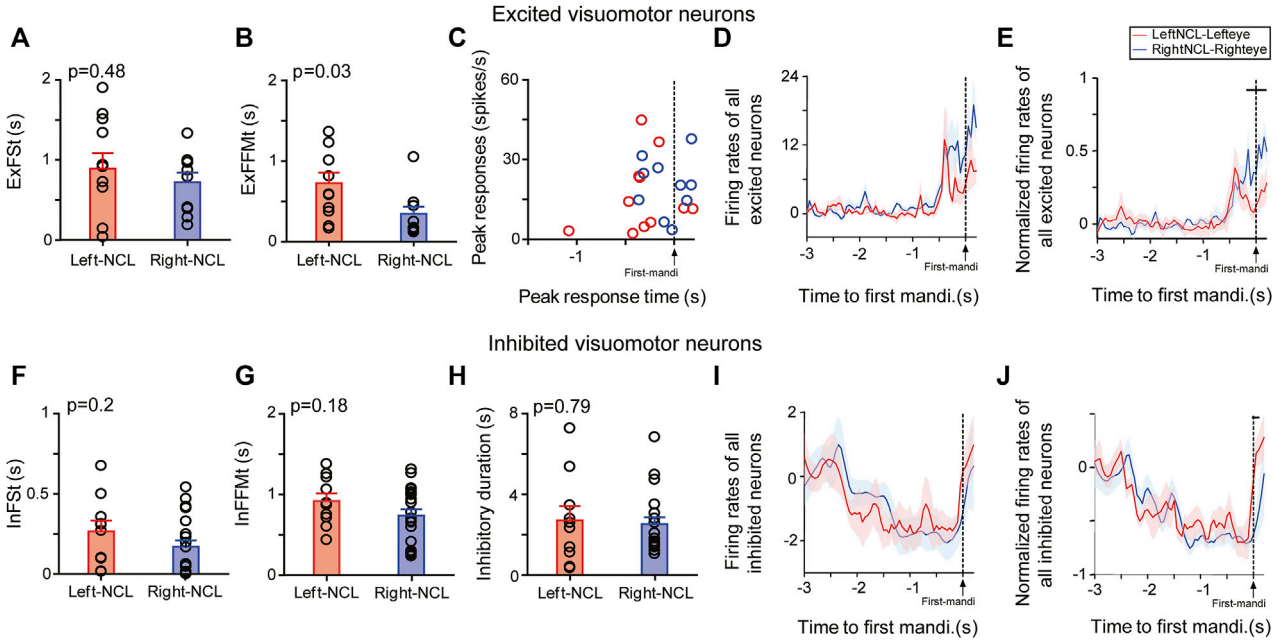


**FIGURE 7 |** Responses of visuomotor neurons to Go-stimuli presented to the ipsilateral eye. Response characteristics of excited **(A–E)** and inhibited neurons **(F–J)** in the NCL to stimuli presented to the ipsilateral eye (ExFSt; InFSt) and their subsequent ability to swiftly activate a motor response (ExFFMt; InFFMt). Mean response onset time (± SEM) of excited **(A)** and inhibited neurons **(F)** to Go-stimuli. Mean latency (± SEM) between neuronal and behavioral onset in excited **(B)** and inhibited neurons **(G)**. **(C)** Peak activities and peak-firing times of excited neurons in left and right NCL. Mean firing rates (± SEM) of excited **(D)** and inhibited visuomotor neurons **(I)** relative to the first response of the animal to ipsilateral stimuli. Normalized mean responses (± SEM) of excited **(E)** and inhibited visuomotor neurons **(J)** relative to the first response of the animal. Firing rates of each neuron were normalized to its peak (excited neuron) or lowest response (inhibited neuron). The horizontal lines in E and J indicate the significance level of the comparison of left and right NCL neurons ($p < 0.05$). **(H)** Duration of inhibitory responses of inhibited neurons.

Consequently, the projections from the entopallial system to the arcopallium create similar asymmetries of visual representation in the arcopallium (Xiao and Güntürkün, 2018). These are partly re-balanced by the exchange of left and right arcopallia through the commissura anterior (Xiao and Güntürkün, 2021).

Since NCL receives inputs from both the entopallial system as well as from the arcopallium (Kröner and Güntürkün, 1999), we expected some left-right differences in the bilateral visual integration between two hemispheres. To test this hypothesis, we used receiver-operating characteristic (ROC) analyses to compare the discrimination ability of each visuomotor neuron to Go-/NoGo-stimuli from the ipsilateral or the contralateral eye. To reiterate, an AUROC-value of 0.5 indicates that the neuron does not differentiate between Go- and NoGo-stimuli, whereas values of 0 or 1 indicate perfect separation. If the AUROC-value of an excited neuron is close to 1, it implies a higher selectivity of this neuron to the Go-than to the NoGo-stimulus. An AUROC-value of 0 indicates a perfect discrimination of an inhibited neuron.

For contralateral inputs, the excited visuomotor NCL neurons did not show inter-hemispheric differences in AUROC-values (left: 0.8 ± 0.01, n = 23; right: 0.82 ± 0.02, n = 26; two-tailed *t*-test: $p = 0.58$). With respect to ipsilateral inputs, left NCL neurons reached higher AUROC-scores than those on the right (left: 0.81 ± 0.02, n = 11; right: 0.74 ± 0.02, n = 10; $p = 0.02$). Overall, left excited neurons showed comparable discrimination performances for contralateral and ipsilateral inputs ($p = 0.45$). On the contrary, right sided excited neurons evinced higher AUROC-scores for contralateral than ipsilateral inputs ($p = 0.01$; **Figure 6**). This was to be expected based on the more pronounced bilateral input of the left hemisphere (**Figure 1**).

For inhibited visuomotor neurons, no inter-hemispheric differences were observed in their AUROC-scores for contralateral inputs (left: 0.37 ± 0.02, n = 24; right: 0.41 ± 0.01, n = 26; two-tailed *t*-test: $p = 0.58$). Only for ipsilateral input, we observed superior discrimination performances of left NCL inhibited neurons compared with the right ones (left: 0.35 ± 0.04, n = 10; right: 0.42 ± 0.01, n = 20; $p = 0.04$). NCL inhibited neurons of each hemisphere showed comparable discrimination abilities for input from each eye (left: $p = 0.65$; right: $p = 0.5$; **Figure 6**).

We then turned our attention to possible hemispheric speed differences in the integration of ipsilateral and contralateral input. Both excited (ExFSt: left: 0.89 ± 0.19 s, n = 11; right: 0.72 ± 0.12 s, n = 10; mean ± SEM, two-tailed *t*-test: $p = 0.48$; **Figure 7A**) and inhibited visuomotor neurons (InFSt: left: 0.27 ± 0.07s, n = 10; right: 0.17 ± 0.04 s, n = 20; $p = 0.2$; **Figure 7F**) of both hemispheres responded equally fast to Go-stimuli presented to the ipsilateral eye. Overall, inhibited neurons started their responses earlier than excited neurons (all $p$'s < 0.03).

Up to now we presented data on stimulus-driven neuronal responses. We now concentrated on whether there was a potential correlation between the response speed of NCL neurons and

animals' behavioral responses. When the ipsilateral eye was stimulated, the ExFFMt-values of excited visuomotor neurons in the right NCL were smaller than those in the left (left: 0.72 ± 0.13 s, n = 11; right: 0.34 ± 0.09 s, n = 10; mean ± SEM. two-tailed *t*-test: $p = 0.03$; **Figure 7B**). Thus, right NCL was faster in translating an ipsilateral Go-stimulus input into an appropriate motor response.

The excited neurons in both hemispheres did not differ much in their general response properties to ipsilaterally presented stimuli. They had comparable peak activities (left: 16.5 ± 4.2 spikes/s; right: 20 ± 3.4 spikes/s; $p = 0.53$; **Figure 7C**), evinced comparable peak-firing times (left-NCL: 0.31 ± 0.1 s; right-NCL: 0.07 ± 0.07 s; $p = 0.07$; **Figure 7C**), and showed no hemispheric differences before ($p = 0.08$) and during the animal's first response ($p = 0.09$; **Figure 7D**). However, after firing rates of excited neurons were normalized relative to their peak responses, we found that more left than right NCL neurons reached their peak responses before the animal's first mandibulation (left: 82%, 9/11; right: 60%, 6/10; **Figures 7C,E**).

The inhibited visuomotor neurons had higher spontaneous activities during ITI than excited ones (both left and right NCL, *p*'s = 0.04). Inhibited neurons had comparable InFFMt scores between both hemispheres (left: 0.92 ± 0.1 s, n = 10; right: 0.74 ± 0.08 s, n = 20; mean ± SEM, two-tailed *t*-test: $p = 0.18$) as well as in their durations of inhibitory responses (left: 2.71 ± 0.69 s, right-NCL: 2.53 ± 0.3 s, $p = 0.79$; **Figures 7G,H**). After the animal's first response, the effect of inhibition significantly similarly increased over time for both left and right inhibited neurons (both *p*'s < 0.05; **Figure 7I**), but evinced a rebound during the response (**Figure 7J**).

In summary, both excited and inhibited visuomotor neurons evinced comparable discrimination performances between two hemispheres for visual input from the contralateral eye. An asymmetry emerged, however, when visual stimuli were given to the eye ipsilateral to the recorded hemisphere. Here, left sided neurons had an advantage. In addition, the right hemisphere was more efficient in activating a motor output after ipsilateral right-eye input.

# DISCUSSION

In the current study, we conducted single unit recordings from the "prefrontal" NCL while pigeons were working on a color discrimination task. As expected, our birds learned the discrimination faster and also responded more quickly to the Go-stimuli when using the right eye. Our recordings uncovered two main asymmetrical neuronal properties of the NCL that possibly constitute parts of the neuronal foundations of this lateralized behavior. First, both left and right NCL visuomotor neurons responded equally fast to the Go-stimuli in this study, while the response properties of left hemispheric neurons suggested that they possibly play a more important role in controlling the operant response than right NCL neurons. Thus, behavioral asymmetry of response speed does not seem to be related to encoding stimuli but to action systems. Second, the processing of stimuli from the ipsilateral eye differed between two hemispheres, with the left NCL being more efficient in representing inputs from both eyes. We will discuss these points one by one and will at the end combine them with insights from previous publications (Verhaal et al., 2012; Güntürkün et al., 2018; Xiao and Güntürkün, 2018; Xiao and Güntürkün, 2021) to propose a model of lateralized color discrimination learning and task execution in birds.

## Behavioral Asymmetries

Most studies on visual discrimination of various visual features in birds revealed faster learning and response speeds as well as higher discrimination scores when using the right eye/left hemisphere (Galliformes, domestic chicken: Vallortigara, 1989; Regolin et al., 2003; Rogers 2014; quail: Valenti et al., 2003; Gülbetekin et al., 2009; Columbiformes, pigeon: Güntürkün, 1985; Prior et al., 2004; Passeriformes, zebra finch: Alonso, 1998). Thus, most but not all avian species (Templeton and Gonzalez, 2004) evince a left hemispheric superiority in learning and discriminating visual features during appetitive tasks. Our results accord with this general pattern. It is important to mention that these findings do not signify an overall left hemispheric "visual dominance" but hint to hemisphere specific differences in the processing of visual object features. Visually guided social (Rosa-Salva et al., 2012) and spatial tasks (Tommasi et al., 2012) often reveal an inverted asymmetry pattern (Güntürkün, 1997b; Vallortigara, 2000; Regolin et al., 2005). This implies that the lateralized neural processes that we describe are related to our task contingencies and are thus amenable to change when pigeons work on a different kind of visual discrimination.

## The Left Hemisphere's Advantage in Activating the Response

Visuomotor neurons of both left and right NCL responded with equal speed to the onset of the Go-stimuli. Thus, we did not reveal any asymmetries of stimulus encoding in the NCL. However, we observed asymmetries of response initiation. Normalized firing rates of the left visuomotor neurons increased shortly *before* the animals' response onset while those on the right increased their spike frequencies *after* the onset of the behavioral response. Thus, if both hemispheres would rush to ignite motor areas in order to activate the behavioral response, the right NCL would mostly be too late to determine the action of the animal. This finding has two implications. First, the time advantage of the left hemisphere is, at least with respect to the NCL, not due to left-right differences of stimulus encoding but due to asymmetries of motor activation. Second, our results from the NCL are practically identical to findings from the arcopallium (Xiao and Güntürkün, 2018) and so possibly result from a similar mechanism.

As depicted in **Figure 1**, the arcopallium projects to subpallial motor areas and has commissural projections through the commissura anterior to the contralateral arcopallium. Xiao and Güntürkün (2018) observed that blocking neural activities of the leading left arcopallium drastically increased the variability of the ExFSt and ExFFMt values of right arcopallial neurons, but blocking right arcopallium had no such effects on left NCL

neurons. This implies that the left (but not the right) arcopallium was able to control the temporal activity structure of contralateral arcopallial neurons. As a result, the left arcopallium was able to delay peak activity times of right arcopallial neurons, thereby monopolizing the control of the animals' behavior during color discrimination. This mechanism depended on the arcopallial interactions through the commissura anterior. The NCL has no commissural projections to the other hemisphere and thus cannot invoke a similar mechanism to its homotopic side (Letzner et al., 2016). This implies that the asymmetries of the spike peak times of left and right NCL neurons possibly result from projections of the arcopallium onto the ipsilateral NCL (**Figure 1**).

A comparison of the current study on the NCL with those on the arcopallium (Xiao and Güntürkün, 2018) reveals an important difference in the proportion of excited visuomotor neurons. In the NCL (current study), 49% of left and 50% of right hemispheric neurons were excited by the Go-stimuli. In the arcopallium, however, the comparable numbers were 67% on the left and 38% on the right (Xiao and Güntürkün, 2018). Since the experimental procedures were identical in the current and the previous study, it is likely that the proportion of excited visuomotor neurons is indeed symmetric in NCL but asymmetric in the motor arcopallium. The small proportion of excited right hemispheric arcopallial neurons could constitute a further mechanism with which behavioral responses during visual discrimination are primarily controlled by the left hemisphere.

## Asymmetries of Visual Integration

As depicted in **Figure 1**, the left hemispheric entopallium receives a strong input from both the left and the right tectum through the n. rotundus (Güntürkün et al., 1998; Letzner et al., 2020). This is different from the right entopallium that mostly receives input from the contralateral eye *via* the right tectorotundal system. This anatomical asymmetry results in a higher level of bilateral representation in the left hemispheric tectofugal system (Letzner et al., 2020). The left-right difference of visual representation is also visible at the neuronal processing level (Verhaal et al., 2012; Xiao and Güntürkün, 2018) and concomitantly affects behavioral performances (Güntürkün and Hahmann, 1999; Valencia-Alfonso et al., 2009). Since the tectofugal system feeds into the NCL, we could also demonstrate an asymmetry of visual representation when comparing the input from the ipsilateral eye between left and right NCL. While AUROC values from the contra- and the ipsilateral eye did not differ in the left NCL, those in the right NCL were worse for input from the ipsilateral eye. Thus, asymmetries of bilateral visual representation of left and right NCL resemble those of the entopallium.

Xiao and Güntürkün (2021) observed a different pattern in the arcopallium. Here, the commissura anterior was the gateway to exchange visual stimuli in a reversed asymmetrical manner such that the left arcopallium provided the right side with more information about its ipsilateral visual half-field than vice versa. Thus, the arcopallial interhemispheric communication acted in an opposite direction to asymmetries of the tectofugal system, thereby leveling left-right differences of visual representation at arcopallial level. The fact that we did not observe anything comparable in the NCL implies that this symmetrizing of visual representation is confined to the arcopallium and is not projected back onto the NCL.

## A Working Hypothesis for Lateralized Discrimination Learning and Task Execution at Pallial Level in Birds

Based on the results of the present study as well as previous publications we will now outline a hypothesis on the neuronal processes during visual feature discrimination in pigeons (Verhaal et al., 2012; Güntürkün et al., 2018; Xiao and Güntürkün, 2018; Xiao and Güntürkün, 2021). We will proceed in four steps that are related to the numbers depicted in **Figure 1**.

First, the tectofugal system of the left hemisphere shows a higher propensity to spontaneously respond to color stimuli - an asymmetry that is drastically increased once a color is associated with reward (Verhaal et al., 2012). This is especially visible for the initial phasic burst of entopallial activity (Colombo et al., 2001) that is only visible in the left entopallium (Verhaal et al., 2012). Learning to associate a visual feature with a strong appetitive value quickly recruits a large number of left entopallial neurons that start to respond to the Go-stimuli. This left-skewed quantitative asymmetry in neuronal population size could give the left tectofugal system an important advantage to ignite neuronal networks in downstream associative and motor areas.

Second, entopallial neurons respond to a large number of visual features (Scarf et al., 2016; Clark and Colombo, 2020; Clark et al., 2022) that are represented as population codes (Koenen et al., 2016; Azizi et al., 2019). If some of these features become associated with reward, left entopallial neurons excel in discriminating between rewarded and non-rewarded stimuli and this left hemispheric superiority correlates with the lateralized responses of the animals when working under monocular conditions (Verhaal et al., 2012). Thus, visual processes and response patterns become linked.

Third, the previous point implies that activity patterns of the left tectofugal system gain a higher prediction for the upcoming reward during choice situations. Since the increase of associative strength between a visual feature and reward is proportional to the magnitude of error between prediction and outcome, we would expect a higher gradient descent of error for visuo-associative and visuo-motor networks in the left hemisphere (Rescorla and Wagner, 1972; Kruschke, 1992; Soto and Wasserman, 2010). NCL, arcopallium and striatum are densely innervated by dopaminergic fibers from the brainstem (Wynne and Güntürkün, 1995; von Eugen et al., 2020) that activate local D1A- and D1D-receptors (Herold et al., 2011; 2018) which mediate synaptic stimulus-response associations in birds (Izawa et al., 2001; Yanigahara et al., 2001; Herold et al., 2012). Thus, the capacity of the left entopallium and its associated nidopallial and mesopallial territories (Stacho et al., 2020) to better discern between the rewarded and non-rewarded features would result in a dopamine-mediated higher synaptic

coupling between the visual tectofugal, "prefrontal" and arcopallial motor neurons in the left hemisphere (Güntürkün et al., 2018).

Fourth, the commissura anterior plays a key role in the lateralized transduction of these visual and associative processes into the animal's response pattern. Xiao and Güntürkün (2018) could show that the left arcopallium can adjust the responses of right arcopallial neurons such that their motor command is generated too late to influence the choice of the pigeon. As a consequence, the left hemisphere dominates choices and responses of the animal. A further function of the commissura anterior is to re-balance the asymmetries of bilateral visual representation between two hemispheres such that left and right arcopallia have equal access to ipsi- and contralateral information during motor execution (Xiao and Güntürkün, 2021). Thus, different functions of which some increase and others decrease functional asymmetries are accomplished *via* the commissura anterior.

Our working hypothesis covers the loop that starts with the midbrain tectum, proceeds to the thalamic n. rotundus and the pallial entopallium and runs up to the "prefrontal" NCL and the motor arcopallium. Our ideas are obviously speculative at the moment but can serve as a working hypothesis for future experiments. These could, for example, use a meta-conflict paradigm, where both hemispheres are occasionally brought into a response conflict (Adam and Güntürkün, 2009; Ünver and Güntürkün, 2014; Manns et al., 2021). Such conflicts result in slower response times and an outcome in which the perceptual specialization of one hemisphere often dominates that of the other. In this case, we would expect the left hemisphere to more often dominate the animals' behavior. The situation is different, when both eyes synergistically see their specific Go-stimulus. This condition was dubbed "super-stimulus" in the study of Ünver and Güntürkün (2014) and resulted in faster response times. All of these studies were purely behavioral and thus it is speculative what the neuronal response patterns would look like. But such a design is appropriate to test our hypothesis of a commissural mechanism that swiftly enables a shift between interhemispheric competition and cooperation.

Overall, this scenario uncovers many open questions that remain to be answered in order to come up with fully mechanistic explanations of the processes that govern avian visual asymmetries. Foremost is the question why left tectofugal neurons are more apt to respond to neutral visual cues and are subsequently superior in discriminating between visual features, once discrimination training has started. Answers to these and similar questions are needed to achieve a truly comprehensive account that goes beyond the preliminary hypothesis that the current study can provide.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were proved by the state committee (North Rhine-Westphalia, Germany).

## AUTHOR CONTRIBUTIONS

QX performed experiments and data analyses. QX and OG designed experiments, interpreted results, and jointly wrote the manuscript.

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# Behavioral Training Related Neurotransmitter Receptor Expression Dynamics in the Nidopallium Caudolaterale and the Hippocampal Formation of Pigeons

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Learning and memory are linked to dynamic changes at the level of synapses in brain areas that are involved in cognitive tasks. For example, changes in neurotransmitter receptors are prerequisite for tuning signals along local circuits and long-range networks. However, it is still unclear how a series of learning events promotes plasticity within the system of neurotransmitter receptors and their subunits to shape information processing at the neuronal level. Therefore, we investigated the expression of different glutamatergic NMDA (*GRIN*) and AMPA (*GRIA*) receptor subunits, the GABAergic *GABARG2* subunit, dopaminergic *DRD1*, serotonergic *5HTR1A* and noradrenergic *ADRA1A* receptors in the pigeon's brain. We studied the nidopallium caudolaterale, the avian analogue of the prefrontal cortex, and the hippocampal formation, after training the birds in a rewarded stimulus-response association (SR) task and in a simultaneous-matching-to-sample (SMTS) task. The results show that receptor expression changed differentially after behavioral training compared to an untrained control group. In the nidopallium caudolaterale, *GRIN2B*, *GRIA3*, *GRIA4*, *DRD1D*, and *ADRA1A* receptor expression was altered after SR training and remained constantly decreased after the SMTS training protocol, while *GRIA2* and *DRD1A* decreased only under the SR condition. In the hippocampal formation, *GRIN2B* decreased and *GABARG2* receptor expression increased after SR training. After SMTS sessions, *GRIN2B* remained decreased, *GABARG2* remained increased if compared to the control group. None of the investigated receptors differed directly between both conditions, although differentially altered. The changes in both regions mostly occur in favor of the stimulus response task. Thus, the present data provide evidence that neurotransmitter receptor expression dynamics play a role in the avian prefrontal cortex and the hippocampal formation for behavioral training and is uniquely, regionally and functionally associated to cognitive processes including learning and memory.

**Keywords: hippocampus, prefrontal cortex, stimulus-response learning, reward learning, decision-making, associative learning, avian, quantitative real-time PCR**

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## INTRODUCTION

Learning and cognitive processes in mammals result in changes the way neurons communicate to support memory and/or other cognitive functions (Snowball et al., 2013; Kelly et al., 2014; Vijayvagharan et al., 2017; Cools and Arnsten, 2021; Faraza et al., 2021). Different mechanisms like long-term potentiation (LTP) and long-term depression (LTD) play a pivotal role in synaptic plasticity to strengthen or lower synaptic contacts of neurons. These processes are strongly linked to changes of the neurotransmitter receptor composition at the synapse, while receptors are differentially expressed in different brain areas and networks (Kandel et al., 2021 [1981]; Lein et al., 2007; Zilles et al., 2015; Zilles and Amunts, 2009; Zilles and Palomero-Gallagher, 2017; Palomero-Gallagher et al., 2020).

The prefrontal cortex and the hippocampus are two key brain areas involved in learning and cognitive processes. While the prefrontal cortex supports functions like working memory, response selection, decision making and reward-guided learning, reward evaluation and prediction (Miller and Cohen, 2001; Fuster, 2015 [1980]; Roberts et al., 1998; Friedman and Robbins, 2021), the hippocampus is predominantly involved in episodic memory, memory consolidation, spatial memory and navigation, pattern separation, context-dependent and associative learning (Brasted et al., 2003; Broadbent et al., 2004; Eichenbaum, 2004; Suzuki, 2005; Andersen et al., 2007; Bird and Burgess, 2008; Tyng et al., 2017). In mammals, it has been shown that different cognitive functions and the performance in cognitive tasks are associated with changes in neurotransmitter receptor binding (Zilles et al., 2000; Takahashi et al., 2008; McNab et al., 2009; Paoletti et al., 2013; Wass et al., 2018). Those studies have demonstrated in mammals that learning and consecutive exercise of cognitive tasks can result in an increase or a decrease of neurotransmitter receptors that was correlated to different behavioral tests, while less information was provided about altered subunit composition or even subtypes, which may change the signal propagation at the synapse. Further, it is yet still not fully understood how different learning processes and cognitive tasks are accompanied by receptor expression dynamics to support a certain behavioral change *via* learning processes and how this is associated to certain brain areas like the prefrontal cortex and the hippocampus.

In the avian brain, the nidopallium caudolaterale (NCL) is a brain structure at the caudal end of the forebrain that shares many cellular, anatomical, neurochemical, electrophysiological and functional similarities with the mammalian prefrontal cortex, although the evolution of the pallial organization and neuronal development in birds was separated from that of mammals during the past 310 million years (Waldmann and Güntürkün, 1993; Kumar and Hedges, 1998; Puelles et al., 2000; Güntürkün and Bugnyar, 2016; Medina et al., 2019; Stacho et al., 2020). Like the mammalian prefrontal cortex, the avian NCL is a highly interconnected associative forebrain area. It receives projections from all sensory modalities (Kröner and Güntürkün, 1999), projects to premotor areas (Kröner and Güntürkün, 1999), is one of the main targets of dopaminergic innervation (von Eugen et al., 2020) and shows similarities to the neurotransmitter receptor profile of prefrontal cortical areas of mammals (Herold et al., 2011). Similar to the prefrontal cortex, lesions of the NCL resulted in deficits in delayed alternation tasks, reversal learning and response selection (Mogensen and Divac, 1982; Hartmann and Güntürkün, 1998; Diekamp et al., 2002).

Electrophysiological recordings in the NCL proved working memory neurons firing during delay (Diekamp et al., 2002; Veit et al., 2014), which is accompanied by an increase of dopamine (Karakuyu et al., 2007), as well as firing of more general "executive control" neurons that encode what to forget and what to remember (Rose and Colombo, 2005). Further, reward-related neurons (Kalenscher et al., 2005; Koenen et al., 2013; Starosta et al., 2013; Dykes et al., 2018) and neurons that encode the association of new stimuli (Veit et al., 2015), numbers, categories or abstract rules were detected in the NCL (Kirsch et al., 2009; Veit and Nieder, 2013; Ditz and Nieder, 2015; 2016). Additionally, blockade of dopamine $D_1$-receptors in the NCL has been shown to decrease performance in reversal learning and working memory (Diekamp et al., 2000; Herold et al., 2008), and infusion of glutamatergic NMDA receptor antagonists showed a role for NMDA receptors in response selection and cognitive flexibility (Lissek and Güntürkün, 2004; Herold, 2010). It has also been shown that dopamine $D_1$-receptors were differentially expressed in the NCL and the striatum after behavioral training in a set of learning paradigms that distinguished stimulus-response association, response selection and maintenance of stimuli information like color, i.e., working memory (Herold et al., 2012). Based on these many resemblances between the mammalian prefrontal cortex and the NCL, the NCL is considered to be the avian prefrontal cortex (Güntürkün, 2005; Güntürkün and Bugnyar, 2016).

The avian hippocampal formation (HF) develops from the medial pallium and shares the origin with the mammalian hippocampus (Medina and Abbelan, 2009; Puelles, 2011). However, the avian hippocampal formation and the mammalian hippocampus show a different morphology (Atoji and Wild, 2004; Herold et al., 2014; Striedter, 2016). It is still under debate which subdivision of the avian hippocampal formation corresponds to its mammalian counterpart or even exists (Aboitiz et al., 2003; Colombo, 2003; Kempermann, 2012; Tosches et al., 2018; Bingman and Muzio, 2017; Barnea and Smulders, 2017; Herold et al., 2015; 2019). Apart from these controversial views, of what is known so far, cell types, connectivity and functionality of the hippocampal formation in birds largely matches the mammalian situation (Wieraszko and Ball, 1991; Colombo and Broadbent, 2000; Macphail, 2002; Bingman et al., 2005; Atoji and Wild, 2006; Shanahan et al., 2013; Herold et al., 2015, 2019; Gagliardo et al., 2020). Similar to mammals, the hippocampal formation of birds is involved in spatial cognitive processes and episodic memory and orientation that includes learning new routes/maps or food caches as well as recall familiar locations or behavioral inhibition (Sherry et al., 1992; Hampton and Shettleworth, 1996; Clayton and Dickinson, 1998; Mayer et al., 2010; Scarf et al., 2014; Gagliardo et al., 2020). These functions are further accompanied by adaptions in volume and adult neurogenesis (Smulders et al., 1995; Barnea and

Pravosudov, 2011; Barkan et al., 2017). Recordings of neurons in the hippocampal formation of birds revealed visually responsive cells (Scarf et al., 2016; Ditz et al., 2018), spatially responsive cells (Hough and Bingman, 2004; Sherry et al., 2017), place cells (Payne et al., 2021) and head direction cells (Ben-Yishay et al., 2021). Properties of avian hippocampal cells seem to be very similar to mammalian hippocampal cells, which is also reflected in the neurotransmitter receptor fingerprints of hippocampal regions across species (Kahn et al., 2008; Herold et al., 2014; 2015). In addition, pharmacological approaches have shown that the blockade of noradrenergic receptors results in deficits of memory formation, while NMDA-receptor blockade seems to be involved in memory consolidation but not formation (Gibbs et al., 2008). This is in line with the finding that LTP occurs in the hippocampus of birds, but seems to be independent of NMDA-receptor activation. This in turn implies a possible other form of synaptic plasticity in the hippocampus of birds (Margrie et al., 1998).

Taken together, both the avian prefrontal cortex and the hippocampal formation seem to be relevant for learning and memory, while the functional resemblance between prefrontal and hippocampal areas of mammals and birds and the underlying mechanisms are still poorly understood.

Here we aim to disentangle the role of receptor plasticity in the NCL and the HF after learning a rewarded response-association task and a stimulus-selection and -comparison task. The latter task includes the components of the first task but adds the component of stimulus comparison and selection, i.e., decision-making. Therefore, we measured the expression levels of different glutamatergic NMDA (*GRIN*) and AMPA (*GRIA*) receptor subunits, GABAergic *GABARG2* subunit, dopaminergic *DRD1A* (*DRD1*), DRD1B (*DRD5*), *DRD1D* serotonergic *5HTR1A* and noradrenergic *ADRA1A* in both areas with quantitative real-time PCR after training the birds in these two tasks. Apart from the above-mentioned involvement of these receptor types in different behavioral tasks tested with pharmacologically manipulated animals, we choose them for our investigation out of three different reasons:

(1) The ionotropic glutamatergic NMDA and AMPA receptors are known key receptors involved in LTP and LTD during learning (Bliss and Cooke, 2011). The function of NMDA glutamate receptors changes according to their sub composition which is rather complex as seven subunits with different isoforms exist. In a nutshell, NMDA receptors form heteromeric complexes (tetramers) with different subunits that can change the affinity for ligands and channel properties (Seeburg, 1993). Particularly the GRIN1/GRIN2B composition has been implicated in learning and memory of mammals and GRIN2B mediates higher cognitive function in the prefrontal cortex (Wang et al., 2013). For example, higher amounts of the GRIN2B subunit result in higher opening probabilities of the ion-channel compared to GRIN2C and both exhibit lower opening probabilities compared to GRIN2A, which results in different steady-state activities or "burst" activities (Wyllie et al., 2013). Further, reduced levels of GRIN2C in the medial prefrontal cortex of mice lead to higher inhibition, reduced dendritic spine density and impairments in working memory, associative learning and sensorimotor gating (Gupta et al., 2016). GRIN3A is critical to from long-term synaptic plasticity for memory consolidation, while the function of GRIN3B is less clear and mostly confined to motor neurons, where they are highly expressed in contrast to low cortical expression sites in mammals (Das et al., 1998; Niemann et al., 2007; Roberts et al., 2009; Pachernegg et al., 2012). AMPA receptors also form heterotetramers comprised out of the four subunits GRIA1-4 (Hollmann and Heinemann, 1994). They mediate synaptic strengthening subunit specific due to LTP and LTD (Kessels and Malinow, 2009). For example, increased levels of GRIA2/GRIA3 can be found in cortical synapses of mice when deprived from experience-dependent stimulation (Makino and Malinow, 2011). In the hippocampus of mammals, GRIA1 has been further described to be important for LTP maintenance (Jiang et al., 2021) and GRIA1 expression is important for short-term memory, while depletion of GRIA1 results in profound long-term memory (Bannerman et al., 2014).

(2) Neurotransmitters like noradrenaline, dopamine and serotonin act via their specific receptors as modulators of synaptic transmission and play a key role for different aspects in learning and memory. Both, the NCL and the HF receive dopaminergic, noradrenergic and serotonergic input (Reiner et al., 1994; Challet et al., 1996; Durstewitz et al., 1999). Thereby, metabotropic ADRA1A regulate hippocampal interneurons by depolarizing and releasing GABA, adult neurogenesis and short-term plasticity, while increased levels of ADRA1A improved LTP in the mouse hippocampus and cognitive performance (Perez, 2020). All dopamine receptor D1 subtypes are metabotropic and involved in reward-related learning processes and goal-directed behavior, in both, prefrontal and hippocampal areas (Goto and Grace, 2005; Herold et al., 2012; Puig et al., 2014). Hippocampal metabotropic 5HTR1A play a role in mice adult neurogenesis (self-renewal of precursor cells; Gould, 1999; Klempin et al., 2010), spatial learning (Glikmann-Johnston et al., 2015) and can be modulated in birds by ingestive and sleep behavior (Hoeller et al., 2013; Dos Santos et al., 2015).

(3) Because we found that *DRD1A* (*DRD1*), *DRD1D* receptor expression was altered in the NCL by a similar training protocol (Herold et al., 2012), we wanted to know if further interacting neurotransmitter receptors that can shape and spatially tune the signal transfer along dendrites are additionally affected, i.e., GRIN1 interacts with DRD1 and GABARG2 interacts with DRD5 (Wang et al., 2008; Puig et al., 2014).

We hypothesized that if receptor plasticity plays a role in these components of learning and cognitive functions, we would see altered expression levels as compared to untrained controls. Moreover, if the NCL and the HF would play a differential role in these functions, we would expect that the expression

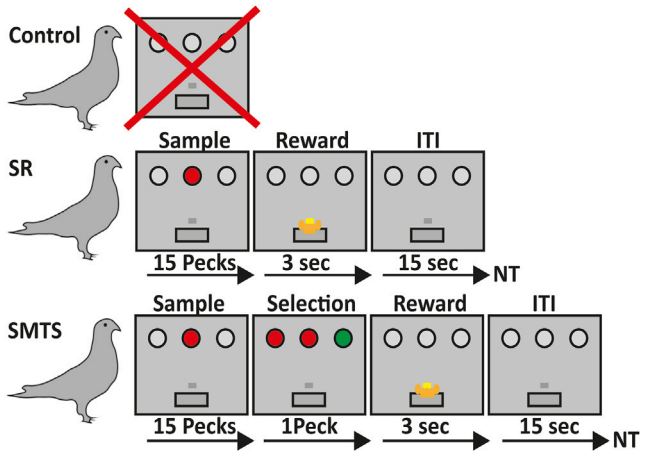


**FIGURE 1 |** Overview of the learning paradigms for the three pigeon groups. Birds in the control group did not participate in any operant task (line 1). Pigeons of the stimulus-response (SR)-group were trained to associate a stimulus with a reward after 15 pecks on either a red or green operant key on one of the three positions at the back wall of the Skinner box, which was then rewarded for 3 s with access to food *via* a food hopper. After that an intertrial interval (ITI) of 15 s began before the next trial (NT) started (line 2). In the simultaneous-matching-to-sample (SMTS)-group pigeons were trained to peck 15 times on the either red or green illuminated center key. Afterwards a choice period started, during which they had to select the lateral key that matched the sample color. During this phase all keys were simultaneously illuminated so that the birds were able to compare the stimuli and decide. Selection of the correct response resulted into 3 s access to food that was followed by an ITI of 15 s before the NT started (line 3).

levels change specifically according to the task. Additionally, we would be able to discover in detail which receptors or subunits are involved in these processes in the avian brain and compare it to mammals, to gain more insights into the fundamentals of learning.

# MATERIALS AND METHODS

## Experimental Design

Thirty adult, age-matched (3–4 years), unsexed pigeons (*Columba livia*) of the local stock were used in the experiments. Each test-group included ten pigeons. At the beginning, none of the birds had participated in an experiment before. The pigeons of the control group did not undergo any behavioral training, while the remaining two groups, the stimulus-response group (SR) and the simultaneous-matching-to-sample (SMTS) group, performed task dependent training sessions (**Figure 1**). During the experiments, all pigeons were housed in individual cages in a temperature-controlled room on a 12-h light-dark cycle. One week before the experiment started, all birds were food-deprived to 80% of their normal free feeding weights but had *ad libitum* access to water and grit. Animals were trained four to 5 days a week in an operant chamber. After each training session animals were compensated for food to nullify the differences between food-intake in the operant chamber between animals. The duration of sessions was equal for all stages according to the training task protocol. After reaching the learning criterion of the SR or the SMTS pigeons were directly used for brain tissue preparation. In parallel, pigeons of the control group were housed in individual cages for 8 weeks under the same conditions like the pigeons of the SR and the SMTS group before they were sacrificed. All pigeons were handled, controlled for weight every day and fed.

## Ethics Statement

The animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under adherence to the German laws to protect animals, and hence, the European Communities Council Directive of 18 June 2007. The experimental protocol was approved by national authorities and the Ethics Committee of the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) of North Rhine-Westphalia, Germany.

## Learning Environment

Two operant chambers (34 cm × 33 cm × 36 cm) were utilized for the learning procedures and behavioral training. Each chamber was controlled *via* a digital input-output board (CIO-PDISO8; Computer Boards, Inc. United States) and illuminated by a 24 W, centrally fixed light bulb. Three opaque operant keys (2 cm in diameter) with a distance of 10 cm in-between were located at the back panel of each box, 22 cm above the floor. The pecking keys were homogeneously illuminated either by white, red or green light, without matching the brightness of colors. White lights were used in the operant conditioning and pre-training sessions, while red and green lights were used during the test sessions in SR and SMTS tasks. The food-hopper, which was combined with a light-emitting diode, was fixed in the center of the back panel, 5 cm above the floor.

## Behavioral Tasks

The rational and the sequential procedure of the learning tasks is depicted in **Figure 1**.

### Pre-Training

During the first sessions pigeons received auto-shaping. After they started pecking the key during auto-shaping sessions, they were trained to peck reliably on the center key, whenever it was illuminated with white light. After a single peck, the light of the center key turned off, and the pecking was reinforced with three seconds access to food, followed by an inter-trial interval of 15 s. In the next steps, each trial began with the illumination of the center key. One peck on the lateral keys during this phase terminated the trial that was then followed by an inter-trial interval of 15 s and a retry of the trial. One training session included 80 trials with a 15 s inter-trial interval between each trial. In the following training sessions, the number of pecks required on the center key to extinguish the center light was constantly increased from 1 to 15 pecks. The criterion for the pre-training was 100% correct responses in one session. After this training, pigeons were randomly divided into two groups. One group was trained further in a colored stimulus-response task and the other group had to learn a simultaneous-matching-to-sample task.

### The Colored Stimulus-Response Task

After pre-training, pigeons were trained for a stimulus-response (SR) task with colored operant keys. For this step, they learned to peck reliably on one of the three keys (two lateral, on central), whenever it was illuminated with colored light. No discrimination or response selection of colors was involved. After 15 pecks, the light was turned off, and birds were rewarded with 3 s' access to food, followed by an inter-trial interval of 5 s. Illumination of the three keys was randomized to exclude a spatial bias for one of the keys. Pecking one of the dark keys caused punishment by a 10 s' time-out period during which all lights in the operant chamber were turned off. One session included 80 trials with a 15 s inter-trial interval between each trial. Before decapitating the animals for quantification of the different neurotransmitter receptor mRNA levels, all pigeons had to reach an overall criterion of 80% correct responses on three subsequent training days (SR-group). Here, the SR task included the association of a colored stimulus with a response, i.e., track a location of the colored key, repeat pecking to it 15 times to then obtain a reward, which further includes processes like certain reward expectation and reward consumption because a classical operant conditioning procedure was applied here (**Figure 1**).

### SMTS Task

After pre-training, the operant keys were illuminated with colored light and pigeons were trained to peck on the colored key like the SR-group. After reaching the criterion, the illumination of the central stimulus with either red or green light started each trial. The center light stayed on until the pigeon had pecked the key 15 times. Immediately thereafter, the two lateral choice keys were illuminated simultaneously, one in red and the other in green light, while the central key stayed on. Pigeons were rewarded after pecking the lateral illuminated choice key that matched the color of the simultaneously illuminated central key with 3 s' access to food, and were punished after pecking the non-matching key with a 10 s' time-out in darkness. No working memory of stimulus color information was required to perform the task because during the choice phase all keys stayed on. One session included 80 trials with a 15 s inter-trial interval between each trial. Training was conducted until the pigeons reached a performance level of 80% correct responses on three subsequent days. The colors of the keys were randomly presented to avoid that the pigeons learn a fixed sequence of presentation of the stimuli. Here, the SMTS task required the same motor skills like the SR task until the 15th peck on the central key. The learning component that came on top, in comparison to the SR-group, was that immediately after they pecked the central key 15 times, pigeons had to match the color of the central key to one of the lateral choice keys and then select and initiate a response to the identified key before they had the chance to obtain the reward (**Figure 1**). Thus, relative to the SR birds, the SMTS group had to learn a color dimension, discriminate and match between the two colors, which further included a response selection and decision-making task component. In contrast to the SR task, in the SMTS task the reward was not guaranteed but choice dependent.

## RNA Preparation and Quantitative Real-Time RT-PCR

For brain tissue preparation pigeons were deeply anesthetized with Equithesin (0.5 ml/100 g body weight, i.m.) and decapitated. Brains were quickly removed and stored on ice. The NCL and the HF (**Figure 2**) were dissected out, frozen in liquid nitrogen and stored at −80°C for later use. For the dissection, the pigeon brain was adjusted under a binocular microscope with a µm scale and cut into 2 mm thick slices. The NCL was prepared according to Herold et al. (2011). Because a large part of the half-moon-shaped NCL is located caudal from the stereotactic coordinate A 6.25, we used the slices caudal from this coordinate and removed the ventrally positioned arcopallium/amygdala complex (Herold et al., 2018). The tractus dorso-arcopallialis served for orientation because it is well visible in the native preparation. Next, we cut off the medial parts of the nidopallium, i.e., the nidopallium caudolaterale central and the nidopallium caudomediale as well as the overlaying dorsolateral corticoid area region that is, like the HF, separated from the NCL by the ventricle. Therefore, this sample consists mostly of NCL material. The HF was prepared according to Herold et al. (2014) as the most medial portion above the ventricle from atlas coordinates A 6.25 to A 3.00 along the anterior-posterior axis and M 0.00 to M 2.00 along the medial-lateral extension in coronal slices (Karten, 1967). Total RNA of all probes was extracted to process for real-time RT-PCR by using the NucleoSpin®RNA II Kit (Macherey-Nagel, Düren, Germany). RNA quality was checked for each probe photometrically. Because of technical issues, one HF-probe of the SR group and two HF-probes of the SMTS group were lost (not enough RNA could be eluted). For the rest of the probes, cDNA was obtained with the Superscript™II RT First Strand Synthesis System for RT-PCR (Invitrogen, Karlsruhe, Germany). For each probe 600 ng of total RNA was used for the RT reaction. Each probe was replicated twice.

Real-time PCR was performed on a LightCycler® 480 system (Roche, Mannheim, Germany) to determine the mRNA expression in the NCL or HF. For the preparation of the PCR standard reaction the protocol from LightCycler® FastStart DNA Master$^{PLUS}$ SYBR Green I (Roche, Mannheim, Germany) at a total volume of 20 µl was used. For each sample 1 µl cDNA diluted with 4 µl PCR-grade water was used as template for the reaction, with 10 µm forward and backward primers. Both targets and reference amplifications were performed in doublets in separate wells on a 96 well plate. The primers for the different neurotransmitter receptor subtypes and the "housekeeping genes" H3 histone3B (*H3-3B*) and Glyceralaldehyde-3-Phosphate dehydrogenase (*GAPDH*) used in the real-time PCR are listed in **Table 1**. The thermal cycling conditions included 10 min at 95°C pre-incubation, followed by 40 amplification cycles comprising 95°C for 10 s, 62°C for 10 s, and 72°C for 20 s, and one cycle for melting curve analysis comprising 95°C for 0 s, 65°C for 15 s, and 95°C with a slope of 0.1°C/s, followed by cool-down to at least 40°C. Under these conditions the efficiency for all primers was in the range of two, i.e., at maximum. In addition, the expression of the reference

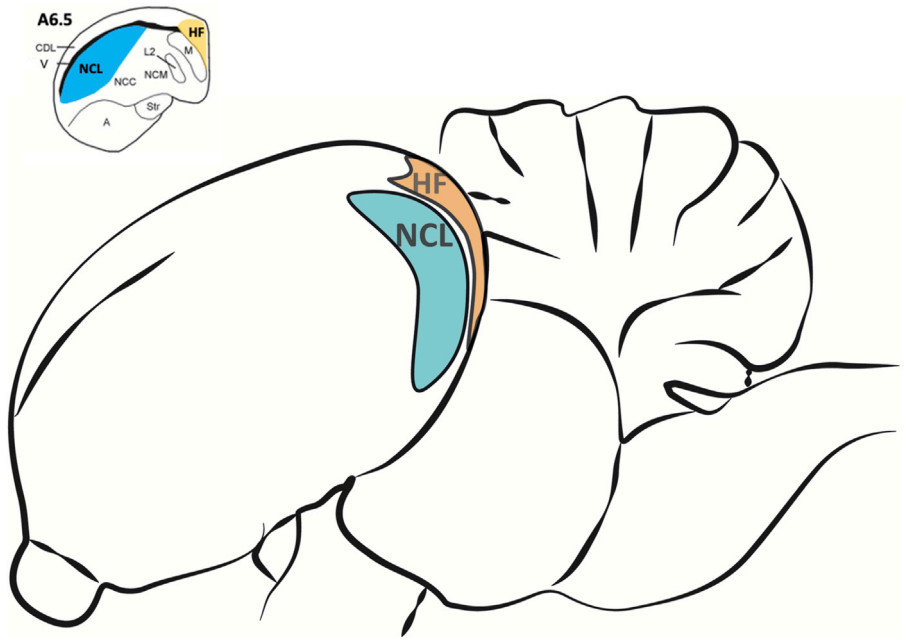


**FIGURE 2 |** Regions of interest for the analysis of different neurotransmitter receptor mRNA levels in the pigeon brain after learning. The nidopallium caudolaterale (NCL, blue) of pigeons is a half-moon shaped structure located laterally beneath the forebrain ventricle at the end of the caudal forebrain (see upper left). It is involved in executive functions and reward processing, comparable to the prefrontal cortex in mammals. The hippocampal formation (HF, orange) is located above the forebrain ventricle and begins at the most caudal level end of the avian telencephalon along the medial wall up to the anterior level A 9.00 (Herold et al., 2014). Here we only used HF samples caudal from A 6.50 (see upper left) because the largest portions of both, NCL and HF, are located beyond.

**TABLE 1 |** Primerpairs used for quantitative RT-PCR. Each primer pair binds specifically the indicated gene without cross-reactions. The obtained fragments were verified by sequence analysis.

| Gene | Forward primer 5′-3′ | Reverse primer 5′-3′ | GenBank accession # for amplicon | Size (bp) |
|---|---|---|---|---|
| *DRD1(D1A)* | ATACGCCGCATCTCAGCCTT | TCTGTTGCCGGTCGTGTTCT | XM_021289222.1 | 72 |
| *DRD5(D1B)* | TAGTCATGCCCTGGAAGGCG | ATGGAGGCCGTGGAACACAT | XM_021285849.1 | 106 |
| *DRD1D* | AGCCCCAAGAGCCATCAGAC | GGGTGATCGGGTTCCACACA | XM_021288368.1 | 84 |
| *GRIA1* | GCACTGAGAGGTCCCGTAAA | TAGAAAACCCCGGCCACATT | NM_001282812.1 | 170 |
| *GRIA2* | TACGGCATCGCCACACCTAAA | GGGCGCTGGTCTTCTCCTTAC | NM_001315518.1 | 165 |
| *GRIA3* | AAGGGCAAGTTCGCCTTCCT | CTTGGAATCCAGGTTGCCGC | XM_021290293.1 | 102 |
| *GRIA4* | CGTGTCCGCAAATCCAAGGG | TCCTTGGAGTCACCTCCCCC | XM_021286317.1 | 272 |
| *GRIN1* | GGAGGAAGATGCCCTGACCC | CCTTCTCCGATGCCGGAGTT | XM_013370181.2 | 78 |
| *GRIN2B* | GCCATGGCCCTCAGTCTCAT | GCCATGTTCTTGGCTGTCCG | XM_013367952.1 | 245 |
| *GRIN2C* | CGTCATACCGGGAGGCTTGT | CAGGTAGAGGGGCAGGTTGG | XM_021288660.1 | 98 |
| *GRIN3A* | GCTTTGCCGTCACAGAGACC | ATTCGTGGTAGGACCAGCCG | XM_021281606.1 | 139 |
| *GRIN3B* | CGACTCCGACTGCAAACTGC | AGATGCCCATCTGCAGGGTC | XM_021296971.1 | 221 |
| *GABAAG2* | GCTGCCTGAGCTGACGTTTC | AGAATGCAGTGCTCCCCAGG | XM_005503517.2 | 273 |
| *5HTR1A* | AAGCGGAGGATGGCTCTGTC | GCCACTTGGGCATGTAGCAC | XM_021284843.1 | 148 |
| *ADRA1A* | CCATCGGGCCTCTTTTTGGC | GGGCTCCTCGGTGATCTGAC | XM_021280945.1 | 74 |
| *GAPDH* | CCATGCCATCACAGCCACAC | GGCTGGTTTTTCCAGACGGC | NM_001282835.1 | 226 |
| *H3.3B* | GTGCAGCCATCGGTGCGCT | TGCGAGCCAACTGGATATCT | EU196043.1 | 128 |

genes was controlled in all groups. None of the groups showed regulation in *H3-3B* or *GAPDH* expression, and all expression data of the receptor subtypes were normalized to both of these reference genes.

Real-time PCR products were verified by melting curve analysis, 2% agarose gel electrophoresis (ethidium bromide staining), and sequence analysis on an ABI PRISM Genetic Analyzer 3100C (Applied Biosystems, Darmstadt, Germany). All products matched the expected base pair length and sequence and showed no cross reaction.

## Data Analysis

For quantitative analysis of real-time RT-PCR data, the levels of target gene expression were normalized to the levels of the

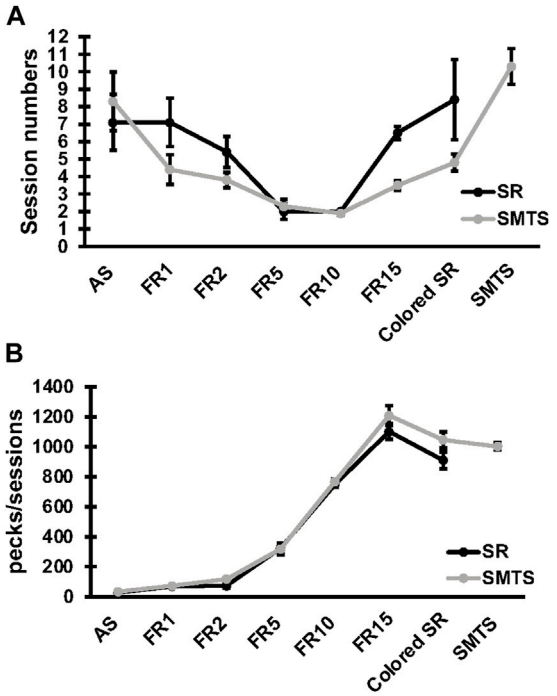


**FIGURE 3 |** Learning curve and pecking activity of the two pigeon groups. The learning curve in **(A)** shows the session numbers for each step of the behavioral training by means ± SEM for each group (N = 10, SR and SMTS). At the end of the training all pigeons reached a performance level of 80% correct responses in both tasks (SR and SMTS). Both groups performed an equal total amount of sessions. In parallel, in **(B)** the pecking activity during the learning periods is presented by means ± SEM for each group (N = 10, SR and SMTS). Abbreviations: AS, autoshaping; FR1 to FR15, fixed ratio 1 to 15; Colored SR, Stimulus-Response task including color; SMTS, Simultaneous-Matching-To-Sample task.

housekeeping genes histone *H3-3B* and *GAPDH* according to the following formula $2^{-\Delta CT}$ with ΔCT = (Average of target gene $CT_T$− average of housekeeping genes $CT_{HK}$ for each probe). The resulting values were multiplied with 100 to achieve the percentage expressed values of each target gene from the two housekeeping genes. These values for each receptor subtype or subunit and each region were than statistically compared between groups with a non-parametric analysis with group as an independent factor (Kruskal-Wallis-ANOVA) because data was not normally distributed (Shapiro-Wilks's test). When a group effect with Kruskal-Wallis was confirmed, a post-hoc analysis with Mann-Whitney- *U*-tests with continuity correction was conducted for pair-wise group comparisons resulting in corrected *p*- and z-values using Statistica 13.1 (StatSoft Europe GmbH, Hamburg, Germany). For the initial Kruskal-Wallis analysis between groups the *p*-level was set at 0.05. The post-hoc analysis results were additionally controlled with the Benjamini-Hochberg/Simes procedure for multiple comparisons for each individual gene and three groups that were tested. The analysis revealed that the acceptance *p*-level for a significant result of post-hoc analysis for the first comparison was $p < 0.0166$, for the second $p < 0.033$ and for the third $p < 0.05$.

# RESULTS

Specific changes in expression for receptor subtypes and subunits in the NCL and the HF were observed in both experimental groups after a learning period of 9 ± 3 weeks (mean ± SD) of training (SR-group: 39 ± 13 total number of sessions in all learning tasks; SMTS-group: 40 ± 6 total number of sessions in all learning tasks, **Figure 3A**) when compared to control pigeons, which were untrained but had the chance to randomly peck grit and move in their cages. In addition, the number of pecks per session in each task was elucidated (**Figure 3B**). The mean number (over all learning tasks) of pecks per session was 3,252 ± 103 in the SR-group and 4,568 ± 136 in the SMTS-group, which additionally had to perform in the SMTS-task compared to the SR-group und thus carried out more pecks (**Figure 3B**). Until this point no differences in pecking activity could be observed (**Figure 3B**).

In the NCL the glutamatergic, dopaminergic and noradrenergic receptors showed significant changes, while in the HF the glutamatergic and the GABAergic receptors were altered. All expression data is presented as relative expression (%) of the two housekeeping genes *H3-3B* and *GAPDH*.

## Neurotransmitter Receptor Plasticity in the NCL After Associative Learning and Stimulus-Selection/Discrimination

The individual analysis with Kruskal-Wallis tests between groups for glutamatergic NMDA- and AMPA-receptor expression of the NCL showed significant differences for the *GRIN2B* subunit ($H = 16.42$, $df = 2$, $N = 30$, $p = 0.0009$), *GRIA2* ($H = 6.89$, $df = 2$, $N = 30$, $p = 0.0319$), *GRIA3* ($H = 15.25$, $df = 2$, $N = 30$, $p = 0.0005$) and *GRIA4* ($H = 8.19$, $df = 2$, $N = 30$, $p = 0.0166$), while the other glutamatergic receptors showed no effects between groups (*GRIN1*, *GRIN2C*, *GRIN3A*, *GRIN 3B*, *GRIA1*; $p > 0.05$).

Post-hoc analysis with Benjamini-Hochberg/Simes correction revealed that *GRIN2B* expression decreased in both experimental groups (SR: $z = 3.74$, $p = 0.0002$; SMTS: $z = 3.14$, $p = 0.0017$) as compared to the control condition (**Figure 4B**). *GRIA2* decreased in the SR-group ($z = 2.68$; $p = 0.007$), while no effect was observed after SMTS-training (**Figure 4G**). *GRIA3* (SR: $z = 3.59$, $p = 0.0003$; SMTS: $z = 2.99$, $p = 0.0028$) and *GRIA4* were decreased in both experimental groups (SR: $z = 2.53$, $p = 0.0113$; SMTS: $z = 2.31$, $p = 0.0211$; **Figures 4H,I**).

The monoaminergic receptor expression differed between the three pigeon groups for the *ADRA1A* ($H = 13.22$, $df = 2$, $N = 30$, $p = 0.0013$), the *DRD1A* ($H = 8.83$, $df = 2$, $N = 30$, $p = 0.0121$) and *DRD1D* ($H = 9.45$, $df = 2$, $N = 30$, $p = 0.0089$) mRNA levels as revealed by Kruskal-Wallis tests.

The post-hoc analysis with subsequent Benjamini-Hochberg/Simes correction showed that ADRA1A were less expressed in the SR-group ($z = 3.36$, $p = 0.0008$) and SMTS-group ($z = 2.61$, $p = 0.0091$; **Figure 4L**) than in the control group. *DRD1A* decreased in the SR-group ($z = 2.98$, $p = 0.0028$; **Figure 4M**), while *DRD1D* was down-regulated in the SR- and the SMTS-group (SR: $z = 2.49$, $p = 0.0126$; SMTS: $z = 2.68$, $p = 0.0073$; **Figure 4O**) More general, in control pigeons, the highest expression levels were observed for

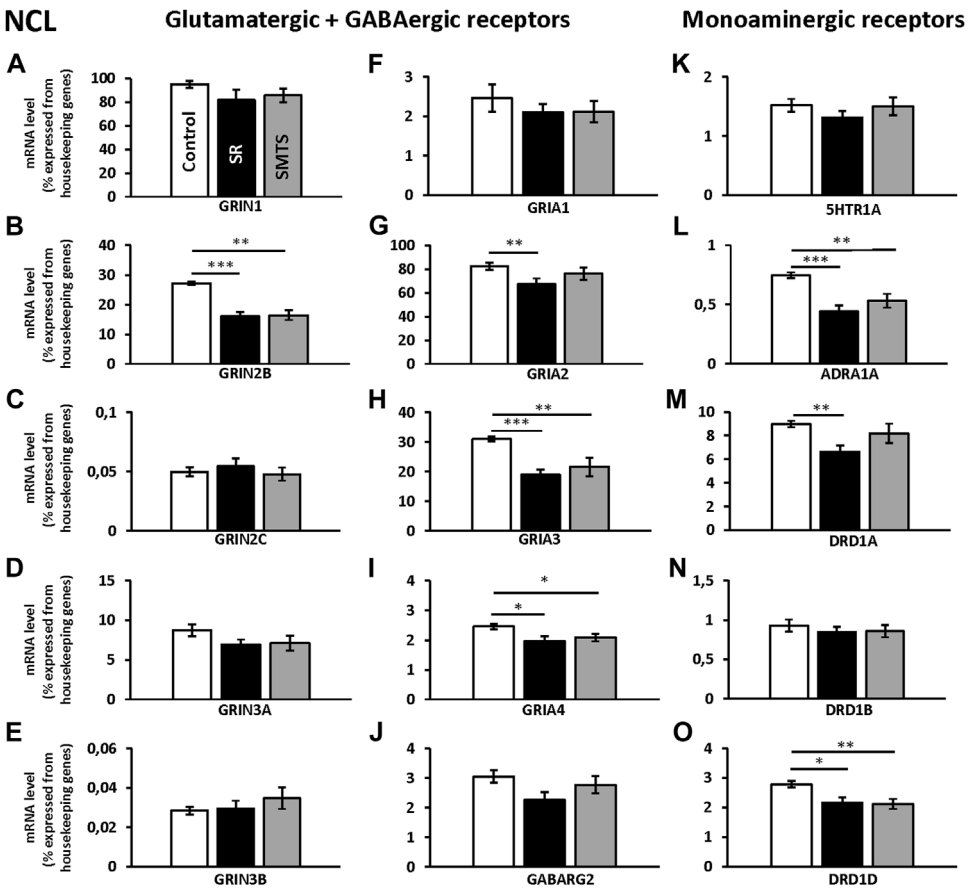


**FIGURE 4 |** Neurotransmitter receptor expression in the nidopallium caudolaterale (NCL) after learning different tasks. The mRNA levels of 15 neurotransmitter receptors and subunits were quantified as relative expression levels in percent expressed of the two housekeeping genes *H3-3B* and *GAPDH*. Results for glutamatergic and GABAergic receptor mRNA levels in the control group (Control, white), the stimulus-response group (SR, black) and the simultaneous-matching-to-sample group (SMTS, grey) are presented in **(A–J)** and monoaminergic receptor mRNA levels in **(K–O)** (mean ± SEM; N = 10 for each group). Significant differences between the different learning groups are highlighted with asterisks (*$p < 0.03$; **$p < 0.01$; ***$p < 0.001$).

*GRIN1* and *GRIA2* (**Figures 4A,G**), while the *GABARG2* subunit, i.e., was about 32 times lower expressed compared to *GRIN1*, and 27 times lower compared to *GRIA2* in the NCL (**Figure 4J**). From the monoaminergic receptors *DRD1A* showed the highest expression levels in the control group, followed by *DRD1D* and *5HTR1A* (**Figures 4K,M,O**).

## Changes in Expression Rates of Receptors in the HF After Associative Learning and Stimulus-Selection/Discrimination.

The comparisons with Kruskal-Wallis tests for the receptor expression rates in the HF of the three experimental pigeon groups showed differences for *GRIN2B* (H = 9.79, df = 2, N = 27, $p = 0.0075$), *GRIN3A* (H = 6.68, df = 2, N = 27, $p = 0.0354$), *GRIA3* (H = 6.05, df = 2, N = 27, $p = 0.0484$), *GABARG2* (H = 8.26, df = 2, N = 27, $p = 0.0161$) and *5HTR1A* (H = 6.42, df = 2, N = 27, $p = 0.0404$).

Post-hoc analysis and subsequent Benjamini-Hochberg/Simes correction showed that glutamatergic *GRIN2B* (SR: z = 2.74, $p = 0.0062$; SMTS: z = 2.44, $p = 0.0145$) were decreased under both training conditions compared to the control group, while *GRIN3A* levels have to be considered as not significant different (Control vs. SMTS: z = 2.36, $p = 0.0185$, n.s; **Figures 5B,D**). After correcting for multiple comparisons, *GRIA3* expression levels also showed no significant differences (Control vs. SR: z = −2.25, $p = 0.0247$, n.s; **Figure 5H**). GABAergic *GABARG2* subunit expression was increased in the SMTS-group (SMTS: z = −2.71, $p = 0.0067$; **Figure 5J**), while the comparison to the SR-group failed barely the significance criterion (SR: z =−2.08, $p = 0.0373$, n.s). The expression rates for *5HTR1A* receptors after SR-training had to be further considered equal after pair-wise comparison (SR: z = −2.25, $p = 0.0247$, n.s.; **Figure 5K**), while the other monoaminergic receptors also showed no effects (**Figures 5L–O**).

Regarding the glutamatergic receptors, the highest relative expression was detected for *GRIA2* (**Figure 5G**) followed by *GRIN1* (**Figure 5A**) and *GRIA3* (**Figure 5H**), while the lowest levels were measured for *GRIN2C* (**Figure 5C**) and *GRIN3B* (**Figure 5E**) in the HF of the control group. Overall,

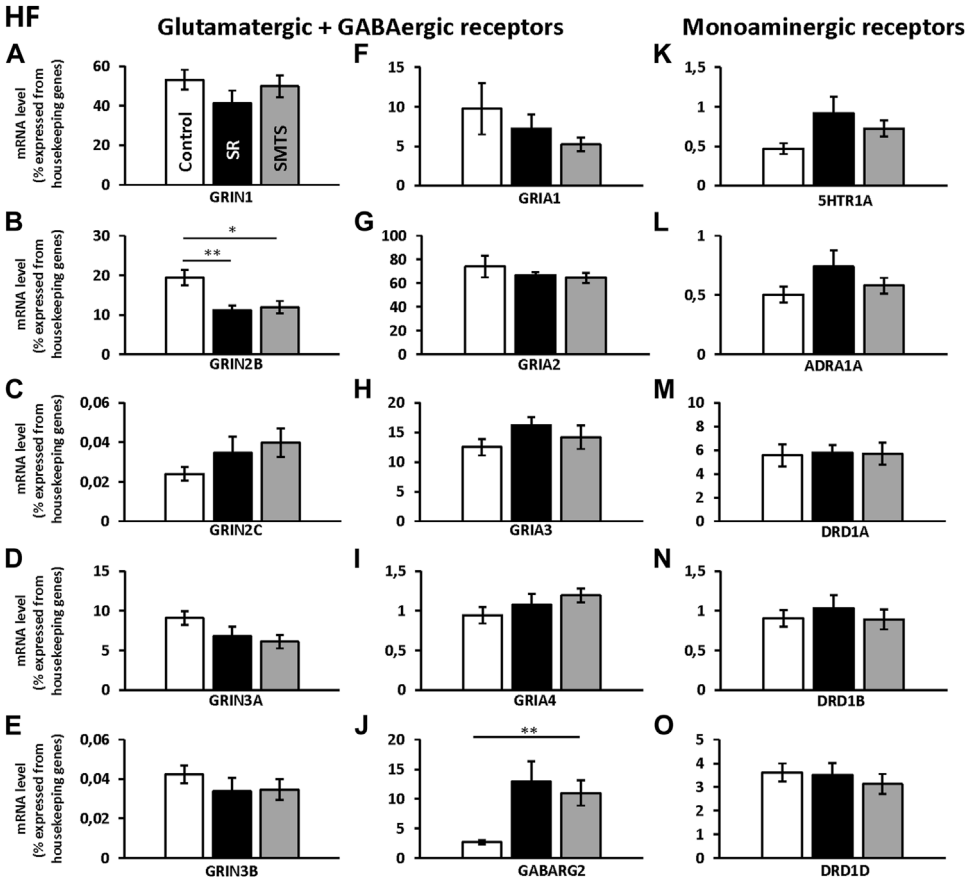


**FIGURE 5 |** Neurotransmitter receptor expression in the Hippocampal formation (HF) after learning different tasks. The mRNA level of 15 neurotransmitter receptors and subunits were quantified as relative expression levels in percent expressed of the two housekeeping genes *H3-3B* and *GAPDH*. Results for glutamatergic and GABAergic receptor mRNA levels in the control group (Control, white), the stimulus-response group (SR, black) and the simultaneous-matching-to-sample group (SMTS, grey) are presented in **(A–J)** and monoaminergic receptor mRNA levels in **(K–O)** (mean ± SEM; Control: N = 10; SR: N = 9; SMTS: N = 8). Significant differences between the different learning groups are highlighted with asterisks (∗$p < 0.03$; ∗∗$p < 0.01$).

GABAergic *GABARG2* subunit expression was 28 times lower compared to *GRIA2* and 20 times lower compared to *GRIN1* expression. From the monoaminergic receptors, *5HTR1A*, *ADRA1A* and *DRD1B* showed the lowest expression rates in the HF of the control group (**Figures 5K,L,N**) and highest levels were seen for *DRD1A* and *DRD1D* (**Figures 5M,O**).

## DISCUSSION

The study demonstrated receptor changes of different ionotropic and metabotropic neurotransmitter receptor subunits and subtypes in the NCL and HF of pigeons that were trained in two cognitive tasks: a rewarded color stimulus-response association task and a simultaneous matching to sample task that includes cognitive processes like stimulus-comparison and selection.

In the NCL, the NMDA-receptor subunit *GRIN2B*, the AMPA-receptor subunits *GRIA2*, *GRIA3* and *GRIA4*, the noradrenergic receptor subtype *ADRA1A* and the dopaminergic receptor subtypes *DRD1A* and *DRD1D* gene expression responded with a decrease after forming the rewarded stimulus-response association and behavioral training. The expression of these receptor was also decreased after SMTS training when compared to the control group, except for *GRIA2* and *DRD1A*, which showed no longer a difference to control levels after ongoing behavioral training to discriminate between two stimuli. In the HF, the mRNA levels of the NMDA-receptor subunit *GRIN2B* decreased after both, SR and SMTS training, while the $GABA_A$-receptor subunit *GABARG2* increase was indicated after training of the operant stimulus-response task, but was statistically confirmed only after SMTS training.

To our knowledge, this is the first study that explored receptor expression dynamics of different neurotransmitter systems in the avian brain in dependence on learning and training of different tasks that constitute fundamentals in learning and cognition. The results underpin the importance of the avian prefrontal cortex and the HF to form stimulus-response associations that combines aspects of reward expectation and reward consumption with

learning to respond to a certain stimulus on the one side, and to perform selective responses and stimulus-comparisons after the association has been established on the other. In both structures, receptor expression was initially altered after SR training. This "receptor state" seems to be mostly sustained and only slight adjustments have been found after SMTS training, while no differences between both learning paradigms were observed. However, specific receptor subtypes and subunits were altered to shape synaptic contacts according to the behavioral training and the expression levels changed differentially in the NCL and the HF. These results would argue against effects that could have been observed due to handling of the pigeons or higher pecking activity of the SR and SMTS group when compared to the control group. Further, pecks per sessions were equal between the two training groups until pigeons had to perform the SMTS-task. Nevertheless, we have to admit that SMTS- pigeons, although they did not perform in more sessions, executed more pecks in total compared to the SR-group and thus we cannot completely exclude that pecking (physical) activity influenced the expression of receptors in the SMTS-group. However, we would see other brain structures like the basal ganglia, the cerebellum or parts of the pre-motor arcopallium/amygdala complex predominantly involved in such motor behavior (Goodman et al., 1982; Yuan and Bottjer, 2020; Tian et al., 2022). Moreover, an earlier study of dopamine receptor expression after SR, SMTS and Delay-Matching-to-Sample training has shown that expression levels of receptors can change specifically according to different demands of cognitive tasks or learning components (Herold et al., 2012). Together, receptor expression changes can be associated with behavioral training and altered receptor expression may influence signal propagation at the neuronal level differentially in dependence of the structural involvement in a specific task.

## Receptor Plasticity in the Nidopallium Caudolaterale After Associative Learning and Stimulus Discrimination Training

The study showed that receptor plasticity in the NCL is linked to associative learning, which includes processes such as pairing the stimulus and the response, reward expectation and consumption and attention. In line with the present data, a role for the NCL in these processes has been confirmed in behavioral, pharmacological as well as electrophysiological experiments in different bird species (Koenen et al., 2013; Starosta et al., 2013; Veit et al., 2015; Kasties et al., 2016). Under the control condition, high levels of the NMDA receptor *GRIN1*, *GRIN2B*, AMPA receptor *GRIA2* and *GRIA3* subunits were detected, while the monoaminergic receptors were relatively low expressed, with the highest amounts found for *DRD1A* receptors. These findings are comparable to the receptor protein levels of NMDA and AMPA receptors, which were earlier investigated with non-selective ligands for specific subunits or subtypes (Rehkämper and Zilles, 1991; Herold et al., 2011). Additionally, low expression levels for the $GABA_A$ receptor *GABARG2* subunit in the NCL were found, which corresponds to the general lower receptor protein concentrations of $GABA_A$ receptors in the NCL compared to NMDA and AMPA receptors (Herold et al., 2011).

## Glutamatergic Receptors

After stimulus-response learning the receptor subunit/subtype expression profile of the NCL changed. From the nine investigated glutamatergic receptor subunits, four decreased (*GRIN2B, GRIA2, GRIA3, GRIA4*). Here, further evidence is provided that the decrease of different neurotransmitter receptors may be evoked by learning of the operant conditioning task and consecutive training to respond to a visual stimulus.

Considering that the changed glutamatergic neurotransmitter receptors are usually involved in increasing neuronal activity, it can be assumed that a decrease in transcripts would lead to a decrease in excitation, signaling or global firing activities. Accordingly, a lower activity has been reported in electrophysiological recordings of NCL neurons responding to familiar stimuli compared to novel stimuli in crows (Veit et al., 2015). In rabbits and rodents, it has been shown that the mPFC controls the performance of selected behaviors against unwanted ones by decreasing or increasing global firing activities of prefrontal projection neurons and that high frequency stimulation in the mPFC evokes significant long-term depression (Leal-Campanario et al., 2013; Gruart et al., 2015). Particularly, LTD is associated with both, the expression of the *GRIN2B* receptor subunit and the internalization of AMPA receptors (Brigman et al., 2013; Paoletti et al., 2013; Shin et al., 2020). Both receptors also play an important role in synaptic plasticity that is linked to associative learning (Gray et al., 2011; Paoletti et al., 2013; Mukherjee et al., 2017), which may explain decreased levels of *GRIN2B*, *GRIA2*, *GRIA3* and *GRIA4* in the NCL observed here. However, all glutamatergic receptors are also expressed in glia cells and proliferation and gliogenesis was increased in the medial prefrontal cortex of rats during learning of an operant stimulus-response task (Rapanelli et al., 2010). Because our tissue sampling method did not allow for differentiation between glia and neuronal cells, the glutamatergic receptor mRNA of glia cells might have additionally contributed to our measurements. So far, at least in mammals, it is known that astrocytes play an important role to regulate network plasticity and the function of neuronal networks (Mederos et al., 2018; Durkee and Araque, 2019; Lyon and Allen, 2021). In birds less is known about the role of glia cells during learning, but it was shown that a food caching bird like the blacked-capped chickadees exhibited more glial cells in the hippocampal formation if they had lived in the wild compared to lab reared birds (Roth et al., 2013). Roth and colleagues concluded that this may rely on individual experience and environment. Thus, the exact role of glia cell function in learning and memory in birds might be a very interesting topic for future research.

## Monoaminergic Receptors

The decrease in *DRD1A* and *DRD1D* receptors after SR training confirmed earlier results and is likely associated to regular bouts of rewards and dopaminergic stimulation in the NCL after extended periods of training (Herold et al., 2012). This

assumption is further supported by expression analysis of D1 and D2 receptors in the nucleus accumbens of zebra finches that show relative expression changes with regard to the experience of a rewarding behavior (Alger et al., 2022).

The finding of a decrease of the noradrenergic receptor subtype *ADRA1A* after SR training adds a new aspect. In general, *ADRA1A* receptors are involved in certain aspects of synaptic plasticity, learning and memory like regulation of adult neurogenesis (which occurs in the nidopallium of birds (Melleu et al., 2016)), gliogenesis, LTP and LTD (Gupta et al., 2009; Perez, 2020). Apart from neurons, where *ADRA1* are expressed at all sides but predominantly at axon terminals (Mitrano et al., 2012), *ADRA1A* can be also expressed in stem cells or glia cells, while they were not detected *in vivo* in cerebral blood vessels, in contrast to the periphery where the receptor can be found in vascular smooth muscle (Papay et al., 2006; Perez, 2020). Thus, we cannot exclude that a small amount of our probe constitutes also stem cell and glia cell *ADRA1A* mRNA.

In birds, treatments with non-subtype-selective ADRA1 agonists have been shown to inhibit the consolidation of memory if applied to the intermediate medial mesopallium of chicks (Gibbs and Summers, 2001). In addition, application of low doses into the nucleus interfascialis of the nidopallium (NIf) of zebra finches increased the auditory response of neurons in the higher vocal center (HVC), which is a major target structure of Nlf neurons, while higher doses suppressed activity in both, NIf and HVC (Cardin and Schmidt, 2004). A non-subtype-selective ADRA1 antagonist infused in the NIf prevented the arousal mediated suppression of HVC auditory responses that demonstrated the critical involvement of these receptor types in attentional modulation (Cardin and Schmidt, 2004). Consistent with the latter finding it seems to be very likely that *ADRA1A* plasticity in the NCL is modulated by the attentional requirements of the SR task and that the decrease is a result of persistent training to pay attention to a certain stimulus. It may also modulate local as well as long range neural activity, but this has to be further studied as well as further noradrenergic receptor subtypes.

## Stimulus-Response Versus Simultaneous-Matching-To-Sample

In contrast to SR learning, adding new task components, i.e., training the discrimination between two stimuli, including processes like stimulus comparison and response selection (or decision-making), did not affect the expression profile of the analyzed receptors in the NCL differentially, when compared to the SR-group. Receptors in the SMTS-group were either decreased similar to the SR-training or showed no changes in expression levels like *GRIA2* and *DRD1A* as compared to the untrained pigeons. This is in line with earlier data for concentrations of different D1 receptor subtypes and the D2 receptor in the NCL and striatum (Herold et al., 2012). Therefore, we would assume that receptor plasticity in the NCL is more important for the initial operant training and consolidation of relevant reward-related stimulus information, but not to stimulus comparison and selection *per se* that may depend on other neural mechanisms. Finally, changes in favor of the SR-task compared to the SMTS-task, which has a higher cognitive load might also reflect a difference in encoding the reward value, which is guaranteed in the SR-task after pecking a visual stimulus, while the SMTS-task holds the chance to fail. This point would be in line with electrophysiological recordings that have shown that the encoding of the reward value was most prominent during the sample period, i.e., presentation of a visual key that is associated to reward (Dykes et al., 2018).

## Receptor Plasticity in the HF After Associative Learning and Stimulus Discrimination Training

The receptor subtype/subunit expression was also specifically altered after associative learning in the HF but revealed different expression dynamics compared to the NCL. In addition to the considerably plastic nature of the avian HF forming constantly new neurons in association with learning and memory and showing volume changes (Patel et al., 1997; Barnea and Pravosudov, 2011; Hall et al., 2014), the present study indicates that the function of the avian HF further relies on changes in neurotransmitter receptor expression. These findings confirm that receptor changes in the HF of birds are involved in training of an associative stimulus-reward task (Reilly and Good, 1989; Bingman, 1993). Control pigeons showed high mRNA levels of the glutamatergic NMDA receptor subunits *GRIN1* and *GRIN2B* and the AMPA receptor subunits *GRIA2* and *GRIA3*, while from the monoaminergic receptors, the dopaminergic *DRD1A* receptor subtype showed the highest concentration in the HF. This fits well with the receptor protein profile obtained by receptor autoradiography (Herold et al., 2014) and detection of all AMPA receptor types in the HF of pigeons (Rosinha et al., 2009). The low expression levels for the $GABA_A$ receptor *GABARG2* subunit in the HF is also in line with a general lower receptor concentration of $GABA_A$ receptors in the HF that was reported with Muscimol-binding to the $\alpha$-subunits of the $GABA_A$ receptor when compared to NMDA and AMPA receptors (Herold et al., 2014). The low mRNA levels of *5HTR1A* in the HF of pigeons support earlier findings of an *in-situ* hybridization study of expression patterns for *5HTR1A* in the brain of chicks (Fujita et al., 2020). The discrepancy between mRNA levels and protein levels of the HTR1A, which showed high densities of binding sites in both, HF and the NCL compared to other monoaminergic receptors, can be explained by the fact that the ligands access the protein levels binding both, pre- and postsynaptic sites, while *5HTR1A* mRNA cannot be detected in axon terminals (Vilaro et al., 2020). Overall, *GRIN1*, *GRIN2C*, *GRIA3*, *5HTR1A* and *DRD1A* levels were lower, *GRIN3B* and *GRIA1* were higher and *GRIN2B*, *GRIN3A*, *GABARG2*, *ADRA1A*, *DRD1B* (*DRD5*) and *DRD1D* were similarly expressed in the HF of the control group when compared to the NCL. These differences in receptor expression profiles are in line with the findings of individual receptor fingerprints for both brain areas (Herold et al., 2011; Herold et al., 2014).

## Glutamatergic NMDA2B Receptors

After training in the SR task, we found decreased levels for the glutamatergic subunit *GRIN2B* in the HF. Here, *GRIN2B* showed a

comparable decrease to the NCL. Therefore, it could be argued that the decrease may serve a similar function for SR and SMTS in both regions. In mice, the loss of GRIN2B receptors in the hippocampus and cortex resulted in abolished NMDA-dependent LTD as well as in impairment in several cortico-hippocampal mediated tasks like T-Maze spontaneous alternation, water maze and pavlovian trace fear conditioning (Brigman et al., 2010; Shin et al., 2020). Additional findings in the hippocampus also described the critical contribution of GRIN2B for NMDAR channel function and the maintenance and formation of dendritic spines (Akashi et al., 2009). In humans, *GRIN2B* also plays a role in response inhibition which is mainly controlled by cortico-basal ganglia circuits (Beste et al., 2010). In our view, we can only speculate that the reduction in *GRIN2B* in the HF and the NCL could have facilitate general learning-dependent, but task-independent processes that were evoked by global glutamate release during the training that can result in decreased *GRIN2B* levels. This may be supported because in hippocampal slices of rats it was observed that specifically GRIN2B containing NMDA receptors monitor glutamate released from multiple sources, i.e., afferent inputs, and thus monitor the level of overall activity level in a network and regulate the strength of glutamatergic synapses (Scimemi et al., 2004). In contrast, *GRIN2A* containing NMDA receptors seem to be more important to sense "local" glutamate signaling (Scimeni et al., 2004) and so it would be of interest for future studies to investigate the expression of *GRIN2A* as well. In addition, electrophysiology studies revealed that although stimulus-responsive neurons have been detected in the HF of crows and pigeons (Scarf et al., 2016; Ditz et al., 2018), studies in the HF of pigeons have revealed less stimulus-specific neurons (Scarf et al., 2016). It has to be also considered that stimulus discrimination in the HF of pigeons is further highly associated with a spatial component (Coppola et al., 2014). Further, a new study suggested that the function of the HF in memorizing properties, or more precisely probabilities of reward occurrences, seems to be associated with encoding of the spatial location (Sizemore et al., 2021). Thus, this might also support the theory that a reduction of *GRIN2B* is more correlated to global behavioral learning phenomena.

## GABAergic $GABA_A$ Receptors

It is well accepted that $GABA_A$ receptor signaling is critically involved in regulating synaptic inhibition in the hippocampus of mammals and that the GABARG2 subunit is important for controlling efficacy of synaptic inhibition and spatial learning (Kittler et al., 2008; Tretter et al., 2009). Moreover, $GABA_A$ receptors and expression of the *GABARG2* subunit is necessary for developmental neurogenesis as well as for adult neurogenesis in the hippocampus (Earnheart et al., 2007; Jacob et al., 2008). The present data of increased levels of *GABARG2* in the pigeons HF after SR as well as after SMTS training demonstrate that this might also apply for pigeons and perhaps more general, in birds. Whether the increase was a result of increased neurogenesis after learning or shaped the efficacy of synaptic inhibition needs to be further investigated. In fact, adult neurogenesis in the HF of birds was stimulated by learning and memory processes (Patel et al., 1997; Barnea and Pravosudov, 2011; Hall et al., 2014) as well as environmental enrichment (Melleu et al., 2016), which makes it likely that the training and stimulation of neurogenesis in the cognitive tasks played a role for increased *GABARG2* subunit expression in the pigeon HF. Because our probe also included glia cells that can express $GABA_A$-receptors the increase might be partly induced by gliogenesis (Mederos and Perea, 2019). However, in rats it has been shown that learning and training of a rewarded operant conditioning task resulted only in increased astrogliogenesis in the medial prefrontal cortex, but not in the hippocampus where neurogenesis occurred (Rapanelli et al., 2011). The findings of Rapanelli and colleagues would then speak against a participation of glial *GRIN2B* subunit expression to the decrease observed in the HF here.

## Stimulus-Response Versus Simultaneous-Matching-To-Sample

Both, *GRIN2B* and *GABARG2* subunits were either reduced or increased after SMTS training comparable to the effects observed after SR training. Similar as for the NCL, no significant differences were observed between the SR and SMTS condition. This may imply that the behavioral training in general resulted in changes of receptor expression in the HF and NCL but changed the level of neurotransmitter receptors and their subunits differentially. According to our results the changes occurred mainly in favor of the SR-task. It has to be emphasized that the SMTS-task included all behavioral skills and cognitive requirements of the SR-task plus cognitive functions like stimulus comparison, response selection (i.e., decision-making) and reward evaluation (uncertainty). Therefore, it may be hypothesized that the absence of differences between SR and SMTS training suggests that neurotransmitter receptor changes in the NCL and the HF do not have such a high value for encoding functions like response selection or stimulus-comparison. Because damage to the pigeon hippocampus has been shown to have an impact on operant-conditioning (Good and MacPhail, 1994; Colombo et al., 2001), it is in line with our data that we found effects after the stimulus-response learning and after the SMTS-learning (because the operant-conditioning procedure preceded both tasks) but no effects between both SR- and SMTS. This would further correspond with lesion data that show that the hippocampus is not involved in visual-discrimination or response selection per se (see Colombo and Broadbent, 2000 for review and; Colombo et al., 2001). On the other hand, this might imply that synaptic processes that increase performance in those functions might be possibly driven by other neural mechanisms or factors like for example brain derived neurotrophic factor (BDNF) or cAMP response element binding protein (CREB) expression and do not underly plastic changes at the level of neurotransmitter receptors. Clearly, further research in the field is needed to elucidate this question.

# CONCLUSION

The data of the present study emphasize the role of NCL and HF in stimulus-response learning and related neurotransmitter receptor expression and plasticity in both brain structures. This adds new insights regarding information processing and learning mechanisms

in the bird brain. Many parallels between mammalian and avian prefrontal and hippocampal structures have been elucidated for the first time including the expression of specific receptor subtypes and subunits as well as their role in stimulus-response learning and attentional processes in an avian model that is frequently used for comparative studies: the pigeon. The basic principles of learning seem to be conserved between species with some degrees of freedom may exist that besides adaption and specialization still result in incredible abilities of learning and memory of birds.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) of North Rhine-Westphalia, Germany.

## AUTHOR CONTRIBUTIONS

CH designed the experiments. PO conducted the real-time PCR experiments. CH and PO analyzed the data. CH wrote the manuscript and designed the figures. CH, PO, and KA contributed to the interpretation of the data. All authors contributed to the manuscript.

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# The Acute Pharmacological Manipulation of Dopamine Receptors Modulates Judgment Bias in Japanese Quail

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We have studied the effects of dopamine antagonists and agonists on Japanese quail behavior in the spatial judgment task. Twenty-four Japanese quail hens were trained in the spatial discrimination task to approach the feeder placed in the rewarded location (Go response, feeder containing mealworms) and to not approach the punished location (No-Go response, empty feeder plus aversive sound). In a subsequent spatial judgment task, the proportion of Go responses as well as approach latencies to rewarded, punished, and three ambiguous locations (near-positive, middle, near-negative, all neither rewarded nor punished) were assessed in 20 quail hens that successfully mastered the discrimination task. In Experiment 1, each bird received five treatments (0.1 and 1.0 mg/kg of dopamine D1 receptor antagonist SCH 23390, 0.05 and 0.5 mg/kg of dopamine D2 receptor antagonist haloperidol, and saline control) in a different order, according to a Latin square design. All drugs were administered intramuscularly 15 min before the spatial judgment test, with 2 days break between the treatments. Both antagonists caused a significant dose-dependent increase in the approach latencies as well as a decrease in the proportion of Go responses. In Experiment 2, with the design analogous to Experiment 1, the hens received again five treatments (1.0 and 10.0 mg/kg of dopamine D1 receptor agonist SKF 38393, 1.0 and 10.0 mg/kg of dopamine D2 receptor agonist bromocriptine, and saline control), applied intramuscularly 2 h before the test. The agonists did not have any significant effect on approach latencies and the proportion of Go responses in the spatial judgment task, as compared to the saline control, except for 10.0 mg/kg SKF 38393, which caused a decrease in the proportion of Go responses. The approach latency and the proportion of Go responses were affected by the cue location in both experiments. Our data suggest that the dopamine D1 and D2 receptor blockade leads to a decrease in the reward expectation and the negative judgment of stimuli. The effect of dopamine receptor activation is less clear. The results reveal that dopamine receptor manipulation alters the evaluation of the reward and punishment in the spatial judgment task.

**Keywords: cognitive bias, spatial judgment task, decision-making under uncertainty, dopamine, Japanese quail**

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## INTRODUCTION

The complex interactions between emotions and cognition offer new opportunities for the study of affective states (emotions) in animals. Emotions can influence cognitive processes by modifying attention, memory, or judgment in a short- or long-term manner. Cognitive processes can therefore provide a useful tool for the assessment of emotions in animals (Harding et al., 2004; Paul et al., 2005; Boissy et al., 2007; Mendl et al., 2009; Mendl and Paul, 2020).

Judgment bias tasks represent a group of tools for the assessment of the emotional states of animals based on decision-making under ambiguity (uncertainty). In this type of task, the judgment bias is estimated according to animals' responses to ambiguous cues that have the spatial, visual, auditory, and/or olfactory attributes intermediate between the cues associated with positive and negative events (Harding et al., 2004; Roelofs et al., 2016). Biases in the judgment of ambiguous cues may provide a proxy measure of the valence of affective states in animals (Düpjan et al., 2013).

The spatial judgment task, originally introduced in rats (Burman et al., 2008), represents one of the alternatives how to test the judgment bias. In this task, animals are trained to expect a positive event in one location and a negative event in another. To determine whether animals are in a relatively positive or negative affective state, their response to ambiguous cues of intermediate spatial location is measured. Variations of this task have been used in many species, including birds (Košťál et al., 2020). The underlying mechanisms of the cognitive biases in animals (and man) are far from being understood. Many brain areas, such as the amygdala, prefrontal cortex, *nucleus accumbens*, ventral tegmental area, and various brain neurotransmitter systems, such as the dopamine, noradrenaline, opioid, and serotonin systems, appear to be involved in the processing of affective information (Mendl et al., 2009; Pessoa et al., 2019).

Principles from reinforcement learning theory can provide a model of the links between animal affect and decision-making (Mendl and Paul, 2020). Reinforcement learning is an adaptive process in which an animal utilizes its previous experience to improve the outcomes of future choices. The goal of reinforcement learning is to maximize future rewards. The reinforcement learning theories describe how the animal's experience alters its value functions, which in turn influence subsequent decision-making (Lee et al., 2012). Activity related to reward expectancy has been found in many different brain areas. There is a lot of evidence supporting the idea that the phasic activity of dopaminergic neurons in the ventral tegmental area signals a discrepancy between the expected and actual reward outcomes (Schultz et al., 1997; Schultz, 2007). The reward prediction error signaling is not limited to the ventral tegmental area but has also been found in the prefrontal cortex (Oya et al., 2005), including the avian prefrontal cortex homolog (Packheiser et al., 2021).

Dopamine plays an essential role in reward in both birds and mammals, and it is found in analogous brain regions (Emery and Clayton, 2015; Durstewitz et al., 1999; von Eugen et al., 2020; Reiner et al., 2004). There is topographical, neurochemical, developmental, and hodological evidence in support of putative homologies of the mesolimbic reward system in vertebrates (O'Connell and Hofmann, 2011). Dopamine receptors D1A, D1B, and D1C called also D1D [see (Yamamoto et al., 2013)], belong to the D1 family, and the receptors D2, D3, and D4 belong to the D2 family (Kubikova et al., 2010; Kubikova and Košťál, 2010). The receptors D1A, D1B, and D2 are found in the avian brain highly expressed particularly in the striatum. The receptors D1C and D3 are expressed throughout the pallium and within the mesopallium, respectively, and the D4 receptors are found mainly in the cerebellum (Kubikova et al., 2010; Kubikova and Košťál, 2010). In red junglefowl chicks, it has been shown recently that individuals with the higher expression of dopamine D1 receptors in the prefrontal cortex are more optimistic (Boddington et al., 2020). Judgment bias was also related to the dopamine turnover rate in the mesencephalon of domestic chicks, with higher activity in individuals that had a more optimistic response (Zidar et al., 2018).

Pharmacological manipulations of affective states alter judgment bias [see (Neville et al., 2020) for review] although the evidence concerning the effects of dopaminergic drugs is sometimes controversial. Administration of the catecholamine precursor L-DOPA increased an optimism bias in humans (Sharot et al., 2009; Sharot et al., 2012). In contrast, the L-DOPA treatment of rats made them less likely to interpret ambiguous tone as a cue predicting reward, i.e., produced pessimism. This applied only to rats that were classified as "optimistic", while the L-DOPA treatment did not affect the individuals classified as "pessimistic" (Golebiowska and Rygula, 2017). On the other hand, the acute administration of D-amphetamine induced positive bias in rats (Rygula et al., 2014a). Haloperidol, the D2 dopamine receptor antagonist, induced a negative bias in rats classified as "optimistic", while it induced a positive bias in "pessimistic" rats (Golebiowska and Rygula, 2017).

This study aimed to test the hypotheses that blockade of dopamine receptors by antagonists induces a negative (pessimistic) bias while the activation of dopamine receptors by agonists induces a positive (optimistic) bias in Japanese quail. To test these assumptions we used spatial judgment task and pharmacological manipulation of the dopamine D1 and D2 receptors with selective antagonists and agonists. The choice of drugs was based on our previous study of kinetics and pharmacology of dopamine receptors in Japanese quail (Kubíková et al., 2009), as well as on the previous use of these drugs in avian behavioral studies (SCH 23390 (Akins et al., 2004; Schroeder and Riters, 2006), haloperidol (Moe et al., 2011; Moe et al., 2014), SKF 38393 (Balthazart et al., 1997; Balthazart et al., 1997) and bromocriptine (Kostal and Savory, 1994; Reddy et al., 2007)).

## MATERIAL AND METHODS

### Animals

The experiment was carried out using 24 adult Japanese quail (*Coturnix japonica*) hens. The animals were housed in wire cages

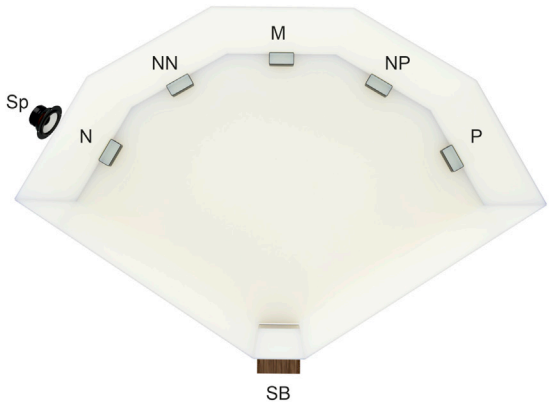


**FIGURE 1 |** The experimental training/testing arena. Five possible feeder locations—positive (P, rewarded by the mealworms), negative (N, punished, empty feeder plus white noise *via* speaker Sp), and the three ambiguous locations (neither rewarded nor punished), near positive (NP), middle (M), and near negative (NN), start box (SB). For half of the quails, the positive location was on the right side of the test arena (as on the figure), and for the other half, it was on the left. During the training and testing, the feeder was placed at only one location per trial.

with floor space 5620 $cm^2$ in groups of maximally 12 quails per cage. Housing conditions were as follows: ambient temperature 20°C, relative humidity 55%, and light:dark cycle 16:8 h. The food (feeding mash for layers, 15% of crude protein, 11.5 MJ $kg^{-1}$ metabolizable energy) and water were available *ad libitum* during the whole experiment. All procedures were approved by the State Veterinary and Food Administration of the Slovak Republic and by the Animal Ethics Committee of the institute.

## Testing Arena

The method of judgment bias testing used in this study is a modification of the spatial judgment task introduced by Burman et al. (Burman et al., 2008) in rats. The testing arena was built from white plastic boards (PVC 800 mm × 400 mm × 3 mm; **Figure 1**). The wooden start box (150 mm × 150 mm × 150 mm) with a plexiglass guillotine door, that opened into the arena, was operated manually by the experimenter sitting out of the animals' sight. The video camera (Microsoft LifeCam Cinema, Microsoft, United States) was placed above the arena allowing remote observation and recording of quail behavior. During training and testing, an opaque plastic cup feeder was placed close to the wall in one of the five locations 80 cm from the start box with a 40 cm distance between the neighboring locations. In any training or testing session, only one cup feeder was placed in one of the five possible locations.

The experiment consisted of the training phase, pharmacological manipulation, and testing phase. All animals were trained to consume mealworms used as a reward, habituated to the testing arena, and trained to associate one location with the reward and another location with the punishment. After this training, the animals were subjected to pharmacological manipulation using dopamine D1 and D2 receptor antagonists and agonists followed by the spatial judgment task (details described below).

## Habituation

During 3 days, the quails were habituated to mealworms as a food item, to the testing arena, and to transport from their home cage to the testing room in the start box. At this stage, the feeder containing a small amount of standard food and three mealworms were placed in the middle of the arena. Birds should leave the start box after opening the guillotine door, approach the feeder and consume the food. Each quail was subjected to one session with one trial per day for a maximum of 10 min.

## Positive Association Training

Birds were trained to associate the positive (P) feeder location with the reward (3 mealworms) for 4 days. This positive location was on the left side of the testing arena for half of the animals and on the right side of the arena for the rest of them. The birds were trained to leave the start box and enter the arena after opening the guillotine door, approach the feeder and consume the mealworms within 5 min. Each quail was subjected to one session per day. The first training session consisted of one trial while the next three sessions consisted of three trials. A trial was terminated once the animal reached the feeder and consumed the mealworms. In case the animal did not approach the feeder within the 60 s period, the maximum latency (60 s) was assigned for the trial. The interval between consecutive trials was 60 s. In each trial, the latency to approach the feeder (crossing the imaginary decision line 5 cm around the feeder) was recorded for each quail.

## Discrimination Task

During the spatial discrimination task training, the quails were trained to associate the positive feeder location (P) with the reward and the negative feeder location (N) with the punishment. The quails were trained to approach the P location and consume mealworms and refrain from approaching the N location to avoid punishment (empty feeder and 5 s of 80 dB white noise). For half of the animals

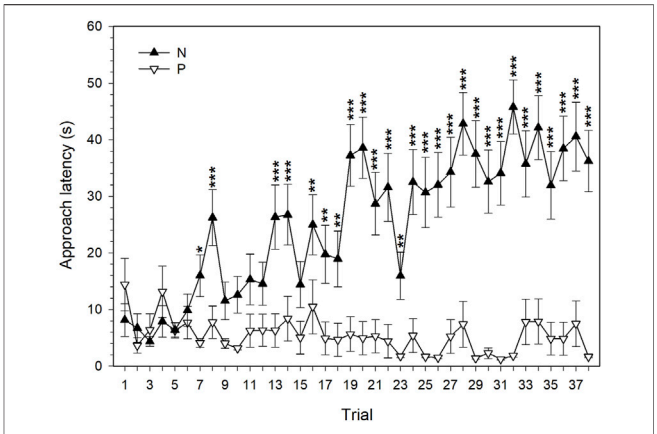


**FIGURE 2 |** The latency to approach the rewarded positive (P) and punished negative (N) feeder locations (mean ± SEM) by quail hens ($n$ = 20). *–$p$ < 0.05, **–$p$ < 0.01, ***–$p$ < 0.001.

the P location was on the left side and the N location on the right side of the arena, while for the other half of the animals the positions of the feeders were reversed. Each bird was subjected to one training session per day. The first five training sessions consisted of four trials (2 P, 2 N) presented in random order, i.e., together 10 P and 10 N trials (**Figure 2**, trials 1–10). In the consecutive 14 sessions, the number of trials was increased to five. The initial trial of the session, not included in the analyses, was P, followed by four measured trials (2 P, 2 N) in random order, i.e., 28 P and 28 N trials (**Figure 2**, trials 11–38). The order of the feeder locations was the same for all animals within the training day but it differed in each session. For each animal, the latency to approach the feeder (crossing the imaginary decision line 5 cm around the feeder) after leaving the start box was recorded. In case the animal did not approach the feeder within this time, a trial was terminated, the animal was returned to the start box and the next trial started after 60 s. A correct response was defined as approaching the feeder (i.e., a Go response) within 60 s following a positive cue and not approaching the feeder (i.e., a No-Go response) within 60 s following a negative cue.

## Spatial Judgment Task

Four quails were excluded from further testing since they failed to discriminate between the P and N locations after the end of the discrimination training, i.e., the number of birds included in the judgment bias tests was reduced to 20. The spatial judgment task testing included the presentation of the feeder in P and N locations and also in three ambiguous intermediate spatial locations (NP—near positive, M—middle, NN—near negative; **Figure 1**) that were neither rewarded nor punished. The animals were subjected to one session after each pharmacological treatment (see Drug Treatment below). The sessions consisted of seven trials in total, starting with one trial with P and one N location to foster the learned discrimination (data from these two trials were not included in the analyses), and after that, the feeder was placed in all five locations in random order. The order of the locations differed between the sessions. Again, the latency to approach the feeder i. e., the Go response (crossing the imaginary decision line 5 cm around the feeder) within 60 s after leaving the start box was recorded. In case that animal did not approach the feeder within this time (No-Go response) a trial was terminated, the animal was returned to the start box and after the 60 s break, the next trial started.

## Drug Treatment

Dopaminergic drugs were selected based on their pharmacological properties and receptor binding in the quail brain (Kubíková et al., 2009). All drugs were obtained from RBI/Sigma-Aldrich (St. Louis, United States). Drug treatments were divided into two experiments, Experiment 1 using dopamine receptor antagonists SCH 23390 for D1 and haloperidol for D2 and Experiment 2 using dopamine receptor agonists SKF 38393 for D1 and bromocriptine for D2.

Experiment 1 included five treatments: 0.1 mg/kg of SCH 23390, 1 mg/kg of SCH 23390, 0.05 mg/kg of haloperidol, 0.5 mg/kg of haloperidol, and saline control. All solutions were injected intramuscularly in the amount of 1 ml/kg 15 min before the start of the spatial judgment task. Experiment 2 included five treatments: 1.0 mg/kg of SKF 38393, 10.0 mg/kg of SKF 38393, 1.0 mg/kg bromocriptine, 10.0 mg/kg bromocriptine, and the saline control. All solutions were injected intramuscularly in the amount of 1 ml/kg 2 h before the start of the spatial judgment task. The doses of drugs, their dissolving, and the timing of injection were based on published data (Kostal and Savory, 1994; Moe et al., 2014). The time-course of bromocriptine effects on the behavior of rats (Pizzolato et al., 1985) as well as chickens (Kostal and Savory, 1994) is biphasic, with initial suppression and delayed stimulation. Biphasic locomotor effects were also reported after the treatment with SKF 38393 in mice (Tirelli and Terry, 1993). Therefore the treatments in Experiment 2 were applied 2 h before the behavioral tests. In both experiments, all animals received all five treatments in a different order, according to a Latin square design. To minimize the carry-over effect, there was a 2-day break between each treatment.

## Data Analysis

Data were analyzed using the GLIMMIX procedure for generalized linear mixed models in SAS (version 9.04; SAS Institute Inc. Cary, NC, United States). Latency data from the spatial discrimination training were analyzed using the model with two fixed factors, feeder position (P, N) and trial. Latency data from Experiment 1 and Experiment 2 were analyzed with the feeder position (P, NP, M, NN, N), treatment, and their interaction as fixed effects. The proportions of the Go responses based on binary data (Go response = 1, No-Go response = 0) were also analyzed using the GLIMMIX procedure but with binomial distribution and logit link function. In all analyses, individual bird identity was taken into account as a random effect. Multiple post-hoc comparisons were done using the Tukey–Kramer test.

# RESULTS

## Discrimination Learning

There was a significant effect of the feeder position ($F_{1,1425}$ = 537.98, $p < 0.001$), trial ($F_{37,1452}$ = 4.75, $p < 0.001$), and their interaction ($F_{37,1452}$ = 6.25, $p < 0.001$) on the approach latency during the discrimination learning. The first significant difference between the latencies to approach the P and N cues during the discrimination training was observed in the 7th trial (**Figure 2**). Although there were fluctuations in the discrimination performance of quail hens, from the 16th trial on the mean latency to reach the feeder in the P location was significantly shorter than the latency to reach the feeder in the N location. Beginning from the 19th trial, the mean difference between the latencies to approach the P and N location of the feeder was, with one exception (the 23rd trial), more than 20 s. The maximum mean difference of 43.95 s between the latencies to reach the P and N location was observed in the 32nd trial (**Figure 2**).

## Spatial Judgment Task

In Experiment 1, there was a significant effect of the feeder position ($F_{4,456}$ = 40.20, $p < 0.001$) and treatment ($F_{4,456}$ =

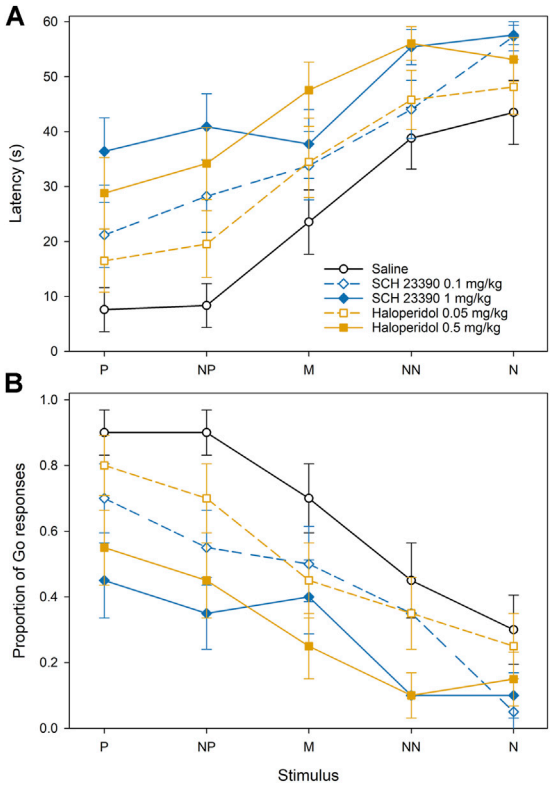


**FIGURE 3 |** The effect of dopamine D1 and D2 receptor antagonists on the approach latencies **(A)** and a mean proportion of Go responses **(B)** of Japanese quail hens in the spatial judgment task. Mean ± SEM of the approach latencies to the positive (P), near-positive (NP), middle (M), near-negative (NN), and negative (N) cues.

17.54, $p < 0.001$), but not their interaction, on the approach latencies (**Figure 3A**). There were dose-dependent increases in approach latencies after the treatment with both antagonists, SCH 23390 (D1) as well as haloperidol (D2). The post-hoc comparisons showed that the approach latencies after the treatment with 0.1 mg/kg SCH 23390 were significantly longer in comparison with control ($p < 0.001$), while the latencies after the treatment with 1 mg/kg SCH 23390 latencies were significantly longer in comparison with treatment with 0.1 mg/kg SCH 23390 ($p < 0.05$) as well as with the saline control ($p < 0.001$). The post-hoc comparisons also showed that the approach latencies after the treatment with 0.05 mg/kg haloperidol were significantly longer in comparison with control ($p < 0.05$), and the approach latencies after the treatment with 0.5 mg/kg haloperidol were significantly longer in comparison with 0.05 mg/kg haloperidol ($p < 0.01$) as well as with the saline control ($p < 0.001$). The pattern of the proportions of the Go responses after the treatment with dopamine antagonists in Experiment 1 was reciprocal to latencies. There was a significant effect of the feeder position ($F_{4,456} = 19.19$, $p < 0.001$) and treatment ($F_{4,456} = 12.25$, $p < 0.001$), but not their interaction, on the proportions of the Go responses. The proportion of the Go responses decreased after the treatment with both, SCH 23390 (D1) and haloperidol (D2) (**Figure 3B**). The proportions of the Go responses after the treatment with 0.1 mg/kg as well as 1.0 mg/kg of SCH 23390 were significantly lower in comparison with the control ($p < 0.001$). There was no significant difference in the proportion of the Go responses between the two doses of SCH 23390. The proportions of the Go responses after the treatment with 0.05 mg/kg as well as 0.5 mg/kg of haloperidol were significantly lower in comparison with control ($p < 0.05$ and $p < 0.001$, respectively), and the proportion of Go responses after the treatment with 0.5 mg/kg of haloperidol was significantly lower in comparison with 0.05 mg/kg of haloperidol ($p < 0.01$).

Both, the approach latencies and the proportion of Go responses in Experiment 1 were affected by the cue location. The latency to approach the feeder in the P location was significantly shorter than the latency to approach the feeder in the M, NN, and N locations (all $p < 0.001$), the latency to approach the NP location was shorter than the latency to approach the M ($p < 0.05$), NN, and N ($p < 0.001$), and the latency to approach the M was significantly shorter than the NN and N ($p < 0.001$). The proportion of Go responses to the feeder in the P location was significantly higher than the proportion of the Go responses to the feeder in the M, NN, and N locations (all $p < 0.001$), the proportion of the Go responses to the NP location was higher than the proportion of the Go responses to NN, and N

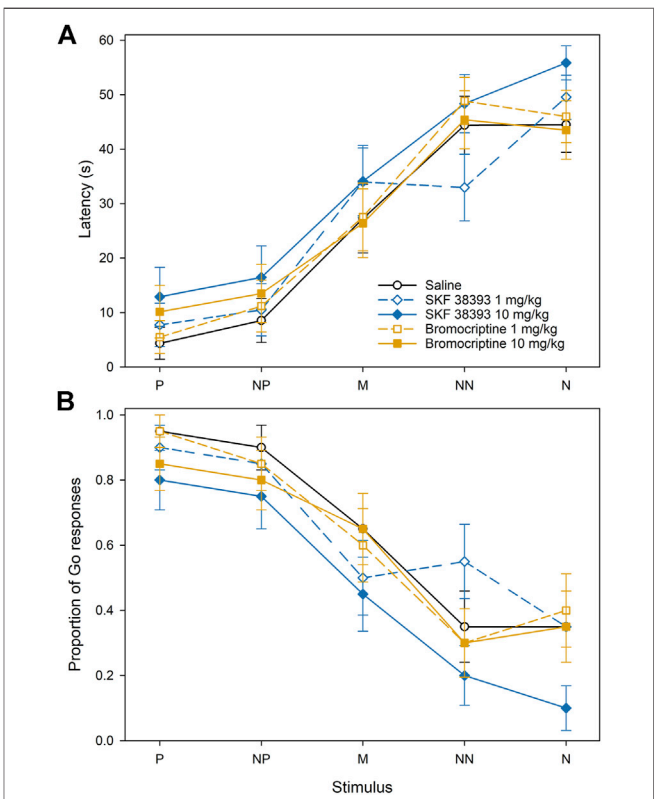


**FIGURE 4 |** The effect of dopamine D1 and D2 receptor agonists on the approach latencies **(A)** and mean proportion of Go responses **(B)** of Japanese quail hens in the spatial judgment task. Mean ± SEM of the approach latencies to the positive (P), near-positive (NP), middle (M), near-negative (NN), and negative (N) cues.

locations ($p < 0.001$), and the proportion of the Go responses to the feeder in the M location was significantly higher than the NN ($p < 0.01$) and N ($p < 0.001$).

In Experiment 2, there was a significant effect of the feeder position ($F_{4,456} = 40.20$, $p < 0.001$), but the effects of treatment and treatment × position interaction on the approach latencies were not significant (**Figure 4A**). However, in case of the proportion of the Go responses, there was a significant effect of both the feeder position ($F_{4,456} = 20.15$, $p < 0.001$) and the treatment ($F_{4,456} = 3.83$, $p < 0.01$). The post-hoc comparisons of treatments showed that the treatment with 10.0 mg/kg of SKF 38393 led to the significant decrease of the proportion of the Go responses in comparison with the control ($p < 0.001$).

The approach latencies and the proportion of the Go responses were affected by the cue location similarly to Experiment 1. The latency to approach the feeder in the P location was significantly shorter than the latency to approach the feeder in the M, NN, and N locations (all $p < 0.001$), the latency to approach the NP location was shorter than the latency to approach the M, NN, and N (all $p < 0.001$), and the latency to approach the M was shorter than the latency to approach the NN and N (all $p < 0.001$). The proportion of Go responses to the feeder in the P location was significantly higher than the proportion of the Go responses to the feeder in the M, NN, and N locations (all $p < 0.001$), the proportion of the Go responses to the NP location was higher than the proportion of the Go responses to M, NN, and N locations (all $p < 0.001$), and the proportion of the Go responses to the feeder in the M location was significantly higher than the NN and N (all $p < 0.001$).

## DISCUSSION

In this study, we were testing the hypothesis that dopaminergic signaling affects decision-making under ambiguity. We were evaluating the response of Japanese quail hens in the spatial judgment task following the blockade or activation of specific dopamine receptors belonging to the D1 and D2 families. The dopamine receptor blockade led to increased response latencies and decreased proportion of the Go responses, indicating possible pessimistic bias. Although the dopamine receptor activation has not affected the latencies of the responses in the judgment bias task, the higher dose of the D1 agonist SKF 38393 decreased the proportion of the Go responses.

The cognitive bias paradigm used for the study of affective states evaluates biases in processing towards reaching a reward or avoiding punishment. Therefore the changes in circuits involved in reward and punishment are expected. Human and animal studies identified that the reward circuits are centered around the dopaminergic neurons in various brain regions (Hales et al., 2014). Dopamine is implicated in the valuation of reward-related cues (Schultz et al., 1997; Schultz, 2007). That is why the dopaminergic system is considered one of the candidate systems involved in the mechanisms underlying judgment bias (Mendl et al., 2009; Noworyta et al., 2021).

The distribution of the dopamine receptors in the avian brain (Kubikova et al., 2010) and their pharmacological properties (Kubíková et al., 2009) are similar to those in mammals. Boddington et al. (Boddington et al., 2020) linked the D1 receptors with optimistic judgment when they found the higher expression of dopamine D1 receptors in the prefrontal cortex of junglefowl chicks which had shorter latencies to approach the ambiguous cues. In the same study, the expression of the D2 receptors in the prefrontal cortex did not correlate significantly with the optimism in the judgment bias test.

Our main prediction in this study was that the treatment with dopamine receptor antagonists will induce the negative/pessimistic judgment bias (prolonged approach latencies and decreased proportion of the Go responses in response to ambiguous cues), while conversely, the treatment with dopamine receptor agonists will induce the positive/optimistic bias (shorter latencies, increased proportions of the Go responses). Blockade of dopamine receptors by antagonists SCH 23390 and haloperidol in our experiment prolonged the latencies to reach the feeders in ambiguous locations in the judgment bias tests and decreased the proportions of the Go responses. However, the effect was not restricted to ambiguous cues only. The latencies to reach the feeder in positive and negative (reference) locations were prolonged too. The same applies to the reduction in proportions of the Go responses. Similar "generalized response bias" was reported by Rygula et al. (Rygula et al., 2014b) in rats after the treatment with the norepinephrine–dopamine reuptake inhibitor mazindol. Rats showed overall decreased positive and overall increased negative responses in an operant Go/Go task generalized to the reference cues. The authors concluded that it may reflect a decreased expectation of reward and increased expectation of punishment, resulting from a change in the perception of the likelihood of receiving the reward and punishment, respectively, or a change in the value of the reinforcement. The combined treatment of rats with the norepinephrine reuptake inhibitor reboxetine and corticosterone had a similar effect. It also decreased the proportion of positive responses in an operant Go/Go task to all, ambiguous as well as both reference cues (Enkel et al., 2010; Anderson et al., 2012). Anderson et al. (Anderson et al., 2012) hypothesized that the treatment caused a reduction in motivation to respond for reward. Similarly, in chicks in the depression-like state, not only the latencies to reach the ambiguous cues, but also the latency to reach the aversive cue in the runway test were increased (Salmeto et al., 2011). More generally, the meta-analysis of pharmacological manipulations of judgment bias confirmed that such manipulations do not influence only the response to ambiguous cues, but also the response to reference cues, or more precisely, these manipulations exerted a similar effect at the negative reference cue compared with the probe cues (Neville et al., 2020). On the other side, in the seminal study of Harding et al. (2004), rats in a mild depression-like state were slower to press the lever and tended to show fewer responses in an operant Go/NoGo task not only in reaction to the ambiguous cues but also to the positive one.

If we accept the "generalized response bias" idea, then both dopamine receptor antagonists in our study resulted in a negative/pessimistic bias in Japanese quail judgment. Such results were found in several other studies. The acute haloperidol treatment in rats caused a "pessimistic" shift in "optimists", but an "optimistic" shift in "pessimists"

(Golebiowska and Rygula, 2017). Haloperidol had also a negative effect on spatial reference and working memory in the case of another type of spatial task in rats, the spatial cone field task (Blokland et al., 1998). Dopamine antagonist fluphenazine, with the affinity for the D2 site slightly greater than for the D1 (Morgan and Finch, 1986), abolished optimistic judgment in bumblebees after sucrose consumption (Mendl and Paul, 2016; Solvi et al., 2016). On the other hand, the acute haloperidol treatment has been shown to enhance dopamine turnover in the rat striatum (Lerner et al., 1977; Rastogi et al., 1982) as well as in the domestic chicken prefrontal cortex homolog (Gruss et al., 2003), but the increased dopamine turnover in the mesencephalon domestic fowl females was associated with a more optimistic response in a judgment bias test (Zidar et al., 2018). Microinfusion of the D1 receptor antagonist SCH 23390 into the *nidopallium caudolaterale*, a prefrontal cortex homolog of pigeon, caused an impairment of a visual discrimination reversal (Diekamp et al., 2000) but did not affect the performance in a delayed-matching-to-sample task (Herold et al., 2008). In our experiment, the blockade of D1 receptors caused a similar "generalized response bias" as the blockade of D2 receptors.

A possible explanation of the prolonged latencies to reach the feeder locations after the treatment with dopamine receptor antagonists could be sedation, changes in motor function, which can complicate interpretation of results (Bushnell and Levin, 1993). Haloperidol at 0.5 mg/kg for example decreased the response rate of pigeons in a delayed-matching-to-sample task but failed to impair accuracy (Poling et al., 1984). However, 0.5 mg haloperidol/kg body weight did not have any clear sedative effect in laying hens (Kjaer et al., 2004). Similar results were reported by Moe et al. (Moe et al., 2014), who found that the latency to walk to the reward and start to eat in laying hens was affected only by the high dose of haloperidol (2 mg/kg) but not by the 0.5 mg/kg dose. Zarrindast and Namdari (Zarrindast and Namdari, 1992), who used the highest dose of SCH 23390 in chickens corresponding to our lower dose state that SCH 23390 did not induce catalepsy or marked sedative effect.

The treatment with dopamine receptor agonists SKF 38393 and bromocriptine have not affected significantly the latencies to approach the cues by quail hens in the spatial judgment task. However, the acute treatment with the SKF 38393 at the dose of 10.0 mg/kg led to the decreased proportions of Go responses. The published data on the influence of drugs with agonistic effects on the dopaminergic system on the judgment bias are inconsistent. Although the L-DOPA induced optimistic bias in humans (Sharot et al., 2009; Sharot et al., 2012), it failed to induce optimism in rats, where the acute administration of L-DOPA induced "pessimism" in animals classified as "optimistic" and did not affect the judgment bias of "pessimistic" animals (Golebiowska and Rygula, 2017). The indirect dopamine agonist D-amphetamine induced optimistic bias in rats (Rygula et al., 2014a). On the other hand, the lack of effect of the SKF 38393 on judgment bias in quail can be maybe attributed to the fact that it is a partial D1 dopamine receptor agonist. It has been shown in rats that it has a lower affinity for the specific dopamine receptors than the full D1 receptor agonists and also its effects on learning and memory differ (Watts et al., 1993; Amico et al., 2007).

Behavioral effects of SCH 23390 and SKF 38393 do not always correspond to their established receptor subtype selectivity at D1 receptors (Zarrindast et al., 2011). For example, it was shown that both SKF 38393 and SCH 23390 induce inhibition of feeding in rats (Zarrindast et al., 1991). In our experiment, both SCH 23390 and SKF 38393 (at the high dose) decreased the proportion of Go responses of quail in the spatial judgment task. Terry and Katz (Terry and Katz, 1994) called into question the use of SKF 38393 as a D1 agonist in studies of feeding, and other contexts as well. Inhibition of feeding in rats by SKF 38393 and SCH 23390 can be according to some studies mediated *via* serotonergic mechanisms (Zarrindast et al., 1991; Zarrindast et al., 2011), that also play a role in a judgment bias (Golebiowska and Rygula, 2017; Neville et al., 2020).

Affective states differ in their duration. Mood differs from shorter-term (acute) emotions in that it represents a state that usually lasts over a longer period of time such as days or weeks (Mendl et al., 2010; Webb et al., 2019). A recent theory argues that mood reflects the cumulative impact of differences between obtained outcomes and expectations. The judgment bias tests were developed to assess mood (Raoult et al., 2017). That points out to the possible importance of duration of treatment in case of pharmacological induction of affective states. The acute and chronic pharmacological treatments influence them in a different ways. The study investigating the affect-induced cognitive bias in rats using systemic treatments with anxiolytic (diazepam) and antidepressant drugs (reboxetine or fluoxetine) suggested that judgment bias may be sensitive to chronic but not acute antidepressant treatment (Anderson et al., 2012). Hales et al. (Hales et al., 2017) state that experiments to date have generally failed to observe consistent effects with conventional antidepressants following acute administration. That can be also a possible explanation of the minor effects of the dopamine agonists used in this study. The acute treatment might not induce large enough differences in the affective state detectable by the spatial judgment task.

## CONCLUSIONS

The inhibition of dopamine D1 and D2 receptors by SCH 23390 and haloperidol led to prolonged latencies to approach the feeder in ambiguous locations in the spatial judgment task, but also latencies to approach positive and negative locations. It also decreased the proportions of Go responses. This suggests the induction of negative, pessimistic judgment bias, but also a generally lower expectation of the reward. On the other hand, the activation of dopamine D1 and D2 receptors by SKF 38393 and bromocriptine did not have any significant effect on the spatial judgment task latencies within the range of doses applied. However, the larger dose of the SKF 38393 decreased the proportion of the Go responses. The present results demonstrate that dopamine signaling is involved in the underlying mechanisms of judgment bias in Japanese quail.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the State Veterinary and Food Administration of the Slovak Republic.

## AUTHOR CONTRIBUTIONS

KP: investigation, writing; LKU: methodology, writing; LKO: conception and design of the study, funding acquisition, data analysis, writing. All authors contributed to the article and approved the submitted version.

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# Effect of Darkness on Intrinsic Motivation for Undirected Singing in Bengalese Finch (*Lonchura striata Domestica*): A Comparative Study With Zebra Finch (*Taeniopygia guttata*)

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The zebra finch (ZF) and the Bengalese finch (BF) are animal models that have been commonly used for neurobiological studies on vocal learning. Although they largely share the brain structure for vocal learning and production, BFs produce more complex and variable songs than ZFs, providing a great opportunity for comparative studies to understand how animals learn and control complex motor behaviors. Here, we performed a comparative study between the two species by focusing on intrinsic motivation for non-courtship singing ("undirected singing"), which is critical for the development and maintenance of song structure. A previous study has demonstrated that ZFs dramatically increase intrinsic motivation for undirected singing when singing is temporarily suppressed by a dark environment. We found that the same procedure in BFs induced the enhancement of intrinsic singing motivation to much smaller degrees than that in ZFs. Moreover, unlike ZFs that rarely sing in dark conditions, substantial portion of BFs exhibited frequent singing in darkness, implying that such "dark singing" may attenuate the enhancement of intrinsic singing motivation during dark periods. In addition, measurements of blood corticosterone levels in dark and light conditions provided evidence that although BFs have lower stress levels than ZFs in dark conditions, such lower stress levels in BFs are not the major factor responsible for their frequent dark singing. Our findings highlight behavioral and physiological differences in spontaneous singing behaviors of BFs and ZFs and provide new insights into the interactions between singing motivation, ambient light, and environmental stress.

**Keywords: motivation, birdsong, zebra finch, Bengalese finch, stress, vocal learning, darkness, voluntary behavior**

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## INTRODUCTION

Songbirds have been widely used in behavioral and neurobiological studies because of their remarkable abilities of vocal learning. Bengalese finches (BFs) and zebra finches (ZFs), the two most commonly used songbird species in neurobiology, learn to sing by imitating the songs of other conspecifics during a critical period of development and share many aspects of neural circuits and mechanisms of song production and learning (Murphy et al., 2017). However, despite these common features, BFs and ZFs show considerable differences in their song structure: ZFs produce songs with

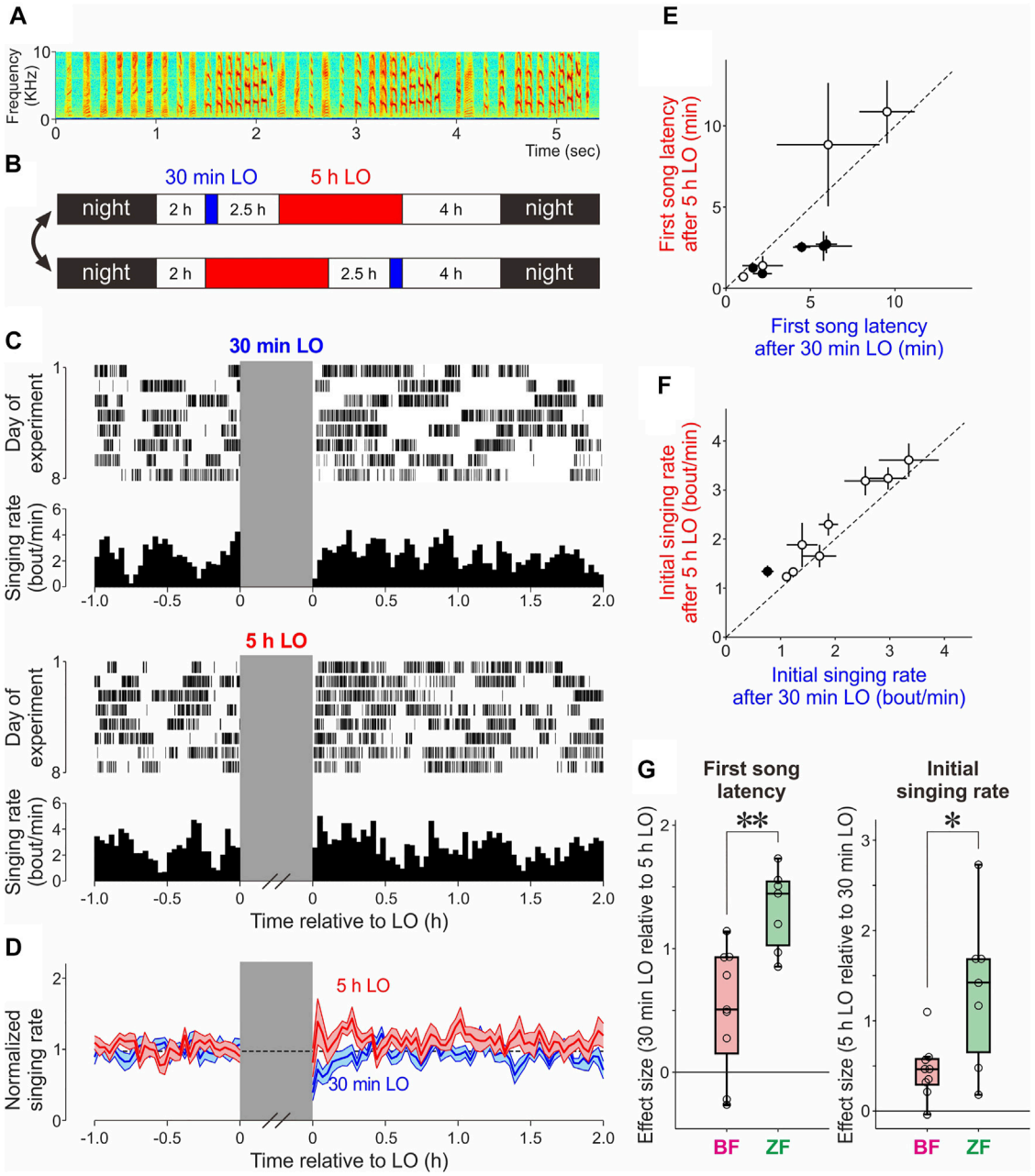


**FIGURE 1 |** Effects of short (30 min) and long (5 h) lights-out (LO) on undirected singing in BFs and their comparison with those in ZFs. **(A)** Spectrogram showing one bout of undirected song in a representative BF. **(B)** Daily schedule of 30 min and 5 h LO periods. After a 2 h light period (white area) in the morning, a 30 min LO (blue area) and a 5 h LO (red area) were given with a 2.5 h intervening light period, followed by a 4 h light period. The order of the 30 min and 5 h LO was switched every 1–3 days. Each row indicates the schedule on one day. **(C)** Raster plot of song bouts produced before and after a 30 min LO (top) and 5 h LO (bottom) and corresponding singing rate histograms (bin size is 2 min) in a representative bird. **(D)** Time course of instantaneous singing rate before and after a 30 min (blue) and 5 h LO (red), normalized to the mean singing rate before LO (mean ± SEM, $n = 9$ birds). **(E)** The first song latencies (mean ± SEM) after a 5 h LO plotted against those after a 30 min LO ($n = 9$ birds). Filled circles indicate birds with statistical significance between a 5 h and 30 min LO data ($p < 0.05$). The dashed lines indicate unity. **(F)** Initial singing rates after a 5 h LO plotted against those after a 30 min LO ($n = 9$ birds). Conventions are as in E. **(G)** The effect sizes [Hedges' g for the first song latency (left) and the initial singing rate (right) between a 30 min and 5 h LO in BFs and ZFs. *$p < 0.05$; **$p < 0.01$].

repetitions of a highly stereotyped sequence of song notes or "syllables," whereas BFs produce much longer songs with highly variable syllable sequencing (**Figure 1A**) (Okanoya, 2004) with real-time control using auditory feedback (Okanoya and Yamaguchi, 1997). Such differences in song structure between BFs and ZFs despite the similarities in their brain structure make excellent models for comparative studies to understand how animals learn to produce and control complex motor behaviors.

In both BFs and ZFs, males spontaneously produce more than hundred renditions of songs a day throughout their life even when they are isolated from other individuals (Dunn and Zann, 1996; Derégnaucourt et al., 2005; Tumer and Brainard, 2007). Such spontaneous singing in a solo context, referred to as "undirected singing," is thought to serve, at least in part, as vocal practice by which birds develop and optimize song structure (Konishi, 1965; Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001; Tumer and Brainard, 2007). Undirected songs are produced in the absence of any immediate external rewards and appear to be driven by intrinsic motivation to obtain internal reward (Riters and Stevenson, 2012), providing a unique opportunity to study the mechanisms of intrinsic motivation that drives the learning and production of complex motor skills.

We recently found in young adult ZFs that temporary suppression of undirected singing by turning off the ambient light enhances intrinsic motivation for undirected singing to an extent that depends on the duration of singing suppression (Kim et al., 2021): birds sing much sooner and more intensely after relatively long (5 or 10 h) periods of lights-out (LO) than after shorter LO periods. In the present study, we use the same procedure in BFs to examine the possible differences in the enhancement of intrinsic motivation for undirected singing between BFs and ZFs. Our results revealed substantial differences between the two species in LO-induced enhancement of singing motivation as well as singing behavior during LO periods. We also investigated the physiological mechanisms underlying such behavioral differences between the two species by measuring blood corticosterone (CORT) levels under both dark and light conditions.

# MATERIALS AND METHODS

## Subjects

Subjects were adult male BFs [*Lonchura striata domestica*, 91–128 days post-hatching (dph)] and ZFs (*Taeniopygia guttata*, 87–119 dph). Birds were raised in our colony with their parents and siblings until ~60 dph and then housed with other conspecifics until the experiments started. Care and treatment of animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Brain Research Institute. All experiments were performed following the relevant guidelines and regulations.

## Song Recording

Birds were housed individually in sound-attenuating chambers (MC-050, Muromachi Kikai) under a 14/10 h light/dark cycle throughout the experiments. Undirected songs were recorded as previously reported (Kim et al., 2021). Briefly, the output from a microphone (PRO35, Audio-Technica) positioned above the cage was amplified using a mixer (402-VLZ4, Mackie) and digitized via an audio interface (Octa-Capture UA-1010, Roland) at 44.1 kHz (16-bit). The recording was controlled by a custom-written song recording program (R. O. Tachibana of the University of Tokyo), which triggered recording if it detected four or five consecutive sound notes, each of which was defined based on the sound magnitude, sound duration, and intervening gap duration. Each recording ended when the silent period lasted longer than 0.5 s. Birds with sufficient singing rates (>300 song bouts per day) were used for further experiments (2/19 ZFs and 0/17 BFs were rejected because they did not satisfy this criterion). All ZF song data were originally collected for a previous study (Kim et al., 2021) and re-analyzed for comparison with the BF data.

## Temporary Singing Suppression and Song Analysis

To suppress undirected singing, the light in the sound-attenuating chambers was turned off using digital timers. The duration and schedule of lights-out (LO) periods varied depending on experimental paradigms (from 30 min to 10 h; see Results); individual birds received LO of the same duration 6–8 times. Singing behaviors following individual LO periods were quantified using two measures, the first song latency and initial singing rate (Kim et al. (2021)). The first song latency was measured as the time interval from the offset of a LO period to the onset of the first song recorded. We visually inspected the spectrograms of the sound files recorded after the LO periods to identify the first file that included at least one song motif. The initial singing rate was measured as the mean singing rate over a 30 min period starting at the onset of the first song produced after an LO period (the timing of the 30 min period varied across trials, depending on the first song latencies). To measure singing rates, we screened all sound files recorded during the periods of interest to exclude non-song files using a previously reported semi-automated method (Kim et al., 2021). Briefly, we sorted song files (files that included at least one full song motif) and other sound files by focusing on the temporal structure of two acoustic features: sound amplitude and Weiner entropy. We compared the trajectories of these features between a canonical song motif and all sound files recorded by calculating the cross-correlation function. Sound files that had relatively low correlation coefficients in both the amplitude envelope and envelope trajectory were then excluded from the analysis.

To quantify the degree of singing motivation enhancement caused by LO, we calculated the Hedges' g effect sizes for both the first song latency and the initial singing rate between 30 min and 5 h LO or 30 min and 10 h LO. For each bird, the mean and SD of either the first song latency or the initial singing rate were computed across trials for both short (30 min) LO and long (5 h or 10 h) LO, and the effect size was calculated using the following formula, which includes a correction for small sample sizes (Hedges, 1981):

$$g = \frac{\bar{x}_1 - \bar{x}_2}{SD^*} \times \left(\frac{N-3}{N-2.25}\right) \times \sqrt{\frac{N-2}{N}},$$

where $\bar{x}_1$, $\bar{x}_2$, $SD^*$, and $N$ denote the mean of the data 1, the mean of the data 2, the pooled standard deviation, and the sum of the sample sizes (i.e., the number of trials) of the data 1 and 2, respectively. The $SD^*$ was computed as:

$$SD^* = \sqrt{\frac{(n_1 - 1)SD_1^2 + (n_2 - 1)SD_2^2}{n_1 + n_2 - 2}},$$

where $n_1$, $n_2$, $SD_1$, $SD_2$ denote the sample size of the data 1, the sample size of the data 2, the standard deviation of the data 1, and the standard deviation of the data 2, respectively. When calculating the effect sizes for the first song latency, which tends to be relatively long after a short LO in ZFs (Kim et al., 2021), the data with 30 min LO were assigned to the data 1 and the data with longer LO (5 or 10 h) were assigned to the data 2 so that the effect size of most ZFs become positive. For the same reason, when calculating the effect sizes for the initial singing rate, which tends to be relatively large after a long LO in ZFs, the data with longer LO (5 or 10 h) were assigned to the data 1 and the data with 30 min LO were assigned to the data 2.

## Corticosterone Assays

Blood CORT levels under both dark and light conditions were measured for each experimental bird. Before blood sampling, the birds were housed individually in sound-attenuating chambers for >3 days. On the day of blood sampling in dark conditions ("Dark day" in **Figure 4A**), 6 h LO was conducted in the middle of the day, and 50–200 μl of blood was collected 1 h before the end of the LO period by puncturing the brachial wing vein with a sterile needle (23G). On the day of blood sampling in light conditions ("Light day" in **Figure 4A**), blood was collected at the same time of the day, but no LO was given. To minimize the potential stress caused by the blood sampling operation, all blood samples were collected within 3 min after opening the sound-attenuating chambers to take the birds out of their cages. The blood samples were centrifuged at 3,500 rpm for 20 min at ~20°C, and the sera were stored at −20°C until the assay. The dark and light days were repeated 3–5 times in an interleaved manner until a total of >100 μl of serum was collected under both dark and light conditions (2/14 BFs and 4/15 ZFs were rejected because we could not collect enough serum samples from them). CORT levels were measured using enzyme immunoassay kits (Cat. ADI-900-097, Enzo Life Sciences). All serum samples (1:10 dilution) and standards were analyzed according to the manufacturers' instructions. The plates were read on an absorbance 96-plate reader (SpectraMax® M2, Molecular Devices). CORT levels were determined using 5-parameter logistic regression software of CORT standards ranging from 32 to 20,000 pg/ml.

## Statistical Analysis

To analyze the effects of LO on singing behavior, we compared first song latencies and initial singing rates between 30 min LO and 5 h LO for each bird using a Wilcoxon signed-rank test ($\alpha$ = 0.05), which was also used for the group data. We examined the effects of LO with four different durations on singing behavior using Friedman's test. To compare the enhancement of intrinsic singing motivation between BFs and ZFs, Fisher's exact test was used for the ratio of birds with statistical significance and a Mann–Whitney U test for the effect sizes (Hedges' g). For the data of blood CORT levels, we used a Mann–Whitney U test to compare the data between BFs and ZFs and a Wilcoxon signed-rank test to compare the data between dark and light conditions. All statistical analyses were performed using the MATLAB software.

# RESULTS

## Bengalese Finchs Exhibit Suppression-Dependent Enhancement of Undirected Singing Motivation to a Smaller Degree Than Zebra Finchs

Our previous study in ZFs demonstrated that temporary suppression of undirected singing by turning off the ambient light enhances intrinsic motivation for undirected singing to an extent that depends on the duration of singing suppression: ZFs sing much sooner and more intensely after relatively long (5 h) periods of lights-out (LO) than after shorter (30 min) LO periods (Kim et al., 2021). Using the same experimental procedures, we examined whether BFs exhibit such differences in post-LO singing behavior between 5 h LO and 30 min LO to degrees similar to those observed in ZFs. As in the previous study in ZFs (Kim et al., 2021), young adult male BFs (91–128 dph) were subjected to both 30 min and 5 h LO periods on each day (they were separated by a 2.5 h light period and their order was switched every 1–3 days; **Figure 1B**). We found that BFs exhibited differences in post-LO singing behavior between 5 h LO and 30 min LO that were much less clear than those observed in ZFs. BFs appeared to recover singing rates to the pre-LO levels faster after 5 h LO than after 30 min LO on average (**Figures 1C,D**), and the latency to the first song after LO periods ("first song latency") was significantly smaller after 5 h LO than after 30 min LO in more than half of birds (5/9 birds, Wilcoxon signed-rank test, $\alpha$ = 0.05 was used for each bird) (**Figure 1E**). As a group, however, no significant difference in first song latency was observed between 30 min LO and 5 h LO (**Figure 1E**, $n$ = 9 birds, $p$ = 0.25, Wilcoxon signed-rank test), sharply contrasting with the striking and significant difference in the same measure in ZFs (Kim et al., 2021). To compare the degrees of singing motivation enhancement between BFs and ZFs more directly, we re-analyzed the ZF song data that were originally collected for our previous study (Kim et al., 2021) to calculate the effect sizes (Hedges' g; see Methods) of first song latencies between 30 min LO and 5 h LO, and compared them with the effect sizes of BF data. We found that the effect sizes were significantly smaller in BFs than in ZFs (**Figure 1G**, left, $n$ = 9 BFs and 7 ZFs, $p$ = 0.0021, Mann–Whitney U test). Moreover, although the initial singing rate (see Methods), another measure of singing motivation used in our previous study (Kim et al., 2021), was significantly greater after 5 h LO compared to that after 30 min LO in BFs (**Figure 1F**, $p$ = 0.0078, $n$ = 9 birds) as seen in ZFs (Kim et al., 2021), their effect sizes were significantly smaller than those in ZFs (**Figure 1G** right, $n$ = 9 BFs and 7 ZFs, $p$ = 0.031). In addition, the ratio of birds with a statistical difference in initial singing rate between 30 min and 5 h LO was significantly lower in BFs than in ZFs (1/9 BFs vs. 5/7 ZFs, $p$ = 0.035, Fisher's exact test); no significant difference was observed for first song latency (5/9 BFs vs. 6/7 ZFs, $p$ = 0.31).

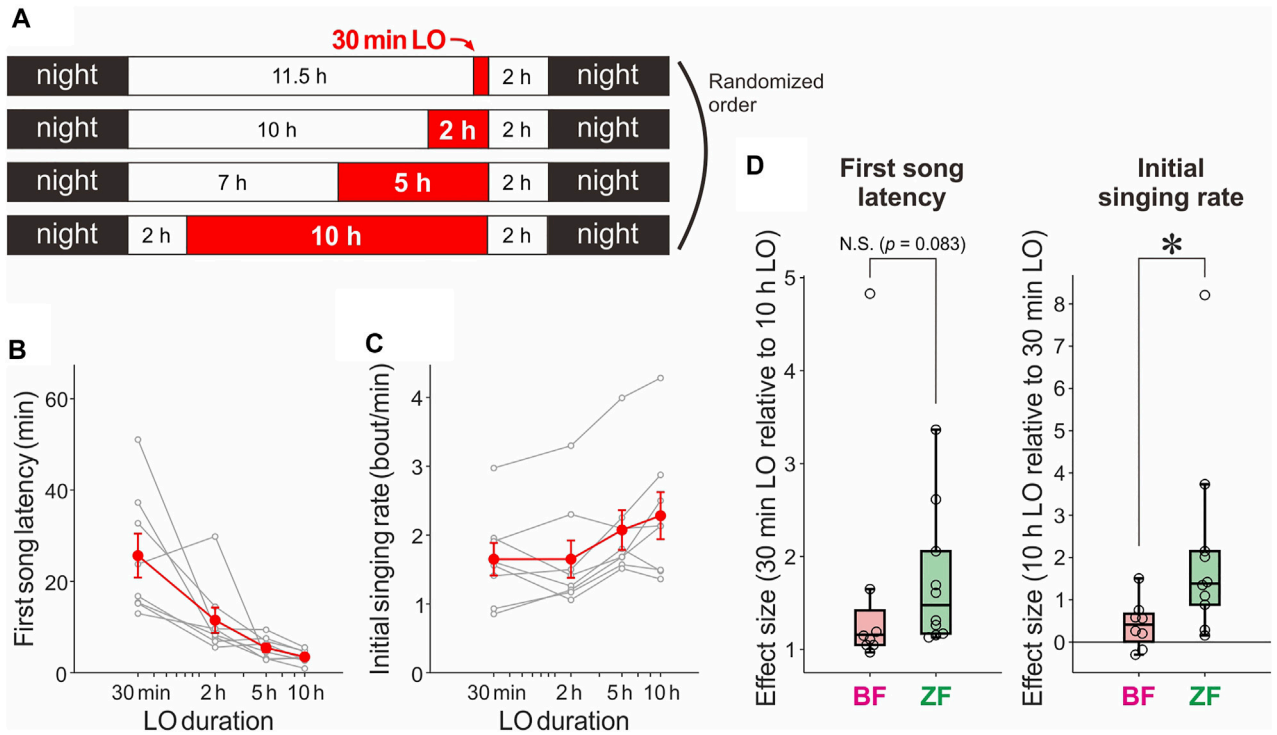


**FIGURE 2 |** Effects of LO with 4 different durations (30 min, 2, 5, and 10 h) on singing motivation in BFs and their comparison with those in ZFs. **(A)** Daily schedule of LO periods (red areas) with four different durations. On each day, a single LO period with one of the four different durations was given with the offset at 2 h before night; the onset was varied depending on the LO duration. Birds received LO periods with four different durations in a randomized order. **(B)** First song latencies plotted against LO durations in BFs ($n$ = 8 birds). Gray lines indicate data for individual birds and red lines represent mean ± SEM across all birds. **(C)** Initial singing rates plotted against LO duration in BFs ($n$ = 8 birds). Conventions are same as in B. **(D)** The effect sizes for the first song latency (left) and the initial singing rate (right) between a 30 min and 10 h LO in BFs and ZFs *$p$ < 0.05.

Taken together, these results indicate that LO-dependent enhancement of intrinsic singing motivation is much smaller in BFs than in ZFs.

## Effects of Lights-Out With Four Different Durations on Singing Motivation in Bengalese Finchs and Their Comparison With Those in Zebra Finchs

Kim et al. (2021) also characterized the suppression-dependent enhancement of singing motivation in ZFs using LO periods with four different durations (30 min, 2, 5, and 10 h). We used the same procedure in BFs and compared the results with those obtained in ZFs. On each experimental day, birds received a single LO period with one of the four durations; all LO periods ended 2 h before nighttime regardless of the duration to exclude possible circadian effects (**Figure 2A**). Consistent with the results of 30 min and 5 h LO shown in **Figure 1**, BFs exhibited enhancement of singing motivation depending on the duration of LO periods to a smaller degree than ZFs. Although the first song latencies monotonically decreased as the LO duration increased (**Figure 2B**, $n$ = 8 birds; $p$ = 0.00036, Friedman's test) in a manner similar to those in ZFs (Kim et al., 2021), the effect sizes (Hedges' g) calculated between the 30 min LO and 10 h LO data tended to be smaller in BFs than in ZFs (**Figure 2D**, left, $n$ = 8 BFs and 10 ZFs, $p$ = 0.083, Mann–Whitney U test; $p$ = 0.014 after removing an apparent outlier in BF data). Moreover, unlike ZFs, which exhibit significant increases in initial singing rates depending on LO duration (Kim et al., 2021), BFs did not show significant increases in initial singing rates (**Figure 2C**, $n$ = 8 birds; $p$ = 0.054). Consistent with this, the effect sizes of the initial singing rate between the 30 min LO and 10 h LO data in BFs were significantly smaller than those in ZFs (**Figure 2D**, right, $n$ = 8 BFs and 10 ZFs, $p$ = 0.021).

## Bengalese Finchs Produce Undirected Songs More Frequently Than Zebra Finchs During Lights-Out Periods

To investigate the possible reasons for the difference in LO-dependent enhancement of singing motivation between BFs and ZFs, we examined singing activity during LO periods ("dark singing") in both species. Interestingly, we found that a subset of BFs exhibited dark singing during relatively long LO periods: In the BFs that received the 30 min/5 h LO schedule shown in **Figure 1B**, 55.6% (5 out of 9 birds) produced at least one bout of undirected song during 5 h LO periods and one of those 5 birds also sung during 30 min LO periods (**Figures 3A–C**). Moreover,

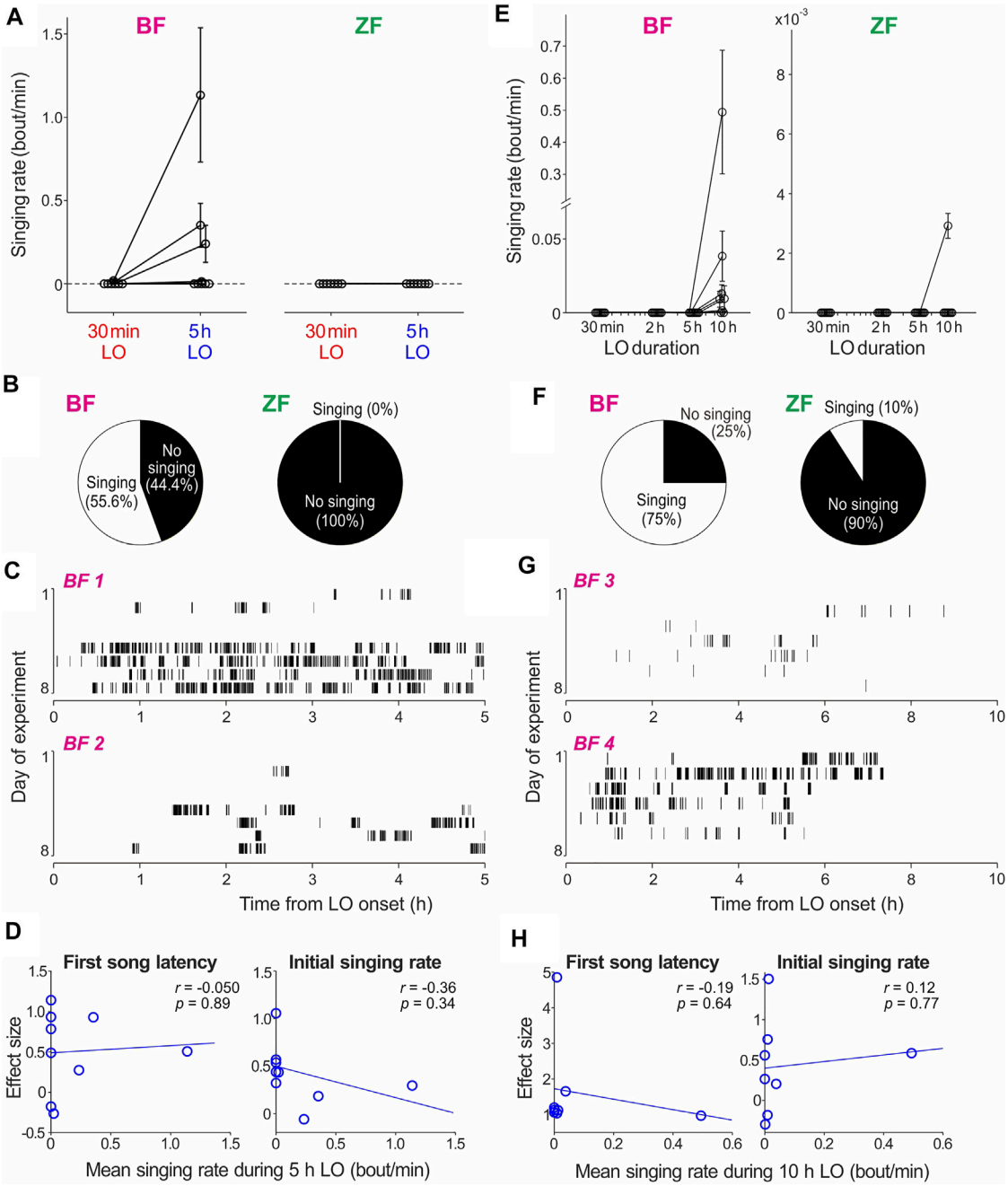


**FIGURE 3 |** Comparison of BF and ZF singing behavior during LO periods. **(A)** Mean singing rates during 30 min and 5 h LO periods in BFs and ZFs ($n$ = 9 BFs and 7 ZFs). **(B)** Percentage of BFs and ZFs that sung during 5 h LO periods. **(C)** Raster plots of song bouts produced during a 5 h LO in 2 representative BFs. **(D)** For all BFs examined, the effect sizes of first song latency (left) and initial singing rate (right) between the 30 min and 5 h LO are plotted against mean singing rate during 5 h LO periods. **(E)** Mean singing rates during LO periods with four different durations in BFs and ZFs ($n$ = 8 BFs and 10 ZFs). **(F)** Percentage of BFs and ZFs that sung during 10 h LO periods. **(G)** Raster plots of song bouts produced during a 10 h LO period in two representative BFs. **(H)** Effect sizes of first song latency (left) and initial singing rate (right) between a 30 min and 10 h LO, plotted against mean singing rate during a 10 h LO.

in the BFs that received the four different LO durations shown in **Figure 2A**, 75.0% (6 out of 8 birds) exhibited dark singing during 10 h LO periods, but no birds sang during any other LO periods (**Figures 3E–G**). Mean rates of dark singing during LO periods were highly variable across birds, ranging from 0 to 1.14 (mean = 0.19) bout/min for the 5 h LO and 0 to 0.49 (mean = 0.070) bout/min for the 10 h LO. In sharp contrast with BFs, almost none of the examined ZFs exhibited dark singing during LO periods. In the experiments with the 30 min/5 h LO schedule, none of the ZFs sung during any LO period ($n$ = 7 birds; **Figures 3A,B**).

Furthermore, in the experiments with four different LO durations, only 1 out of 10 ZFs (10%) sung during 10 h LO periods (**Figures 3E,F**), and the singing rate was relatively low (0.0029 bouts/min) compared to those of BFs. Thus, darkness almost completely suppresses undirected singing in ZFs, but much less strongly in BFs, revealing a striking difference in singing behavior between these species. This differential inhibitory effect of darkness on undirected singing may contribute to the difference in the LO-dependent enhancement of intrinsic singing motivation.

Our results so far in the current study and our previous study (Kim et al., 2021) demonstrate that longer and almost complete suppression of undirected singing causes greater enhancement of intrinsic singing motivation in ZFs whereas many BFs exhibited incomplete singing suppression and smaller degrees of singing motivation enhancement compared to ZFs. Given these findings, we wondered whether the frequency of dark singing (i.e., incompleteness of singing suppression) and the degrees of singing motivation enhancement are negatively correlated. More specifically, we assumed that BFs producing more frequent dark singing exhibit a smaller degree of LO-dependent enhancement of singing motivation, and vice versa. To test this possibility, we examined the relationships between the effect sizes of the LO-dependent enhancement of singing motivation (**Figures 1G**, **2D**) and the amount of dark singing (**Figures 3A,E**) in BFs. However, we did not find any significant correlations between them either when the effect sizes were calculated from the first song latencies or the initial singing rates (**Figure 3D** for 5 h LO; **Figure 3H** for 10 h LO).

## Blood Corticosterone Levels Are Higher in Bengalese Finchs Than in Zebra Finchs

The differential inhibitory effects of darkness on undirected singing between BFs and ZFs suggest that darkness affects the internal physiological state that regulates song initiation differently between the two species. One possible mechanism is that darkness differentially induces stress responses in these species. As singing in the dark generally increases the risk of predation (Lima, 2009), reducing the singing rate in the dark could be a stress reaction to cope with predation. BFs have been shown to exhibit lower degrees of anti-predator stress reactions induced by physical restraint than ZFs and BF's wild ancestor, the white-rumped munia (WRM) (Suzuki et al., 2013; Suzuki et al., 2014), raising the possibility that more frequent dark singing in BFs than in ZFs is attributable to lower degrees of stress responses to darkness. To test this possibility, we measured the blood concentration of corticosterone (CORT), the main stress hormone in birds, in both dark and light conditions (**Figure 4A**) and compared them between BFs and ZFs. Previous studies have demonstrated that environmental stresses, which include the risk of predation, induce CORT release (Scheuerlein et al., 2001; Clinchy et al., 2004), and ZFs with higher CORT levels produce fewer songs (Wada et al., 2008). Therefore, if the more frequent dark singing of BFs is attributable to lower degree of stress responses to darkness, BF CORT levels in dark conditions must be lower than those of ZFs, and darkness would increase CORT levels to a smaller degree in BFs than in ZFs.

We found that blood CORT levels in dark conditions were significantly lower in BFs than in ZFs (**Figure 4B**, left; $n = 12$ BFs and 11 ZFs, $p = 0.0019$, Mann–Whitney U test). These results are consistent with the idea that the more frequent dark singing in BFs is attributable to the lower degrees of stress reactions caused by darkness. However, lower CORT levels in BFs than in ZFs were observed even under light conditions (**Figure 4B**, right; $p = 0.0056$). Moreover, the dark/light ratios in CORT levels were not significantly different from 1 (**Figure 4C**; $p = 0.73$ for BFs and 0.32 for ZFs, Wilcoxon signed-rank test), and there was no significant difference between BFs and ZFs (**Figure 4C**; $p = 0.28$). These results indicate that BFs have lower stress levels than ZFs regardless of whether they are in dark or light conditions, suggesting that stress responses are not a major factor suppressing undirected singing in dark conditions. Thus, frequent dark singing in BFs is unlikely to directly result from their lower degree of stress responses to darkness.

# DISCUSSION

In the present study, we highlighted important differences in singing behavior between BFs and ZFs, the two most commonly used songbirds in neurobiological studies of vocal learning and communication. We found that the LO-dependent enhancement of intrinsic motivation for undirected singing was much smaller in BFs than in ZFs. Also, in contrast with almost no dark singing in ZFs during LO periods, a substantial portion of BFs exhibited frequent dark singing during relatively long LO periods, providing a plausible explanation for the smaller degree of LO-induced enhancement of intrinsic singing motivation in BFs. Moreover, although blood CORT levels were significantly lower in BFs than in ZFs in both light and dark conditions, no significant differences were observed between the conditions in either species. These comparative data in BFs and ZFs demonstrate that darkness differentially influences intrinsic motivation and production of undirected song between these species, and highlight the difficulty in manipulating undirected song production and motivation using darkness in BFs. Our results also provide new insights into the interactions between song production, intrinsic singing motivation, ambient light, and environmental stress.

Both BFs and ZFs have been extensively used to investigate the behavioral and neural mechanisms of vocal learning and communication. For example, a commonly used experimental manipulation to induce adaptive changes in the acoustic structure of adult song elements was first reported in BFs (Tumer and Brainard, 2007) and the neural mechanisms of such song changes have been intensively studied in both BFs and ZFs (Andalman and Fee, 2009; Charlesworth et al., 2012; Gadagkar et al., 2016; Hisey et al., 2018; Xiao et al., 2018; Tachibana et al., 2022). Because of the vocal learning ability and underlying neural circuits largely shared between these species, most studies have

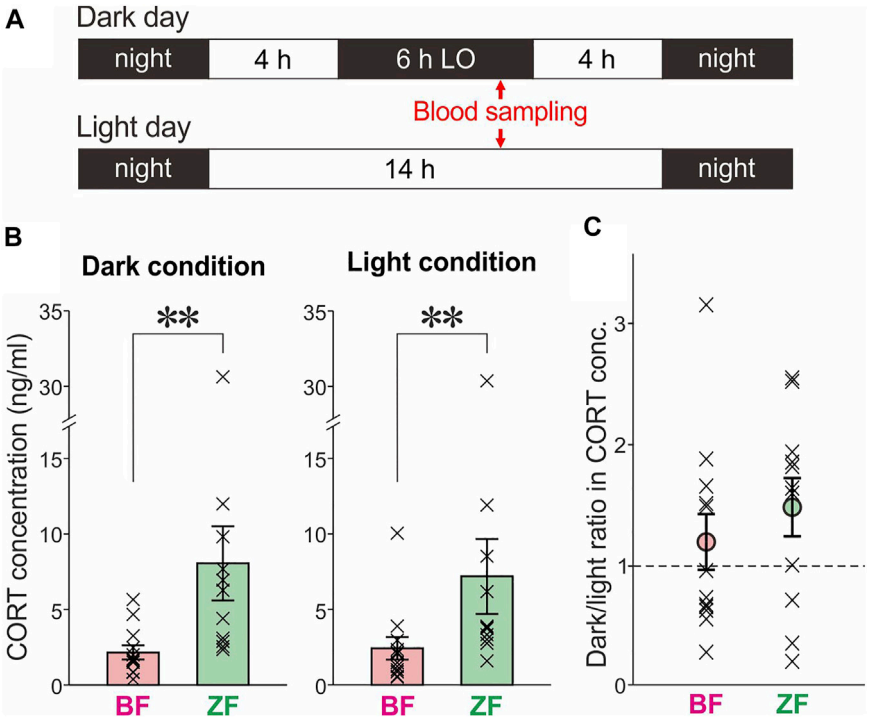


**FIGURE 4 |** Comparison of blood CORT levels in dark and light conditions between BFs and ZFs. **(A)** Daily schedules of blood sampling with and without LO. **(B)** Blood CORT levels in BFs and ZFs under dark (left) and light (right) conditions. BFs showed significantly lower CORT levels than ZFs in both conditions (**$p < 0.01$). **(C)** Dark/light ratios in CORT levels were not significantly different from 1 either in BFs ($p = 0.73$) or in ZFs ($p = 0.32$). No significant difference was found between BFs and ZFs ($p = 0.28$).

used them with little attention to species differences, although a limited number of studies took advantage of variable syllable sequencing in BF songs (Okanoya, 2004; Warren et al., 2012; Lipkind et al., 2013; Polomova et al., 2019; Veit et al., 2021). The current study systematically compared the behavioral properties of singing between the two species and investigated the underlying mechanisms, revealing previously unknown differences between them. Given the widespread use of both BFs and ZFs in neurobiological studies of vocal learning and communication, our findings, as well as other comparative studies in those species, including our recent study (Tachibana et al., 2022), broaden our understanding of the behavioral and neural mechanisms underlying learning and production of complex motor behaviors.

BF is a domesticated strain of the wild ancestor WRM, and a growing body of research suggests that in the course of the domestication process, BFs have acquired behavioral, anatomical, and neuroendocrine changes, including reduced sensitivity to environmental stresses (Suzuki et al., 2014; Suzuki et al., 2021; Suzuki and Okanoya, 2021; Tobari et al., 2022). In particular, BFs exhibit reduced levels of fearfulness related to coping with predation (Suzuki et al., 2013) and lower fecal CORT levels than WRM (Suzuki et al., 2012). We hypothesized that such reduced sensitivity to predation-related environmental stresses in BFs is a crucial factor underlying their frequent dark singing, and that hypothesis was partially supported by our results: The lower CORT levels in BFs than in ZFs in dark conditions (**Figure 4B**) are consistent with the idea that frequent dark singing in BFs is attributable to their reduced sensitivity to environmental stresses caused by darkness. Because darkness generally increases predation risk (Lima, 2009), this hypothesis also predicted that CORT levels would be higher under dark conditions than under light conditions in both species, but we did not find such cross-condition differences in either species (**Figure 4C**). This implies that singing suppression by darkness is mainly mediated by a physiological process that does not critically involve stress responses. Although darkness-induced reduction in singing is presumably an adaptive behavior that has evolved to reduce predation risk at night, it may be an automatic response to darkness that normally occurs independently of environmental stresses. This notion is also supported by our results in ZFs showing that intrinsic singing motivation is enhanced while singing is suppressed by darkness, because strong environmental stresses such as predation risk are likely to reduce intrinsic singing motivation rather than enhance it. Taken together, our CORT results lead to the conclusion that the frequent dark singing in BFs is not directly attributable to their reduced sensitivity to environmental stresses, but rather may reflect adaptive behavioral changes that BFs have acquired through domestication in captive environments with low predation risk. Of course, we cannot rule out the possibility that our blood sampling operations substantially affected CORT levels

under both dark and light conditions and attenuated dark-light differences. However, this is unlikely because the stress responses caused by blood sampling, if any, should affect CORT levels equally in both dark and light conditions, and thus would not severely attenuate dark-light differences. In addition, our blood sampling procedure, which was completed within 3 min, is considered appropriate for measuring the baseline levels of blood CORT concentrations (Wada et al., 2008).

The neurophysiological mechanisms by which song production is regulated by intrinsic motivation, ambient light, and environmental stress remain unclear and should be investigated in further studies. Our results in ZFs that LO suppresses song production but enhances intrinsic singing motivation strongly suggest that darkness affects only the song initiation process, but not intrinsic singing motivation, which normally drives song initiation in light conditions. In addition, the enhancement of intrinsic singing motivation depending on the duration of LO periods suggests that intrinsic singing motivation spontaneously and gradually increases over time while song production is suppressed, but substantially decreases after the song is produced. Moreover, unlike darkness, strong environmental stresses (such as predation risk) appear to suppress song production, presumably by decreasing intrinsic singing motivation. Because modulations of intrinsically motivated behaviors by ambient light and environmental stresses are universal phenomena observed not only in songbirds but also in many other bird and animal species, understanding the neurophysiological mechanisms of how undirected singing is regulated by intrinsic motivation and modulated by ambient light and environmental stress would provide great insights into the physiology of animal behavior and cognition.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the Korea Brain Research Institute.

## AUTHOR CONTRIBUTIONS

YK and SK conducted the experiments and data analyses; SK designed the experiments; YK, CM, and SK wrote the paper.

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# Developmental-Based Classification of Enkephalin and Somatostatin Containing Neurons of the Chicken Central Extended Amygdala

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The central extended amygdala, including the lateral bed nucleus of the stria terminalis and the central amygdala, plays a key role in stress response. To understand how the central extended amygdala regulates stress it is essential to dissect this structure at molecular, cellular and circuit levels. In mammals, the central amygdala contains two distinct cell populations that become active (on cells) or inactive (off cells) during the conditioned fear response. These two cell types inhibit each other and project mainly unidirectionally to output cells, thus providing a sophisticated regulation of stress. These two cell types express either protein kinase C-delta/enkephalin or somatostatin, and were suggested to originate in different embryonic domains of the subpallium that respectively express the transcription factors Pax6 or Nkx2.1 during development. The regulation of the stress response by the central extended amygdala is poorly studied in non-mammals. Using an evolutionary developmental neurobiology approach, we previously identified several subdivisions in the central extended amygdala of chicken. These contain Pax6, Islet1 and Nkx2.1 cells that originate in dorsal striatal, ventral striatal or pallidopreoptic embryonic divisions, and also contain neurons expressing enkephalin and somatostatin. To know the origin of these cells, in this study we carried out multiple fluorescent labeling to analyze coexpression of different transcription factors with enkephalin or somatostatin. We found that many enkephalin cells coexpress Pax6 and likely derive from the dorsal striatal division, resembling the off cells of the mouse central amygdala. In contrast, most somatostatin cells coexpress Nkx2.1 and derive from the pallidal division, resembling the on cells. We also found coexpression of enkephalin and somatostatin with other transcription factors. Our results show the existence of multiple cell types in the central extended amygdala of chicken, perhaps including on/off cell systems, and set the basis for studying the role of these cells in stress regulation.

**Keywords: bed nucleus of stria terminalis, central amygdala, intercalated amygdalar cells, stress regulation, embryonic origin, pain regulation**

## INTRODUCTION

The stress response is triggered by coordinated activation of the neuroendocrine and the autonomic nervous systems, with the participation of specific subsets of neurons of the hypothalamus and brainstem (reviewed by Ulrich-Lai and Herman, 2009). These systems are regulated by the telencephalon, where the central extended amygdala, including the central nucleus of the amygdala and the lateral bed nucleus of the stria terminalis (BSTL), plays critical roles (Phelps and LeDoux, 2005). It appears that the central amygdala is an integration center that plays a key role in systemic stress (involving life threatening challenges), and less so in psychogenic stress that is mostly regulated by the medial amygdala (Ulrich-Lai and Herman, 2009). However, both the central amygdala and the BSTL are very complex in terms of subdivisions, cell composition, chemoarchitecture and connections (Cassell et al., 1986; Gray and Magnuson, 1992; Dong et al., 2001; Dong and Swanson, 2003). Based on its projections, central amygdala regulation of the hypothalamus and brainstem can be direct or indirect by way of the BSTL (Gray and Magnuson, 1987, 1989, 1992; Dong et al., 2001; Davis et al., 2010). By way of its direct projections, the medial part of the central amygdala appears to play a relevant role in phasic (short) fear responses to discrete cues (both conditioned and unconditioned), mostly through control of autonomic and somatic responses (Walker and Davis, 2008; Davis et al., 2010). However, the BSTL (and the central amygdala cells projecting to BSTL) appears to play a prevalent role in sustained (long lasting, anxiety-like) fear responses to contextual conditioned stimuli, mainly by controlling the hypothalamic-pituitary-adrenal axis (Pêgo et al., 2008; Davis et al., 2010). To understand how the central extended amygdala regulates stress it is essential to dissect this structure at molecular, cellular and circuit levels. This has started to be done in mammals, mostly using mouse and rats as models. Tract-tracing studies combined with immunohistochemistry or immunofluorescence showed that both the central amygdala and the BSTL contain different neuropeptidergic cell types involved in internal connections (between central amygdala and BSTL, and viceversa) and/or in projections to the hypothalamus and brainstem. For example, the central amygdala contains enkephalinergic (ENK) neurons and somatostatin (SST) neurons that project to the BSTL (McDonald, 1987; Rao et al., 1987). In addition, the central amygdala and the BSTL contain SST, neurotensin, substance P, and/or corticotropin-releasing factor (CRF) expressing neurons that project to the hypothalamus, the periaqueductal gray, the parabrachial nucleus and the nucleus of the solitary tract (Moga and Gray, 1985; Moga et al., 1989; Gray and Magnuson, 1987, 1992; Gray, 1993). More recently, using optogenetics combined with pharmacological and electrophysiology approaches in mouse, two distinct cell populations of the central amygdala were found to become active (on cells) or inactive (off cells) during conditioned fear responses (Ciocchi et al., 2010). They include two subtypes of inhibitory neurons located in the capsular/lateral subdivisions of the central amygdala, which are able to inhibit each other, and project in a mostly unidirectional manner to output neurons of the medial subdivision of the central amygdala (Ciocchi et al., 2010). It appears that the off cells express the protein kinase C-delta (PKCδ) (Haubensak et al., 2010), many of which are enkephalinergic (more than 40%, Supplementary Table S1 in Haubensak et al., 2010). In contrast, the on cells do not contain PKCδ, but express SST (Penzo et al., 2014). It also appears that in mouse PKCδ positive cells play a role in promoting anxiety-like behavior (Douceau et al., 2022), while SST neurons become active by threat-predicting sensory cues after fear conditioning, and promote passive defensive behaviors (Yu et al., 2016). The ENK/PKCδ cells of the mouse central amygdala have also been involved in stress-induced pain regulation (Paretkar and Dimitrov, 2019). Understanding the neural mechanisms regulating the stress response in non-mammals, and how they help animals to cope with changing environmental conditions, is critical for identifying general principles on stress regulation in vertebrates, but also for improving animal welfare (Broom, 1987). This is a big concern in farm animals, including poultry, which represents one of the most intensive farming systems in the European Union, and produces the second most consumed meat, after pig meat (Augère-Granier and Members' Research Service, 2019). However, brain-behavior relationships are poorly understood in farm animals, including chicken. One of the most challenging problems is to identify in non-mammals the homologues of telencephalic areas known to regulate the hypothalamic-pituitary-adrenal axis and the autonomic nervous system in mammals. This is particularly difficult in birds due to the highly divergent evolution of their telencephalon when compared to that of mammals (Reiner et al., 2004). To solve this problem, our group has been using a very powerful approach based on the evolutionary developmental neurobiology (Medina et al., 2011, 2017). This is based on the fact that, at early embryonic stages, the brain of different vertebrates is more similar, and fundamental divisions-homologous across species-are easily identified based on their topological position together with the combinatorial expression of developmental regulatory genes. This can help to follow these divisions from these early embryonic stages and trace their derived cells throughout development. Since the embryonic origin of cells conditions much of their phenotype, this information is also relevant to better understand the logic behind the connections of different neurons and, overall, the functional networks of the mature amygdala (Medina et al., 2011, 2017; Sokolowski & Corbin, 2012; Morales et al., 2021). We used this approach to identify the cell populations that constitute the central extended amygdala in the chicken and zebra finch telencephalon (Vicario et al., 2014, 2015, 2017). Basically, we found three major cell types derived from the dorsal striatal embryonic division (expressing the transcription factor Pax6), the ventral striatal division (expressing the transcription factor Islet1), and the pallidal division (expressing the

transcription factor Nkx2.1), which grouped in different combinations across several areas and nuclei located above the lateral branch of the anterior commissure, between the ventral part of the lateral ventricle and the arcopallium (Vicario et al., 2014, 2015, 2017). From medial to lateral, these included: 1) the BSTL, medially; 2) the peri-intrapeduncular island field (pINP), the oval central nucleus (Ceov), and the perioval zone (Pov), at intermediate levels; and 3) the capsular central amygdala (CeC) and the intercalated cells (ITC), laterally. These cell populations were argued to be homologous to those of the mammalian central extended amygdala (Vicario et al., 2014, 2015, 2017), which include similar cell types with identical embryonic origin (Bupesh et al., 2011). In mouse, SST cells of the telencephalon, including those of the central amygdala, appear to originate from Nkx2.1-expressing progenitors of the pallidal embryonic division, including its ventrocaudal or diagonal subdomain (García-López et al., 2008; Xu et al., 2008; Real et al., 2009; Bupesh et al., 2011; Puelles et al., 2016). Based on this, we suggested that the on cells, previously found to express SST in mouse, have a pallidal origin (Bupesh et al., 2011). Regarding the ENK cells (more than 70% of which express PKCδ in mouse and are thus off cells; Haubensak et al., 2010), based on correlation in their distribution with that of the Pax6 cells, we suggested that they might originate in the dorsal striatal division (Bupesh et al., 2011). Based on the finding of similar cells in birds and turtles, we suggested the presence of on/off cell systems in the central extended amygdala of sauropsids (Medina et al., 2017). However, data on colocalization of ENK or SST with region-specific transcription factors of the subpallium, such as Pax6 (dorsal striatal), Islet1 (ventral striatal) and Nkx2.1 (pallido-preoptic), in cells of the central extended amygdala are missing in mammals and non-mammals. Without this, we cannot know if ENK and SST of the central extended amygdala include one or different subpopulations with different origins. The aim of this study was to analyze coexpression of different transcription factors in ENK or SST cells of the chicken central extended amygdala, and to provide a developmental-based classification of neurons that can be useful in future studies on amygdalar function in chicken.

# MATERIALS AND METHODS

## Animals

Fertilized eggs of domestic chicks (*Gallus gallus domesticus*; Leghorn strain) were obtained from a commercial hatchery (Granja Santa Isabel, Cordoba, Spain; Authorization ES140210000002), which were incubated at 37.5°C and 55%–60% relative humidity, with rocking. Fertilized eggs were selected by a light test on the day of experiment and only those that contained live embryos were used. The first day of incubation was considered embryonic day 0 (E0).

All animals were treated according to the regulations and laws of the European Union (Directive 2010/63/EU) and the Spanish Government (Royal Decrees 53/2013 and 118/2021) for the care and handling of animals in research. The protocols used were approved by the Committees of Ethics for Animal Experimentation and Biosecurity of the University of Lleida (reference no. CEEA 08-02/19), as well as that of the Catalonian Government (reference no. CEA/9960_MR1/P3/1 for embryos, and CEA/9960_MR1/P4/1 for post-hatchlings).

## Tissue Collection and Fixation

Embryos at embryonic day 16 (E16) and 18 (E18), as well as post-hatchlings until day 2 (P2) were used ($N = 31$). At the right day, animals were anaesthetized as follows. First, for embryos a small hole was made in the egg shell and membrane at the level of the air sac, and then the egg was placed in a camera containing Halothane (2-Bromo-Chloro-1,1,1-trifluoro-ethane, Sigma-Aldrich, Germany; 1 ml Halothane/1,000 ml of chamber volume). Post-hatchlings were also placed in a camera containing Halothane in the same concentration as above, until inducing anesthesia. After, the embryos and post-hatchlings received a euthanasic dose of Dolethal (100 mg/kg of sodium pentobarbital; intraperitoneal). Following this, the animals were perfused transcardially with cold saline solution (0.9% NaCl) containing Heparin (Sigma-Aldrich), followed by phosphate-buffered (PB) 4% paraformaldehyde (PFA 4%, pH 7.4, PB 0.1 M). After dissection and post-fixation (24 h at 4°C), brains were sectioned (100 μm-thick) in coronal plane using a vibratome (Leica VT 1000 S). All brain sections were maintained at 4°C (for short storage) or at−20°C (for longer storage) in hybridization buffer, until being processed as described below.

## Single and Double Chromogenic Labeling: *In Situ* Hybridization and Immunohistochemistry

Some series of brain sections were processed for single *in situ* hybridization, whereas other series of sections were processed for double labeling, doing first *in situ* hybridization, followed by immunohistochemistry.

Brain sections were processed for *in situ* hybridization using digoxigenin-labelled riboprobes, following the procedure previously described by Abellán et al. (2014) and Vicario et al. (2014). The antisense digoxigenin-labelled riboprobes were synthesized using Roche Diagnostics (Switzerland) protocols from cDNAs containing a fragment of the gene of interest:

- Pro-enkephalin (*pENK*; bp 1–862; Genbank accession no. XM_419213.3; BBSRC ChickEST Database, clone ChEST140a9; Boardman et al., 2002).
- Somatostatin precursor (*SST*; bp 1–707; Genbank accession no. NM_205336.1; BBSRC ChickEST Database, clone ChEST114E9; Boardman et al., 2002).

Free floating sections were prehybridized in hybridization buffer (HB; as described by Abellán et al., 2014; Vicario et al., 2014), for 2–4 h at 58°C. Then, the sections were hybridized overnight at 61°C in HB containing 0.5–1 μg/ml of the riboprobe, depending of the age of the embryo. For pENK, the standard HB

was used throughout the procedure. For SST, a different, viscous hybridization buffer (HBv) was used during the hybridization step, to optimize the labelling. HBv contained 50% formamide molecular (Sigma-Aldrich), 10% dextran sulfate (Sigma-Aldrich), 1 mg/ml of yeast tRNA (Sigma-Aldrich), 0.2% Tween-20, 2% Denhardt solution (Sigma-Aldrich), and 10% salt solution in RNase and DNase free water (Sigma-Aldrich). The following day, the hybridized sections (both pENK and SST) went through a series of washes: first in HB at 58°C, then in a mix 1:1 of HB and MABT (1.2% Maleic acid, 0.8% NaOH, 0.84% NaCl, and 0.1% Tween-20) at 58°C, and finally in MABT at room temperature. Then, the sections were blocked to avoid unspecific binding, using a blocking solution containing 10% blocking reagent (Roche Diagnostics, Switzerland) and 10% of sheep serum (Sigma-Aldrich) in MABT, for 4 h at room temperature. Following this, the sections were incubated overnight, at 4°C, with a sheep anti-digoxigenin antibody conjugated with alkaline phosphatase (AP coupled anti-DIG, Roche Diagnostics), diluted 1:3,500 in blocking solution, followed by washing with MABT. Signaling was revealed by incubation with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche Diagnostics) for 4–8 h at room temperature. After washing in MABT followed by an overnight step in 4% PFA, some of the sections were then processed for the immunohistochemistry, as follows.

For immunohistochemistry, three different antibodies were used:

- Mouse anti-Pax6, raised against recombinant protein containing aminoacids 1–223 of chick Pax6, made in E. coli (Developmental Studies Hybridoma Bank, University of Iowa, Iowa, IA, United States; catalog reference: Pax6); diluted 1/1,000.
- Mouse anti-Islet1, raised against the C-terminal residues 178–349 of rat Islet1 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa, IA, United States; catalog no. 40.2D6); diluted 1/1,000.
- Rabbit anti- Nkx2.1 (anti- TTF-1), raised against the N-terminal residues 110–122 of rat Nkx2.1 (Biopat Immunotechnologies, Italy; catalog no. PA0100); diluted 1/4,000.

Briefly, sections were first processed to inhibit endogenous peroxidase activity by incubating in 1% $H_2O_2$ and 10% methanol in phosphate buffered saline (PBS) for 30 min. Then, the tissue was permeabilized by washing with PBS containing 0.3% Triton X 100 (PBST; pH 7.4; 0.1 M), followed by an incubation with a blocking solution, containing 10% normal goat serum (NGS; Vector Laboratories Ltd., United Kingdom) and 2% of bovine serum albumin (BSA) in PBST, for 1 h at room temperature. Then, the sections were incubated in the primary antibody, diluted in blocking solution, for about 64 h at 4°C and gentle agitation. After incubation in the primary antibody, the sections were rinsed in PBST and then incubated in a biotinylated secondary antibody diluted in PBST overnight and under gentle agitation.

The secondary antibodies used were:

- Goat anti-mouse, biotinylated, diluted 1/200 (Vector, Burlingame, CA, United States).
- Goat anti-rabbit, biotinylated, diluted 1/200 (Vector, Burlingame, CA, United States).

Then, the sections were rinsed and incubated with the Vectastain Elite ABC Kit (PK- 6100, Vector Laboratories). After rinsing, the immunoreactivity was revealed by a color reaction, incubating the sections in a DAB solution (SIGMAFAST, 3,3′-Diaminobenzidine tablets, Sigma-Aldrich) diluted in water, following the manufacturer instructions. Finally, the sections were rinsed in Tris buffer (0.05 M, pH 8) and then mounted on gelatinized glasses, dehydrated and cover slipped with Permount (Fisher Scientific, United States).

## Double and Triple Fluorescent Labeling: Fluorescent *In Situ* Hybridization and Immunofluorescence

To check if there is coexpression of different markers at cellular level, different series of parallel sections were processed either for double immunofluorescence or for indirect fluorescent *in situ* hybridization combined with single or double immunofluorescence.

The protocol used was previously described by Morales et al. (2021) and Metwalli et al. (2022). Briefly, floating sections were pre-hybridized in HB for 2–4 h at 58°C. Then, the sections were hybridized overnight at 61°C in the HB or HBv buffers previously described containing 1 μg/ml of the riboprobes *pENK* or *SST* (as explained above). The following day, the sections were washed with HB for 30 min at 58°C. After that, a series of washes were done using saline sodium-citrate buffer (SSC; pH 7.5, 0.2 M), 3 times for 20 min, at 58°C, followed by 1 wash in the same buffer for 15 min at room temperature and then 1 wash with Tris buffer (TB, 0.1 M, pH 8) for 15 min at room temperature. The activity of the endogenous peroxidase was inhibited as described above, diluting the hydrogen peroxide in TB. After, the sections were washed with Tris-NaCl-Tween buffer (TNT; 10% TB, pH 8, 0.1 M; 0.9% NaCl; 0.05% Tween-20) for 15 min at room temperature (RT). Following this, the sections were incubated in a blocking solution (TNB) consisting of 20% BBR and 20% of sheep serum in TNT for 2–4 h at RT, followed by incubation in sheep anti-digoxigenin antibody conjugated to the peroxidase enzyme (anti-DIG POD; diluted 1:200; Roche Diagnostics, Basel, Switzerland) in TNB, overnight at 4°C and under gentle agitation. After washing with TNT 3 times for 10 min each, the slices were incubated in Cy3 tyramide complex (AATBioquest, United States), prepared in TB containing 0.003% of $H_2O_2$ (diluted 1/50 and 1/200 for pENK and SST, respectively), for 10 min. Finally, the sections were rinsed in TB and processed for single of double immunofluorescence to detect the same epitopes previously described (Pax6, Islet1 or Nkx2.1).

After tissue permeabilization and blocking of unspecific binding (the same described above for the immunohistochemistry), the sections were incubated in the primary antibodies previously described (rabbit anti-Nkx2.1, diluted 1/4,000; mouse anti-Pax6, diluted 1/100; mouse anti-

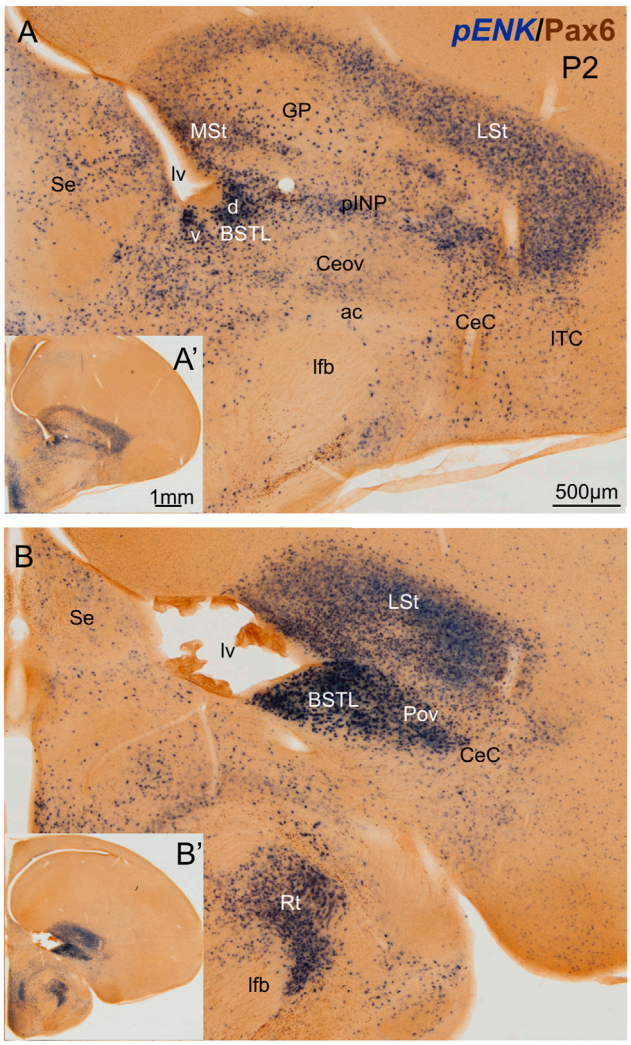


**FIGURE 1 |** Chromogenic double labeling of pro-enkephalin (*pENK*) and Pax6 in the chicken central extended amygdala at P2. **(A,B)** Details of the subpallium, with the central extended amygdala, taken from frontal sections (insets) of the chicken embryonic telencephalon, at commissural **(A)** and post-commissural **(B)** levels, hybridized for *pENK* (blue color) and immunostained for Pax6 (brown color). Note the high amount of *pENK* cells in the BSTL and pINP. Both areas also contain many Pax6 cells, which are better appreciated in the fluorescent images (**Figures 2**,**5**,**7**). See text for more details. For abbreviations see list. Scale: bar in **(A)** = 500 µm [applies to **(A,B)**]. **(A')** = 1 mm (applies to **(A',B')**].

Islet1, diluted 1/100), and combining them in different ways to obtain the triple labelling staining. Following this, sections were washed and then incubated in one or two of the following fluorescence secondary antibodies, diluted in PBST overnight at 4°C.

- Alexa fluor 488 goat anti-mouse Ig (G+L), Invitrogen-Thermo Fisher Scientific (United States), diluted 1/500
- Alexa fluor 488 goat anti-rabbit Ig (G+L), Invitrogen-Thermo Fisher Scientific (United States), diluted 1/500
- Alexa fluor 405 goat anti-rabbit Ig (G+L) Invitrogen-Thermo Fisher Scientific (United States), diluted 1/500.

Finally, the sections were rinsed in PBS, mounted as explained above, and cover slipped using an antifading mounting medium with or without DAPI (Vectashield Hardset Antifade mounting medium and Vectashield Hardset Antifade mounting medium with DAPI, Vector Laboratories Ltd., United Kingdom).

## Digital Photographs and Figures

Digital microphotographs from chromogenic experiments were taken on a Leica microscope (DMR HC, Leica Microsystems GmbH) equipped with a Zeiss Axiovision Digital Camera (Carl Zeiss, Germany), using ×1.6 and ×5 magnification objectives. Serial images from fluorescent material were taken with a confocal microscope (Olympus FV1000, Olympus Corporation, Japan) using ×10 and ×40 objectives; Z-series stacks were taken at 2 µm-step to allow analysis of co-expression. The fluorescent images were adjusted for brightness and contrast, and extracted using Olympus FV10-ASW 4.2 Viewer (Olympus Corporation). Some of the images at ×40 were used to estimate degree of colocalization in selected areas. Counting was done manually using ImageJ Fiji (Schindelin et al., 2012) at a single z-level, selected for displaying the best labeling for all markers. All the figures were mounted using CorelDraw 2012 (Corel Corporation, Canada).

# RESULTS

## Enkephalinergic Cells

We first analyzed the distribution of ENK cells in the central extended amygdala of chicken, ranging from E16 to P2. As previously described (Vicario et al., 2014), ENK cells were abundant and densely grouped in the BSTL and pINP, moderately abundant and sparser in CeC and ITC, and scarce in Ceov (**Figure 1**). We also observed that the BSTL was particularly complex and showed variations along its antero-posterior, mediolateral, and dorsoventral axes. Based on ENK cell distribution, it showed dorsal and ventral subdivisions. The first was larger at anterior levels (**Figure 1A**), while the second became predominant at posterior levels (**Figure 1B**). In the dorsal BSTL subdivision, ENK cells were more densely grouped in the intermediate zone of the nucleus, and sparser in the lateral zone (**Figure 1A**). In the dorsal BSTL subdivision, the periventricular zone was poor in ENK cells, and the ENK cells found in the intermediate zone showed continuity with those in the ventralmost part of the striatal division. In the ventral BSTL subdivision, ENK cells were densely grouped from periventricular to lateral levels. This was especially visible at posterior levels, when pINP had already disappeared (**Figure 1B**), and ENK cells of BSTL were continuous with those of the perioval zone (Pov) (see also Vicario et al., 2014).

Double chromogenic labeling of ENK with Pax6, Islet1 or Nkx2.1 confirmed overlapping of ENK cells with: 1) Pax6 cells mainly in CeC, pINP and BSTL; 2) Islet1 cells mainly in pINP and BSTL; and 3) Nkx2.1 cells mainly in Pov and BSTL. To know if

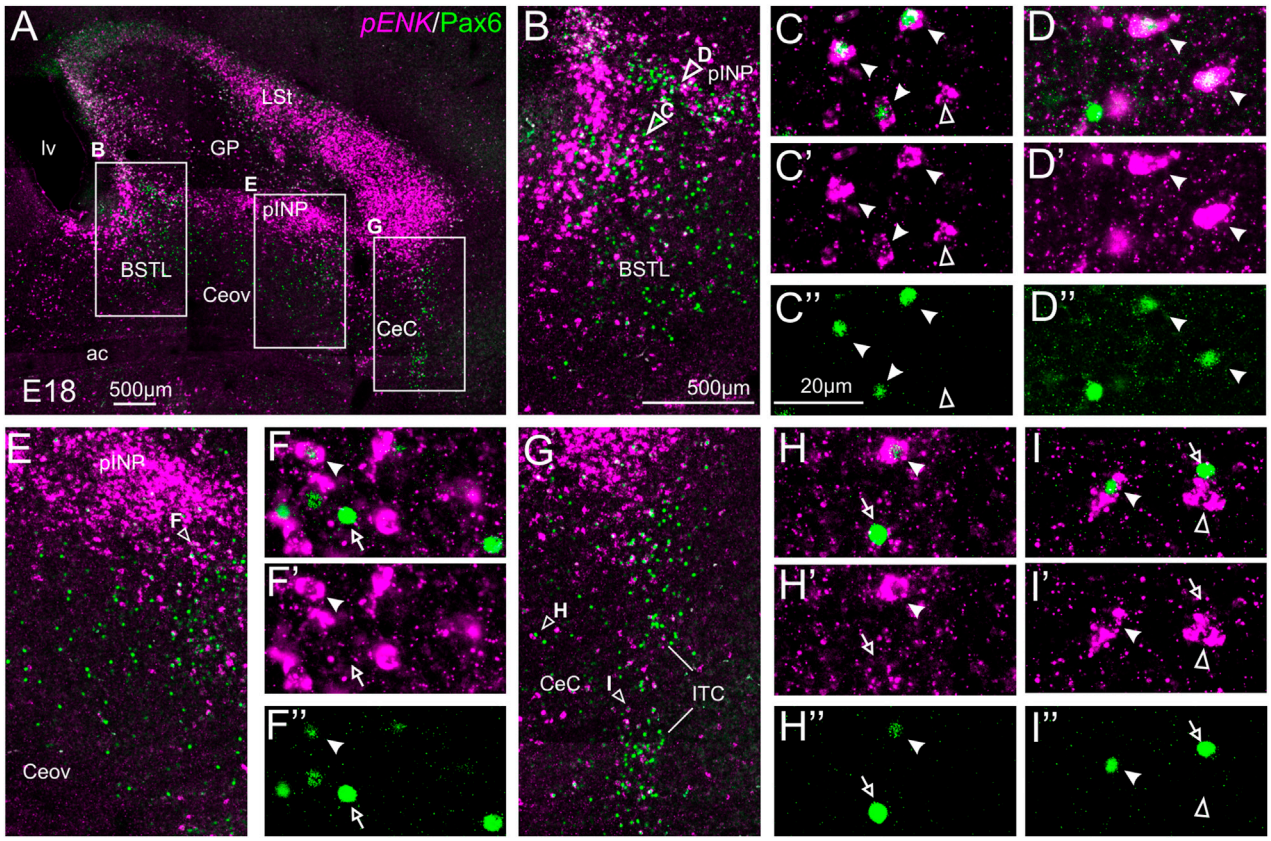


**FIGURE 2 |** Double fluorescence labeling of pro-ENK (*pENK*) and Pax6 in the chicken central extended amygdala at E18. **(A)** General view (×10 objective) of the subpallium in a frontal section at the level of the anterior commissure, processed for indirect fluorescent *in situ* hybridization for *pENK* (magenta) and immunofluorescence for Pax6 (green). **(B,E,G)** show amplifications of the squared areas in **(A)**, at the level of the BSTL and medial pINP **(B)**, lateral pINP **(E)** or CeC and ITC **(G)**. Details (×40 objective, only one single z level of the confocal stack) of the areas pointed with arrowheads in **(B,E)**, and **(G)** are shown in **(C–C′′)** (for BSTL), **(D–D′′)** (for medial pINP), **(F–F′′)** (for lateral pINP), **(H–H′′)** (for CeC) and **(I–I′′)** (for ITC) (merged plus separate magenta and green channels are shown). In these details, cells coexpressing *pENK* and Pax6 are pointed with a filled arrowhead, cells single labeled for *pENK* are pointed with an empty arrowhead, while cells single labeled for Pax6 are pointed with an empty arrow (only a few examples are pointed). See text for more details. For abbreviations see list. Scale bars: **(A)** = 500 µm; **(B)** = 500 µm [applies to **(B,E,G)**]; **(C′′)** = 20 µm [applies to **(C–C′′,D–D′′,F–F′′, H–H′′,I–I′′)**].

there is coexpression of ENK and any of the transcription factors, we carried out double and triple fluorescent labeling in animals ranging from E16 until P0 (N = 16; 2 E16, 13 E18, 1 P0).

## Double Fluorescent Labeling of Enkephalin and Pax6

We did indirect *in situ* hybridization for ENK (magenta in **Figure 2**) combined with immunofluorescence for Pax6 (green in **Figure 2**). Observation with the 10X objective allowed confirmation of the known labeling patterns of ENK and Pax6 in the subpallium (**Figure 2A**). Analysis at higher magnification (with 40X objective) allowed distinction of double labeled cells in some of the areas with overlapping of both markers, as follows. Many cases of cells coexpressing ENK and Pax6 were found in the intermediate and lateral zones of dorsal BSTL and medial part of pINP (**Figure 2B**, details in **Figures 2C–C′′,D–D′′**). In these areas, about half of the ENK cells coexpressed Pax6. More laterally, the pINP also contained a few cases of double labeled cells (**Figure 2E**, detail in **Figures 2F–F′′**). Even more laterally, some cases of double labeled cells were observed in the CeC and ITC (**Figure 2G**, details in **Figures 2H–H′′,I–I′′**). Across the areas, some of the double labeled cells showed light intensity of Pax6 immunofluorescence, while other cells were intensely labeled for this transcription factor (for example, **Figures 2H–H′′**). In all of these areas, we also observed many single labeled ENK cells and Pax6 cells.

## Double Fluorescent Labeling of Enkephalin and Islet1

We also carried out indirect *in situ* hybridization for ENK (magenta in **Figure 3**) combined with immunofluorescence for Islet1 (green in **Figure 3**). Using the 10X objective, we first confirmed that the labeling patterns of ENK and Islet1 in the subpallium were in accordance with previous descriptions (**Figure 3A**, amplifications of the squared areas are shown in **Figures 3B,E**). Analysis at higher magnification (with 40X objective) (**Figures 3C–C′,D–D′′** in BSTL; **Figures 3F–F′′** in Ceov; **Figures 3G–G′′** in CeC; and **Figures 3H–H′′** in pINP) showed extremely few cases of coexpression of ENK and Islet1 in cells of the chicken central extended amygdala, which were

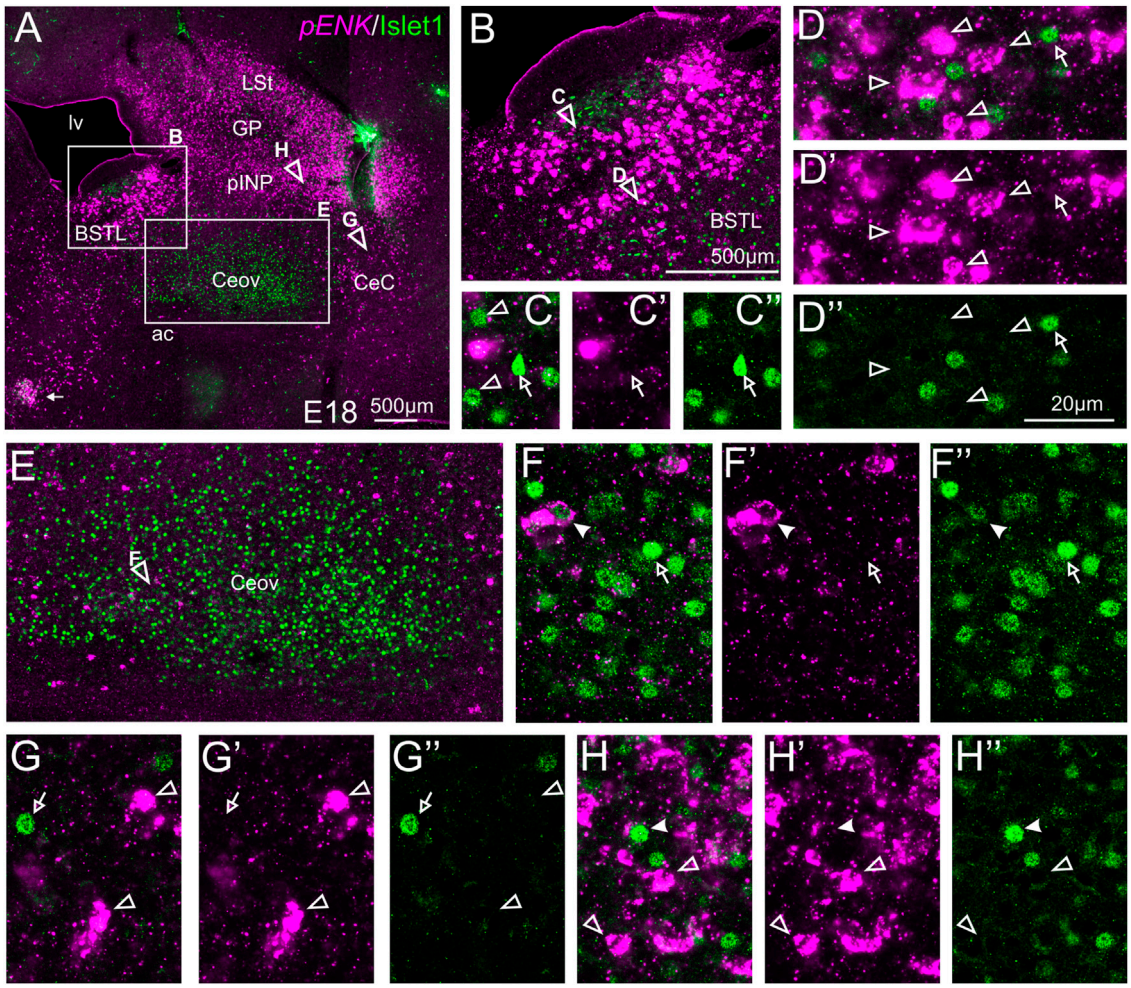


**FIGURE 3 |** Double fluorescence labeling of pro-ENK (*pENK*) and Islet1 in the chicken central extended amygdala at E18. **(A)** General view (10X objective) of the subpallium in a frontal section at the level of the anterior commissure, processed for indirect fluorescent *in situ* hybridization for *pENK* (magenta) and immunofluorescence for Islet1 (green). **(B,E)** show amplifications of the squared areas in **(A)**, at the level of the BSTL **(B)** and Ceov **(E)**. Details (×40 objective, only one single z level of the confocal stack) of the areas pointed with arrowheads in **(B,E)** are shown in **(C–C′′)** (for medial zone of BSTL), **(D–D′′)** (for intermediate zone of BSTL), **(F–F′′)** (for Ceov). Additional details of the areas pointed with arrowheads in A are shown in **(G–G′′)** (for CeC) and **(H–H′′)** (for pINP) (merged plus separate magenta and green channels are shown). In these details, cells coexpressing *pENK* and Islet1 are pointed with a filled arrowhead, cells single labeled for *pENK* are pointed with an empty arrowhead, while cells single labeled for Islet1 are pointed with an empty arrow (only a few examples are pointed). See text for more details. For abbreviations see list. Scale bars: **(A)** = 500 µm; **(B)** = 500 µm [applies to **(B,E)**]; **(D′′)** = 20 µm [applies to **(C–C′′,D–D′′,F–F′′,G–G′′,H–H′′)**].

almost restricted to Ceov (**Figure 3E**, detail in **Figures 3F–F′′**) and intermediate parts of pINP (**Figures 3H–H′′**).

## Double Fluorescent Labeling of Enkephalin and Nkx2.1

We also performed indirect *in situ* hybridization for ENK (magenta in **Figure 4**) combined with immunofluorescence for Nkx2.1 (green in **Figure 4**). Since the downregulation of Nkx2.1 appears to begin in preterm embryos, we also carried out double labeling at an earlier age, E16. Observation with the 10X objective allowed confirmation of the known labeling patterns of ENK and Nkx2.1 in the subpallium (**Figure 4A** at E16, amplification of the squared area is shown in **Figure 4C**; and **Figure 4B** at E18, amplification of the squared area is shown in **Figure 4E**). Nkx2.1 labeling was more intense at E16 than at E18. Double-labeling of ENK and Nkx2.1 allowed distinction of a "striatal-like" subregion of the dorsal BSTL, not adjacent to the Nkx2.1-expressing ventricular zone (**Figures 4C,E**). This "striatal-like" subregion was present from rostral to caudal levels of BSTL. The continuity of the ENK cells of the intermediate zone of dorsal BSTL with those found in the striatal division was clearer in this double-labeled material. Analysis at higher magnification (with 40X objective) (**Figures 4D–D′′,4F–F′′** in BSTL; and (**Figures 4G–G′′** in pINP) showed extremely few cases of cells coexpressing ENK and Nkx2.1, which were restricted to BSTL (filled arrowhead in **Figures 4F–F′′**), especially at ventral and posterior levels.

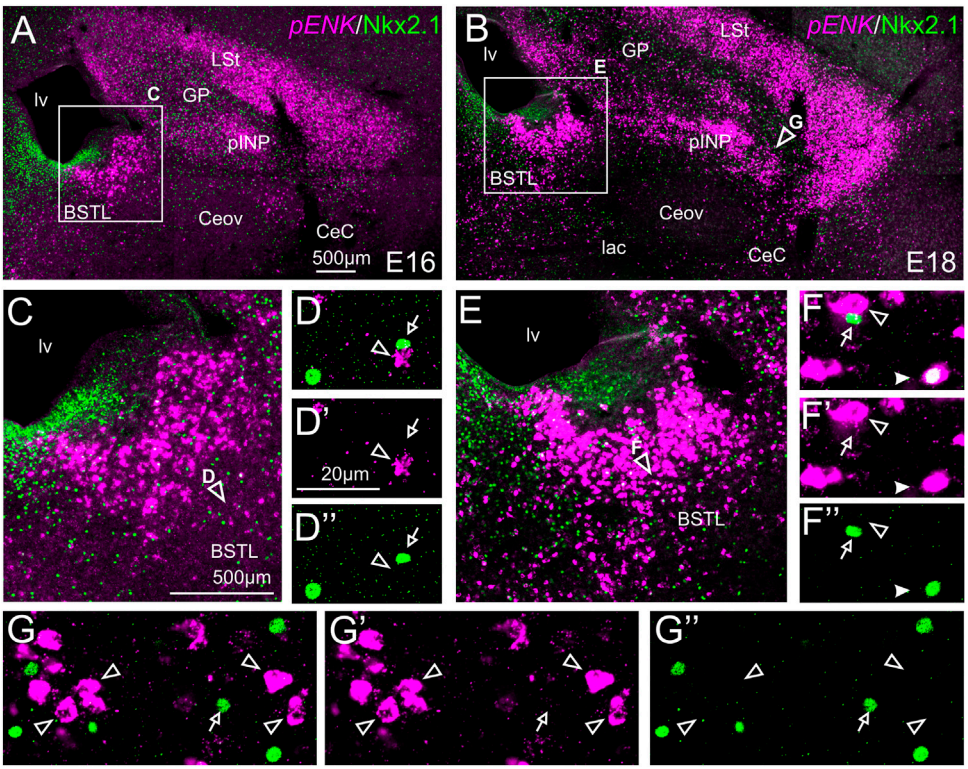


**FIGURE 4 |** Double fluorescence labeling of pro-ENK (*pENK*) and Nkx2.1 in the chicken central extended amygdala at E16 and E18. **(A,B)** General views (×10 objective) of the subpallium in frontal sections at the level of the anterior commissure, from E16 **(A)** or E18 **(B)** chicken brain, processed for indirect fluorescent *in situ* hybridization for *pENK* (magenta) and immunofluorescence for Nkx2.1 (green). **(C,E)** show amplifications of the squared areas in **(A)** or **(B)**, respectively, at the level of the BSTL. Details (×40 objective, only one single z level of the confocal stack) of the areas pointed with arrowheads in **(C,E)** are shown in **(D–D′′,E–E′′)** (merged plus separate magenta and green channels are shown). Additional details of the areas pointed with arrowheads in **(B)** are shown in **(G–G′′)** (for pINP). In these details, cells coexpressing *pENK* and Nkx2.1 are pointed with a filled arrowhead, cells single labeled for *pENK* are pointed with an empty arrowhead, while cells single labeled for Nkx2.1 are pointed with an empty arrow (only a few examples are pointed). See text for more details. For abbreviations see list. Scale bars: **(A)** = 500 µm [applies to **(A,B)**]; **(C)** = 500 µm [applies to **(C,E)**]; **(D′)** = 20 µm [applies to **(D–D′′, F–F′′,G–G′′)**].

### Triple Fluorescent Labeling of Enkephalin With Pax6 and Nkx2.1

We also performed triple labeling of ENK (magenta, indirect fluorescent *in situ* hybridization), with Pax6 (green) and Nkx2.1 (blue) immunofluorescence (**Figure 5**). In the triple labeling experiments, we found no cases of coexpression of Pax6 with Nkx2.1 in the central extended amygdala (details in **Figures 5B–B‴** for BSTL; **Figures 5C–C‴** for pINP; **Figures 5D–D‴** for CeC; and **Figures 5E–E‴** for ITC; in these images, the empty arrowheads point to examples of Nkx2.1 labeled cells, while the empty arrows and the filled arrowheads point to examples of Pax6 cells), thus showing that they are separate populations. In addition, the triple labeling helped us to confirm the observations with double labeling on abundant ENK/Pax6 colocalization (filled arrowheads in the previous images), but scarce ENK/Nkx2.1 colocalization in cells of the chicken central extended amygdala.

## Somatostatin Cells

We then analyzed the distribution of SST cells in the central extended amygdala of chicken, ranging from E18 until P2 (**Figure 6**). In contrast to the ENK cells, the SST cells were generally dispersed in the central extended amygdala, and their abundance changed depending on the area. We found SST cells in different areas, including BSTL, pINP, Pov, Ceov, CeC and ITC (**Figures 6A,B**). SST cells were relatively more abundant in BSTL and CeC, and quite scarce in Ceov (**Figures 6A,B**). In the BSTL, we observed SST cells in the dorsal and ventral subdivisions. In the dorsal BSTL, SST cells were more abundant in the medial zone, and scattered in the intermediate and lateral zones (**Figure 6A**). In the ventral subdivision, SST cells were mostly scattered at the medial and lateral zones. To know if there is coexpression of SST and any of the transcription factors, we carried out double fluorescent labeling in animals ranging from E16 until P0 (N = 11; 1 E16, 9 E18, 1 P0).

### Double Fluorescent Labeling of Somatostatin and Pax6

We did indirect *in situ* hybridization for SST (magenta in **Figure 7**) combined with immunofluorescence for Pax6 (green in **Figure 7**). Analysis with the 10X objective allowed confirmation of the known labeling patterns of SST and Pax6

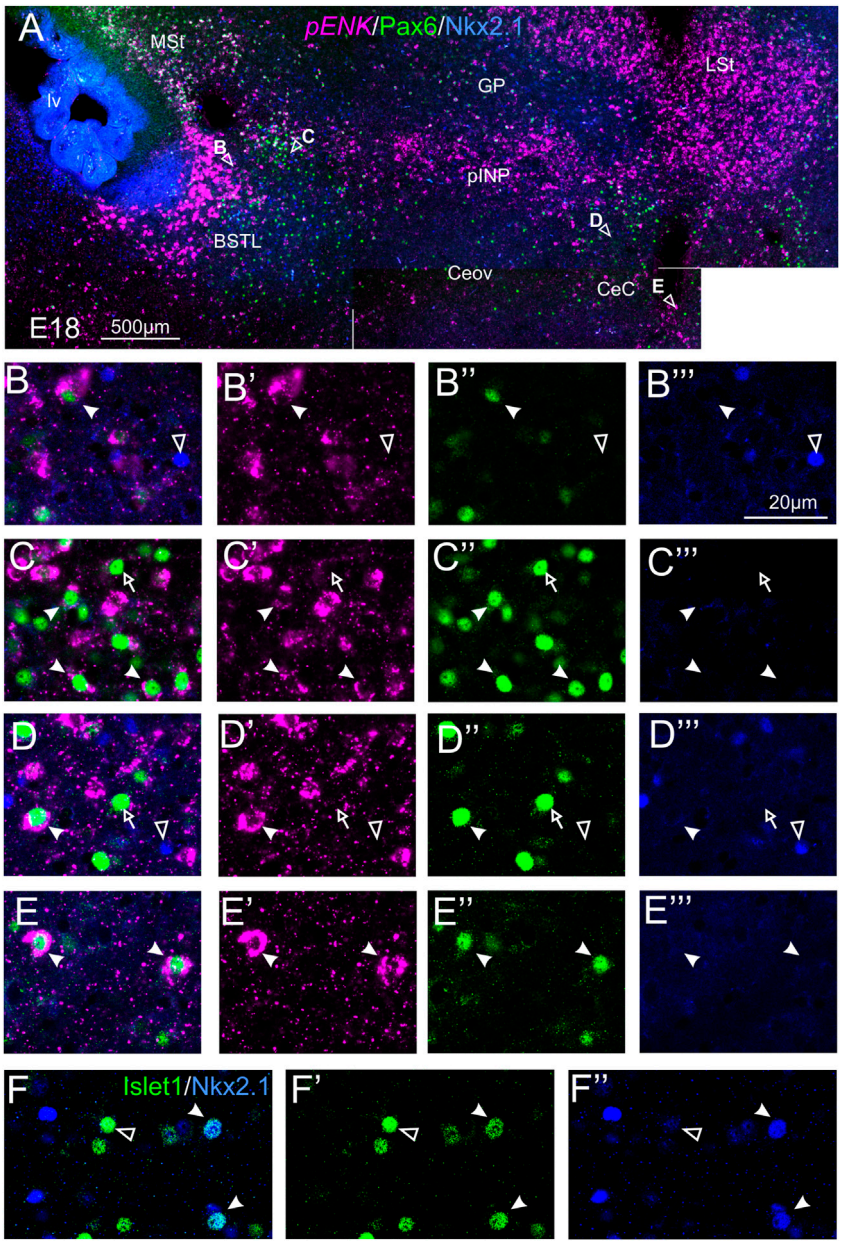


**FIGURE 5 |** Triple and double fluorescence labeling of pro-ENK (*pENK*), Pax6 and Nkx2.1, or Islet1 and Nkx2.1 in the chicken central extended amygdala at E18. **(A)** General view (×10 objective) of the subpallium in frontal sections at the level of the anterior commissure, processed for indirect fluorescent *in situ* hybridization for *pENK* (magenta), immunofluorescence for Pax6 (green), and immunofluorescence for Nkx2.1 (blue). Details (×40 objective, only one single z level of the confocal stack) of the areas pointed with arrowheads in A are shown in **(B–B‴)** (for BSTL), **(C–C‴)** (for medial pINP), **(D–D‴)** (for lateral pINP, and **(E–E′′)** (for CeC) (merged plus separate magenta and green channels are shown). In these details, cells coexpressing *pENK* and Pax6 are pointed with a filled arrowhead, cells single labeled for Pax6 are pointed with an empty arrow, while cells single labeled for Nkx2.1 are pointed with an empty arrowhead (only a few examples are pointed). No coexpression was seen between Pax6 and Nkx2.1. **(F–F′′)**: Details of the BSTL with double fluorescence of Islet1 (green) and Nkx2.1 (blue). Most cells did not show coexpression (empty arrow), but we found very few examples (filled arrow). See text for more details. For abbreviations see list. Scale bars: **(A)** = 500 µm; **(B‴)** = 20 µm [applies to **(B–B‴)** to **(F–F‴)**].

in the subpallium (**Figure 7A**, amplifications of the squared areas shown in **Figures 7B,F,H**). Analysis at higher magnification (with 40X objective) allowed distinction of double labeled cells in some of the areas with overlapping of both markers, but not in others. In particular, some double labeled cells were found in the lateral zone of BSTL (**Figure 7B**, detail in **Figures 7D–D′′**), in the adjacent Pov (**Figures 7B,F**, detail in **Figures 7G–G′′**), in CeC (**Figure 7H**, detail in **Figures 7I–I′′**), and ITC (**Figure 7H**, detail in **Figures 7J–J′′**). In all of these areas, we also observed many single labeled SST cells and Pax6 cells (empty arrowheads in

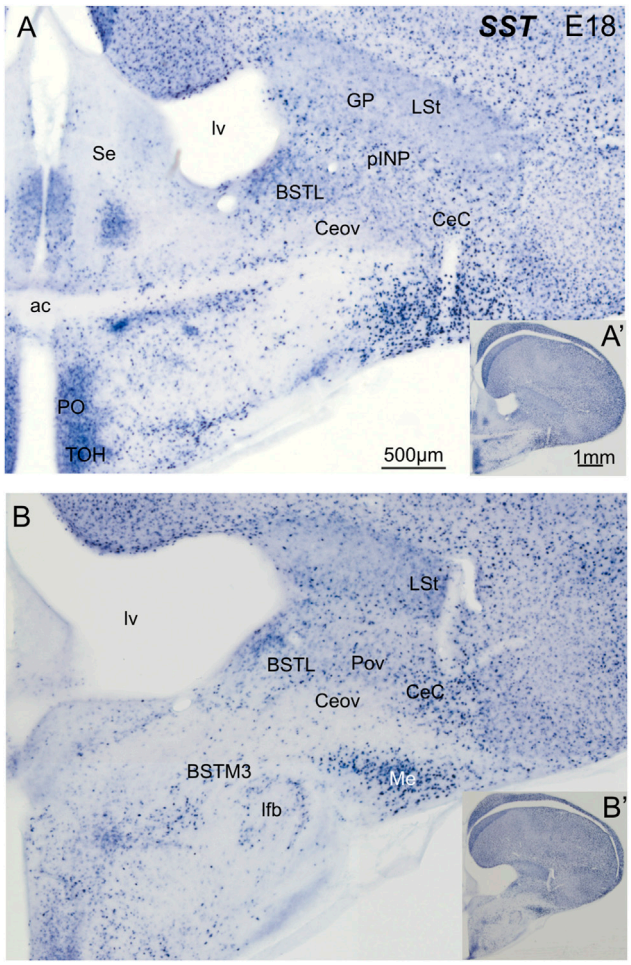


**FIGURE 6 |** Chromogenic double labeling of somatostatin (*SST*) in the chicken central extended amygdala at E18. **(A,B)** Details of the subpallium, with the central extended amygdala, taken from frontal sections (insets) of the chicken embryonic telencephalon, at commissural **(A)** and post-commissural **(B)** levels, hybridized for *SST* (blue color). Note the presence of SST cells in the BSTL, pINP, Pov and CeC. Many cells are also seen in the medial amygdala, and a subpopulation is also seen in the BSTM3. See text for more details. For abbreviations see list. Scale: bar in **(A)** = 500 µm [applies to **(A,B)**]. **(A′)** = 1 mm (applies to **(A′,B′)**].

previous images). In contrast, no double labeled cells were seen in the medial BSTL, pINP and Ceov (**Figure 7B**, details in **Figures 7C–C′′,E–E′′**).

### Double Fluorescent Labeling of Somatostatin and Islet1

We also carried out indirect *in situ* hybridization for SST (magenta in **Figure 8**) combined with immunofluorescence for Islet1 (green in **Figure 8**). Using the ×10 objective, we first confirmed that the labeling patterns of SST and Islet1 agree with previous descriptions (**Figure 8A**). Analysis at higher magnification (with ×40 objective) allowed distinction of double labeled cells in some of the areas with overlapping of both markers. In particular, we found many cases of double labeled cells in the medial zone of BSTL (detail in **Figures 8B–B′′**). We also observed a few double labeled cells in the lateral zone of BSTL (detail in **Figures 8C–C′′**), in pINP (detail in **Figures 8D–D′′**), and in the medial and lateral parts of Pov (details in **Figures 8E–E′′,F–F′′**). In all areas, we also observed many single labeled SST cells and Islet1 cells (empty arrowheads and empty arrows, respectively, in previous images).

### Double Fluorescent Labeling of Somatostatin and Nkx2.1

We also performed indirect *in situ* hybridization for SST (magenta in **Figure 9**) combined with immunofluorescence for Nkx2.1 (green in **Figure 9**) at E16 and E18. Observation with the 10X objective allowed confirmation of the known labeling patterns of SST and Nkx2.1 in the subpallium (**Figure 9A**). Analysis at higher magnification (with 40X objective) showed many cases of double labeled cells in the BSTL (detail in **Figures 9B–B′′**), Pov, pINP (from medial to lateral, details in **Figures 9C–C′′,D–D′′**, respectively), and CeC (detail in **Figures 9E–E′′**). For example, in the lateral zone of BSTL, more than half of the SST cells contained Nkx2.1. A few SST/Nkx2.1 double labeled cells were also observed in Ceov (detail in **Figures 9F–F′′**). In most of these areas, except Ceov, many cases of Nkx2.1 single labeled cells were observed (empty arrows in previous images). Moreover, all of these areas contained a few SST single labeled cells (empty arrowhead in **Figures 9C–C′′,E–E′′,F–F′′**).

## Colocalization of Transcription Factors

To examine the relationship between cells expressing different transcription factors, we also performed double immunofluorescent labeling of either Pax6 or Islet1 with Nkx2.1. In the double labeling experiments of Pax6 with Nkx.2.1, we found no cases of coexpression, thus confirming the results of triple labeling and showing that Pax6 cells and Nkx2.1 cells are separate populations in the chicken central extended amygdala (see details in **Figures 5B–E‴**; in these images, the empty arrowheads point to examples of Nkx2.1 cells, while empty arrows and filled arrowheads point to examples of Pax6 cells). Regarding the double labeling of Islet1 with Nkx2.1, most of the cells were single labeled and appear to represent separate populations, but we also observed very few double labeled cells in the lateral part of BSTL (**Figures 5F–F′′**; filled arrowheads). Based on previous data (Abellán and Medina, 2009; Vicario et al., 2014, 2015), these few Islet1/Nkx2.1 double labeled cells of chicken BSTL likely originate in the preoptic embryonic division.

# DISCUSSION

## A Developmental-Based Classification of ENK and Somatostatin Neurons of the Central Extended Amygdala

Using an evolutionary developmental neurobiology approach, with combinatorial expression of highly conserved region-specific transcription factors and different neuropeptides, we

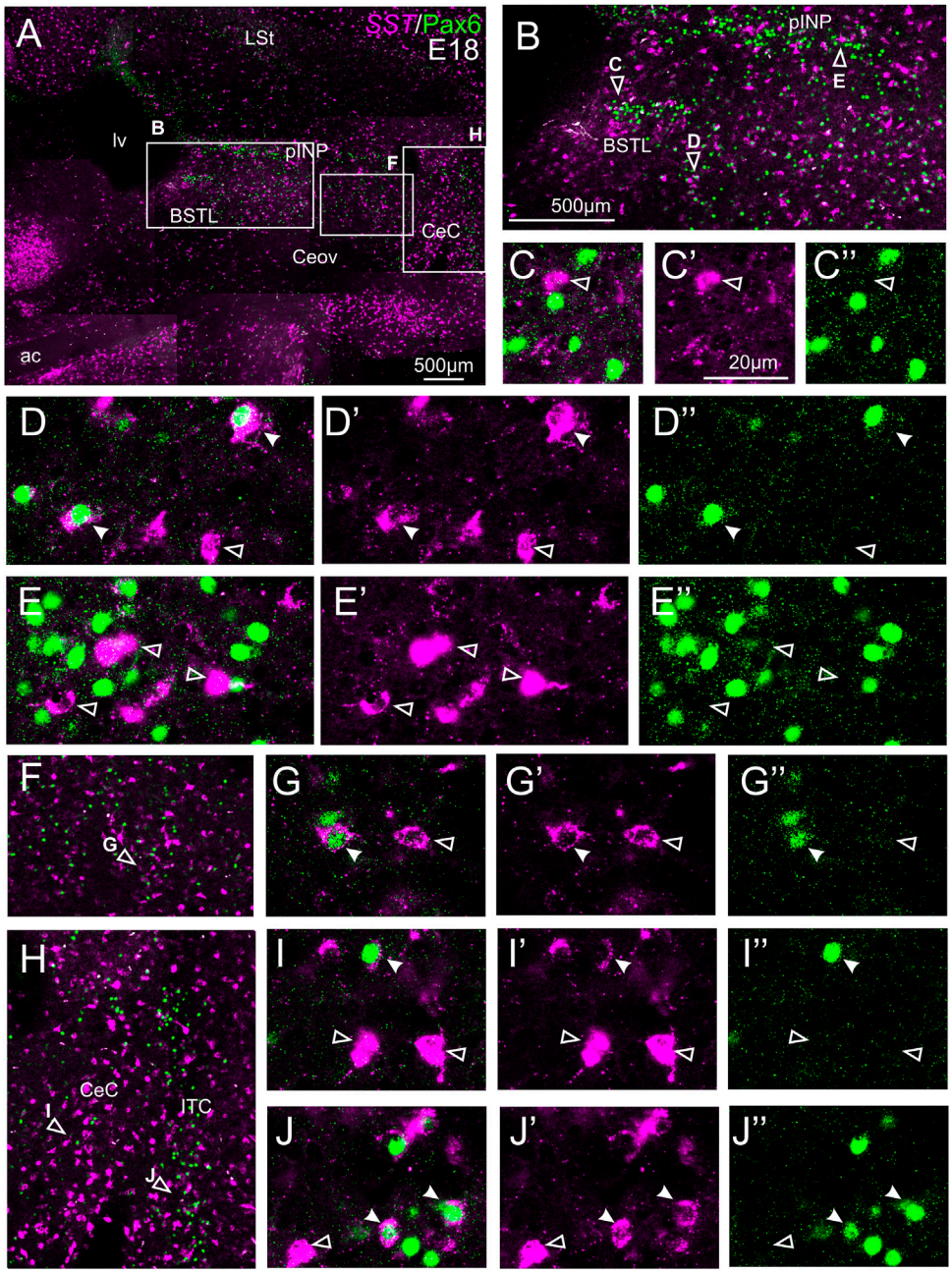


**FIGURE 7 |** Double fluorescence labeling of somatostatin (*SST*) and Pax6 in the chicken central extended amygdala at E18. **(A)** General view (×10 objective) of the subpallium in a frontal section at the level of the anterior commissure, processed for indirect fluorescent *in situ* hybridization for *SST* (magenta) and immunofluorescence for Pax6 (green). **(B**,**F**,**H)** show amplifications of the squared areas in **(A)**, at the level of the BSTL and medial pINP **(B)**, Pov **(F)**, and CeC and ITC **(H)**. Details (×40 objective, only one single z level of the confocal stack) of the areas pointed with arrowheads in **(B,F,H)** are shown in **(C–C′′)** (for medial BSTL), **(D–D′′)** (for lateral BSTL), **(E–E′′)** (for medial pINP), **(G–G′′)** (for Pov), **(I–I′′)** (for CeC) and **(J–J′′)** (for ITC) (merged plus separate magenta and green channels are shown). In these details, cells coexpressing *SST* and Pax6 are pointed with a filled arrowhead, and cells single labeled for *SST* are pointed with an empty arrowhead (only a few examples are pointed). See text for more details. For abbreviations see list. Scale bars: **(A)** = 500 μm; **(B)** = 500 μm [applies to **(B,F,H)**]; **(C′)** = 20 μm [applies to **(C–C′′, D–D′′, E–E′′, G–G′′, I–I′′, J–J′′)**].

previously identified several areas of the subpallium that constitute the central extended amygdala of chicken and zebra finch, which include the BSTL, the pINP, the Pov, the Ceov, the CeC and the ITC (Vicario et al., 2014, 2015, 2017). These areas contain subpopulations of neurons expressing the transcription factors: Pax6, expressed in cells derived from the dorsal striatal embryonic division; Islet1, expressed in cells derived from the ventral striatal embryonic division; and Nkx2.1, expressed in cells

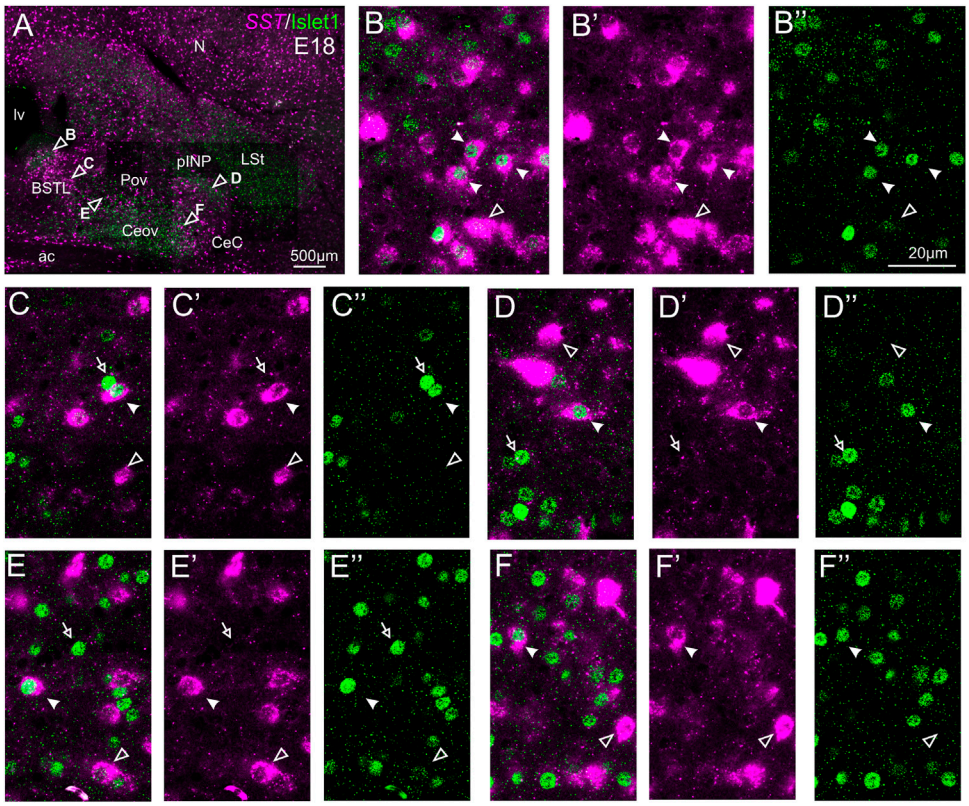


**FIGURE 8 |** Double fluorescence labeling of somatostatin (*SST*) and Islet1 in the chicken central extended amygdala at E18. **(A)** General view (×10 objective) of the subpallium in a frontal section at the level of the anterior commissure, processed for indirect fluorescent *in situ* hybridization for *SST* (magenta) and immunofluorescence for Islet1 (green). Details (×40 objective, only one single z level of the confocal stack) of the areas pointed with arrowheads in A are shown in **(B–B′′)** (for medial BSTL), **(C–C′′)** (for lateral BSTL), **(D–D′′)** (for lateral pINP), **(E–E′′)** (for medial Pov), and **(F–F′′)** (for lateral Pov) (merged plus separate magenta and green channels are shown). In these details, cells coexpressing *SST* and Islet1 are pointed with a filled arrowhead, cells single labeled for *SST* are pointed with an empty arrowhead, and cells single labeled for Islet1 are pointed with an empty arrowhead (only a few examples are pointed). See text for more details. For abbreviations see list. Scale bars: **(A)** = 500 μm; **(B′′)** = 20 μm [applies to **(B–B′′)** to **(F–F′′)**].

derived from the pallido-preoptic embryonic division (Abellán and Medina, 2009; Vicario et al., 2014, 2015). These areas also contain subpopulations of ENK and SST neurons, which were suggested to have different embryonic origins based on their location in relation to the position of cells expressing different transcription factors, and on comparison to similar data in mouse (Bupesh et al., 2011; Vicario et al., 2014, 2015). For example, ENK cells of the central extended amygdala were suggested to include at least three distinct subpopulations that originate in dorsal striatal (mainly those of CeC and pINP), pallidal or preoptic embryonic domains (mainly those of BSTL), while SST cells were suggested to originate in the pallidal embryonic division (including its ventrocaudal or diagonal domain) (see Figure 10 in Vicario et al., 2015). However, until now there were no data on colocalization of region-specific developmental transcription factors and neuropeptides in cells of the central extended amygdala of any amniote species. In this study, we analyzed coexpression of the transcription factors Pax6, Islet1 or Nkx2.1 in ENK and SST cells of the chicken central extended amygdala, aiming to provide a developmental-based classification of ENK and SST cells that can be used in future studies on amygdalar function in chicken and other amniotes (including mammals).

Regarding the ENK cells, we found coexpression with Pax6 in many cells of BSTL (mainly its lateral zone) and medial pINP, and in a few cells of lateral pINP, CeC and ITC. All of these areas were previously noted to contain abundant Pax6 cells (Vicario et al., 2014). In contrast, we found only very few cases of coexpression of ENK with either Islet1 or Nkx2.1. Our results partially agree with our previous suggestions (Vicario et al., 2015), but also provide new unexpected results. In particular, our results agree with the origin of many ENK cells (those expressing Pax6) of the central extended amygdala in the dorsal striatal embryonic division, but we also found very few cells co-expressing Islet1. Islet1 cells have two possible origins: either the ventral striatal embryonic division or the preoptic area (Vicario et al., 2015). The Ceov and pINP, where very few cases of ENK/Islet1 coexpressing cells were found, are rich in Islet1 cells and we previously found that most of those originate in the ventral striatal embryonic division. However, both also included very few cells expressing Nkx2.1 with apparent pallidopreoptic origin (Vicario et al., 2014, 2015). Since the preoptic embryonic division also produces Islet1

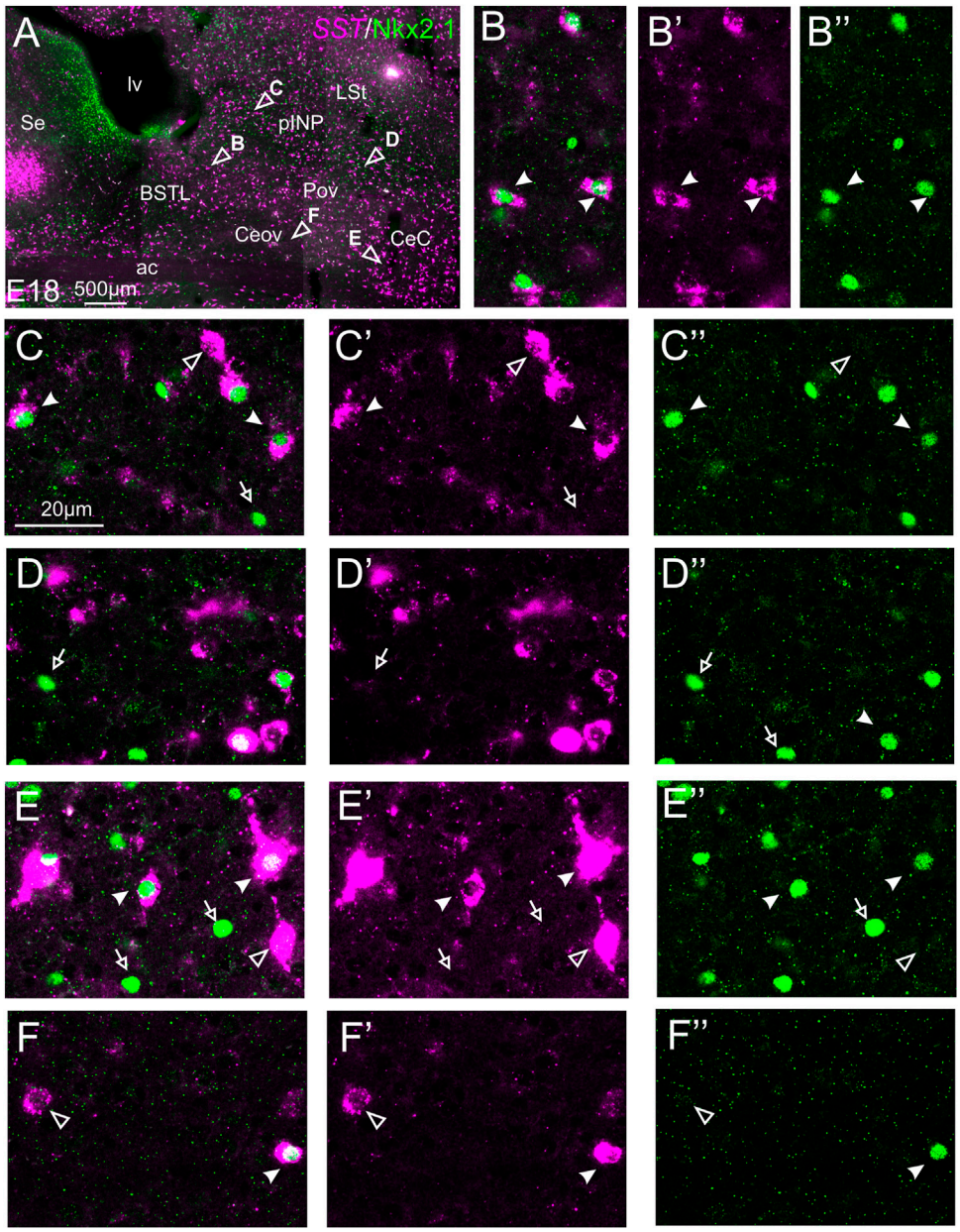


**FIGURE 9 |** Double fluorescence labeling of somatostatin (*SST*) and Nkx2.1 in the chicken central extended amygdala at E18. **(A)** General view (×10 objective) of the subpallium in a frontal section at the level of the anterior commissure, processed for indirect fluorescent *in situ* hybridization for *SST* (magenta) and immunofluorescence for Nkx2.1 (green). Details (×40 objective, only one single z level of the confocal stack) of the areas pointed with arrowheads in **(A)** are shown in **(B–B′′)** (for lateral BSTL), **(C–C′′)** (for medial pINP), **(D–D′′)** (for lateral pINP), **(E–E′′)** (for CeC), and **(F–F′′)** (for Ceov) (merged plus separate magenta and green channels are shown). In these details, cells coexpressing *SST* and Nkx2.1 are pointed with a filled arrowhead, cells single labeled for *SST* are pointed with an empty arrowhead, and cells single labeled for Nkx2.1 are pointed with an empty arrowhead (only a few examples are pointed). See text for more details. For abbreviations see list. Scale bars: **(A)** = 500 µm; **(C)** = 20 µm [applies to **(B–B′′)** to **(F–F′′)**].

and ENK cells, it is likely that this is the source of the few cells coexpressing both found in Ceov and pINP. In contrast to our previous suggestion that most ENK cells of the BSTL may originate in the pallidal division (Vicario et al., 2014, 2015, 2017), we only found extremely few cases of coexpression of ENK and Nkx2.1 in this and other parts of the central extended amygdala. It is surprising considering the high density of ENK cells in BSTL, especially in its ventrocaudal subdivision. As noted above, a part of these cells co-expresses Pax6 and these likely derive from the striatal division. However, the density of ENK cells in the BSTL is very high, and the ENK/Pax6 cells appear to represent only a small fraction of all ENK cells found in this nuclear complex. It is unclear if our finding of only few cases of coexpression of ENK and Nkx2.1 may be due to the

downregulation of Nkx2.1 during intermediate-late embryonic ages (E16-E18 chicken). In agreement with this, we only found very few cases of coexpression of Islet1 and Nkx2.1, which should be expected in cells of preoptic origin (Abellán and Medina, 2009). Additional studies of the central extended amygdala of earlier chicken embryos will be required to further investigate this issue. Alternatively, this could be investigated in transgenic animal models with permanent labeling of Nkx2.1 lineage cells.

With respect to the SST cells, we found that many of them coexpressed Nkx2.1 in most areas of the chicken central extended amygdala, including BSTL, Pov, pINP, CeC and Ceov. This agrees with our previous suggestion that these cells originate in the pallidal embryonic division (Vicario et al., 2014, 2015), and also agrees with previous findings in mouse (Bupesh et al., 2011). However, we previously proposed that most of these cells of the central extended amygdala may originate in the ventrocaudal pallidal (diagonal) division (Vicario et al., 2014, 2015; see also Bupesh et al., 2011; Puelles et al., 2016), but based on the ample distribution of SST cells in dorsal and ventral parts of BSTL, it is possible that other subdomains of the pallidal division also contribute to produce these cells, which would agree with previous findings in mouse regarding the origin of striatal and cortical SST interneurons (Marín et al., 2000; Flames et al., 2007; Fogarty et al., 2007; Xu et al., 2008; Asgarian et al., 2019). Based on data in mouse (Gelman et al., 2011; Asgarian et al., 2019), some SST cells of the chicken telencephalon, including the central extended amygdala, may also originate in the preoptic embryonic domain. These may include the SST cells coexpressing Islet1 found in this study. We also found that not all SST cells of the central extended amygdala of chicken coexpress Nkx2.1. As noted above, this may be due to downregulation of Nkx2.1 at late embryonic stages, but more studies are needed to further investigate if this is so and/or if there is a non-pallidopreoptic source of SST cells for the central extended amygdala. In relation to the latter, in this study we found some cases of SST cells coexpressing Pax6, which origin is unknown. In Nkx2.1-knockout mouse, although most SST cells of the subpallium are missing, a subpopulation of SST cells remains (Marín et al., 2000; Asgarian et al., 2019). It has been suggested that the caudal ganglionic eminence may be the source of such SST cells (Chittajallu et al., 2013), but this finding is controversial (discussed by Asgarian et al., 2019). The mouse caudal ganglionic eminence mostly represents a distinct caudal pole of the striatal embryonic division (i.e., the caudolateral ganglionic eminence) that does not express Nkx2.1 and produces Pax6 cells for the central extended amygdala (Nery et al., 2002; Bupesh et al., 2011). Our results on the existence of SST/Pax6 double labeled cells would agree with the proposal that this caudal pole of the striatal division produces a subpopulation of SST cells for the central extended amygdala, although we cannot discard other sources, such as the prethalamic eminence (PThE), known to give rise to a subpopulation of Pax6 for the extended amygdala in chicken (Abellán and Medina, 2009; see also Alonso et al., 2020, 2021) and mouse (Bupesh et al., 2011; Ruiz-Reig et al., 2017). In addition, other possible sources of SST cells of the central extended amygdala may be the recently described telencephalon-opto-hypothalamic (TOH) domain and the adjacent supraopto-paraventricular hypothalamic (SPV) domain, both of which were found to produce subpopulations of cells expressing the transcription factors Otp and/or Sim1 for the BSTL, the CeC, and ITC in chicken (Metwalli et al., 2022). While SPV and mostly TOH produce subpopulations of cells for the medial extended amygdala in both chicken and mouse, the contribution to the central extended amygdala seems to be specific for chicken, but has not been found in mouse (García-Calero et al., 2021; Morales et al., 2021; Metwalli et al., 2022). It is possible that some of the latter contain SST, since SPV is known to produce SST neurons for the hypothalamus in mouse (Wang and Lufkin, 2000; Díaz et al., 2015), which fail to differentiate in Otp-knockout animals (Wang and Lufkin, 2000). Moreover, our results showed the presence of SST cells in other areas of the extended amygdala that contain cells derived from TOH/SPV, such as the medial bed nucleus of the stria terminalis (BSTM3 subdivision) and the medial amygdala (**Figure 6B**).

Overall, our results provide a developmental-based classification of the ENK and SST neurons in the chicken central extended amygdala, showing the existence of at least three subtypes of ENK cells and three subtypes of SST cells. According to our data, it seems that a large part of the ENK cells coexpress Pax6 and likely originate in the dorsal striatal embryonic division, while the majority of the SST cells coexpress Nkx2.1 and have a pallidal origin, but additional quantitative studies are needed to investigate the exact proportions. However, these studies would have the limitation of the downregulation in expression of the developmental regulatory transcription factors at late embryonic stages, which combined with the often late expression of adult phenotypic markers would lead to an underestimation of the colocalization. This is likely the case in our study in chicken.

In the mouse central amygdala, most ENK cells coexpress PKCδ (about 70%) and represent a separate cell population from the SST neurons (Haubensak et al., 2010). In fact, ENK/PKCδ and SST occupy partially separate positions in the central amygdala (McCullough et al., 2018). However, while it is true that these cell populations are mostly segregated, there is a very small subpopulation of cells coexpressing PKCδ and SST (McCullough et al., 2018). Thus, we cannot discard the possibility that a small subset of ENK cells coexpress SST in chicken too. These may be the ENK cells found to coexpress Nkx2.1 and/or Islet1 (present results). Nevertheless, in chicken, ENK cells and SST cells show different distribution patterns in the subpallium and likely are mostly segregated. Our data on coexpression with region-specific developmental transcription factors also agree with the major segregation of both types of neuropeptidergic neurons. Regarding the SST cells, in the mouse central amygdala they include several subpopulations, coexpressing tachykinin 2 (substance P), neurotensin and/or corticotropin-releasing factor (McCullough et al., 2018). Based on the embryonic origins of the SST cells (discussed above), these different subpopulations may originate in different subdomains of the pallidal embryonic division, in the preoptic embryonic division, in the PThE, in TOH or in SPV (at least). The existence of different subpopulations of peptidergic neurons with different embryonic origin should be taken into consideration for future

studies on the connectivity and function of these different cells. Moreover, cells with origin in the subpallium are GABAergic, while those from PThE, TOH and SPV are glutamatergic (Abellán and Medina, 2009; Ruiz-Reig et al., 2017; Morales et al., 2021). Thus, while most peptidergic neurons of the central extended amygdala originate in the subpallium and are GABAergic, based on the presence of some minor cell subpopulations of the central extended amygdala that may originate in PThE (perhaps some of the SST/Pax6 cells) or the TOH/SPV (perhaps some SST cells that do not express Pax6, Islet1 or Nkx2.1), our results suggest the participation of both GABAergic and glutamatergic networks of the central extended amygdala in the regulation of stress.

## Possible Existence of On/Off Cell Systems for Regulating Stress in the Chicken Central Extended Amygdala

In mammals, the central extended amygdala is known to play a key role in regulation of the stress response (Phelps and LeDoux, 2005; Davis et al., 2010). To understand how the central extended amygdala regulates stress it is essential to dissect this structure at molecular, cellular and circuit levels. Studies in mouse have shown that the central amygdala contains two types of inhibitory neurons that become active (on) or inactive (off) during the conditioned fear response (Ciocchi et al., 2010). These neurons inhibit each other, and project in a mostly unidirectional manner to output neurons of the central amygdala (Ciocchi et al., 2010). It appears that the off cells express the protein kinase C-delta (PKCδ) (Haubensak et al., 2010), and many of them are enkephalinergic (about 40%, Haubensak et al., 2010). In contrast, the on cells do not contain PKCδ, but express SST (Penzo et al., 2014).

In chicken, the BSTL has been shown to become active by stress (Nagarajan et al., 2014), similarly to that of mammals (Davis et al., 2010). Moreover, it projects to the paraventricular hypothalamic nucleus (Atoji et al., 2006), being thus able to regulate the hypothalamic-pituitary-adrenal axis (Smulders, 2021), and receives input from a posterior part of the arcopallium (part of the avian pallial amygdala) (Atoji et al., 2006) that is also involved in control of fear behavior (Saint-Dizier et al., 2009). The subpallial amygdalar area interposed between the arcopallium and the BSTL also projects to the BSTL and seems to belong to the same functional network (Kuenzel et al., 2011). Using an evolutionary developmental neurobiology approach, we recently identified several subdivisions and cell subpopulations within this subpallial amygdala region that, together with the BSTL, appear to form the avian central extended amygdala (Vicario et al., 2014, 2015, 2017). Like that of mammals, this region of chicken also contains a majority of GABAergic neurons (Abellán and Medina, 2009), which originate in identical embryonic subpallial divisions that express Pax6, Islet1 or Nkx2.1 during development (Vicario et al., 2014, 2015). These neurons include subpopulations of ENK and SST neurons. Like in mammals, it is likely that these two types of neurons of the chicken central extended amygdala are involved in inhibitory pathways and inhibit each other to block or release the outputs to the hypothalamus and brainstem centers involved in the stress response. Thus, our data suggest the existence of on/off cell systems in the central extended amygdala of chicken. In addition, our results on coexpression of ENK and SST with different region-specific transcription factors, combined with those from previous experimental studies on the origin of those cells (Bupesh et al., 2011; Vicario et al., 2015), show that many of the ENK cells (including the putative off cells) express Pax6 and originate in the dorsal striatal embryonic division, while most SST cells (including the putative on cells) express Nkx2.1 and derive from the pallidal embryonic division. Based on the presence of Pax6, Islet1 and Nkx2.1 expressing cells in the central extended amygdala of turtles, derived from dorsal striatal, ventral striatal or pallidal embryonic divisions (Moreno et al., 2010), it appears that on/off cell systems may be a common feature in the central extended amygdala of amniotes (discussed by Medina et al., 2017). In the central extended amygdala of amphibians and lungfishes, Pax6 cells of dorsal striatal origin appear to be missing (Moreno et al., 2008; Moreno et al., 2018), suggesting that the on/off cell systems may be an innovation of amniotes, likely contributing to a more sophisticated and plastic regulation of the stress response (Medina et al., 2017).

## Enkephalin Cells of the Central Amygdala and Regulation of Pain

Painful stimuli are known to recruit the amygdala, leading to anxiety-like behavior and to changes in pain sensitivity (Jiang et al., 2014; Thompson and Neugebauer, 2017). Studies in mammals have shown that regulation of pain by the central amygdala is complex, and can lead to either analgesia or algesia depending on the specific cells and subcircuits involved (Veinante et al., 2013; Andreoli et al., 2017; Paretkar and Dimitrov, 2019). Stress can also regulate pain sensitivity, and depending on the duration and intensity can lead to stress-induced analgesia or to hyperalgesia (Parikh et al., 2011; Baker et al., 2019). Stress-induced analgesia can be mediated by endogenous opioids, such as ENK, or by non-opioids (Baker et al., 2019). It appears that more severe, short and continuous stressors involve non-opioid mediated analgesia. On the contrary, less severe, intermittent or long-duration stressors involve opioid-mediated analgesia (Parikh et al., 2011; Baker et al., 2019). The latter type involves activation of opioid receptors in the central amygdala (Zhang et al., 2013). This type of analgesia can also be produced by the chemogenetic activation of ENK neurons of the central amygdala (including the ENK/PKCδ neurons), which results in anxiolysis and increase of pain threshold (Paretkar and Dimitrov, 2019). Activation of these neurons leads to inhibition of output central amygdala neurons that project to the periaqueductal gray (Haubensak et al., 2010; Paretkar and Dimitrov, 2019), a midbrain region critical for orchestrating behavioral responses to internal and external stressors and for modulating pain sensitivity (Oliveira and Prado, 2001; Benarroch, 2012). As a consequence, the ventrolateral

periaqueductal gray is disinhibited and becomes active (Paretkar and Dimitrov, 2019). This indirectly acts on the gate control system of pain in the dorsal horn, in the spinal cord, leading to inhibition of ascending nociceptive transmission, which results in analgesia (Baker et al., 2019).

Data on the central neural regulation of pain in birds is limited, but the avian brain and spinal cord contain areas, networks, and neurotransmitter/neuropeptide systems similar to those involved in pain regulation in mammals (Kuenzel, 2007; Baker et al., 2019). It also appears that in young chickens there is a reduction in pain response following exposure to a stressor (Sufka and Hughes, 1991; Feltenstein et al., 2002), which suggests the implication of the amygdala. Our finding of ENK cells in the central extended amygdala of chicken, comparable to those of mammals, open new venues for investigating if in birds these cells also play a role in stress-induced regulation of pain.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Committees of Ethics for Animal Experimentation and Biosecurity of the University of Lleida (reference no. CEEA 08-02/19), as well as that of the Catalonian Government (reference no. CEA/9960_MR1/P3/1 for embryos, and CEA/9960_MR1/P4/1 for post-hatchlings).

## AUTHOR CONTRIBUTIONS

AP processed all of the chicken brain material as part of her Ph.D. research project, and AHM contributed in the processing. AP photographed and prepared all figures, and analyzed the material with help of LM and ED. AP and LM produced the first draft of the manuscript, and all authors revised it and approved it.

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## GLOSSARY

**ac** anterior commissure
**BST** bed nucleus of the stria terminalis
**BSTL** lateral division of the BST
**BSTM** medial division of the BST
**BSTM3** part 3 of BSTM
**Ce** central amygdala
**CeC** capsular division of the Ce
**Ceov** oval nucleus of the Ce
**CRF** corticotropin releasing factor
**d** BSTLd
**EAce** central extended amygdala
**ENK** enkephalin
**GP** globus pallidus
**ITC** intercalated amygdalar cells
**lac** lateral branch of the anterior commissure
**lfb** lateral forebrain bundle
**LSt** lateral striatum
**lv** lateral ventricle
**Me** medial amygdala
**MSt** medial striatum
**N** nidopallium
**PKCδ** protein kinase C-delta
**pINP** peri-intrapeduncular island field
**PO** preoptic area
**Pov** perioval zone (part of the EAce)
**PThE** prethalamic eminence
**Rt** reticular prethalamic nucleus
**Se** septum
**SST** somatostatin
**SPV** supraopto-paraventricular hypothalamic domain
**TOH** telencephalo-opto-hypothalamic domain
**v** BSTLv

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# Activation of the Nucleus Taeniae of the Amygdala by Umami Taste in Domestic Chicks (*Gallus gallus*)

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In chickens, the sense of taste plays an important role in detecting nutrients and choosing feed. The molecular mechanisms underlying the taste-sensing system of chickens are well studied, but the neural mechanisms underlying taste reactivity have received less attention. Here we report the short-term taste behaviour of chickens towards umami and bitter (quinine) taste solutions and the associated neural activity in the nucleus taeniae of the amygdala, nucleus accumbens and lateral septum. We found that chickens had more contact with and drank greater volumes of umami than bitter or a water control, and that chicks displayed increased head shaking in response to bitter compared to the other tastes. We found that there was a higher neural activity, measured as c-Fos activation, in response to umami taste in the right hemisphere of the nucleus taeniae of the amygdala. In the left hemisphere, there was a higher c-Fos activation of the nucleus taeniae of the amygdala in response to bitter than in the right hemisphere. Our findings provide clear evidence that chickens respond differently to umami and bitter tastes, that there is a lateralised response to tastes at the neural level, and reveals a new function of the avian nucleus taeniae of the amygdala as a region processing reward information.

**Keywords: reward, bitter, taste perception, Immediate early genes (IEGs), accumbens (NAc), lateral septum (LS), c-Fos**

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## INTRODUCTION

Taste perception provides animals with information about the quality of food via five basic tastes: sweet, umami, bitter, salty and sour (Jiang et al., 2012). Bitter can indicate toxic and harmful substances, and aversion to bitter tastes likely evolved as a protective mechanism (Davis et al., 2010). Umami, on the other hand, reflects amino acids in protein (Chandrashekar et al., 2006), and is known to increase appetite, but also to increase satiety (Masic and Yeomans, 2014). Research on the mechanism(s) involved in taste perception will increase our understanding of how animals use taste to make adaptive foraging decisions.

To fully understand taste perception requires comparative physiological studies ranging from receptor mechanisms to brain circuitry, but also psychophysical studies that quantitatively describe the perceptual output of the system (Green, 2021). These types of research can lead to the discovery of new phenomena such as the functional loss or neofunctionalization of taste receptors (Baldwin et al., 2014), which generate new insights and hypotheses about the mechanisms and evolution of taste perception (Zhao et al., 2010; Jiang et al., 2012; Toda et al., 2021). The comparative genetic linkage between taste perception and feeding specializations are well studied (Li and Zhang, 2014;

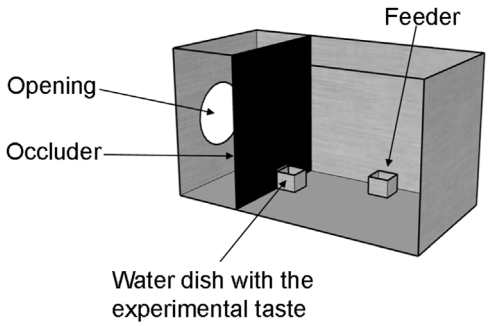


**FIGURE 1 |** Experimental setup. Chicks were habituated in metal cages with food and water *ad libitum*. Prior to the presentation of the experimental taste, chicks were water deprived for 45 min by removing their water dish through an opening in the occluder. Then, an identical water dish with the experimental taste was presented for 10 minutes. A camera was placed above each cage to record chicks behavioural responses to the taste.

Wang and Zhao, 2015; Liu et al., 2016), whereas the cognitive mechanisms that underlie taste perception in animals other than humans, non-human primates, and rodents is less studied.

Among birds, domestic chickens (*Gallus gallus domesticus*) are probably the most intensively investigated species with regards to taste receptor physiology and associated behaviour, which can be attributed to their commercial importance (for a review see Roura et al., 2013). Chickens lack the receptor for sweet taste (Cheled-Shoval et al., 2015), but possess umami and bitter receptors in their oral cavity (Shi and Zhang, 2006; Yoshida et al., 2015). The behavioural responses of chickens to sour, salty, umami, and bitter can be observed during the first post hatch day (Ganchrow et al., 1990). The typical response to bitter includes prolonged head shaking, beak wiping and sticking out the tongue (Nicol, 2004). Chicks also develop conditioned taste aversions and exhibit foraging biases in response to bitter (Skelhorn and Rowe, 2005a; Skelhorn and Rowe, 2005b; Skelhorn et al., 2008). Umami, on the other hand, is palatable for chickens (Roura et al., 2013; Liu et al., 2018; Niknafs and Roura, 2018) and it is suggested that chicks may perceive umami as a salty-and sweet-like taste (Yoshida et al., 2018).

While there has been some research into the neural mechanisms underlying reward in domestic chickens (Yanagihara et al., 2001; Izawa et al., 2002; Aoki et al., 2003; Matsushima et al., 2003; Ichikawa et al., 2004; Amita and Matsushima, 2014), there has been less attention given to the brain regions involved in palatable taste perception. In mammals, the pathway for the gustatory sensory information runs through the nucleus of the solitary tract to the parabranchial nucleus, which in turn projects to various forebrain regions, including the amygdaloid arcopallium and nucleus accumbens (Arends et al., 1988; Wild et al., 1990; Berk et al., 1993). Palatable taste perception involves the mesolimbic rewards system (Dayan and Balleine, 2002; Ramírez-Lugo et al., 2007; Berridge et al., 2009), which includes the nucleus accumbens, ventral pallidum, basolateral and medial amygdala (Ramírez-Lugo et al., 2006; Ramírez-Lugo et al., 2007; Wassum et al., 2009; Berridge and

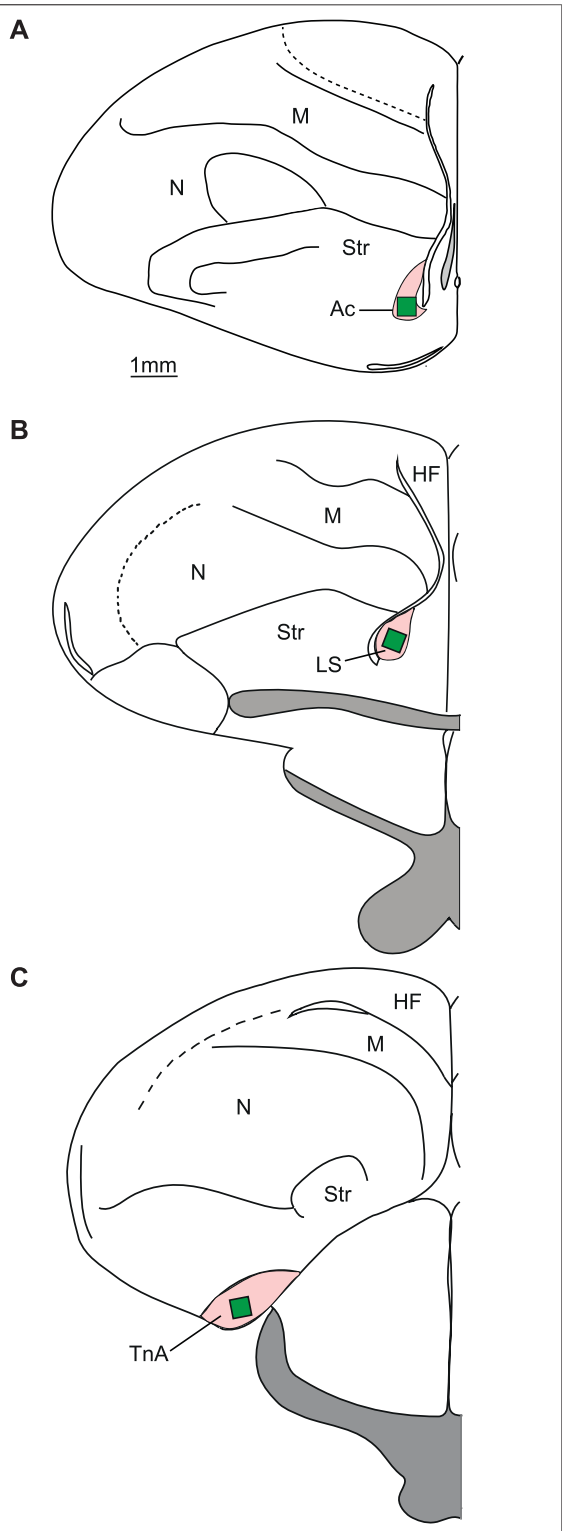


**FIGURE 2 |** Typical placements of cell counting zones (green rectangles) in the regions of interest (pink). **(A)** Schematic representation of a coronal section at the level of the nucleus accumbens. **(B)** Coronal section at the level of the lateral septum. **(C)** Coronal section at the level of nucleus taeniae of the amygdala. TnA—nucleus taeniae of the amygdala; LS—lateral septum; Ac—nucleus accumbens; HF—hippocampal formation; M—mesopallium; N—nidopallium; Str - Striatum.

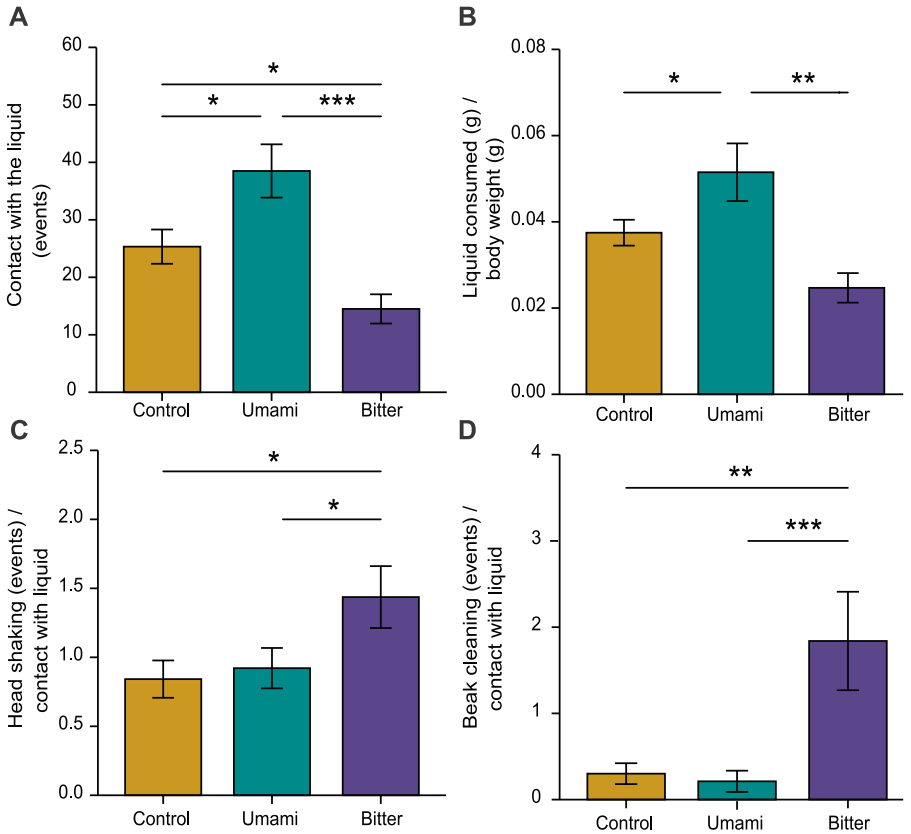


**FIGURE 3 |** Behavioural responses of chicks according to experimental tastes. **(A)** Contact with the liquid, **(B)** liquid consumed/body weight, **(C)** head shaking in relation to contact with the liquid and **(D)** beak cleaning in relation to contact with the liquid. Bar plots indicate mean ± s.e.m. (*$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001).

Kringelbach, 2013; Baumgartner et al., 2021; Hu et al., 2021). Although the mesolimbic reward system is highly conserved in vertebrates (O'Connell and Hofmann, 2011), investigating whether these brain areas are also involved in taste perception in domestic chicks will add to our understanding of comparative taste perception and reward behaviour.

Here we present three different groups of young domestic chicks with either a solution containing monosodium glutamate and inosine 5′-monophosphate (umami taste), quinine solution (bitter taste), or to pure water (as a control) (**Figure 1**). Behavioural reactions to these stimuli were measured in terms of: 1) frequency of contact with the solution, 2) volume of liquid consumed, 3) frequency of head shakes and 4) beak cleaning episodes. Brain activation was measured by immunohistochemical detection of the Immediate Early Gene (IEG) product c-Fos in three interconnected subpallial brain regions: nucleus taeniae of the amygdala (TnA), nucleus accumbens (Ac) and lateral septum (LS) (**Figure 2**) that may directly modulate taste-responsive neurons of the parabrachial complex through an anatomical pathway using neurotensin (Bálint et al., 2011; Bálint et al., 2016), or receive gustatory information indirectly.

# RESULTS

## Behaviour

Contact with the liquid significantly varied among treatments (ANOVA: $F_{33,2}$ = 11.76, $p$ < 0.001). Chicks had more contact with umami (mean ± s.e.m.: 38.5 ± 4.63) than with bitter (14.5 ± 2.54; $p$ < 0.001) or control (25.3 ± 2.98; $p$ = 0.012), and more contact with control than with bitter ($p$ = 0.03) (**Figure 3A**). The amount of liquid consumed was also significantly different among treatments ($F_{33,2}$ = 8.25, $p$ = 0.001). Chicks consumed significantly more umami (0.05 ± 0.003) than control (0.03 ± 0.002; $p$ = 0.041) or bitter (0.02 ± 0.003; $p$ < 0.005) (**Figure 3B**). Head shaking was significantly different among treatments ($F_{33,2}$ = 3.47, $p$ = 0.042). Chicks shook their head significantly more frequently after having contact with bitter (1.43 ± 0.22) than with umami (0.92 ± 0.14; $p$ = 0.04) or control (0.84 ± 0.13, $p$ = 0.02) (**Figure 3C**). Beak cleaning was also significantly different among treatments (Kruskal–Wallis: $X^2$ = 11.629, d.f. = 2, $p$ = 0.002). Chicks cleaned their beak more frequently after having contact with bitter (1.83 ± 0.57) than with control (0.30 ± 0.12; $p$ = 0.008) or umami (0.21 ± 0.12; $p$ = 0.001) (**Figure 3D**).

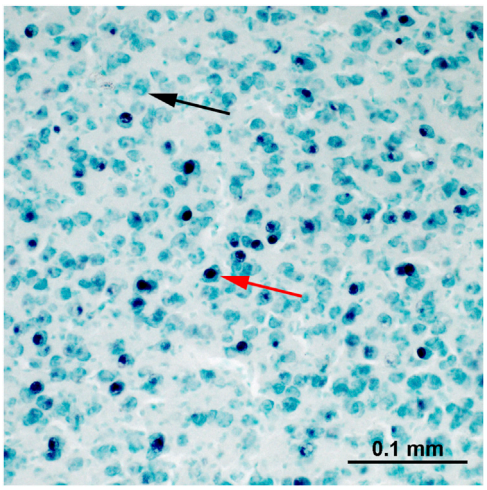


**FIGURE 4 |** An example of c-Fos staining within the nucleus taeniae of the amygdala. The nuclei of c-Fos immunoreactive cells are stained black (red arrow). The c-Fos-negative cells (black arrow) are green due to the methyl-green counterstaining.

## Brain Activity

All 36 brains ($n$ = 12 in each treatment) were successfully stained for c-Fos (**Figure 4**). However, the accumbens region of one brain from the quinine group was damaged and was excluded from further analysis.

In nucleus taeniae of the amygdala there was a significant interaction between treatment and hemisphere ($p$ = 0.008), meaning that in one of the hemispheres, treatment had an effect on c-Fos activation as revealed by ANOVA (**Table 1**). Only in the right nucleus taeniae was c-Fos activation higher in the umami group (Mean ± s.e.m: 1058.33 ± 90.92 cells/mm$^2$) than in the control (813.75 ± 49.38 cells/mm$^2$; $p$ = 0.032), or bitter (742.81 ± 61.39 cells/mm$^2$; $p$ = 0.007) (**Table 1**). Such differences between treatments were not present in the left nucleus taeniae (control 851.45 ± 60.73 cells/mm$^2$; umami: 952.08 ± 97.90 cells/mm$^2$; bitter: 946.35 ± 86.28 cells/mm$^2$) (**Figure 5A**). Moreover, an additional lateralised response was found in nucleus taeniae. c-Fos activation of the bitter group was higher in the left nucleus taeniae than in the right ($p$ = 0.014). Control and umami treatments did not present such differences (**Table 1**).

In the other two brain regions (accumbens and lateral septum), there were no main effects of treatment or hemisphere and no interactions (**Table1**; **Figure 5**). However, patterns of activation qualitatively similar to the right nucleus taeniae of the amygdala were present also in right accumbens (control: 889.89 ± 122.50 cells/mm$^2$; umami: 1104.99 ± 129.71 cells/mm$^2$; bitter: 745.59 ± 82.49 cells/mm$^2$), although the interaction was not significant at the alpha 0.05 level ($p$ = 0.06, **Table 1**). The activation pattern in the left accumbens did not differ (control 848.30 ± 97.34 cells/mm$^2$; umami: 986.27 ± 143.08 cells/mm$^2$; bitter: 1030.36 ± 137.95 cells/mm$^2$), nor did the left lateral septum (control: 536.97 ± 67.73 cells/mm$^2$; umami: 535.52 ± 115.02 cells/mm$^2$; bitter: 589.06 ± 103.74 cells/mm$^2$), or right lateral septum (control: 563.67 ± 54.40 cells/mm$^2$; umami: 562.57 ± 82.53 cells/mm$^2$; bitter: 490.52 ± 62.03 cells/mm$^2$).

## Correlations

There were no significant correlations between c-Fos activation and any of the measured behaviours in any of the brain regions analysed (**Table S1**).

# DISCUSSION

We show that the avian nucleus taeniae of the amygdala responds to gustatory information: at the brain level, c-Fos activation was higher in the right nucleus taeniae in chicks that consumed umami, compared to those that consumed bitter or water control. This is the first evidence to show that the avian nucleus taeniae of the amygdala responds to umami taste. We also show a clear behavioural preference for umami and an aversion to bitter: chicks consumed more umami than bitter and water, and shook their heads and cleaned their beaks more after contact with bitter than with umami or water (see also Gentle, 1975; Ganchrow et al., 1990; Yoshida et al., 2015; Yoshida et al., 2018). The higher activation in the right nucleus taeniae of the amygdala in response to umami, and the greater consumption of umami is likely indicative of reward processing. The avian nucleus taeniae of the amygdala resembles a homolog brain region to the mammalian medial amygdala (Reiner et al., 2004; Yamamoto et al., 2005; Abellán et al., 2009), which in humans and other non-human primates is associated with reward

**TABLE 1 |** Summary of the statistical results of brain activation.

| Brain region | Treatment | Hemisphere | Interaction |
|---|---|---|---|
| Nucleus taeniae | $F_{(1,33)}$=1.699, $p$ = 0.199 | $F_{(1,33)}$=1.266, $p$ = 0.269 | $F_{(2,33)}$=5.620, $p$ = **0.008*** |
| Lateral septum | $F_{(2,33)}$=0.66, $p$ = 0.994 | $F_{(1,33)}$=0.950, $p$ = 0.759 | $F_{(2,33)}$=0.749, $p$ = 0.481 |
| Accumbens | $F_{(2,32)}$=0.766, $p$ = 0.473 | $F_{(1,32)}$=0.215, $p$ = 0.646 | $F_{(2,32)}$=3.075, $p$ = 0.060 |

* indicates the significant interaction based on which the post hoc analyses reported below were performed.

| Treatments | Left hemisphere | Right hemisphere | Left vs. Right |
|---|---|---|---|
| Bitter vs. Umami | $t_{(22)}$=-0.21, $p$ = 0.984 | $t_{(22)}$=-2,955, **$p$ = 0.007** | Control: $t_{(11)}$=0.540, $p$ = 0.600 |
| Bitter vs. Control | $t_{(22)}$=0.812, $p$ = 0.426 | $t_{(22)}$=-1.006, $p$ = 0.325 | Umami: $t_{(11)}$=-1.887, $p$ = 0.086 |
| Umami vs. Control | $t_{(22)}$=0.699, $p$ = 0.492 | $t_{(22)}$=2.292, **$p$ = 0.032** | Bitter: $t_{(11)}$=2.905, **$p$ = 0.014** |

*Significant effects are highlighted in bold (p ≤ 0.05).*

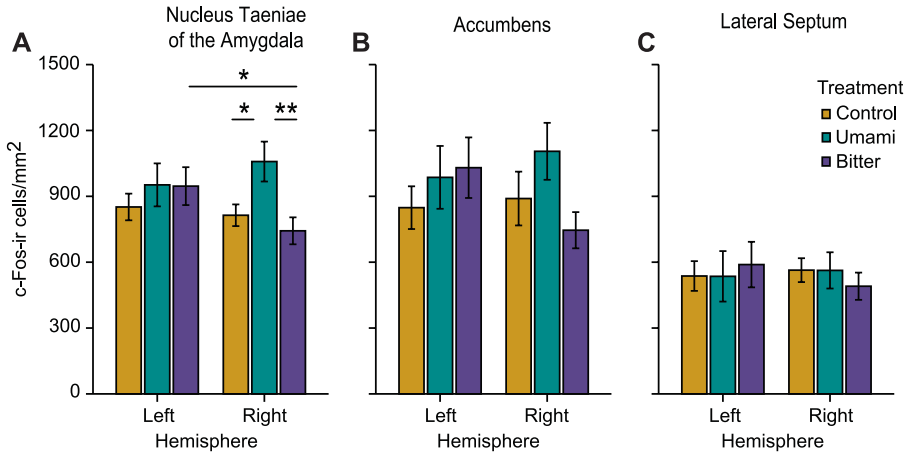


**FIGURE 5 |** Measured c-Fos-ir (immunoreactive) cell densities of the different treatments across regions of interest, in the left and right hemisphere. **(A)** nucleus taeniae of the amygdala, **(B)** nucleus accumbens, and **(C)** lateral septum. Bar plots indicate mean ± s.e.m. (*$p < 0.05$, **$p < 0.01$).

experiences, such as a palatable tastes (Azuma et al., 1984; De Araujo et al., 2003; Wu et al., 2021). Our results suggest a conserved function of avian nucleus taeniae of the amygdala and the medial amygdala in mammals, and the involvement of the mesolimbic reward network in processing taste perception.

In mice, the medial amygdala is associated with processing reward information, at least in a social context (Hu et al., 2021). And in domestic chickens the nucleus taeniae of the amygdala has been reported to respond to social stimuli (Mayer et al., 2019), to novel environments (Morandi-Raikova and Mayer, 2020) and in phobic reactions to novel foods and objects (Perez et al., 2020). In our study we can exclude neophobia from explaining the response of nucleus taeniae of the amygdala because both bitter and umami tastes were novel, only umami was consumed significantly more by chicks, and only umami induced high activation of the right nucleus taeniae of the amygdala. The reward function of nucleus taeniae of the amygdala is further supported by a similar (although not significant) pattern of activity observed in the accumbens (**Figure 5B**). Accumbens in vertebrates is involved in processing reward information and plays an important role in learning and motivation (Day and Carelli, 2007). In domestic chicks the role of accumbens in processing reward information has been investigated only in a few studies (e.g. Yanagihara et al., 2001; Izawa et al., 2002; Aoki et al., 2003; Izawa et al., 2003; Ichikawa et al., 2004; Amita and Matsushima, 2014) which have suggested a role in the control of impulsivity when choosing a predictable food reward (Cardinal et al., 2001). Further studies should assess whether avian nucleus taeniae and nucleus accumbens work together in processing food reward information.

We also found higher c-Fos expression in the left nucleus taeniae of the amygdala than in the right in the chicks presented with bitter taste. This could indicate that some lateralised processing of bitter taste is happening in the nucleus taeniae of the amygdala. In humans, the amygdala plays a role in the recognition, evaluation and response to aversive stimuli (Zald et al., 1998). However, an alternative explanation is that this lateralisation pattern in the nucleus taeniae of the amygdala is not related to the recognition of bitter taste at all. Task independent higher expression of c-Fos in the left nucleus taeniae of the amygdala compared to right was present in our previous study (Corrales Parada et al., 2021), and spontaneous lateralisation of c-Fos activity in young chicks without performing any task has also been found in other brain areas (Lorenzi et al., 2019). Therefore, our results could be indicative of a baseline lateralisation of c-Fos expression independent of bitter taste. These explanations could be teased apart by testing chicks with different concentrations of bitter tastes, and different bitter tastants, and comparing the responses to a water control group.

In chicks, accumbens has been proposed to be involved in the formation of aversive taste memories in a passive avoidance learning paradigm (Patterson and Rose, 1992; Stewart and Rusakov, 1995; Csillag, 1999; Rose, 2000; Bálint et al., 2011). In our study, we did not find a response of accumbens to bitter taste. The paradigm used in our study had some important differences to the classic passive avoidance learning in which chicks are naïve to any visual stimuli, and spontaneously peck on a coloured bead and learn to associate the bitter taste with the colour of the bead. In our study, all chicks were habituated with water, and likely formed a positive association of water taste with the water container. Chicks in our study also had restricted access to water prior to the test to motivate them to drink. When we presented the experimental taste in the same container that was used during habituation, it is possible that the chicks anticipated a rewarding outcome. In humans, altering expectancy reduces neural responses of the gustatory cortex to aversive taste when participants were led to believe that a highly aversive bitter taste would be less distasteful than it actually was (Nitschke et al., 2006). If chicks expected a rewarded experience, this could explain why we did not observe activation of the brain regions associated with aversive taste as we expected. The lack of neural response to quinine in our study is in line with what has been

reported in rats (Yasoshima et al., 2006), where the rats accumbens showed changes in c-Fos expression only during taste aversion retrieval but not after consumption of quinine hydrochloride. Future studies could compare naïve to experienced chicks in the response of the accumbens to bitter tastes, or present different concentration of quinine to naïve newly hatched chicks and investigate c-Fos activation in earlier stations of the gustatory system, such as nucleus of the solitary tract and parabrachial nucleus. In our study, the lateral septum did not show differences between treatments, which confirms a region-specific activation to rewarding taste in nucleus taeniae. To the best of our knowledge, this area has never been reported to process gustatory information. We also found no correlation between the behaviours expressed and c-Fos activation in any of the measured brain areas, which support the conclusion that exposure to appetitive and aversive taste, is associated with activation of the mesolimbic reward system in birds, and that this represents taste perception and not differences in motoric behaviour.

So far, we have only considered the role of gustatory information as a source of our effects. However, olfactory and gustatory information are usually combined in the sensory experience associated with food consumption. Since birds can perceive olfactory information (Jones and Roper, 1997; Krause et al., 2018), it is important to consider also the potential role of olfaction in our results. Chicks respond to odours predominantly when the right nostril is used (Vallortigara and Andrew, 1994; Rogers et al., 1998; Burne and Rogers, 2002). This has been interpreted as reflecting a predominant use of the right side of the brain to process olfactory information (Vallortigara and Andrew, 1994; Rogers et al., 1998; Burne and Rogers, 2002). The primary olfactory sensory area (the olfactory bulb) is a bilateral structure located within the frontal telencephalon of both hemispheres. The bulb projects to further telencephalic areas, without passing the thalamus (a unique feature of the olfactory pathway). It is reasonable to assume that the right bulb would predominantly project to the right hemisphere. However, projections to contralateral areas have also been reported (Reiner and Karten, 1985; Atoji and Wild, 2014). Intriguingly, the nucleus taeniae of the amygdala receives a direct projection from the olfactory bulb (Reiner and Karten, 1985). Thus, the similar rightward bias found in behavioural olfaction studies and in nucleus taeniae's responses to umami, could indeed suggest a role of olfactory cues in our results. While we cannot exclude this interpretation, at present there is no evidence that chicks are actually able to smell umami. It is also not known if the nucleus taeniae process odour information at all. To date, only one study investigated if this structure would respond to odours in any bird species (Golüke et al., 2019). While in this study hippocampus responded to social odours, no evidence was found for the involvement of nucleus taeniae in odour processing (Golüke et al., 2019). Moreover, it has been shown that in another galliform species, gustatory information plays a more prominent role than olfactory information in responses to aposematic signals associated with aversive taste (Marples et al., 1994). Thus, while we cannot exclude an involvement of olfactory information in the activation of nucleus taeniae, we still believe that gustatory information played a major role in this effect. Future studies should be devoted to clarify this issue, e.g., by testing anosmic animals. In any case, whether through purely gustatory information or a combination of taste and olfaction, our results demonstrate that exposure to umami was a rewarding experience for chicks and that nucleus taeniae is involved in the processing of this rewarding information.

In conclusion, we show that umami taste is associated with higher activation of the right nucleus taeniae of the amygdala in domestic chicks. Our results allows us to speculate that reward processing in birds nucleus taeniae is an ancestral function shared with the mammalian medial amygdala and provides new insights into the regulation of gustatory rewards.

# METHODS

## Subjects

We tested 36 four days old female chicks (*Gallus gallus domesticus*) of the Aviagen ROSS 308 strain. Fertilized eggs were obtained from a commercial hatchery (CRESCENTI Societ`a Agricola S.r.l. –Allevamento Trepola–cod. Allevamento127BS105/2). Eggs were incubated from embryonic day one (E0) to seventeen (E17) in complete darkness. On the morning of E18 to the evening of E19, eggs were light stimulated following (Lorenzi et al., 2019). This procedure has an important impact on the lateralisation of chicks visual system (Rogers, 1982; Rogers and Sink, 1988; Rogers, 1990; Rogers and Bolden, 1991; Rogers and Deng, 1999), on the maturation of visual responses (Costalunga et al., 2021) and on several aspects of chicks behaviour (Daisley et al., 2009; Salva et al., 2009). Chicks hatched on E21 in darkness at a constant temperature of 37.7°C and a humidity of 60%. On the day of hatching, chicks were placed in individual metal cages (28 cm× 32 cm× 40 cm; W × H × L), where they were housed for three days with food and water *ad libitum* at a room temperature of 30–32°C and light conditions of 14 h light and 10 h dark.

## Apparatus

On the third day after hatching (24 h prior to the experiment), chicks were taken to the experimental room and housed individually in metal cages (**Figure 1**), identical to their home cages. The experimental cages differed to the home cage only by the addition of a black plastic wall (occluder) that allowed us to make two compartments in the cage. Chicks were located on the larger section of the cage (25 cm L); while the smaller section (15 cm L) was used for keeping the water dishes separated from the chicks until the start of the experiment, and prevented chicks from observing the experimenter (see below). The two compartments were connected by a closable opening (5 × 4.8 cm) in the plastic wall. Above every cage (76 cm), there was a video camera (Microsoft LifeCam Cinema for Business) for recording the experiment. Illumination of the cages was provided by top lights (25 W warm light) at

67.5 cm above the cage, while the rest of the experimental room was dark.

## Habituation

Chicks were left in their individual experimental cages for 24 h prior to the experiment with water and food *ad libitum*. Room temperature was 26.4–28.7°C, humidity 24–41%, and light conditions of 14 h light and10 h dark.

## Experiment

For the experiment, we assigned 12 chicks to each treatment. Treatments consisted of three experimental tastes (control, umami and bitter). We used water as a control, since chicks were habituated to drink it. As a palatable taste, we used umami in a combination of 2.5% monosodium glutamate (MSG, AJI-NO-MOTO >99%, CEE: E621, France) + 0.25% inosine 5′-monophosphate (IMP, ≥98%, Sigma-Aldrich I2879-1G diluted in water). Yoshida et al. (2018) showed that at this concentration, MSG is an enhancer of the umami taste (IMP) in chicks. As an unpalatable bitter taste, we used quinine (Alfa Aesar A10459 99%) 10 mM dissolved in water. The experimental solutions were prepared the day prior to the experiment and kept in the experimental room to adjust their temperature to the room temperature. Experimental tastes were colourless and were presented to chicks in identical containers with 80 ml solution in each. Before the experiment chicks were deprived of water for 45 min. After this period, the experimental taste was presented for 10 min, the behaviour of the chicks was recorded, and after that, the experimental container was removed. The containers with the experimental solutions were weighted before and after the experiment in order to estimate the amount of liquid consumed by each chick. We ran the experiment in 4 days, testing nine chicks per day. Every testing day, we assigned three individuals to each treatment in randomized order.

## Video Analysis

We analysed the videos for seven minutes after the first contact with the experimental taste. In this period, we recorded the total number of the following events: contact with the liquid (when chicks peck the experimental solution), head shaking, and beak cleaning. The category 'beak cleaning' includes two behaviours: beak wiping, when the animal wipes its beak on the floor and beak scratching, performed with a foot. Video analysis was conducted blinded to the experimental conditions using the VLC media player software (v. 3.0.12).

## Histology and Immunohistochemistry

Seventy min after the first contact with the experimental taste, all chicks were weighted and received a lethal dose (0.8 ml) of a ketamine/xylazine solution (1:1 ketamine 10 mg/ml + xylazine 2 mg/ml). They were then perfused transcardially with phosphate-buffered saline (PBS; 0.1 mol, pH = 7.4, 0.9% sodium chloride, 4°C) and paraformaldehyde (4% PFA in PBS, 4°C). The head was post-fixed and stored in 4% PFA for at least 8 days until processing. All the following brain processing steps were performed blind to the experimental treatments. To ensure that the subsequent coronal brain sections would have the same orientation (°45), brains were removed from the skulls following the procedures described in the chick brain atlas (Kuenzel and Masson, 1988). The left and the right hemispheres were separated and processed independently. Each hemisphere was embedded in a 7% gelatine in PBS solution containing egg yolk at 40°C and post-fixated for 48h at 5°C in 20% sucrose in 4% PFA/PBS and further 48 h in 30% sucrose in 0.4% PFA/PBS solution. Four series of 45 μm coronal sections were cut on a Cryostat (Leica CM1850 UV) at −20°C. Two out of the four series were collected in cold PBS. The sections of the first series were used for labelling. The sections of the second series were kept as backup. For free-floating immunostaining, sections were incubated in 0.3% H2O2 in PBS for 20 min to deplete endogenous peroxidase activity. Between every of the following steps of the procedure the sections were washed in PBS. After the sections were treated with 3% normal goat serum (S-1000, Vector Laboratories, Burlingame, CA) in PBS for 30 min at room temperature, they were transferred to the c-Fos antibody solution (c-Fos antibody, 1: 2000; rabbit, polyclonal K-25, Santa Cruz Biotechnology, Santa Cruz, CA) containing 0.1% Bovine Serum Albumin (BSA, SP-5050, Vector Laboratories) in PBS and incubated for 48 h at 5°C on a rotator. The secondary antibody reaction was carried out using a biotinylated anti-rabbit solution (1:200, BA- 1000, Vector Laboratories) in PBS for 60 min at room temperature on a rotator. The ABC method was used for signal amplification (Vectastain Elite ABC Kit, PK-6100, Vector Laboratories). Cells with concentrated c-Fos protein were visualized with the VIP substrate kit for peroxidase (SK-4600, Vector Laboratories), at a constant temperature of 25°C for 25 min. This produced a dark purple reaction product confined to the nuclei of c-Fos immunoreactive (-ir) cells. Brain sections of right and left hemispheres were mounted with the same orientation on gelatine-coated slides, enabling the coder to be blind to the hemisphere's identity while counting. Slides were then dried at 50°C, counterstained with methyl green (H-3402, Vector Laboratories) and cover slipped with Eukitt (FLUKA).

## Brain Anatomy

Brain sections were analysed blind to the experimental condition and hemispheres. c-Fos-ir cells were stained purple-black and were easily discerned from the background and non-activated cells, which were stained light-green (**Figure 4**). Photos were taken with a Zeiss microscope (objective magnification: ×20 with a numerical aperture of 0.5) connected to a digital camera (Zeiss AxioCam MRc5) and the Zeiss imaging software ZEN. Exposure time of the camera and the light conditions of the microscope were kept constant for all photos, while the contrast was slightly adjusted for individual photos to match their visual appearance if it was needed.

To analyse brain activation within the regions of interest a spot with the highest number of c-Fos-ir cells was chosen, by visual observation under the microscope. Then a counting area (400 × 400 μm) was positioned over this spot by keeping a minimum distance of 10 μm from the borders. A snapshot was taken, cropped around the counting area and saved. Automatic counting of the c-Fos immunoreactive cells was performed using the "analyse particle" function of the ImageJ software

(Schneider et al., 2012). All photos were analysed with a predefined macro, where the image was transformed into 8bit, threshold was set to 120, circularity of particles to 0.5–1.0 and particle size to 2–200.

For the analysis of nucleus taeniae of the amygdala five sections of both hemispheres were selected from an area corresponding to the A8.8 to A6.4 of the brain atlas (Kuenzel and Masson, 1988) (**Figure 2C**). To quantify c-Fos-ir cells in accumbens (Bálint et al., 2016), five sections of both hemispheres were selected from A10.0 to A9.2 (Kuenzel and Masson, 1988; **Figure 2A**). Lateral septum was measured on five selected sections from A8.8 to A7.6 (Kuenzel and Masson, 1988) in the lower half of septum starting from the ventral border of the lateral ventricle (**Figure 2B**). The lateral septum was further delineated from the medial septum based on anatomical landmarks that are visible after methyl green counterstain. The cell densities obtained from the different brain sections were averaged to estimate overall activity in each measured area. These individual bird means were employed for further statistical analysis.

### Statistical Analysis

All statistical analysis were conducted with R v. 4.0.4 (R Core Team, 2021). We analysed whether behavioural responses varied according to treatment. The response variables were contact with the liquid, liquid consumed per body weight, head shaking and beak cleaning. The raw values were divided by the number of contact with liquid episodes. This allowed to reveal how much the contact with liquid elicited the behaviours of interest. ANOVA was run on the first three variables with Fischer's LSD tests for *post-hoc* analysis. Given that for beak cleaning the residuals were not normally distributed (Shapiro-Wilk test, $W = 0.7382$, $p < 0.05$), even after square root or logarithmic transformation, a non-parametric Kruskal–Wallis test was used. Significance between the comparisons was assessed with *post-hoc* Dunn tests.

In order to test whether c-Fos activation (response variable) varied according to treatment and hemisphere (factors), we conducted a repeated measures ANOVA for each brain region. We used a between-subject factors with three levels (Treatment: control, umami, bitter), and within-subject factor with two levels (Hemisphere: left and right). Given that for nucleus taeniae of the amygdala and accumbens residuals were not normally distributed (Shapiro-Wilk Test; nucleus taeniae: $W_{71} = 0.955$, $p = 0.013$; accumbens: $W_{71} = 0.952$, $p = 0.009$), we used a square root transformation. This procedure significantly improved the normality of residuals (nucleus taeniae: $W_{71} = 0.979$, $p = 0.272$; accumbens: $W_{71} = 0.982$, $p = 0.393$), and further analysis were conducted with transformed data. Between treatment comparisons were assessed with independent *post-hoc* t-tests, while between hemispheres comparisons for each treatment was assessed with paired t-tests. In order to test whether behaviour and motoric activity is correlated with c-Fos expression, we performed Pearson correlations between c-Fos expression in the brain regions analysed and the behaviours recorded for each of the treatments and hemispheres. All figures were created or postprocessed using Adobe Photoshop and Illustrator software.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Ministero della Salute, Dipartimento Alimenti, Nutrizione e Sanita' Pubblica Veterinaria (permit number 60/2020-PR).

## AUTHOR CONTRIBUTIONS

FP-S: conceptualisation, methodology, formal analysis, investigation, data curation, writing–original draft. CDCP: software, investigation, formal analysis. UM: conceptualisation, methodology, validation, formal analysis, resources, data curation, writing–review and editing, visualization, supervision, project administration. HMR: conceptualisation, methodology, validation, resources, writing–review and editing, supervision, project administration, funding acquisition.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.897931/full#supplementary-material

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REVIEW
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# Birdsong and the Neural Regulation of Positive Emotion

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Birds are not commonly admired for emotional expression, and when they are, the focus is typically on negative states; yet vocal behavior is considered a direct reflection of an individual's emotional state. Given that over 4000 species of songbird produce learned, complex, context-specific vocalizations, we make the case that songbirds are conspicuously broadcasting distinct positive emotional states and that hearing songs can also induce positive states in other birds. Studies are reviewed that demonstrate that that the production of sexually motivated song reflects an emotional state of anticipatory reward-seeking (i.e., mate-seeking), while outside the mating context song in gregarious flocks reflects a state of intrinsic reward. Studies are also reviewed that demonstrate that hearing song induces states of positive anticipation and reward. This review brings together numerous studies that highlight a potentially important role for the songbird nucleus accumbens, a region nearly synonymous with reward in mammals, in positive emotional states that underlie singing behavior and responses to song. It is proposed that the nucleus accumbens is part of an evolutionarily conserved circuitry that contributes context-dependently to positive emotional states that motivate and reward singing behavior and responses to song. Neural mechanisms that underlie basic emotions appear to be conserved and similar across vertebrates. Thus, these findings in songbirds have the potential to provide insights into interventions that can restore positive social interactions disrupted by mental health disorders in humans.

**Keywords: nucleus accumbens (NAc), emotion, positive affect, reward, motivation, songbird, communication, vocal behavior**

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## INTRODUCTION

Birds are admired for many qualities, including impressive migratory feats, complex singing behavior, and highly developed systems of mating and parental behavior; yet birds are not traditionally admired for being emotional. Darwin mentioned birds in only a few cases in *The Expression of the Emotions in Man and Animals* and in these cases focused on feather-ruffling and freezing responses as expressions of negative emotion (Darwin, 1897). More recent studies on avian emotion have also focused on negative states, such as fear or frustration (Papini et al., 2018), and some of the early research on the neural regulation of animal emotion was performed using distress calls in socially separated domestic chicks to assess negative affect (Panksepp et al., 1978, 1980; Vilberg et al., 1984). These studies demonstrate negative affective or fear states in birds; however, positive emotions are also necessary to elicit feeding, sexual, and social behaviors. These

behaviors are essential for survival and reproductive success, but until recently the extent to which birds express positive emotions has been overlooked (Marino, 2017; Bertin et al., 2018).

There are many examples of birds engaging in behaviors that appear playful or pleasurable (Burghardt, 2005; Emery and Clayton, 2015; Pessoa et al., 2019). For example, there are reports of raptors dropping and catching objects, crows sledding, swans surfing, and parrots producing an emotionally contagious play call that is comparable to human laughter (Hewitt, 2013; Emery and Clayton, 2015; Schwing et al., 2017; Kaplan, 2020). Vocal behavior is considered a direct reflection of an individual's emotional state (Cheng, 2003; Burgdorf et al., 2008; Panksepp, 2010; Briefer and Le Comber, 2012), which suggests that contrary to common perceptions, birds, and in particular songbirds, are among the most emotionally expressive species in the animal kingdom. When it comes to vocal production, few animals are as impressive, with approximately 4000 songbird species producing high rates of complex, species typical, learned songs. These songs convey information related to an individual's motivational and emotional state and are designed to alter the emotional states of other birds.

Emotion is challenging to study from a neuroethological perspective. The term "emotion" is not consistently defined, we lack a common language around it, and it is often thought of as a completely internal experience. Indeed, conscious emotional feelings can only be confirmed in humans. However, emotions can be powerful motivators of behavior and emotional behaviors and responses can be quantified in non-human animals when conscious emotional feelings cannot. Here we define emotion as a mental state caused by neurophysiological changes associated with feelings. These include states of pleasure or reward and states of motivated reward seeking (Panksepp, 2011a), that are the focus of this review. Emotion, motivation, and reinforcement are tightly interrelated concepts that can be thought of as components of an integrated process that occurs when an animal faces a challenge or an opportunity. Emotion can occur in response to either innate or learned (i.e., conditioned) stimuli. Innate stimuli are intrinsically aversive or rewarding and can directly elicit emotional responses. Conditioned stimuli could serve as incentives (stimuli that motivate behavior) or reinforcers (stimuli that increase the probability that a learned behavior will occur) and can elicit emotional responses in anticipation of a challenge or an opportunity (LeDoux, 1996; Debiec et al., 2014). Thus, positive emotional states can occur in response to an intrinsic stimulus, can serve to bring an animal into proximity with a goal object, or can promote behaviors that increase opportunity.

Numerous studies on songbirds have provided fundamental insights into the neural regulation of vocal learning and production, but until recently emotion had not become an experimental variable in studies of birdsong (Cheng, 2003). In this review we make the case that birds are ostentatiously broadcasting positive emotional states and that the unique communication and social qualities of songbirds are ideal for providing fundamental insights into the neural regulation of positive emotional states. Many mental health disorders in humans, including depression, social anxiety, and autism spectrum disorder, are characterized by deficits in communication, and interactions that were once positive can become aversive leading to social withdrawal (APA, 2000; Pearlman-Avnion and Eviatar, 2002; Noens and van Berckelaer-Onnes, 2005; Knowland and Lim, 2018; Fox and Lobo, 2019; Gellner et al., 2021). Songbirds are one of only a few animals to produce complex, context-appropriate, learned vocalizations (Petkov and Jarvis, 2012; Vernes et al., 2021). Because neural mechanisms that underlie basic emotions appear to be conserved and similar across vertebrates (Panksepp, 2011a,b; Pessoa et al., 2019), findings in songbirds have the potential to provide insights into interventions that can restore positive social interactions disrupted by mental health disorders in humans.

## BIRDSONG AS AN EXPRESSION OF AN ANTICIPATORY STATE OF REWARD SEEKING

Songbirds sing in multiple contexts to attract and defend mates, repel rivals, to learn and practice songs, and in some cases for no immediate, obvious reason. There are two contexts in which song is strongly modulated by positive emotional states that will be the focus of this review. The first is the mating context. In seasonally breeding songbirds during the breeding season and in opportunistic breeders, males respond to the presentation of females with high rates of courtship song [e.g., (Jarvis et al., 1998; Riters et al., 2000)]. This song is critical for mate attraction. It reflects a male's state of sexual motivation and is driven by the anticipation of female attraction and mating. The emotional state associated with sexually motivated song has been assessed using conditioned place preference (CPP) tests, which are commonly used to assess positive states in animals associated with feeding, drug use, sexual and playful behaviors (Carr et al., 1989; Tzschentke, 2007; Trezza et al., 2009). In brief, a bird is allowed to sing in an aviary and then immediately transferred to one of two distinct chambers separated by a divider. The following day the divider is removed and the amount of time a bird spends on either side of the apparatus is recorded. It is assumed that if the affective state associated with song is positive the bird will associate that positive state with the side of the chamber in which it is placed after singing and therefore spend most of its time in that chamber. This method was developed based on similar tests used to evaluate emotional states associated with sexual behavior in rodents (Agmo and Berenfeld, 1990; Tenk et al., 2009); its use in songbirds, interpretations and caveats have been discussed elsewhere in detail (Riters and Stevenson, 2012; Riters et al., 2014).

Although it is a common public perception that spring song is an expression of joy, experimental CPP tests suggest that it would be more accurate to interpret spring song as a reflection of a motivated, anticipatory state. This is supported by the finding that male European starlings, *Sturnus vulgaris,* do not develop preferences for places (i.e., CPPs) that had been paired previously with their own production of sexually motivated song (Riters and Stevenson, 2012) (**Figures 1A,B**).

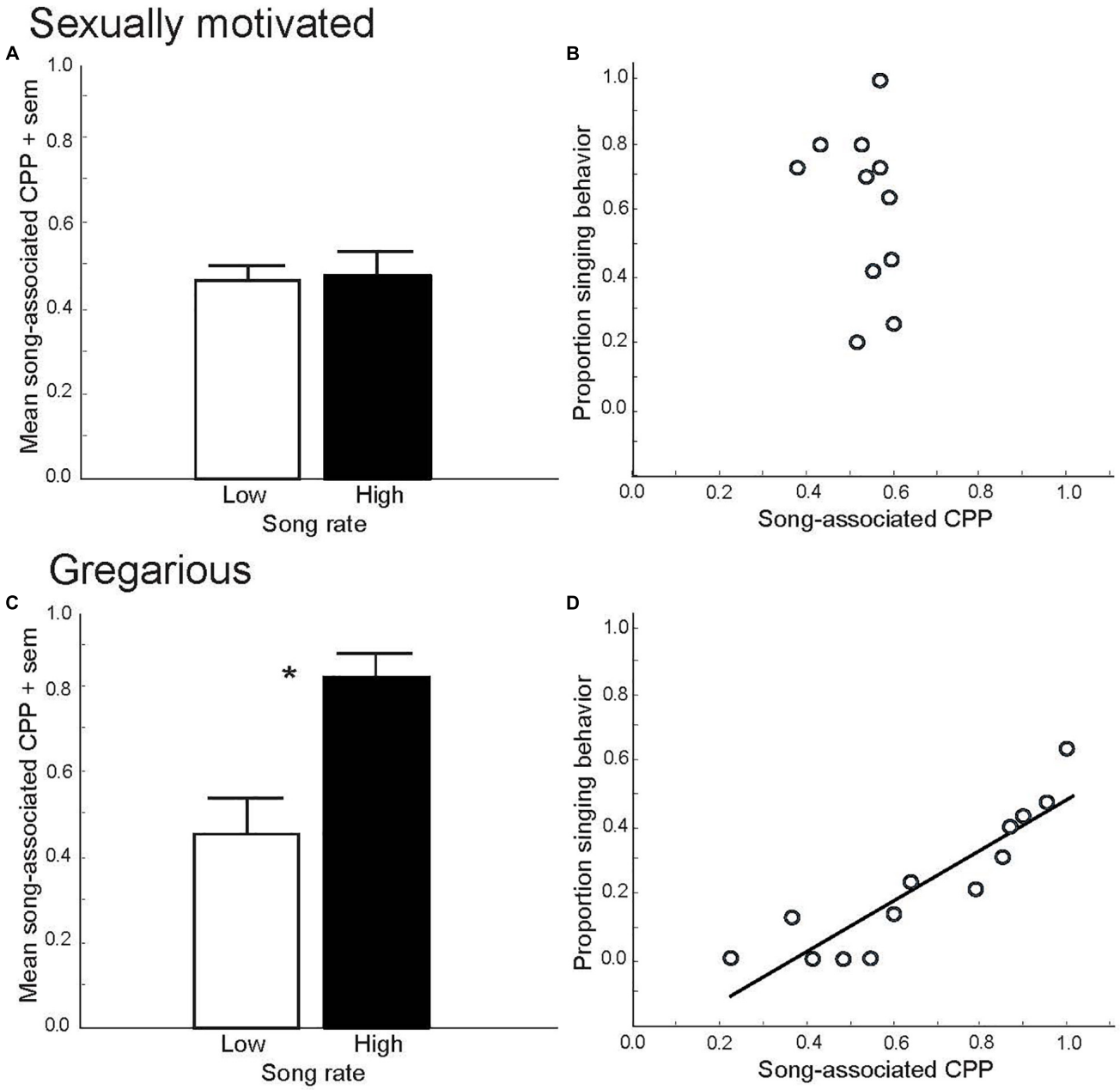


**FIGURE 1 |** Conditioned place preference (CPP) tests demonstrate that sexually motivated and gregarious singing behavior reflect distinct emotional states. Mean proportion time spent in a location (i.e., song-associated CPP) that had been paired previously with either low (open bar) or high (filled bar) rates of singing behavior in male starlings singing in response to a female in spring **(A)** and singing in a flock in fall, $*p = 0.006$ **(C)**. Figures A and C replotted to show correlations between song and CPP in male starlings in spring, ns **(B)** and fall, $p = 0.000002$ **(D)**. *Y*-axis represents the proportion of all vocal behaviors that were songs produced by males during and just prior to being placed into a uniquely decorated place (i.e., a birdcage). The *X*-axis represents the proportion of time males later spent in that place the following day (CPP, considered a reflection of song-associated reward). Each point represents data from a single male. Figures redrawn from Riters and Stevenson (2012).

This indicates that the act of singing in this context is not itself an expression of a positive emotional state. Rather, males appear to sing in this context because they are seeking the pleasure induced by copulation. This idea is supported by numerous studies in birds that demonstrate copulation-induced place preferences (Domjan et al., 1992; Akins, 1998; Balthazart et al., 1998; Riters et al., 1998, 1999). Although males are well known for their singing behavior, there is a growing appreciation that females of many species also sing (Odom et al., 2014). For example, within a breeding context once a female pairs with a mate, female song may play a role in pair bonding, reproductive synchronization, extra-pair copulation or territorial

defense and mate guarding (Sandell and Smith, 1997; Langmore, 1998; Collins, 2004; Catchpole and Slater, 2008). The affective states associated with song in these contexts has yet to be studied.

## BIRDSONG AS AN EXPRESSION OF INTRINSIC POSITIVE EMOTION

The second context in which song has been linked to a positive affective state is in a non-breeding context. Some seasonally breeding birds, such as starlings, form large mixed sex flocks outside their breeding seasons (Feare, 1984), and some opportunistically breeding birds, such as zebra finches, *Taeniopygia guttata*, spend much of their lives in large flocks (Zann, 1996; Goodson et al., 2012). While in these flocks, birds sing high rates of non-sexually motivated song. Song in this context is facilitated by the presence of flock mates (Gochfeld, 1977; Petrinovich, 1988; Jesse and Riebel, 2012), but unlike sexually motivated song it is commonly produced by birds facing away from other birds and it appears to be ignored (Feare, 1984; Zann, 1996; Riters et al., 2000). The immediate function of song in these flocks is not clear, but it is important for song learning and practice and proposed to play a role in flock cohesion (Riters et al., 2019). This is a type of song commonly referred to as undirected song in zebra finches (Dunn and Zann, 1996). Here we will refer to it as "gregarious" song when describing song in starling flocks and "undirected" when we discuss studies on zebra finches because, although these songs are produced in non-sexual contexts, they are not identical. Females of some species also sing in flocks. For example, the gregarious song described here is also produced by female starlings and studies reviewed below suggest that neural mechanisms underlying this type of song in males and females are shared (Stevenson et al., 2020; Maksimoski et al., 2021).

Although not part of his book on animal emotion, in *The Origin of Species*, Darwin proposed that although during the mating season male songs function mainly to attract females, outside the mating season males continue to sing for their own "amusement" (Darwin, 1871). Experimental CPP tests support this idea. Specifically, both male zebra finches and male and female starlings develop strong, positive preferences for places that were paired previously with their own production of gregarious or undirected song in flocks, and these preferences are linearly correlated with song rate (Riters and Stevenson, 2012; Riters et al., 2014; Hahn et al., 2017; Stevenson et al., 2020) (**Figures 1C,D**). These findings suggest that the act of singing in this context is tightly coupled to a positive emotional state, and it has been suggested that a natural function of the singing may be to strengthen group cohesion through a conditioned preference for a flock (Riters et al., 2019).

It has been proposed that song in gregarious contexts can be considered a form of rewarding play behavior similar to forms of play observed in multiple young animals as they practice sequences of motor events that are used later in adult reproductive contexts (Thorpe, 1956; Hartshorne, 1973; Ficken, 1977; Fagen, 1981; Burghardt, 2005; Riters et al., 2017). Performing behaviors for "amusement" (i.e., because they induce a positive emotional state) essentially defines play, and similar to other forms of playful behavior, gregarious song is facilitated by reunion with social partners, and it is initiated when an animal is fed, healthy and free from stress (e.g., in the absence of predators) (Panksepp and Beatty, 1980; Burghardt, 2005; Siviy et al., 2006; Yamahachi et al., 2020; Kim et al., 2021). This suggests that like other forms of play behavior, safety and the presence of flock mates may induce a positive emotional state that is conducive to singing, which may then be maintained by reward induced by the act of singing itself.

## EMOTIONAL STATE IS REFLECTED IN SONG STRUCTURE

Structural attributes of songs differ in association with emotional states. In numerous species, sexually motivated songs are found to be longer than non-breeding songs and to be more highly structurally and temporally stereotyped [e.g., (Sakata and Vehrencamp, 2012; Alger et al., 2016)]. Females prefer to mate with males that produce long, structurally stereotyped songs and males tend to avoid these males during competitions over breeding territories (Mountjoy and Lemon, 1991; Searcy and Yasukawa, 1996; Sakata and Vehrencamp, 2012). Downplaying these features in non-breeding contexts may function to promote social tolerance and group cohesion. The changes in structural features of song thus convey information to other birds about emotional state, and these vocal features can strongly influence the motivational state of others, as reviewed next.

## BIRDSONG AS A STIMULUS THAT INDUCES A POSITIVE EMOTIONAL STATE IN OTHERS

Several studies demonstrate that hearing song can induce positive emotional states. For example, young male zebra finches readily learn to peck keys that trigger tutor song playback during the sensitive period for song learning, which demonstrates that hearing song alone can act as a reinforcer in a young male (Adret, 1993; Houx and Ten cate, 1999). Operant tasks that use song as a reinforcer also indicate that positive emotional states induced by hearing song play a role in adaptively shaping female mate choices. Female canaries, *Serinus canaria*, preferentially perform copulation solicitation displays in response to certain "sexy" male song syllables, and operant key peck tasks indicate that these songs are more reinforcing than songs that lack these syllables (Salvin et al., 2018). Female starlings trained to land on a perch-apparatus to play recorded male song spend more time on perches that play longer songs (Gentner and Hulse, 2000). These sexy elements appear costly to produce, suggesting that they accurately reflect male quality (Suthers et al., 2012). Thus, the positive emotional state induced by these elements adaptively shapes female mating choices by promoting preferential responses to high quality males. The fact that birds are willing to work (i.e., hop onto perches or peck keys) to hear song playback also indicates that hearing

song, in combination with other morphological and behavioral traits, social and environmental context, history and individual differences (Kaplan, 2019; Fujii et al., 2022), may induce an anticipatory state of reward seeking in sexually motivated females. The positive emotional states induced by song likely in combination with these other variables also play a role in maintaining pair bonds in monogamous zebra finches, with operant responses to song reinforcement in females observed exclusively in response to a mate's song (Tokarev et al., 2017; Coleman et al., 2019; Day et al., 2019).

The degree to which male song induces a positive emotional state in females can also depend on the reproductive state of the female. For example, female starlings that are sexually motivated and prepared to breed, as evidenced by active exploration of nesting sites, develop strong CPPs for places paired with playback of male courtship song (Riters et al., 2013) (**Figure 2**). Females that are not actively nesting do not develop similar place preferences (**Figure 2**). This suggests that the emotional state induced by hearing male song is plastic in adulthood and coordinated with female reproductive state such that song only induces a positive emotional state when females are prepared to breed. These studies demonstrate that song alone can serve as a reinforcer in sexually motivated females and that the emotional state induced by song plays a role in adaptive female mating decisions.

Emotional states induced by hearing gregarious song have not been well studied. To date, a single study in non-breeding condition female starlings demonstrated that on average females developed conditioned aversions to places that had been paired with playbacks of recordings of gregarious male song (Hahn et al., 2019). This was unexpected given the hypothesis that gregarious song-associated place preferences function to enhance flock cohesion. However, the prior CPP studies focused on the act of producing rather than hearing song [in females as well as males (Stevenson et al., 2020)]. It may thus be that the production of gregarious song in flocks is self-rewarding, but that hearing song alone is not. A form of vocal self-stimulation occurs in ring doves, in which a female's own coos alter her endocrine physiology and behavior (Cheng, 2003). It may be that for birds in flocks, in females that sing (e.g., starlings), singing is also a form of self-stimulation and that producing song is more rewarding than hearing song. However, past studies also show that female starlings in overwintering flocks share song types with close neighbors (Hausberger et al., 1995). This raises the possibility that neighbor or familiar flock song may be more rewarding than the unfamiliar song stimuli that were used in the CPP study. The emotional valence of hearing flock songs requires additional research.

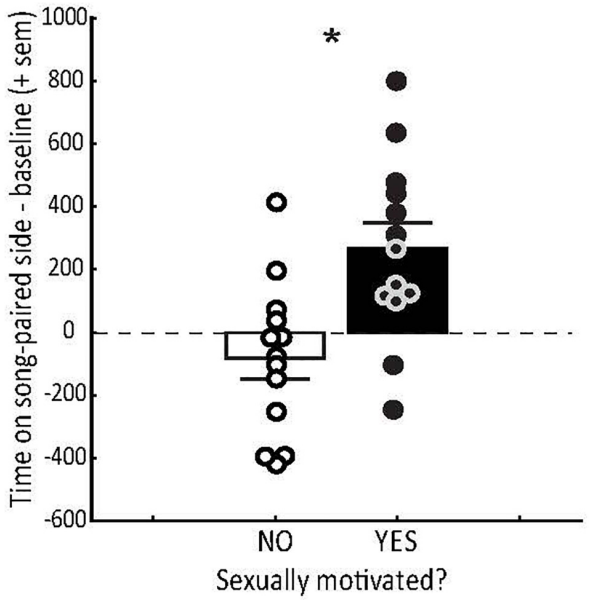


**FIGURE 2 |** Conditioned place preference (CPP) tests demonstrate that hearing sexually motivated song induces a positive emotional state in sexually motivated females. Mean (+ sem) time spent (in the absence of song playback) on the side of the CPP apparatus previously paired with male starling courtship song (minus the baseline preference for that side during habitation) in female starlings that were not (open bar) or were (filled bar) sexually motivated as indicated by exploration of nesting sites. Each point represents data from a single female. Zero on the y axis indicates no preference. * = $p < 0.0027$. Figure redrawn from Riters et al. (2013).

## A ROLE FOR THE NUCLEUS ACCUMBENS IN POSITIVE EMOTION AND SONG PRODUCTION

We make the case above that sexually motivated and gregarious singing behavior advertise and induce distinct emotional states. Studies in mammals indicate that basic emotions, including "seeking" and "play" (emotions associated with sexually motivated and gregarious song, respectively) are generated by subcortical structures that are considered highly evolutionarily conserved across vertebrates (Panksepp, 2011a,b). When it comes to positive emotional states the nucleus accumbens (NAc) is considered among the most important emotional pleasure centers in the brain (Berridge and Kringelbach, 2015), although it is important to note that NAc has many critical functions beyond reward (e.g., feeding, cognition, locomotion, and even the regulation of aversive responses) [reviewed in Floresco (2015)]. Here we focus on the role of NAc in reward-related processes.

An extensive body of literature demonstrates a role for NAc in both an anticipatory state of reward seeking and the experience of positive emotional states (e.g., playful states) in mammals (Berridge, 2007; Smith and Berridge, 2007; Trezza et al., 2011; Vanderschuren et al., 2016). Both dopamine and opioid neuromodulators act in NAc to modulate these emotional processes, with dopamine released into NAc from projections from the ventral tegmental area (VTA) implicated in anticipatory, reward seeking behaviors (Ikemoto and Panksepp, 1999; Berridge, 2007). This suggests a potential role for dopamine in NAc in male sexually motivated song production as well as female motivated responses to playbacks of male courtship song, reflected in operant responses, reviewed above.

In contrast to dopamine, opioids underlie the experience of an intrinsic positive or pleasurable emotional state, with activation

of mu opioid receptors in specific "hedonic hotspots" in NAc found to induce reward in rats (Pecina et al., 2006; Berridge and Kringelbach, 2015). This suggests a potential role for opioids in NAc in the positive emotional state associated with gregarious or undirected singing behavior as well as the positive state induced by hearing male song as reflected in the finding that song playback alone can serve as an operant reinforcer and induce CPPs in females.

Despite over 40 years of research on the contribution of the NAc to positive emotional processes in mammals (Mogenson et al., 1979, 1980; Floresco, 2015), very little is known about the NAc in songbirds, perhaps because positive emotions in birds had not until recently become a serious research topic. Indeed, for many years the location of NAc in birds was in dispute, and in many atlases subregions of what is currently considered NAc were labeled as lobus parolfactorius or the lateral part of the bed nucleus of the stria terminalis (Karten and Hodos, 1967; Stokes et al., 1974; Youngren and Phillips, 1978; Kuenzel and Masson, 1988). In the early 2000s, NAc was newly delineated based on tract tracing and immunolabeling (Mezey and Csillag, 2002; Carrillo and Doupe, 2004; Reiner et al., 2004; Balint and Csillag, 2007; Balint et al., 2011; Husband and Shimizu, 2011; Gutierrez-Ibanez et al., 2016) (**Figure 3**), and recently in male starlings the rostral, shell, and core subdivisions of NAc were found to contain both dopamine- and opioid-related proteins, similar to mammals (Polzin et al., 2021) (**Figure 4**). These findings suggest potential functional homology between the avian and mammalian NAc and raise the possibility that dopamine and opioids in the songbird NAc may underlie emotional states of reward seeking and pleasure reflected in birdsong. Below we review studies that are beginning to provide support for this hypothesis and then integrate the NAc with a neurocircuitry that may provide a core, conserved system that generates positive emotional states reflected in birdsongs.

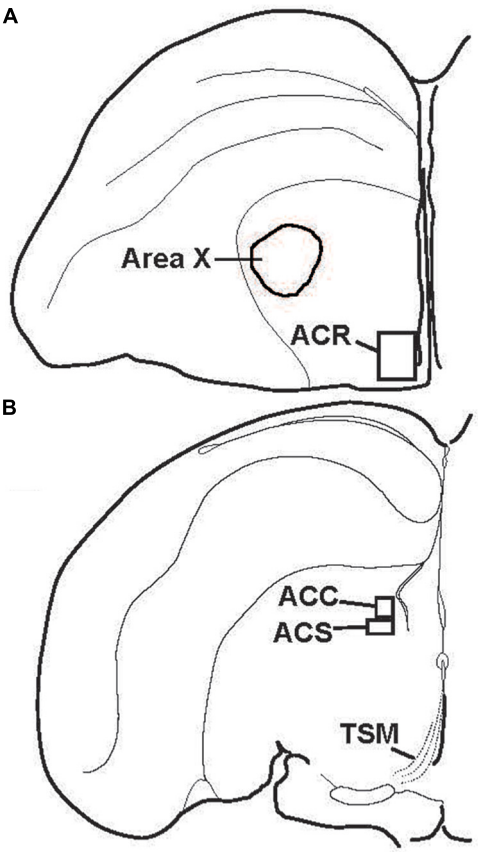


**FIGURE 3 |** Location of subdivisions of the songbird NAc. Illustrations showing locations of **(A)** the rostral pole of the nucleus accumbens (ACR) and **(B)** the nucleus accumbens core (ACC) and shell (ACS) in the left hemisphere of coronal sections. TSM = tractus septomesencephalicus.

## NUCLEUS ACCUMBENS AND BIRDSONG AS AN EXPRESSION OF AN ANTICIPATORY STATE OF REWARD SEEKING

Dopamine in NAc is implicated in motivated, reward-directed behaviors, and several studies in rodents demonstrate that dopamine is released in NAc in males in response to the presentation of a sexually receptive female (Damsma et al., 1992; Wenkstern et al., 1993; Fiorino et al., 1997). This leads to the prediction that activity in NAc, and in particular dopamine release, may underlie anticipatory, seeking emotional states that motivate males to sing to attract females. Although the VTA, which is the major source of dopaminergic projections to NAc, is strongly implicated in song in this context (Yanagihara and Hessler, 2006; Hara et al., 2007; Heimovics and Riters, 2008; Nordeen et al., 2009; Kubikova and Kostal, 2010; Matheson and Sakata, 2015; Merullo et al., 2015), in a recent study no significant correlations were found between the production of sexually motivated song in male starlings presented with females and numbers of cells labeled for either the immediate early gene FOS or Egr-1 in NAc (Polzin et al., 2021) (**Figures 5A,C,E**). The absence of immediate early gene expression does not indicate the absence of neuronal activity, and not every immediate early gene is expressed in every brain area (Heimovics and Riters, 2005). Thus, these results certainly do not preclude a role for NAc in song in this context, which would be expected based on studies in rodents; however, this prediction must be tested in future research.

## NUCLEUS ACCUMBENS AND BIRDSONG AS AN EXPRESSION OF INTRINSIC POSITIVE EMOTION

Research on the role of NAc in song in non-sexual contexts is also in its early stages, but in a recent study in male starlings, numbers of cells labeled for the immediate early gene FOS in each subdivision of the NAc correlated positively with the production of gregarious song (Polzin et al., 2021) (**Figures 5B,D,F**). This contrasts with the absence of a correlation for sexually motivated song (**Figures 5A,C,E**), highlighting a potential role for NAc

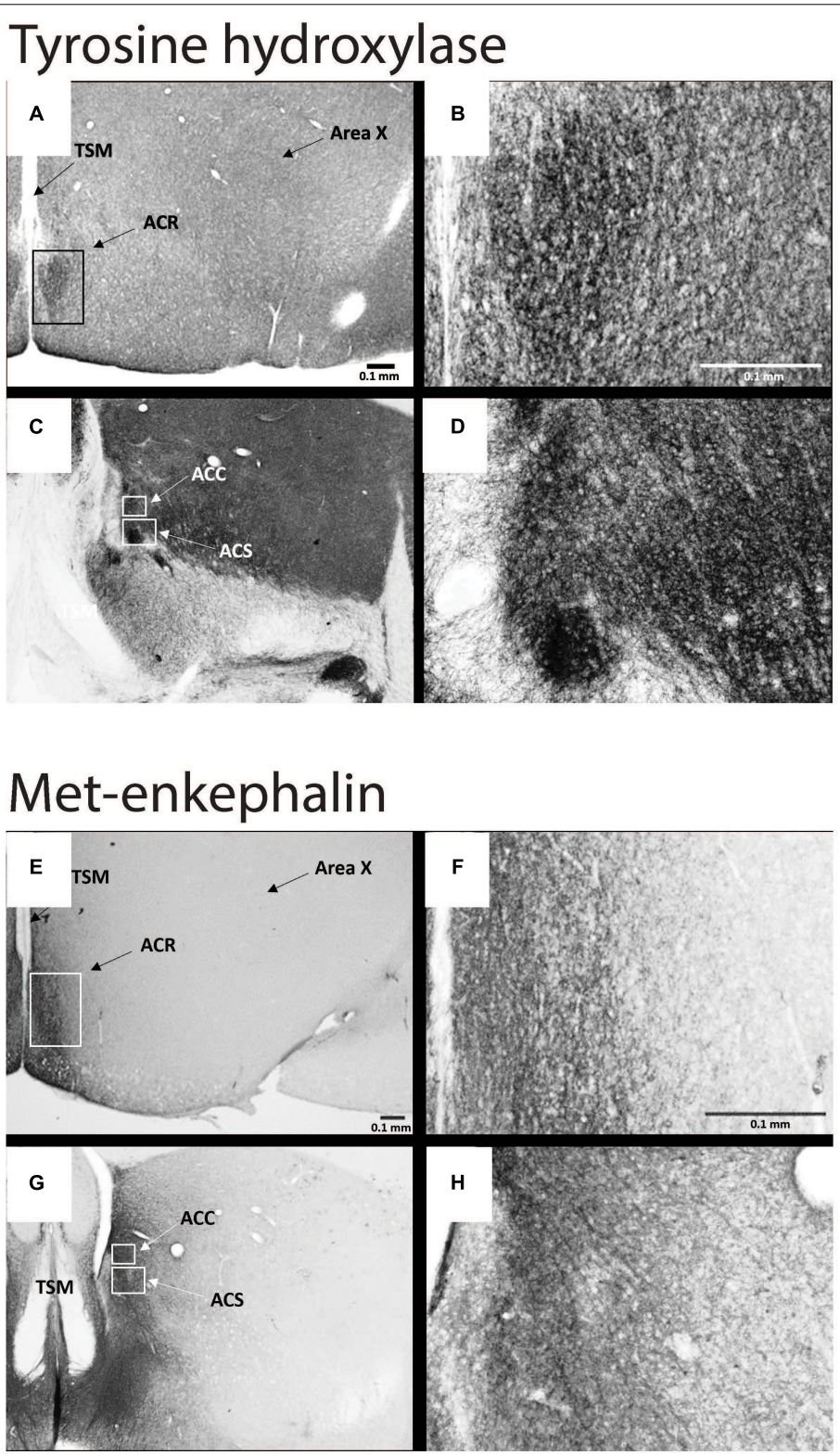


**FIGURE 4 |** Subdivisions of NAc contain dopamine- and opioid-related immunolabeling in male starlings. Photomicrographs of immunolabeling for tyrosine hydroxylase at low **(A,C)** and high **(B,D)** magnification and of met-enkephalin at low **(E,G)** and high **(F,H)** magnification. See **Figure 3** for abbreviations. Figures modified with permission from Polzin et al. (2021).

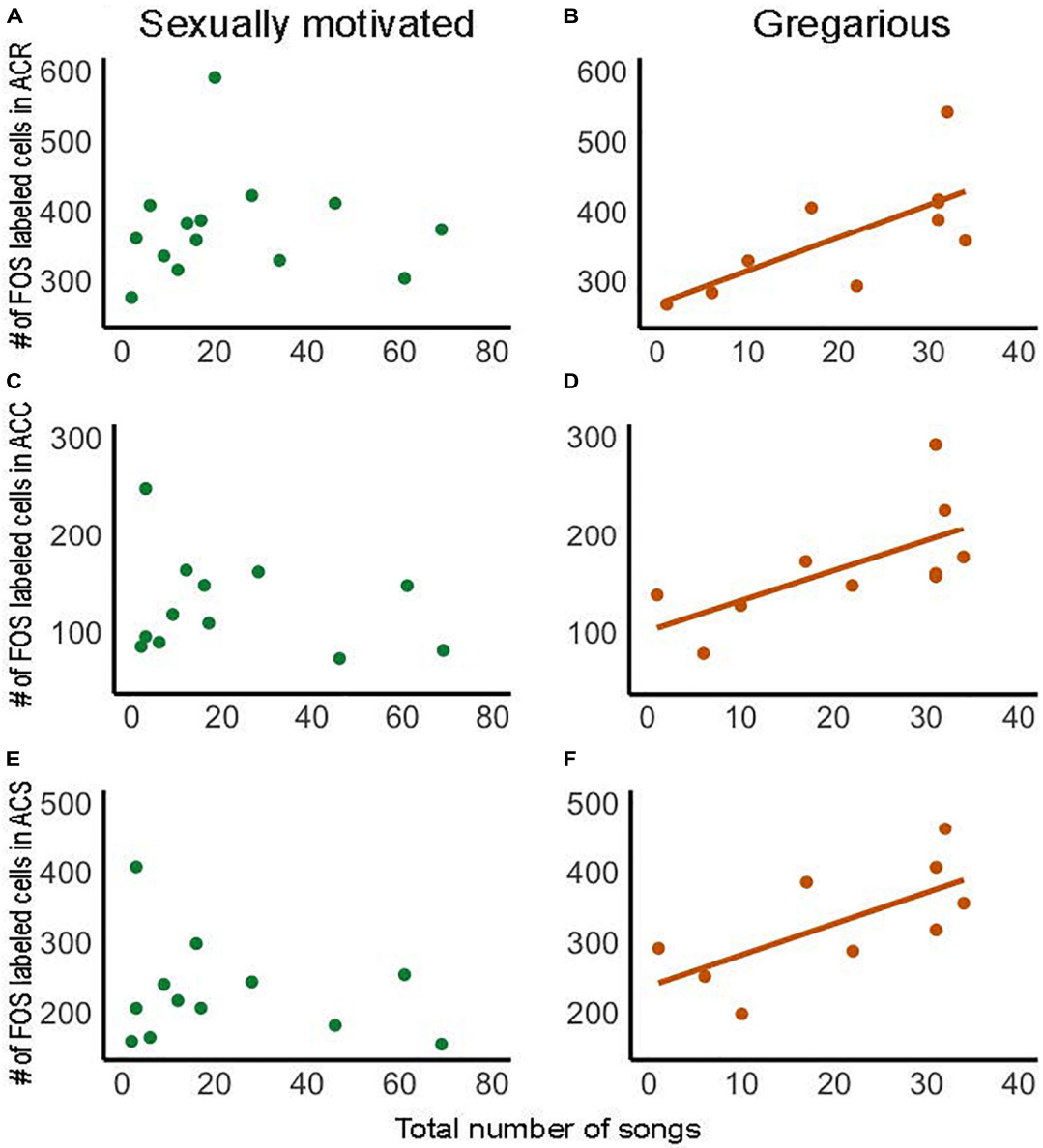


**FIGURE 5 |** Numbers of FOS labeled cells within each subdivision of NAc correlate positively with the production of gregarious but not sexually motivated song in male starlings. Correlations in panel **(A,B)** show the rostral pole of the nucleus accumbens (ACR), **(C,D)** the accumbens core (ACC), and **(E,F)** the accumbens shell (ACS). Each point represents an individual bird. Presence of regression line indicates statistical significance [**(B)**: $R^2 = 0.49$, $p = 0.022$; **(C)**: $R^2 = 0.42$, $p = 0.041$, **(D)**: $R^2 = 0.46$, $p = 0.046$]. Figure redrawn with permission from Polzin et al. (2021).

in gregarious song that differs from sexually motivated song. As reviewed above, unlike sexually motivated singing behavior, which is potentially rewarded later by mate attraction and copulation, gregarious singing behavior is positively coupled in a strongly linear fashion to an intrinsic positive emotional state (**Figures 1C,D**). It is thus possible that the linear positive relationships between gregarious, but not sexually motivated song, and immediate early gene activity in NAc reflect a difference in mechanisms that underlie song that reflects an ongoing intrinsic positive emotional state compared to a state of anticipatory reward seeking.

Song is clearly indicative of a highly motivated state within a mating context, yet birds must be motivated to initiate song in any context. Thus, given the role of dopamine in NAc in motivation, it would be expected that dopamine may play a role in initiating song in non-mating contexts as well. Consistent with this idea, there is evidence that dopamine increases in the striatal region area X in male zebra finches during undirected song, albeit to a lesser extent than during sexually motivated song (Sasaki et al., 2006). Moreover, a study in male zebra finches demonstrated that the latency to initiate undirected singing behavior (with latency considered a variable indicative

of motivation) is inhibited by dopamine receptor antagonists (Kim et al., 2021).

Dopamine in NAc binds to receptors in the D1 and D2 families (Missale et al., 1998). These dopamine receptor subtypes activate opposing intracellular systems (Beaulieu and Gainetdinov, 2011; Soares-Cunha et al., 2016), and have different, sometimes opposing, impacts on behavior, including social behaviors [e.g., pair bond formation and maintenance in prairie voles, *Microtus ochrogaster* (Aragona et al., 2006; Aragona and Wang, 2007; Resendez et al., 2016; Lei et al., 2017)]. This suggests that ratios of D1 to D2 receptors in NAc may modulate motivational states. Consistent with this possibility, in long-term pair bonded zebra finches, ratios of D1 over D2 dopamine receptor expression in NAc related negatively to undirected song production (Alger et al., 2021) (**Figures 6A,C**). These findings potentially implicate dopamine in the intrinsic motivation to sing in any context and raise the possibility that D2 receptors in NAc play a predominant role intrinsically rewarding song production. D1 and D2 specific manipulations in NAc are now needed to test this possibility.

After dopamine in NAc initiates singing behavior, it may be that the ongoing positive emotional state that accompanies song in non-sexual contexts is maintained by the stimulation of opioid receptors in NAc. Opioids in NAc impact emotional states by binding to both mu and kappa receptors (Le Merrer et al., 2009). These receptors engage distinct second messenger systems (Belcheva et al., 2005) and have distinct effects on affective state,

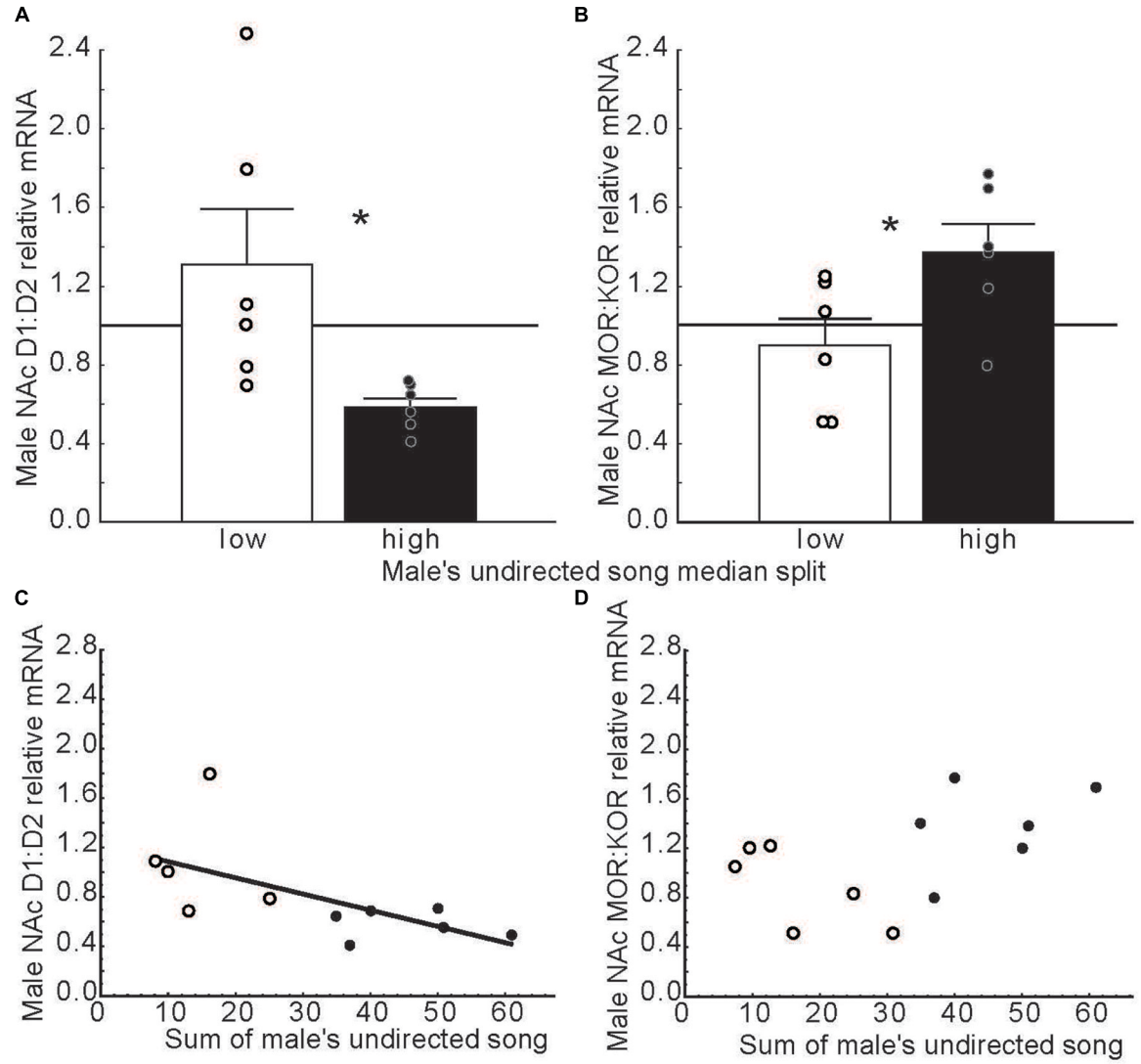


**FIGURE 6 |** Dopamine and opioid receptor mRNA ratios in NAc differ in males that produce low versus high rates of undirected song. **(A,B)** Mean relative mRNA ratios + sem for D1:D2 and MOR:KOR in males that produce low (open bars) or high (filled bars) rates of song as determined using a median split. The point at which ratios diverge from 1:1 is indicated by a horizontal line. * = $p < 0.05$. **(C,D)** Scatterplots illustrating relationships between dopamine and opioid receptor mRNA ratios in NAc and undirected song in males. Each point represents an individual male. Open dots indicate low and filled dots represent high singers in bar graphs. The regression line indicates a significant correlation ($p < 0.05$). Data from Alger et al. (2021). Reproduced with permission.

with mu opioid receptors generally inducing reward (Wise, 1989; Le Merrer et al., 2009) and kappa opioid receptors generally inducing negative affective states in mammals (Mucha and Herz, 1985; Shippenberg and Herz, 1986; Bals-Kubik et al., 1989), but see Castro and Berridge (2014). This suggests that ratios of mu over kappa receptors in NAc may also influence both an individual's emotional state and singing behavior. Studies on male zebra finches support this possibility, with ratios of mu over kappa receptor expression related positively to male undirected song production (Alger et al., 2021) (**Figures 6B,D**). These findings suggest that a predominance of mu signaling in NAc may contribute to a positive emotional state associated with song in this non-sexual context. When combined with studies in mammals, these songbird studies suggest that dopamine, opioids, and receptor subtype ratios in NAc may fine tune emotional states to promote singing behavior in non-sexual social contexts.

The function of dopamine and opioid receptors and plasticity of the receptor subtype ratios in the avian NAc must be examined in future experiments, but progress has been made in the study of mu opioid receptors. In male and female starlings, the selective mu opioid receptor agonist fentanyl facilitates gregarious singing behavior (Stevenson et al., 2020), and in male zebra finches, peripheral injections of the non-selective opioid receptor antagonist naloxone suppress undirected song (Khurshid et al., 2010). A recent study suggests the NAc may be an important site of action. Infusion of the selective mu opioid receptor agonist DAMGO into the rostral NAc in male and female starlings stimulated gregarious singing behavior (Maksimoski et al., 2021) (**Figure 7A**). In addition, DAMGO dose-dependently increased both approach and displacement behaviors considered important for social spacing (**Figures 7B,C**). These results were interpreted to suggest a potential role for mu opioid receptors in the NAc in optimizing the pull of joining a flock with the push of potential agonistic encounters. Because the activation of mu receptors in NAc contributes to the reward value of non-sexual social behaviors in mammals [e.g., social play in young rats (Trezza et al., 2011)], it may be that DAMGO facilitates song and other behaviors important for flock cohesion by facilitating a positive emotional state.

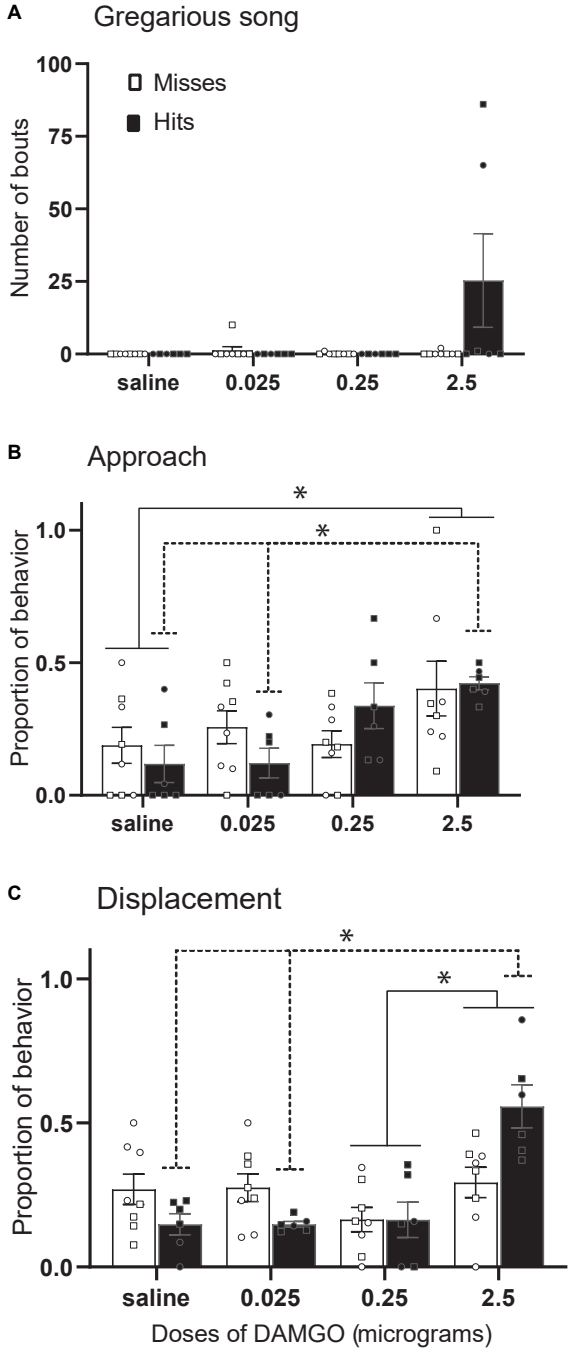


**FIGURE 7 |** Stimulation of MOR in NAc increases gregarious song, approach, and displacements. Mean + sem **(A)** number of song bouts, **(B)** proportion of approach behaviors, and **(C)** proportion of displacements in male (squares) and female (circles) starlings in which the cannula tip missed the NAc (open bars; $n = 8$) or hit the NAc (filled bars; $n = 6$). Lines indicate significant effects (*$p < 0.05$). Misses were in numerous locations outside the NAc. Figure modified from Maksimoski et al. (2021).

## NUCLEUS ACCUMBENS AND BIRDSONG AS A STIMULUS THAT INDUCES A POSITIVE EMOTIONAL STATE IN OTHERS

As introduced above, the fact that hearing song alone can function as a reinforcer in operant tasks indicates that hearing sexually motivated male songs can induce a positive emotional state. The fact that females are willing to work to hear these songs (reviewed above) also indicates that they can induce an emotional state of motivation or anticipation. This leads to the prediction that both dopamine (motivation/anticipation) and opioids (positive emotion) may be released into NAc in response to hearing male courtship song. A growing number of studies implicate NAc and dopamine in NAc in female motivated responses to these male songs. For example, in estradiol treated female white-throated sparrows, *Zonotrichia albicollis,* playbacks of male songs that induce female copulation solicitation also

increase numbers of cells labeled for the immediate early gene Egr-1 in female NAc (Earp and Maney, 2012). In female zebra finches, dopamine transmission increases in rostral NAc selectively in response to the songs of a male mate, but not in response to non-mate songs (Svec et al., 2009; Tokarev et al., 2017). These findings are consistent with studies in female rats that demonstrate dopamine release in response to presentation of sexually active males (Pfaus et al., 1995), consistent with a conserved role for dopamine in NAc in the regulation of female motivated or anticipatory emotional responses to male songs designed to entice them to mate.

With respect to opioids, at least one study relates the opioid met-enkephalin in NAc to female responses to male courtship song. Specifically, in female starlings that are actively nesting (i.e., the females described above who found song to be rewarding in CPP tests), measures of immunolabeling for met-enkephalin in NAc shell and core combined tended to be higher than in females that were not nesting and did not find hearing song to be rewarding (Riters et al., 2013). These findings suggest a potential role for NAc in emotional states induced by male song. Research is now needed to test experimentally the working hypothesis that dopamine in NAc underlies an emotional state of anticipatory motivation while opioids underlie an emotional state of reward associated with responses to sexually motivated songs.

The role NAc in emotional states induced by hearing song outside the context of courtship has not been well studied. One study on this topic indicated that in long-term pair bonded zebra finches, females that were paired with males that produced

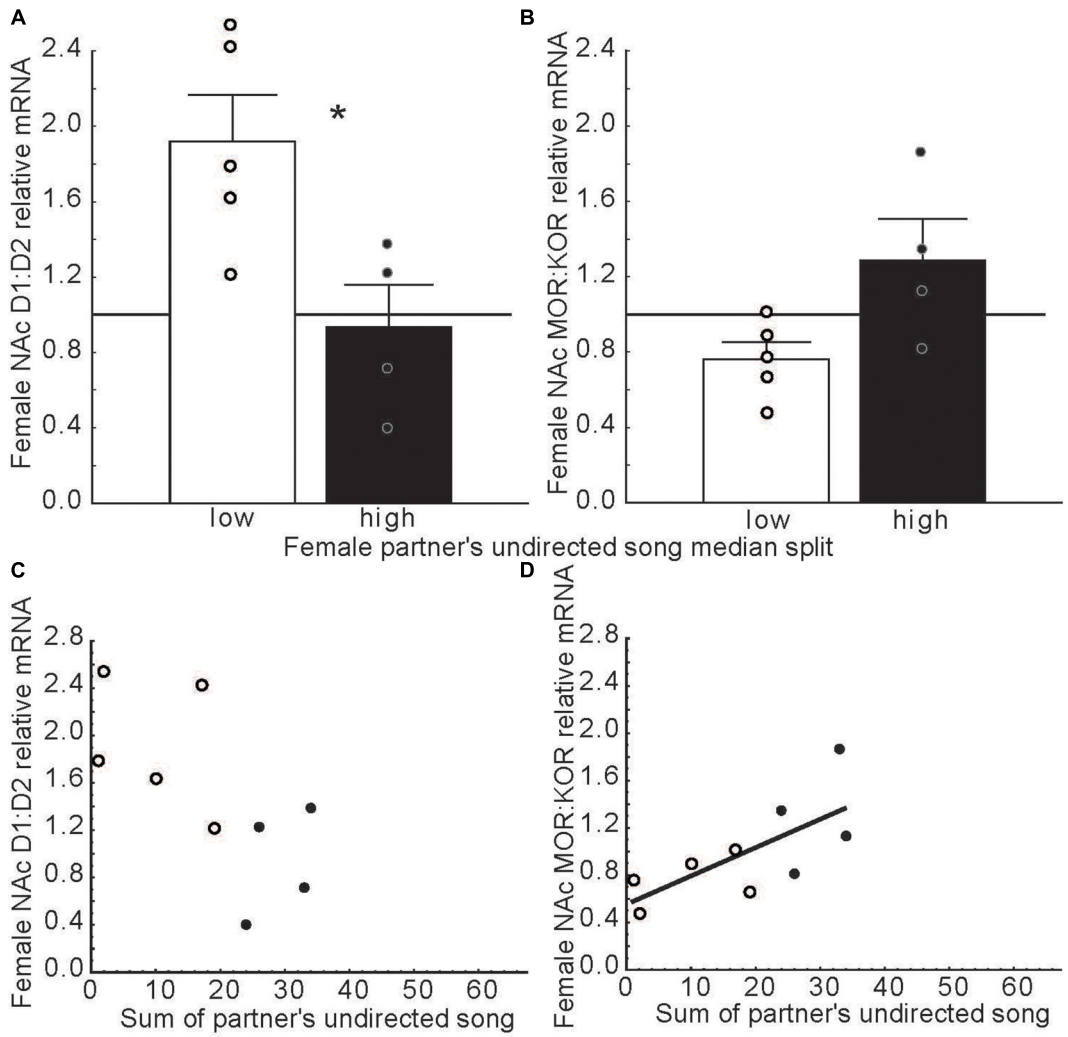


**FIGURE 8 |** Dopamine and opioid receptor mRNA ratios in NAc differ in females whose partners produced low versus high rates of undirected song. **(A,B)** Mean relative mRNA ratios + sem for D1:D2 and MOR:KOR in females paired with males that produced low (open bars) or high (filled bars) rates of song as determined using a median split. The point at which ratios diverge from 1:1 is indicated by a horizontal line. **(C,D)** Scatterplots illustrating relationships between dopamine and opioid receptor mRNA ratios in the female NAc and the number of undirected songs produced by her partner. Each point represents an individual female. Open dots indicate females with male partners that sang at low and filled dots represent partners that sang at high rates in bar graphs. The regression line indicates a significant correlation ($p < 0.05$). Data from Alger et al. (2021). Reproduced with permission.

high compared low rates of undirected song had lower ratios of D1 to D2 dopamine receptor expression and higher ratios of mu to kappa opioid receptor expression in rostral NAc (Alger et al., 2021) (**Figure 8**). The function of undirected song in this context is not clear, but it may play roles in maintaining the pair bond and in female reproductive investment (Silcox and Evans, 1982; Bolund et al., 2012). These findings raise the possibility that hearing undirected song may influence dopamine and opioid receptor expression in NAc to potentially impact female emotional responses to male song, which may reinforce pair bonds in monogamous species or promote flock cohesion in gregarious species. Additional research is needed on this topic.

## NUCLEUS ACCUMBENS AS PART OF A CIRCUIT UNDERLYING POSITIVE EMOTIONS AND BIRDSONG

The research reviewed here brings together for the first-time numerous studies that highlight a potentially important role for the songbird NAc in emotional states that underlie singing behavior. In this section we integrate this new information related to the NAc with prior research on other brain regions to develop a more comprehensive circuit that may infuse emotional state into vocal signals and responses to vocal signals. Much of the research on birdsong is focused on the "song control system", a group of interconnected brain regions that is devoted to song learning, production, perception, and structural aspects of song (Nottebohm et al., 1976). These nuclei were commonly studied without reference to other neural systems, but in the late 1990's studies began to focus on how a bird's motivational state might be communicated to this system so that a bird would sing a structurally appropriate song in an appropriate context.

Because male courtship song is highly sexually motivated it was first proposed that brain regions involved in sexual motivation might directly or indirectly access the song control system to trigger the highly structurally stereotyped songs produced in a sexually motivated context. This led to a focus on the medial preoptic nucleus (POM), a region centrally involved in male sexual behavior across vertebrates (Dominguez and Hull, 2005; Balthazart and Ball, 2007). This region is well positioned neuroanatomically to influence the song control system (Riters and Alger, 2004), and several studies have now demonstrated an essential stimulatory role for the POM in the production of sexually motivated song in starlings and canaries (Riters and Ball, 1999; Alger and Riters, 2006; Alger et al., 2009, 2016; Alward et al., 2013, 2016).

The discovery of a role for POM in sexually motivated song was not surprising given its established role in male sexual motivation. However, it was somewhat surprising to find that the POM was also involved in song outside a sexually motivated context. For example, numerous studies implicate the POM in the production of gregarious song in male, as well as female, starlings (Alger and Riters, 2006; Riters, 2012; Kelm-Nelson and Riters, 2013; Riters et al., 2014; Stevenson et al., 2020), and CPP tests demonstrate the POM to play an important role in the positive emotional state that accompanies gregarious singing

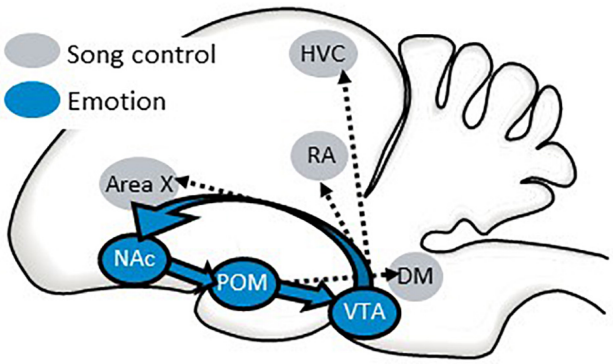


**FIGURE 9 |** A possible pathway by which the NAc may coordinate an individual's emotional state with singing behavior. Sagittal illustration of a songbird brain. A circuit from the preoptic area (POM) to the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and back to the POM is proposed to regulate affective states that can influence singing behavior and perception through its projections to song control nuclei. See text for additional details.

behavior (Riters et al., 2014; Stevenson et al., 2020). These studies demonstrate that the POM is a central node that adjusts singing behavior to reflect an individual's motivational and emotional state (Alger and Riters, 2006; Riters and Stevenson, 2022).

One way in which the POM may infuse singing behavior with reward seeking and intrinsic positive emotion is by accessing the classic "mesolimbic reward system" through projections to the VTA which then sends projections to the NAc (Riters et al., 2019; Riters and Stevenson, 2022). There is evidence that the NAc also sends projections to the POM (Groenewegen and Russchen, 1984) forming a circuit that includes three areas strongly implicated in emotion and birdsong (**Figure 9**). This circuit directly accesses the song control system through projections from the VTA to vocal learning, perception, and production regions [HVC, robust nucleus of the arcopallium (RA), and area X] (Lewis et al., 1981; Appeltants et al., 2000, 2002) and from the POM to a song output region (DM) (Riters and Alger, 2004) (**Figure 9**). This creates a system by which an individual's motivational state can be incorporated into song production and structure. Although this circuitry has not been as well studied in females as it has in males, a few studies implicate the POM in female emotional responses to hearing male song, reflected in CPP studies (Riters et al., 2013; Hahn et al., 2019). This raises the possibility that auditory inputs to this circuitry may also play a role in female emotional responses to male song.

## ADDITIONAL DISCUSSION AND REMARKS

The studies reviewed here demonstrate songbirds to be among the most emotionally expressive species in the animal kingdom, obtrusively advertising states of positive emotion and pleasure seeking. Because song is learned and complex, like human language and unlike vocalizations produced by rodents, songbirds can add layers to emotional communication

that are not present in commonly studied rodent models. For example, in addition to emotional state, structural features of song communicate attributes of male health and past history to females (Nowicki et al., 2002; Peters et al., 2014); and recent studies demonstrate that songbirds can detect and generate prosodic vocalizations (Mol et al., 2017). For example, male zebra finches modify songs when singing to young birds during the period of song learning in a fashion similar to the way that humans alter vocalizations when speaking to young children (Chen et al., 2016). Moreover, gregarious singing in songbirds provides unique opportunities to understand learned vocal communication in relaxed, positive, non-sexual contexts, which are not observed in other commonly studied rodent or primate experimental systems.

We ended this review by highlighting a pathway from the POM to VTA to NAc that appears to contribute context-dependently to positive emotional states that motivate and reward singing behavior (as detailed in prior reviews, Riters, 2011, 2012; Riters et al., 2019; Riters and Stevenson, 2022). This pathway is also implicated in sexually motivated and playful behaviors in mammals (Trezza et al., 2011; Zhao et al., 2020), which underscores the idea that the bird brain has the machinery to regulate positive emotions and the similarity to mammals suggests that findings in birds may generalize to other vertebrates, including potentially humans. Rewarding, positive social interactions in humans are critical for mental health, yet there are few effective treatments for social behavior deficits in humans with mental health disorders. The studies reviewed here are beginning to fill a need for basic, mechanistic information on core emotional circuits that promote positive social interactions and may lay the groundwork for the development of novel treatments to restore positive social interactions in humans.

## AUTHOR CONTRIBUTIONS

LR wrote the first draft of the manuscript. SA contributed to literature review and writing. BP, AM, and SA wrote sections of the manuscript and prepared the figures. All authors contributed to manuscript revision, read, and approved the submitted version.

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