SCHIZOPHRENIA: HUMAN AND ANIMAL STUDIES

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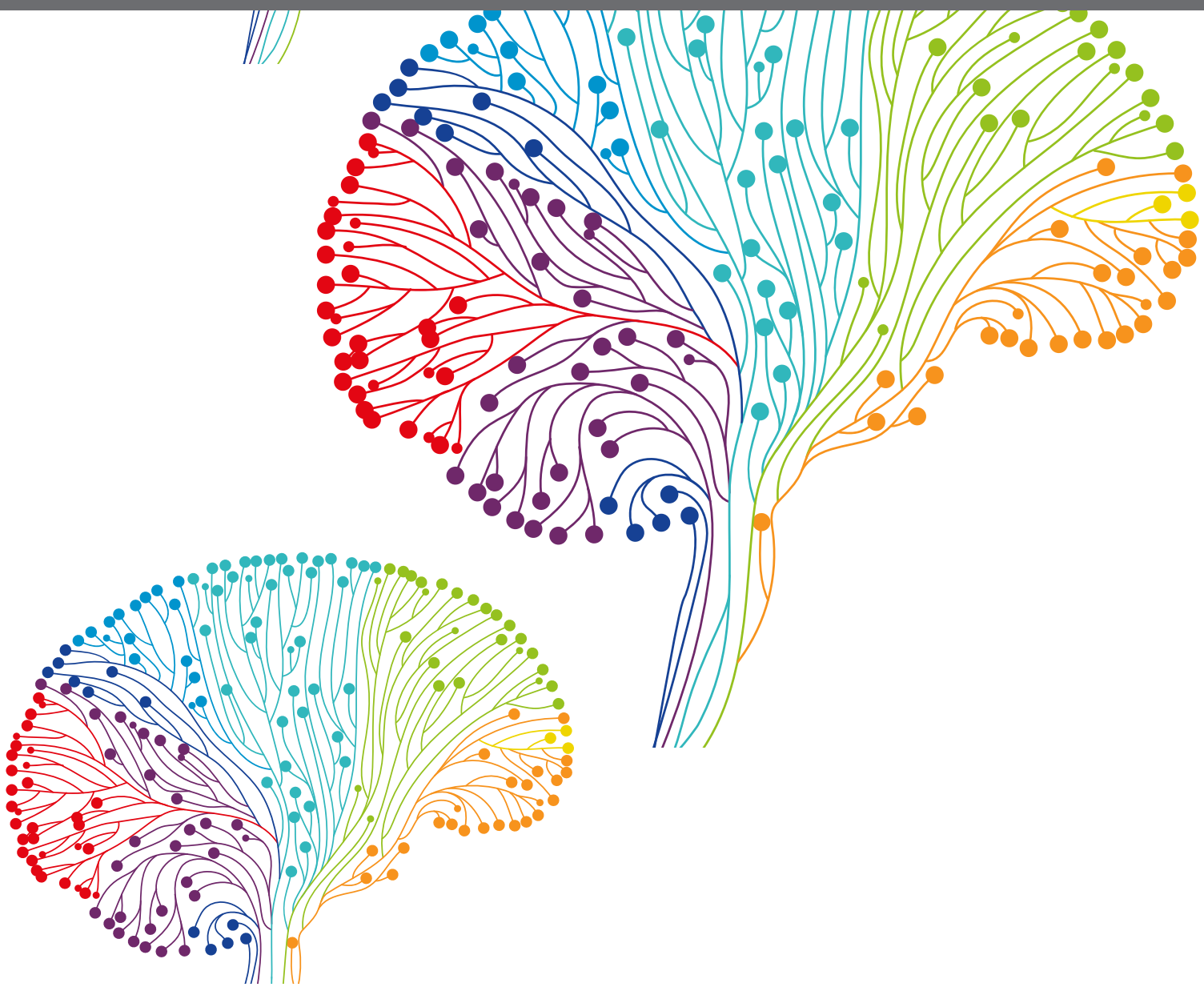

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SCHIZOPHRENIA: HUMAN AND ANIMAL STUDIES

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EDITORIAL
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Editorial: Schizophrenia: Human and Animal Studies

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The Editorial on the Research Topic

Schizophrenia: Human and Animal Studies

Schizophrenia is a complex disorder with relatively stable prevalence rates estimated at about 0.5–1% (Saha et al., 2005). Schizophrenia is characterized by the occurrence of various psychopathological symptoms that have been described as positive symptoms (delusions and hallucinations), negative symptoms (apathy, lack of motivation, flat affect, poverty of speech, and social withdrawal), disorganized symptoms (disorganized speech and behavior), and cognitive impairment (Moustafa et al., 2016). Traditionally, cognitive impairment was thought to be evident only in elderly deteriorated patients with schizophrenia; however, over the past 25 years, a body of evidence challenged this view and showed that cognitive dysfunction is a core feature of schizophrenia (O'Carroll, 2000; Weickert et al., 2000; Bora et al., 2010). Cognitive deficits can be moderate to severe across several domains, including attention, working memory, verbal learning and memory, and executive functions (Weickert et al., 2000; Bowie and Harvey, 2006). These deficits pre-date the onset of psychosis and further decline in cognitive functioning is observed in the course of illness in most patients (Trotta et al., 2015). Numerous rehabilitation programs have been developed in order to alleviate the burden associated with cognitive impairment in schizophrenia since cognitive deficits respond poorly to antipsychotic treatment (Tao et al., 2015).

Below we describe articles in this research topic (mostly focusing on cognitive studies in schizophrenia), dividing them into human vs. animal studies.

HUMAN STUDIES OF SCHIZOPHRENIA

Rodriguez et al. investigated the effect of neuropsychological battery on global functioning and quality of life in 36 first psychotic episode of schizophrenia spectrum disorder (FES) patients. The results indicated that patients with FES were significantly impaired in both visuospatial and verbal functioning compared to healthy controls. The authors concluded that the deficit in verbal functioning was a better predictor of global functioning and quality of life than visuospatial functioning.

Chen et al. investigated the nature of prospective memory using event-related brain potentials in 20 symptomatically remitted patients with schizophrenia and 20 healthy controls. The results indicated that patients with schizophrenia performed worse on the prospective memory

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task compared to the healthy controls. In particular, the prospective positivity amplitude was significantly attenuated in symptomatically remitted schizophrenia patients.

Schott et al. investigated whether dysfunctional processing of novel information by the medial temporal lobe may be a possible neural mechanism that contributes to the pathophysiology of delusions. The authors used fMRI to examine 16 unmedicated patients with paranoid schizophrenia and 20 age-matched healthy controls. Participants completed a visual target detection task that consisted of novel and familiar stimuli, and a standard and a target picture. All of the participants when shown the novel images had activation in the hippocampus, but only healthy controls showed a positive relationship between novelty-related hippocampal activation and recognition memory performance 24 h later, indicating a role of the hippocampus in positive symptoms.

Although previous research (Potvin et al., 2015) suggests that socio-economic status is correlated with cognitive impairments in patients with dementia and schizophrenia, education and income variation may not be the only reasons for such a correlation. Other possible factors include differing life choices, occupations, and environments. The results of Potvin et al. (2015) may lack some experimental control in terms of the dosage and comparison of prescribed antipsychotics. Salem and Moustafa discussed the implications of these findings.

Guell and Bernacer explored the following question: where in the body shall we look for markers of mental disorders? They propose that the constitution of the body's sense organs may be an indicator of mental characteristics. If so, these organs can be affected by mental disorders such as schizophrenia. They suggest the approach of looking for differences in the constitution of the sense organs in order to diagnose mental disorders. Schwitzer et al. provided an insightful commentary on Guell and Bernacer. In their article, they discussed the role played by visual attention in the occurrence of schizophrenia symptoms, focusing on retinal and early visual processing deficits.

Wang et al. investigated the neural correlates of the thalamus and examined whether thalamic resting state networks are different in patients with schizophrenia. The authors used a standard seed-based whole-brain correlation (72 patients with schizophrenia, 73 healthy controls). Patients with schizophrenia had enhanced thalamic connectivity with bilateral precentral gyrus, dorsal medial frontal gyrus, middle occipital gyrus, and lingual gyrus. These patients also had reduced connectivity in bilateral superior frontal gyrus, anterior cingulate cortex, inferior parietal lobe, and cerebellum. The findings indicated that the thalamus is over-connected during resting state in schizophrenia.

Schwab et al. investigated saccadic latency modulation in patients with schizophrenia and their first-degree relatives. Participants (13 patients with ICD-10 schizophrenia, 10 first degree relatives, 24 controls) performed two visual tasks: a color task and a Landolt ring orientation task. Saccadic latency was measured with a video-based oculography. Results indicated that saccadic latencies were not modulated by visual processing of different content for patients with schizophrenia, but saccadic latencies were modulated for relatives and health controls.

Hori et al. investigated the relationship between serum BDNF levels and decision making. They compared patients with schizophrenia (86) to healthy controls (51) in terms of their decision making ability and serum BDNF levels. Scores on the Iowa Gambling Task were lower for patients with schizophrenia compared to the controls. There was a negative correlation between the mean net scores on the final two blocks and serum BDNF levels. The authors concluded that the poor performance of patients with schizophrenia on the Iowa Gambling Task may be related to impaired sensitivity to reward and punishment due to depressive symptoms and reduced serum BDNF levels.

Frydecka et al. compared the level of working memory impairments across patients with schizophrenia ($n = 23$), psychotic bipolar disorder ($n = 19$), non-psychotic bipolar disorder ($n = 24$), and healthy controls ($n = 18$). The results indicated that on more demanding working memory measures the group with schizophrenia and psychotic bipolar disorder performed poorer than the non-psychotic bipolar disorder group and healthy controls. There was a positive correlation of working memory performance with antipsychotic treatment and a negative correlation with depressive symptoms in bipolar disorder and with negative symptoms in the schizophrenia subgroup.

Zhuo et al. investigated changes in resting-state functional connectivity density (rsFCD) in schizophrenia ($n = 95$) and healthy controls (93). The results indicated that patients with schizophrenia had increased rsFCD in the bilateral striatum and hippocampus and decreased rsFCD in the bilateral sensorimotor cortices and right occipital cortex.

Wotruba et al. investigated functional brain correlates in the anticipation and reception of rewards and examined their possible association with symptoms in unmedicated people at risk for psychosis ($n = 21$) and healthy controls ($n = 24$). The results indicated that the same brain areas were recruited during processing of reward information in both at risk individuals and healthy controls. In contrast, during the anticipating rewards stage the at risk individuals showed additional activation in the posterior cingulate cortex and the medio- and superior-frontal gyrus.

Garbarini et al. investigated whether patients with schizophrenia have an altered sense of agency during the observation of others' movements. A circles-lines task was used, in which participants had to draw lines while observing an examiner's hand draw circles. Results indicated an interference effect of the examiner's hand drawing circles on their hand drawing lines.

Moustafa et al. provided an overview of homocysteine metabolism in psychiatric disorders with a focus on cognitive correlates. Several biological mechanisms linking homocysteine to biological underpinnings of psychiatric disorders were discussed and future directions and perspectives were indicated.

ANIMAL STUDIES OF SCHIZOPHRENIA

Below, we summarize animal studies we covered in our research topic.

Falsafi et al. examined which GABAB-containing receptor complexes are involved in a multiple T maze task. Twenty trained and untrained mice were exposed to the multiple T maze task. The results indicated that training increased GABAB1/1A containing receptor complexes in the hippocampus.

Oliveras et al. investigated whether the Roman high-avoidance inbred strain (RHA-I) display prepulse inhibition (PPI) compared to Roman low-avoidance rats (RLA-I) and genetically heterogenous (NIH-HS) rats. More specifically, they examined whether PPI deficits predict spatial working memory impairments. The results indicated that the RHA-I rats had PPI and spatial working memory deficits compared to the other two groups.

Nakao et al. examined the role of beta-anchoring and -regulatory protein (BARP) in higher brain functions. BARP knockout (KO) mice were generated and a comprehensive behavioral test battery was undertaken. The results indicated that these rats showed greatly reduced locomotor activity.

Gruter et al. investigated the cellular mechanisms that may underlie hippocampal dysfunction in psychosis. Male Wistar rats were treated with irreversible NMDAR-antagonist, MK801. After 4 weeks, the treatment led to the loss of hippocampal long-term potentation (LTP). Expression of the early gene (Arc) was enhanced in the hippocampus of controls by spatial learning. The authors concluded that in psychosis, deficits in hippocampus-dependent memory may be due to the loss of hippocampal LTP.

Niu et al. investigated whether the prepulse inhibition (PPI) response can be disturbed by the blockage of olfactory input. The results indicated that the PPI of the startle response was impaired by the blockage of olfactory sensory input. Microinjection of lidocaine/MK801 into the olfactory bulb impaired the PPI. The authors concluded that the olfactory system plays a role in the PPI of the startle response in rats.

Pinnock et al. investigated whether activate muscarinic and potentially nicotine receptors (nAChRs) expressed by pontine receptors are responsible for the nicotine enhancement of prepulse inhibition (PPI). The results indicated that nAChR antagonists had limited effects on PPI. However, PnC microinfusions of the non-α7nAChR preferring antagonist TMPH into the PnC significantly reduced PPI.

DeAngeli et al. investigated whether a change in the concentration of kynurenic acid (KYNA; which is increased in patients with schizophrenia) would affect reward related behavior. The rats that were treated with L-kynurenine showed increased sign-tracking behavior. The authors concluded that exposure to KYNA may increase the salience of cues related to reward. They also examined the effect of exposure to KYNA on hippocampal long-term potentiation (LTP). After a burst of high-frequency stimulation the rats treated with L-kynurenine showed no LTP, whereas vehicle-treated rats showed robust LTP.

Richter et al. translated a human stability-flexibility paradigm to mice. Mice were trained in a touchscreen-paradigm to discriminate visual cues. Results provided evidence for the *Dual State Theory* that assumes an antagonistic relation between cognitive flexibility and stability.

Ding et al. investigated whether *N*-methyl-D-aspartate receptor (NMDR) and Ca^{2+}permeable α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPAR) activation in the striatum is necessary for cognitive flexibility. The findings suggested that NMDR-mediated glutamate transmission in the nucleus accumbens contributes more to cognitive execution than the dorsal striatum.

In short, in this topic, we attempted to cover different aspects of schizophrenia symptoms, using both human and animal studies. The articles covered here explained the complexity of behavioral and neural abnormalities in schizophrenia, and further suggest future work in this area.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH ARTICLE
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Touchscreen-paradigm for mice reveals cross-species evidence for an antagonistic relationship of cognitive flexibility and stability

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The abilities to either flexibly adjust behavior according to changing demands (cognitive flexibility) or to maintain it in the face of potential distractors (cognitive stability) are critical for adaptive behavior in many situations. Recently, a novel human paradigm has found individual differences of cognitive flexibility and stability to be related to common prefrontal networks. The aims of the present study were, first, to translate this paradigm from humans to mice and, second, to test conceptual predictions of a computational model of prefrontal working memory mechanisms, the *Dual State Theory,* which assumes an antagonistic relation between cognitive flexibility and stability. Mice were trained in a touchscreen-paradigm to discriminate visual cues. The task involved "ongoing" and cued "switch" trials. In addition distractor cues were interspersed to test the ability to resist distraction, and an ambiguous condition assessed the spontaneous switching between two possible responses without explicit cues. While response times did not differ substantially between conditions, error rates (ER) increased from the "ongoing" baseline condition to the most complex condition, where subjects were required to switch between two responses in the presence of a distracting cue. Importantly, subjects switching more often spontaneously were found to be more distractible by task irrelevant cues, but also more flexible in situations, where switching was required. These results support a dichotomy of cognitive flexibility and stability as predicted by the *Dual State Theory*. Furthermore, they replicate critical aspects of the human paradigm, which indicates the translational potential of the testing procedure and supports the use of touchscreen procedures in preclinical animal research.

Keywords: cognitive flexibility, cognitive stability, touchscreen chambers, translation, mice, Dual State Theory, neurocomputational models, executive functioning

INTRODUCTION

The abilities to either flexibly adjust behavior according to changing environmental demands (cognitive flexibility) or to maintain it in the face of potential distractors (cognitive stability) form an important component of executive functioning (Diamond, 2013). Along with other higher-level cognitive skills, these abilities are crucial for our daily life when multiple behavioral options exist and demands are shifting (Banich, 2009). Deficits in these cognitive domains, however, are observed among patients suffering from psychiatric diseases (Dirnberger and Jahanshahi, 2013; Etkin et al., 2013; Snyder, 2013). Especially in schizophrenic patients, impairments in executive processing in general and in task switching or cognitive flexibility in particular have been reported (Wylie et al., 2010; Orellana and Slachevsky, 2013; Schirmbeck et al., 2013). Interestingly, impairments in the domains of higher-order cognitive functions are also found in non-affected family members of schizophrenic patients, indicating the presence of a genetic predisposition (Heydebrand, 2006; Snitz et al., 2006). Therefore, deficits in executive functioning may be considered core features of the disease that may even provide the basis on which other symptoms may occur (Barch, 2005; Beck and Rector, 2005).

From a neural perspective, these higher-level cognitive processes are supposed to involve various brain structures, most prominently the prefrontal cortex (PFC) and the mesocortical dopamine (DA) system (Goldman-Rakic et al., 2000; Winterer and Weinberger, 2004; Klanker et al., 2013). In line with these findings, both cognitive flexibility and cognitive stability have been related to PFC functioning (Curtis and D'Esposito, 2003; Floresco et al., 2009; Stelzel et al., 2010, 2013; Toepper et al., 2010;

Kesner and Churchwell, 2011), but it is still not clear whether they depend upon separate or concordant neural networks. Therefore, a theoretical framework has been developed through a biophysically realistic computational model of the PFC-DA network that aims to identify mechanisms underlying cognitive flexibility and stability (Durstewitz and Seamans, 2008). According to this *Dual State Theory,* cognitive stability and cognitive flexibility are regulated by differential activity of the dopaminergic subsystems (D1-receptor-class vs. D2-receptor-class, respectively) in the PFC, possibly relying on differences in either receptor densities, baseline neurotransmitter levels, or the efficiency of neurotransmitter clearance (Durstewitz and Seamans, 2002, 2008; Bilder et al., 2004; Thurley et al., 2008). Specifically, simulation results led to the proposal of two distinct regimes, termed the D1-state and the D2-state, which either result in stable memory representations or flexible switching among representations, respectively. While in a D1-dominated state representations are characterized by a high energy barrier among different system states, resulting in representations that are robust to distraction (= cognitive stability), a D2-state results in low energy barriers between states, allowing for switching between representations (= cognitive flexibility). From a neurocomputational perspective, it can thus be derived that the degree of cognitive flexibility varies between persons due to, for example genetic differences in network properties or neurotransmitter levels, and that cognitive flexibility and stability are antagonistically related and controlled by a common neuronal network. Empirical data seem to support these predictions, in that working memory maintenance has been reliably associated with D1 mediated PFC activity (Goldman-Rakic et al., 2000) and some of our own work has shown that modulation of D2 signaling either genetically Stelzel et al.(2010) or pharmacologically Stelzel et al.(2013) affects the efficiency of cognitive flexibility.

To systematically investigate the predictions made by the *Dual State Theory*, a novel task paradigm has recently been established in a healthy human population, which requires in the same task either cognitive flexibility or cognitive stability (Armbruster et al., 2012; **Figure 1**). Participants had to respond by button press to digits between 1 and 9. In most of the trials, only one digit was presented above a fixation cross and subjects had to decide whether this digit was odd or even (= rule 1). For the remaining 20% of trials, two digits were presented on the screen (above and below the fixation cross). If the upper digit was brighter than the lower digit, the participants had to continue using rule 1 and ignore the second distracting digit. By contrast, if the lower digit was brighter than the upper digit, subjects had to switch the rules and decide whether the lower digit was smaller or larger than 5 (= rule 2). Finally, in an ambiguous condition, the grayscale values of the two digits were indistinguishable, allowing for assessing the individual rate of spontaneous switching in the absence of explicit external cues, as a measure of individual differences in cognitive flexibility (Armbruster et al., 2012). Participants were found to differ substantially in the individual spontaneous switching rate, and more flexible persons were more efficient in task switching but also more distractible during distractor inhibition. These results support the dimensional model of cognitive flexibility and stability described by the *Dual State Theory* (Durstewitz and Seamans, 2008).

However, not all predictions of computational models can be adequately addressed in human subjects. Translational approaches are necessary to understand cellular and molecular mechanisms and identify new treatment options (Van Der Worp and Sandercock, 2012; Homberg, 2013). A high failure rate of preclinically identified compounds in the clinical trials (Kola and Landis, 2004) underscores the importance of reliability and validity of animal models as a premise to draw meaningful translational conclusions from preclinical findings. Some biomedical research efforts therefore now aim at translating clinical findings back to measures in animal disease models (e.g., Garner et al., 2006).

Therefore, the aim of the present study was to introduce a mouse paradigm to assess cognitive flexibility vs. cognitive stability in close analogy to the human paradigm using cues and outcome measures of the same qualities (Armbruster et al., 2012). In contrast to already established set-shifting procedures in mice (Garner et al., 2006; Endo et al., 2011; Bissonette and Powell, 2012; Scheggia et al., 2014), the strength of the present approach is twofold: First, it has a high translational value as it is derived from a behavioral paradigm for humans, and second, it is conceptually based on a biophysically plausible neurocomputational theory that has the potential of linking animal and human behavior across species.

In analogy to the human paradigm, we assessed within one paradigm the individual's task performance in the presence of irrelevant distractor cues and the flexibility required when responding to switching cues, as well as the individual's disposition to spontaneously switch in response to ambiguous stimuli. According to the dimensional model of Durstewitz and Seamans (2008) and in line with the human data, we expected to find opposing trends in behavior among individuals: More flexible subjects with a greater tendency toward switching in an ambiguous situation were thus expected to switch faster and more accurately when explicitly cued, while at the same time being more prone to distraction. By contrast, more stable subjects characterized by less spontaneous switching behavior were expected to resist distraction more efficiently, while at the same time making more errors when cognitive flexibility (i.e., switching) is explicitly required.

In sum, the present study aims at translating a human stability-flexibility paradigm to mice. Via comparison of behavioral results from mice to those previously derived from humans, we both validate the animal paradigm and at the same time provide supportive evidence for the *Dual State Theory* from a different species, i.e., mice.

ANIMALS, MATERIALS, AND METHODS

ANIMALS

The subjects were 24 male C57BL/6N mice (Charles River, Sulzfeld, Germany), approximately 13 weeks old at the onset of the experimental procedure. Mice were single-housed in conventional macrolon cages (Type II, 26 × 20 × 14 cm) with sawdust (Rehofix MK-2000; Rettenmaier & Söhne, Rosenberg, Germany), nesting material, and tap water *ad libitum*. Upon arrival all animals were earmarked with individual patterns to allow precise identification of the individual mouse. Once per week, the cages

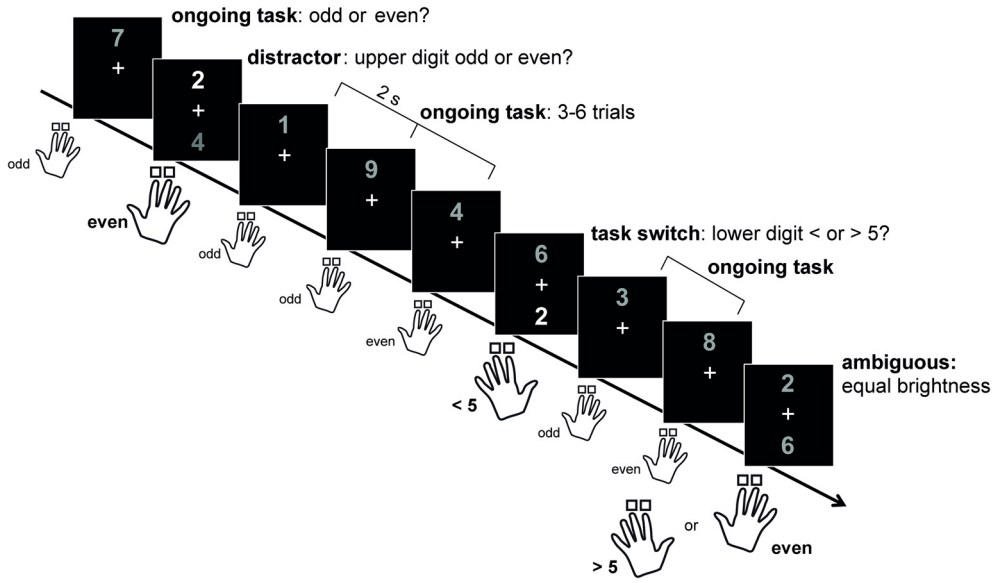

FIGURE 1 | The human STABFLEX test. With permission from: Armbruster et al. (2012). Most of the trials required a response by button press to only one (upper) digit deciding wether it is odd or even. In 20% of the trials a second digit appeared below the fixation cross. Subjects had to ignore this digit if its color was darker than the upper one. If the lower digit was brighter however, subjects had to switch to the bottom digit and respond according to a different rule, i.e., >/<5 with the respective other hand (left and right hand counterbalanced between subjects). In ambiguous trials, brightness differences between stimuli were not detectable, and participants were free to switch or stay. This condition served to assess the individual spontaneous switching rate.

were cleaned, water bottles replaced, and new tissue paper provided. The colony room was maintained at a temperature of 23 ± 2°C, a relative humidity of 50 ± 5% and a reversed 12 h light-dark schedule with the lights off at 7 am.

Prior to testing, *ad libitum* feeding weights were obtained, and mice were food-restricted to 85–90% of their initially measured individual bodyweight. To maintain the animals in a healthy state and to adjust the daily amount of food individually, the weight and health status of each mouse was checked on a daily basis. Food restriction, however, did not cause any observable changes in the animals' behavior.

All experiments complied with the regulations covering animal experimentation within the EU (European Communities Council Directive 2010/63/EU) and were approved by German animal welfare authorities (Regierungspräsidium Karlsruhe). Moreover, all efforts were made to minimize the number of animals used and the severity of procedures applied in the study.

TEST

Apparatus

All animals were tested in Campden Instruments Ltd. (Loughborough, Leics., UK) mouse touchscreen chambers (Model 80614-20). The chambers were equipped with a 3-W house light, a tone generator and several light beams detecting the movement of the mouse. The trapezoid shaped inner chamber (h 19 cm, w 24 respectively 6 cm, d 17 cm) consisted of black Perspex walls and a metal grid floor. At one end, the boxes were equipped with screens surrounded by infra-red detectors to sense touches. As a consequence, the mice were not required to get in direct contact with the screen, but needed to approach the stimuli closely by nose-poke responses. The screens were partly covered by a Perspex mask in order to block access to the display except through three equal response windows that measured 7 × 7 cm each (**Figure 2**). Each window was separated by black Perspex dividers to prevent accidental approaches to the adjacent response window. During the training and testing procedure the outer fields were used to detect touches (left and right *touch fields*), whereas the center field was used to present cues (*cue presentation field*) (**Figure 2**). A food well (2 × 2× 2 cm^3) attached to an externally placed feeder (liquid suspensor) was located centrally at the rear of the chamber. A panel light illuminated the food tray and head entries were detected via a light beam detector. Here, mice were also trained to initiate a trial by breaking the light beam.

Procedure

Using touchscreen chambers, we translated the above described human paradigm (Armbruster et al., 2012) as analogously as possible to the mouse condition. In brief, the mouse-translation of the human paradigm involved side rather than task switches, with repetition trials ("ongoing") occurring with equal likelihood on both sides. A central field was used for the illustration of a reward-indicating cue that could be presented in three different gray intensities in two different positions. According to the position of the cue, the mouse had to touch either on the left or the right *touch field* to get a reward. In some trials, two cues of different gray intensity were presented simultaneously. In these trials, the animal had to solve the task according to the brighter cue, thus ignoring the second distracting cue. In an ambiguous condition two cues of the same gray intensity were presented and the mouse could choose freely between the responses. Thus, taking species-specific differences

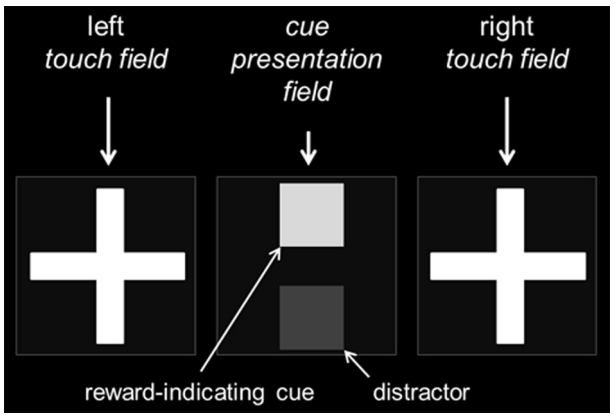

FIGURE 2 | Organization of the cue-presenting and touch-sensitive fields. The touchscreen was covered by a black Perspex mask with three equal response windows. While the central field served as the cue presentation field, the external fields were used as touch fields during training and testing. For the performance of the "cue-position task," a reward-indicating cue was presented either in the top or bottom position of the central cue presentation field and the mouse had to respond by touching either on the left or right cross in the touch fields (position-to-side assignment balanced across subjects). Dependent on the test condition a distractor of a different gray value could be displayed simultaneously to the reward-indicating cue in the central cue presentation field.

into account, we modified the test with respect to five key points:

1. Humans can be instructed and tested in a single session on 1 day, while rodents need to learn various preparatory tasks in an extensive training, before being capable of managing the final test. Thus, the time needed to complete the testing is far greater in the murine than in the human test version.
2. To guarantee a high degree of motivation throughout testing, a rodent test version inevitably relies on a reward-based structure. Thus, rewarding correct behavior and punishing incorrect answers are fundamental strategies in the mouse test version. Moreover, animals need to be food restricted prior to and during the testing to set up the conditions for testing.
3. In the human paradigm, subjects are asked to respond to a visual task by pushing a button with one of four possible fingers (i.e., two fingers per task), while mice have to make a choice by responding directly to the touch-sensitive screen via nose-pokes. Because of the different locations of the *touch fields* this behavioral response contains a larger motoric component that may influence the output measures (e.g., response times).
4. While the human task required the subject to switch between two different rules depending on the position of a cue (cue above the fixation cross > rule 1, cue below the fixation cross > rule 2), the mouse task relied on a simpler discriminative task (Is the cue in position one or two? > touch left or right). Because only the change between two different rules within one test is conventionally considered to be a "task switch" (Brigman et al., 2005; Garner et al., 2006), the mouse test version did not include a "task switch" by definition. However, as the aim of the present work was to investigate the antagonistic nature of cognitive flexibility vs. cognitive stability according to the *Dual State Theory* (and not task switching *per se*) a task switch was not necessarily required. As cue-guided switching of response position within one task may in itself be a demanding challenge on the cognitive systems of a mouse, we replaced the human task switching aspect of the paradigm by the requirement of flexible or stable responses to a discriminative task. Therefore, the procedure was simplified to increase efficacy and practicability in a mouse model without compromising specificity.
5. Since the human paradigm included fMRI imaging in the experimental setup, it was necessary to determine a baseline state from which differences due to behavioral responses could be monitored. The baseline task (= responding to a digit shown in the "upper" position) was presented in 80% of all cases in order to maintain the brain in a steady state. In 20% of the cases a second digit was presented simultaneously in the "bottom" position to allow for assessing brain activation changes under theoretically interesting conditions, i.e., stressing either cognitive flexibility or stability or, in the ambiguous condition, providing no clear cues. An exact translation of this paradigm to the mouse paradigm, however, would have led to a repeated presentation of one cue in the same position, requiring the mouse to respond in always the same way. Thus, to avoid the risk of activating a side bias, animals were trained to both cue positions equally.

The mice were housed under stable conditions for 4 weeks before the onset of testing. The testing procedure was divided into three main phases: habituation, training and testing phase (**Figure 3**). The daily testing order followed a fixed schedule to guarantee a consistent level of motivation due to feeding times. At the beginning of each test session, mice were transported to the test room in their home cages and allowed to acclimatize to the room before testing commenced. The testing was done during the dark phase of the cycle, 2 h after the light change. Food was supplied individually following the testing procedure. Inner chambers were cleaned after the testing of each animal with water. One week ahead the first introduction into the boxes, the animals received sweet condensed milk (SCM) in their home cages in order to avoid later refusal of the reward provided in the touchscreen boxes.

Habituation. During the habituation phase the mice learned basic procedures inside the box, such as touching on the screen, getting a reward, or initiating the next trial (**Figure 3**). Because the procedures have been described in detail elsewhere (see Talpos et al., 2009), we limit the following description to a brief overview: First, subjects were habituated to the boxes once a day with increasing duration (10–40 min) on three consecutive days. During the second session, a liquid reward (7 µl SCM "Milchmädchen," diluted 1:4 in tap water) was placed in the food well that was freely accessible to the subjects. In the third session, mice learned to associate the supply of the reward with the illumination of the food well-light and the presentation of a tone. Following this, they became acquainted with symbols presented

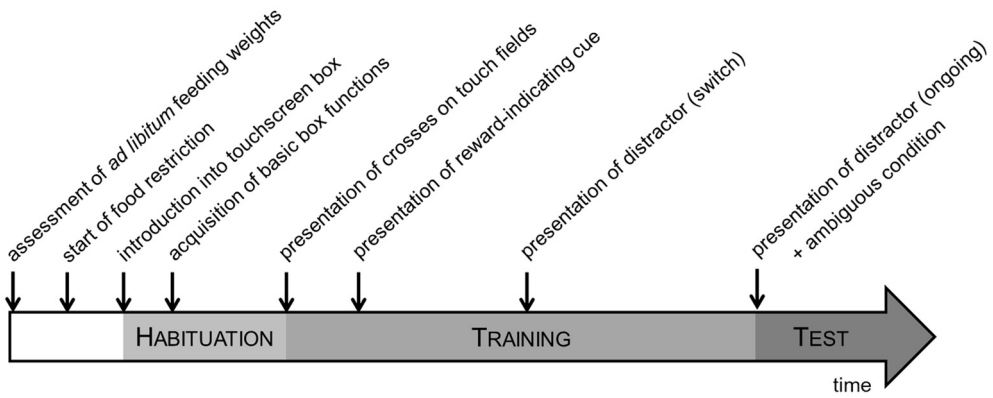

FIGURE 3 | Experimental time schedule. The testing procedure was divided into three main phases: habituation, training and testing phase. During the habituation and training phases, mice were acquainted with the basic box functions and learned to respond to the discriminative task before being tested in the STABFLEX test.

on the *touch fields* and learned to nose-poke them to receive a reward. In the next habituation step the animals learned to initiate each trial by breaking a light beam within the feeder well to produce the stimulus onset. A correct nose-poke to the response screen was followed by the disappearance of the stimuli, presentation of a tone and delivery of the reward. Initiation of the next trial could then occur after a 5 s inter trial interval (ITI). Finally, punishment of incorrect answers was introduced in form of a 5 s timeout with house light illumination before the start of the ITI.

Training. In the training the animals were required to learn a rule of the type "if cue on *cue presentation field* in position A then go left, if cue on *cue presentation field* position B then go right" (**Figure 3**). The position-interpretation was consequently counterbalanced across animals. The training was subdivided into four phases which build on each other introducing single items of the final task needed in the testing. The position-sequence was determined pseudo-randomly based on phase-dependent variations of probability of occurrence.

Phase 1 was named "TWO CROSSES, NO CUE": A nose-poke to crosses presented on *touch fields* was rewarded with a tone, feeder light and a liquid reward. Touches on the *cue presentation field* did not lead to any reaction of the system. The criterion to progress into the next phase was performance of 30 trials in 30 min. In the second phase "CUE-POSITION TASK" a cue on the *cue presentation field* in either top or bottom position indicated which side was counted "correct" and rewarded when touched. The cue was a gray square (2.5 cm) presented in one of three different possible light intensities. Top and bottom position were separated by a gap of 2.0 cm from the boundaries of the squares. Only touches on the *touch field* lead to supply of a reward. Each session included 40 trials or was terminated after 60 min. The criterion to progress into the next phase was performing 40 trials per day on two consecutive days. In 50% of all trials the position of the cue did not change between two consecutive trials ("ongoing" condition), while it changed between the trials in the other 50% ("switch" condition). Touches on the non-reward-indicating *touch field* and the *cue presentation field* did not result in any reaction of the system. During the "CUE-POSITION TASK WITH CORRECTION"-phase touches on the non-reward-indicating *touch field* were counted "incorrect" and lead to a 5 s timeout with house light illumination and initiation of a correction trial, which was counted separately and repeated until the animal responded correctly. Each session consisted of 40 trials (not including correction trials) with equal occurrence probabilities for "ongoing" and "switch" conditions. The session was either finished after the completion of 40 trials or a training time of 60 min. The criterion to progress into the next phase was performance of 40 trials with at least 80% correct responses on two consecutive days.

In the last training phase "CUE-POSITION TASK WITH DISTRACTOR AND CORRECTION," the "distractor switch" condition was introduced. As in the "switch" condition the reward-indicating cue occurred in different positions on two consecutive trials, but was additionally accompanied by the presentation of a distracting cue in the second trial (**Figure 4**). The distracting cue (= distractor) occurred in form of a second gray square on the *cue presentation field* and was presented simultaneously to the reward-indicating cue. Similar to the reward-indicating cue the distractor was presented in three different gray intensities, all darker than the reward-indicating cue and for answering correctly, the mouse needed to solve the task according to the brighter cue. Combination of brightness intensities of the two cues consistently equaled the same mean brightness. "Ongoing," "switch," and "distractor switch" conditions were presented with occurrence probabilities of 50, 25, and 25%, respectively. Incorrect touches (touches according to the distractor) led to a 5 s timeout with house light illumination, followed by correction trials. Initially, the animals had to perform 40 trials a day which was subsequently raised to 50 trials. In both phases the learning criterion was set to 80% correct responses on two consecutive days.

Testing. Once a mouse had successfully reached the final learning criterion, it was tested in the translational stability/flexibility (STABFLEX) test on 14 consecutive days. A testing day consisted of 50 trials per day and mouse, yielding a total amount of 700 trials per animal. A single session ended after the performance of 50 trials or 60 min elapsed time. In contrast to the training phase,

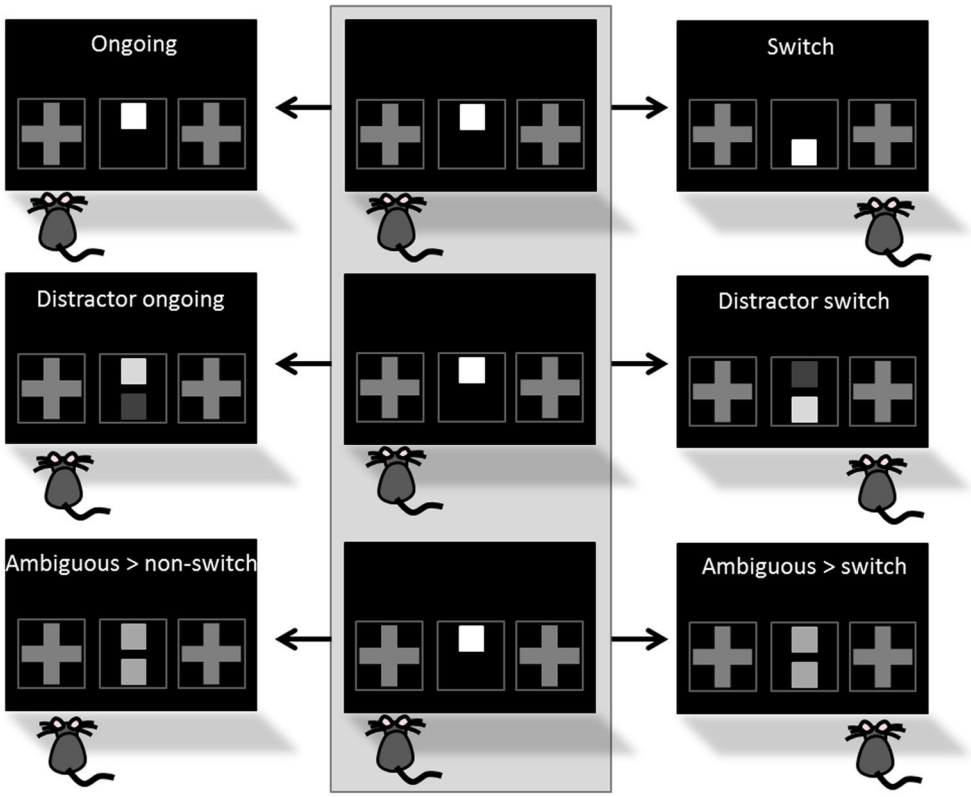

FIGURE 4 | Task conditions. The STABFLEX test combines five different task conditions, including ongoing, switch, distractor ongoing, distractor switch, and ambiguous conditions. Different conditions are defined by the number of cues presented in the cue presentation field (one or two), their position (top or bottom), their relative intensity (different gray values), and the foregoing trial (middle column colored in light gray). **Top row**: While an ongoing trial is characterized by the presentation of only one cue in the same position in two consecutive trials (left), a switch condition describes a change in the position of the cue between the trials (here: cue at the top > cue at the bottom, right). **Middle row**: The distractor conditions, i.e., distractor ongoing (left) and distractor switch (right), are based on the same principle, but are complemented by a second cue of different intensity that is task irrelevant and thus a distractor that renders the task more difficult. For answering correctly, the animal needs to solve the task according to the brighter cue. **Bottom row**: Finally, animals are presented with ambiguous situations that are characterized by the presentation of two cues of the same intensity. The classification of these trials as ambiguous/non-switch (left) vs. ambiguous/switch (right) depends on the behavior of the animal and this condition does not favor one of the two response alternatives. Conditions are presented as a series of two consecutive trials, starting with the foregoing trial in the middle column of the illustration and choices are depicted by the position of the mouse in front of the screen.

testing did not contain any correction trials, while incorrect responses were still punished with house light illumination and 5 s timeout. For all testing days, we used pseudo-randomized sequences with no more than three consecutive "ongoing" trials. Between the "ongoing" conditions one out of four possible other conditions were presented. All conditions, namely "switch," "distractor switch," "distractor ongoing," and "ambiguous" (**Figure 4**) had the same probability to appear (probability of condition appearance: 66% ongoing, 8.5% each switch, distractor switch, distractor ongoing and ambiguous). While "switch" and "distractor switch" conditions were introduced in the training, the "distractor ongoing" and "ambiguous" conditions were completely new to the mice in the testing procedure (**Figure 3**).

In the "distractor ongoing" condition a distractor of another gray intensity was presented simultaneously to the brighter reward-indicating cue on the *cue presentation field* (**Figure 4**; **middle row**). As in the "distractor switch" condition, the subject was required to follow the lighter cue and answer according to the "ongoing" condition. In the "ambiguous" condition two squares with identical gray intensity were presented on the *cue presentation field* (**Figure 4**; lower row). In this condition, the task did thus not unambiguously indicate a rewarded and a non-rewarded side, so that the animal could freely choose between the two possible responses. In any case, however, the choice was followed by a reward.

Behavioral measures

While in the training phase the number of trials required to attain criterion performance in the different training phases was of major interest for the analysis, performance in the STABFLEX test was assessed using the following behavioral measures: (i) number of errors [error rates (ER)], (ii) average choice latencies or response times for correct responses (RT), which were measured as the time from the onset of the cue presentation until the mouse

made a nose-poke response; and (iii) response time costs, which refer to the difference between the RTs of any condition and the baseline condition ("ongoing"). In addition, two further behavioral measures were calculated, the "distractor resistance" and the "individual spontaneous switching score," to analyze the data as analogously as possible to the human condition.

First, a so-called "distractor resistance" was calculated with the aim of describing an animal's ability to maintain a behavior in the face of potential distractors. As more stable subjects are expected to be more resistant to distraction (Durstewitz and Seamans, 2008; Armbruster et al., 2012), the measure was based on correct and incorrect choices made in the "distractor ongoing condition." More precisely, distractor resistance was calculated as a choice score (correct choices—incorrect choices) corrected for the overall individual performance in the "ongoing" condition. By including the correction for the overall success rate under baseline conditions ("ongoing") in the formula, inter-individual differences in learning abilities were taken into account. On a scale from −1 (very distractible) to +1 (resistant to distraction) this score describes an individual's ability to resist distraction.

$$\textit{distractor resistance} = \frac{N_{distractor}\ (correct) - N_{distractor}\ (incorrect)}{N_{distractor}\ (correct\ +\ incorrect\ choices)} \div \textit{success rate}_{ongoing}$$

According to the human test version (Armbruster et al., 2012), the probability of spontaneously switching under ambiguous conditions without external cues was also assessed. This so-called "individual spontaneous switching score" (ISSS) was calculated on the basis of switches and non-switches in the "ambiguous" condition:

$$ISSS = \frac{N_{ambiguous}\ (switches) - N_{ambiguous}(nonswitches)}{N_{ambiguous}\ (switches + nonswitches)}$$

Only responses which followed a correct response in the preceding "ongoing" condition were included in this analysis. Similar to the "individual spontaneous switching rate" in the human paradigm, this measure reflects the tendency of an animal to either continue responding with the same behavioral response (stability) or to switch between the responses and exhibit more flexible characteristics. On a scale from −1 (very stable) to +1 (very flexible) this score thus describes an individual's tendency to behave in a stable or flexible way.

DATA ANALYSIS

Behavioral log files of the conducted experiments contained full information on the course of the experiments. Each trial was represented by a matrix row containing the experimental condition, the response given and the response time. This data was reduced by averaging the latencies over specific condition × decision combinations and by counting the numbers of specific responses as a function of experimental condition and in case of the ISSS also as a function of the condition and response in the preceding trial (see Behavioral Measures). These calculations were performed using custom MATLAB routines.

Latency measures and ER of 22 mice were then entered into the statistical analysis presented below. Latencies were determined by averaging all response times of one condition across all correct trials of this mouse, while errors were summed up per condition and related to the amount of trials per condition to determine individual ER. For inferential statistics, repeated measures ANOVAs were applied to investigate the effects of "condition." on response times and ER. When necessary, *post-hoc* tests were performed using a Bonferroni correction. To test the predictions made by the *Dual State Theory*, correlation analyses were conducted using Pearson's product-moment correlation coefficient (r) and Spearman's rank correlation coefficient (r_s). All statistical tests were conducted using the software package SPSS (version 19.0 for Windows), and differences were considered to be significant at $p \leq 0.05$.

RESULTS

HABITUATION AND TRAINING

Of the 24 mice that were used for the establishment of the STABFLEX test, 22 individuals were entered into the following analyses. The exclusion of two animals was based on either bad or inconsistent performance in the *cue position task* or in the testing phase. Thus, one subject was excluded from the analysis, because its overall success rate did not exceed chance level in the *cue-position task*, although it was trained for 86 days. The second animal succeeded to go through the various training phases, but stopped behaving in an active way after a few days in the testing phase. Because the subject did not show any behavioral abnormalities in the home cage and we could not observe any signs of disease, we considered this behavior to have a motivational cause and excluded the animal due to a lack of data.

During the initial phase of the task (habituation) animals were habituated to the touchscreen-box and required to learn how to initiate trials and to touch on the screen for getting a reward. On average, mice needed 15 days to go through these basic steps with only two animals that needed more than 20 days to reach the criterion (**Table 1**). The subsequent training phase consisted of five different sub-phases that were completed after 63 ± 4 days. Notably, the overall training duration ranged from 29 to 90 days, reflecting considerable inter-individual variability (**Table 1**). Differences in training duration, however, were mainly due to performance differences in the *cue-position task with correction*. While one mouse reached the criterion already after 15 days, another one required 79 days to go through this critical training phase (**Table 1**). In this context, the number of correction trials per day turned out to be a good measure for the assessment of an individual's learning progress throughout this phase (**Figure 5**). While some individuals were characterized by a rapid decrease of correction trials (**Figure 5A**), the performance of other individuals followed a more gradual improvement over several days (**Figure 5C**). Thus, although some animals required more than 2 months learning the *cue-position task with correction*, their performance was characterized by a continuously decreasing number of correction trials.

Furthermore, we were interested in the question whether a "good performer" in the training phase remains a "good performer" in the test phase. We therefore correlated the number of days needed to go through the *cue-position task with correction* in the training with the overall success rate in the testing phase. Indeed, we found a significant negative correlation between

Table 1 | Duration of habituation and training phases in days.

	Habituation	Training (cue-position task with correction)
Mean	15	63 (47)
s.e.m	1	4 (4)
Min.	12	29 (15)
Max.	25	90 (79)

Subjects had to reach specific learning criteria to enter the next phase. Accordingly, habituation and training durations differed widely between the individuals, n = 22.

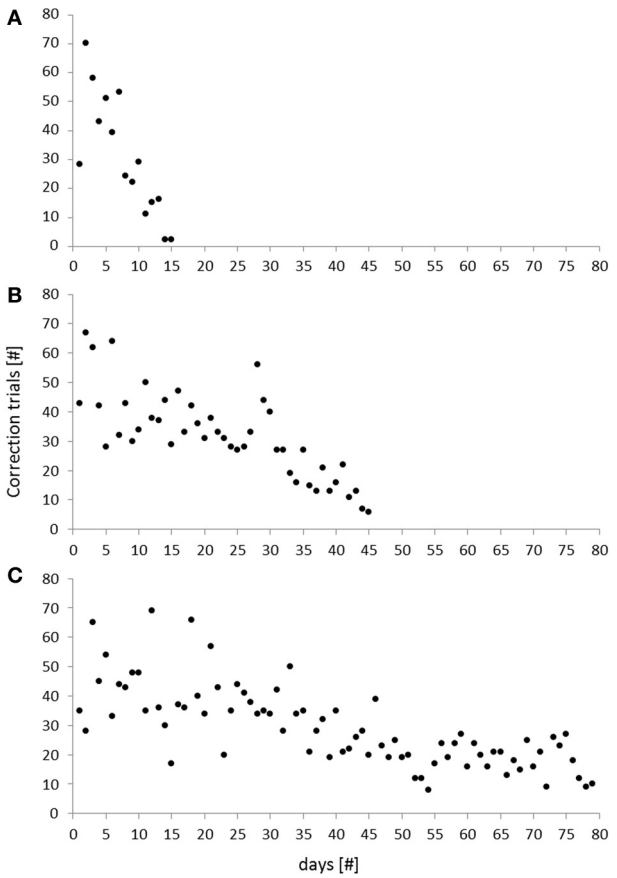

FIGURE 5 | Number of correction trials during the cue-position task with correction of three individual mice. The total number of correction trials turned out to be a good measure for an individual's learning progress in this phase. Different types of learning curves were observed: While some subjects were characterized by a rapid decrease of correction trials, reflecting a steep learning progress **(A)**, others were characterized by a more gradual learning improvement over time **(B,C)**.

the duration of this training phase and the later success rate: The faster an animal reached the learning criterion in the *cue-position task with correction*, the better it was in the testing phase ($r = -0.493$, $p = 0.020$; $r_s = -0.459$, $p = 0.031$).

TESTING COGNITIVE FLEXIBILITY vs. COGNITIVE STABILITY

Testing was successfully done with 50 trials per day on 14 successive days in all mice, yielding a total amount of 700 trials per animal. Although animals were not required to reach a specific learning criterion during the testing phase, the overall success rates were similar to those of the final training phase, indicating robust performances over time. With a mean of 79.3% the overall success rate (= correct choices/700) ranged from 67.5 to 89.1% (**Table 2**). Except of one animal that did not reach the 70% mark, mice solved the task correctly in at least 75% of all trials, demonstrating constantly good performance abilities also during the testing phase. In addition to the overall success rate, behavioral performance in the testing phase was separately analyzed for each condition ("ongoing," "switch," "distractor ongoing," "distractor switch," "ambiguous") using both response times and ER. Concerning the response times, only minimal differences were observed between the five testing conditions, ranging from a mean latency of 1.9 s in the "distractor ongoing" condition to 2.1 s in the "ambiguous" condition (**Table 3**). Accordingly, the observed differences between response times of the specific event conditions and the "ongoing" condition did not exceed ± 130 ms. Notably, we observed negative response time costs in the distractor conditions, indicating that on average it took the mice slightly longer to respond to the "ongoing" than to the "distractor ongoing" and the "distractor switch" conditions (**Table 3**). By contrast, ER clearly differed between the conditions in the expected way, with only 18.6% in the "ongoing" condition and 28.8% in the "distractor switch" condition, probably reflecting an increasing degree of difficulty (**Figure 6**).

Subsequent inferential statistics, using repeated measures ANOVAs, revealed significant effects of the condition on both response times [$F_{(4,\ 84)} = 2.775$, $p = 0.032$] and ER [$F_{(3,\ 63)} = 12.267$, $p < 0.001$]. Further Bonferroni corrected *post-hoc*-analyses of the response times, however, showed that differences were only approaching significance and restricted to the comparison of the "ambiguous" condition with the "distractor ongoing" condition ($p = 0.06$), while all other conditions did not differ significantly. With respect to the ER, a significant difference was found between the "ongoing" and "distractor switch" condition ($p < 0.001$, **Figure 6**), while there were no statistically significant differences between "ongoing," and "switch" and "ongoing," and "distractor ongoing" conditions ($p > 0.1$). Furthermore, ER of the "distractor switch" condition were higher than those of the "switch" condition ($p < 0.001$) and those of the "distractor ongoing" condition ($p = 0.034$, **Figure 6**). A difference between ER of the "switch" and the "distractor ongoing" conditions was not observed ($p > 0.1$).

To test some behavioral predictions derived from the *Dual State Theory*, two further behavioral measures were investigated: The ISSS and the distractor resistance (see Animals, Materials, and Methods). While the ISSS aims to capture the individual level of flexibility/stability in the "ambiguous" condition on a scale from −1 (extremely stable) to +1 (extremely flexible), the distractor resistance was calculated on the basis of correct and incorrect choices in the "distractor ongoing" condition and as such reflects an individual's ability to solve a task correctly in face of distracting stimuli (the higher the value, the more stable the individual, see Animals, Materials, and Methods). Both measures were found to vary substantially between individuals, indicating different degrees of cognitive flexibility and stability

Table 2 | Summary of main behavioral measures.

	Success rate (%)	Individual spontaneous switching score	Distractor resistance
Mean	79.3	−0.057	0.698
s.e.m	1.0	0.041	0.037
Min.	67.5	−0.302	0.293
Max.	89.1	0.349	0.944

On the basis of correct and incorrect choices in five different test conditions, three main behavioral parameters were calculated: overall success rate (correct choices independent of the specific task condition), individual spontaneous switching score, and distractor resistance, n = 22.

Table 3 | Descriptive statistics for response times (RT), and response time costs.

	Response times (ms)	Costs (ms)
	Mean ± s.e.m.	Mean ± s.e.m.
Ongoing	2055 ± 116	
Switch	2057 ± 121	2 ± 58
Distractor ongoing	1926 ± 97	−130 ± 61
Distractor switch	2022 ± 118	−33 ± 46
Ambiguous	2104 ± 118	49 ± 50

While the absolute response times capture mean latency values per condition, the costs are corrected by the time needed to finish an ongoing trial (e.g., RT switch − RT ongoing = Switching costs). Data are separately presented for each test condition and presented as means ± standard error of the mean, n = 22.

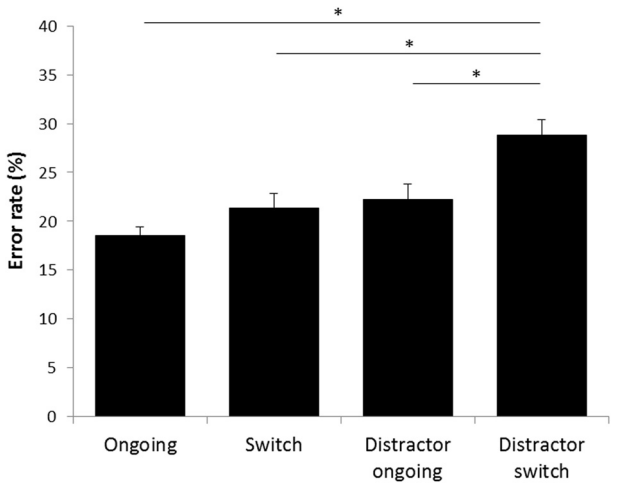

FIGURE 6 | Error rates in the STABFLEX test. The error rates were calculated on the basis of the number of incorrect touches divided by the total number of trials per condition. Conditions were compared using a repeated measures ANOVA with "condition" as within-subjects factor and subsequent Bonferroni-corrected *post-hoc* analyses. Data are presented separately for the four experimental conditions as means ± standard error of the mean. $^*p \leq 0.05$.

in our sample (**Table 2**). Similar to the previous human study, the ISSS thus allowed for identifying more stable (<0) and more flexible (>0) subjects. Furthermore, we observed sufficient variability in the ISSS to conduct individual differences analyses.

In the human paradigm, the individual spontaneous switching rate was negatively correlated with the switching costs (Armbruster et al., 2012), assuming that this rate is not merely a reflection of a perceptual bias but that it indeed reflects a behavioral tendency toward more flexible behavior. Translating the test to the mouse condition led to a similar observation: Here, correlation analyses also revealed a significant negative correlation between the ISSS and the switching costs ($r = -0.480$, $p = 0.012$; $r_s = -0.364$, $p = 0.048$). Thus, the higher the ISSS and, thus, the more flexible the subject behaved in the "ambiguous" condition, the less "costly" it was for the subject to switch between the answers in non-ambiguous conditions when instructed to do so (**Figure 7**). According to further behavioral predictions, one should also expect a correlation between the ISSS and the error rate of the "switch" condition. Indeed, such a correlation was found with more flexible subjects making less switching errors under non-ambiguous conditions (Pearson product-moment correlation coefficient, $r = -0.471$, $p = 0.0013$; Spearman's rank correlation coefficient, $r_s = -0.524$, $p = 0.006$, **Figure 8**), even after the exclusion of one animal with an exclusively high error rate of 44.6% in the "switch" condition ($r = -0.455$, $p = 0.019$; $r_s = -0.483$, $p = 0.013$). However, no similar correlations were found between the ISSS and the ER of the "distractor switch" condition ($p > 0.1$). For distractor inhibition, the results were also in the expected direction, but less robust: Using Spearman's rank correlation coefficient, a trend toward a negative correlation between ISSS and distractor resistance was detected ($r_s = -0.342$, $p = 0.06$, **Figure 9**) that could, however, not be confirmed using Pearson's product-moment correlation coefficient ($r = -0.237$, $p = 0.144$).

DISCUSSION

In the present study, we introduce a novel task paradigm to assess cognitive flexibility vs. cognitive stability in mice. Inspired by neurocomputational modeling of the pFC-DA network, the task was originally established in a healthy human population (Armbruster et al., 2012), before being translated back to preclinical conditions in the present study.

Our aims were, first, to translate the procedure as analogously as possible from the human to the mouse, while at the same time accounting for species-specific demands, and, second, to test core behavioral predictions derived from the *Dual State Theory* (Durstewitz and Seamans, 2008) in a murine wildtype population, namely whether cognitive flexibility and stability are antagonistically related in individual subjects.

TRANSLATING COGNITIVE MEASURES: THE STABFLEX PROCEDURE

Translating tests of human executive functions still is a challenge (Tecott and Nestler, 2004). However, by providing virtually unlimited possibilities for task development, touchscreen approaches extend the repertoire for testing cognitive functions in animals like no other methodology (Bussey et al., 2012; Dickson et al., 2013; Horner et al., 2013; Mar et al.,

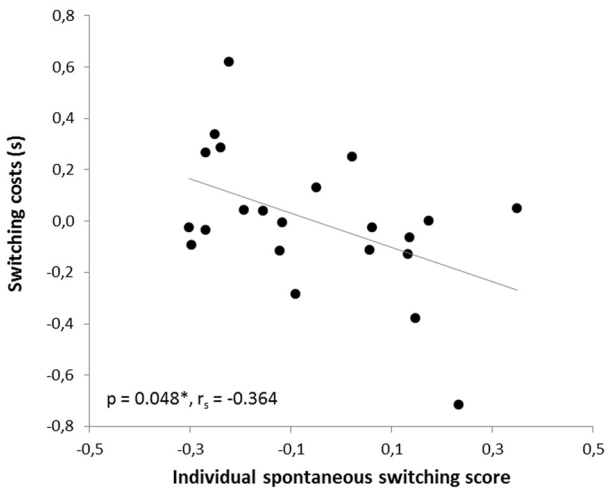

FIGURE 7 | Correlation between switching costs and the individual spontaneous switching rate. Switching costs were calculated by subtracting response times of the ongoing condition from those of the switch condition. According to the predictions of the Dual State Theory, switching costs and the individual spontaneous switching score were negatively correlated: The more flexible the subject, the lower the switching costs in the switch condition. The correlation was calculated using Spearman's rank correlation coefficient.

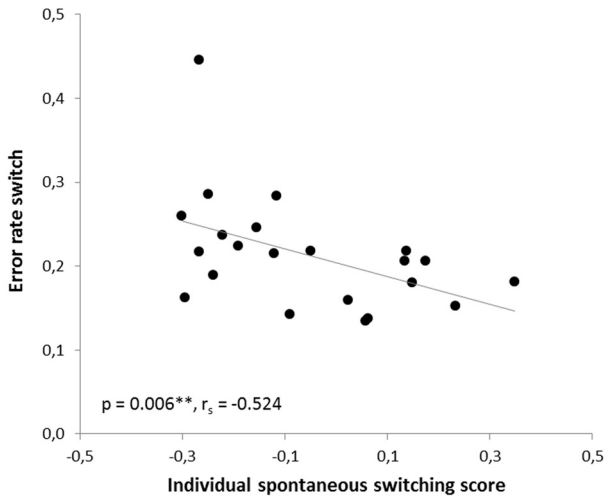

FIGURE 8 | Correlation between the error rate for switching and the individual spontaneous switching rate. According to the predictions of the Dual State Theory, the error rate of the switch condition and the individual spontaneous switching score were negatively correlated: The more flexible the subject, the less errors it made in the switch condition. The correlation was calculated using Spearman's rank correlation coefficient.

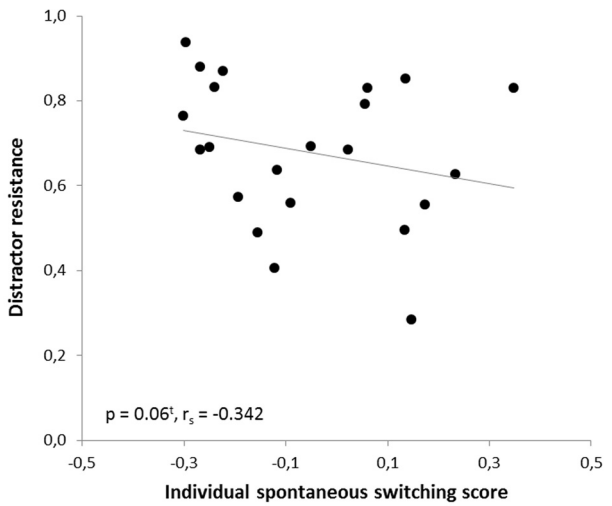

FIGURE 9 | Correlation between distractor resistance and the individual spontaneous switching rate. According to the predictions of the Dual State Theory, the distractor resistance and the individual spontaneous switching score were negatively correlated: The more flexible the subjects, the less resistant to distractors they were. The correlation was calculated using Spearman's rank correlation coefficient.

2013; Oomen et al., 2013; Silverman et al., 2013; Talpos and Steckler, 2013). We carefully aimed to avoid confounding variables: Thus, the procedure is highly automated, avoids experimenter bias, and involves little stress for the animal (Chesler et al., 2002; Wahlsten et al., 2003; De Visser et al., 2006; Bussey et al., 2012; Strickland and Mercier, 2014). Moreover, all subjects were handled gently without physical restraint to reduce anxiety and stress (Hurst and West, 2010).

The cognitive task we established resembled the human version as closely as possible. As in the human paradigm, cues solely rely on visual input, which seems to be in contrast to rodents' low reliance on vision but can also be regarded as the strength of the system: By forcing the animals to solve problems in a "human-like" way, the approach has excellent face validity and prevents the experimental subjects from using secondary cues that we are unaware of (Talpos and Steckler, 2013).

Behavioral differences were assessed using response times and ER as it was done in the human study (Armbruster et al., 2012). By providing direct animal homologs of clinically important measures in human research, we aimed for high construct and predictive validity (Garner et al., 2006, 2011; Gould and Gottesman, 2006; Talpos and Steckler, 2013). Our test systematically investigated two cognitive domains, i.e., cognitive flexibility and cognitive stability, under the same conditions and using the same apparatus, thus completing the "wish-list" for an ideal cognitive testing method (Bussey et al., 2012).

However, there are also slight differences between the human and the animal paradigm, the most important being that while the switch condition of the human paradigm always involved the simultaneous presentation of a distracting cue, the mouse paradigm also involved trials with switching cues without the simultaneous presentation of a distracting cue. This was implemented to reduce complexity and adapt for the cognitive capacities of the mice (see below).

THE DICHOTOMY OF COGNITIVE FLEXIBILITY AND COGNITIVE STABILITY

While behavioral differences between the task conditions could easily be detected on the basis of latency measures in the human paradigm, response time measurements were not sensitive enough to describe differences in the murine task version. Thus, average response times were two- to three-fold bigger in mice than in men, but varied less in mice. Probably, this discrepancy can be explained by the higher motoric component involved in the touching response. Humans were able to respond to the different task conditions by pressing a button within hundreds of milliseconds, while mice had to initiate the trial at the back of the chamber, then turning around, comparing the cues on the screen, before responding to the task by moving forward to either the left or the right cross. The high visual-motoric requirements may therefore overlap with the cognitive operations, challenging the use of response times in this context. This adds to previous discussions about the use of latency measures in cognitive testing (Richter et al., 2012).

ER, on the other hand, were very low and showed little variation in humans, but were well-suited to reflect the cognitive demands in mice. Although overall ER were much higher in mice than in men, similar differences between the conditions could be detected in both species. ER were found to increase from the "ongoing" to the "switch" and "distractor ongoing" conditions. This is in line with the findings of the human study reporting longer response times for task switching (= "distractor switch" in the murine study) than for distractor resistance = "distractor ongoing" in the murine study; Armbruster et al.(2012). Moreover, the data argue for the antagonistic model of cognitive flexibility and stability: Subjects with a higher ISSS were characterized by lower switching costs in the unambiguous "switch" condition. A similar correlation was found in the human task (Armbruster et al., 2012), validating the newly introduced measure of switching in an ambiguous condition (i.e., the ISSS) for mice. Furthermore, there was a significant correlation between the switching score and the error rate in the "switch" condition, indicating that more flexible subjects were able to switch more efficiently. In the "distractor switch" condition however, ER were highest and there was no correlation to the switching score, indicating that this task may be too complex for mice and thus is not suited to describing a mouse's disposition toward more flexible or stable behavior. We therefore believe that the mice's behavioral responses in the simple "switch" condition without distractor more adequately mimic what has been observed in the more complex human switch condition.

Finally, as more flexible mice also tended to be more distractible in the "distractor ongoing" condition, our results confirm the hypotheses that subjects switching more often spontaneously are more distractible to secondary cues, but also more flexible in situations, where cognitive flexibility is required. This is in line with previous findings on humans, showing that higher distractibility occurred in more flexible persons (Dreisbach and Goschke, 2004; Dreisbach et al., 2005; Tharp and Pickering, 2011; Armbruster et al., 2012; Zhang and Chan, 2013) and supports the predictions made by the *Dual State Theory* (Durstewitz and Seamans, 2008). Accordingly, the present study provides additional behavioral support from a different species for two crucial assumptions of the *Dual State Theory*, i.e., that individuals differ in their degree of cognitive flexibility and that cognitive flexibility and stability are antagonistically related—supposedly controlled by a common prefrontal neural mechanism involving differential effects of D1 and D2 receptor activation (Floresco and Phillips, 2001; Floresco and Magyar, 2006; Floresco et al., 2006; Vijayraghavan et al., 2007).

To validate the paradigm and to investigate the involvement of a pFC-DA network in the control of these higher executive functions, future studies are necessary, either working with dopamine-agonists/antagonists or with genetically modified mice to experimentally shift behavior toward a more stable or flexible pattern. In light of clinical research, this may contribute to a better understanding of cognitive dysfunctions in psychiatric diseases like schizophrenia and may even offer new routes for developing efficient pharmacological treatments in the long term.

CONCLUSIONS

Here, we report the successful modification of a human neuropsychological task for use with mice. The results of this study support a dichotomy of cognitive flexibility and cognitive stability and thus confirm some fundamental predictions made by a biophysically realistic computational model of PFC function, i.e., the *Dual State Theory*. Similarities in behavioral patterns between humans and mice indicate the translational potential of the testing procedure and support the use of touchscreen procedures in preclinical animal research. This may launch an exciting new generation of behavioral tests for mice that promise to overcome many limitations of current high-throughput testing, while at the same time providing direct animal homologs of clinically promising measures in human research.

AUTHOR CONTRIBUTIONS

S. Helene Richter, Christian J. Fiebach, Peter Gass, and Barbara Vollmayr conceived this work, S. Helene Richter, Anne S. Vogel, Miriam A. Vogt, Diana J. N. Armbruster-Genç, Marco A. Riva, Christian J. Fiebach, Peter Gass, and Barbara Vollmayr designed it, S. Helene Richter, Anne S. Vogel, Kai Ueltzhöffer, Chiara Muzzillo, and Katja Lankisch acquired data, S. Helene Richter, Anne S. Vogel, Kai Ueltzhöffer analyzed data under supervision and interpreted data together with Diana J. N. Armbruster-Genç, Christian J. Fiebach, Peter Gass, and Barbara Vollmayr. S. Helene Richter and Anne S. Vogel drafted the work, all authors critically revised it and approved the final version.

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N-methyl-D-aspartate receptor-mediated glutamate transmission in nucleus accumbens plays a more important role than that in dorsal striatum in cognitive flexibility

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Cognitive flexibility is a critical ability for adapting to an ever-changing environment in humans and animals. Deficits in cognitive flexibility are observed in most schizophrenia patients. Previous studies reported that the medial prefrontal cortex-to-ventral striatum and orbital frontal cortex-to-dorsal striatum circuits play important roles in extra- and intra-dimensional strategy switching, respectively. However, the precise function of striatal subregions in flexible behaviors is still unclear. *N*-methyl-D-aspartate receptors (NMDARs) are major glutamate receptors in the striatum that receive glutamatergic projections from the frontal cortex. The membrane insertion of Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs) depends on NMDAR activation and is required in learning and memory processes. In the present study, we measured set-shifting and reversal learning performance in operant chambers in rats and assessed the effects of blocking NMDARs and Ca^{2+}-permeable AMPARs in striatal subregions on behavioral flexibility. The blockade of NMDARs in the nucleus accumbens (NAc) core by AP5 impaired set-shifting ability by causing a failure to modify prior learning. The suppression of NMDAR-mediated transmission in the NAc shell induced a deficit in set-shifting by disrupting the learning and maintenance of novel strategies. During reversal learning, infusions of AP5 into the NAc shell and core impaired the ability to learn and maintain new strategies. However, behavioral flexibility was not significantly affected by blocking NMDARs in the dorsal striatum. We also found that the blockade of Ca^{2+}-permeable AMPARs by NASPM in any subregion of the striatum did not affect strategy switching. These findings suggest that NMDAR-mediated glutamate transmission in the NAc contributes more to cognitive execution compared with the dorsal striatum.

Keywords: NMDA receptor, AMPA receptor, striatum, set-shifting, reversal learning

INTRODUCTION

Strategy switching as a cognitive ability is critical for adapting to and surviving in an ever-changing environment. Impaired cognitive flexibility is one of the core symptoms of schizophrenia (Orellana and Slachevsky, 2013). Considerable studies in humans (Hampshire and Owen, 2006; Chamberlain et al., 2008) and animals (Ragozzino et al., 1999; Birrell and Brown, 2000) indicate that the medial prefrontal cortex (mPFC) is involved in switching attention between stimulus dimensions, whereas the orbitofrontal cortex (OFC) mediates reversal learning intradimensionally. The striatum is one of the major brain regions to which frontal cortex glutamatergic neurons project (Reynolds and Zahm, 2005; Xu and Südhof, 2013), but the role of this region in cognitive flexibility is not well-known.

Abbreviations: DMStr, Dorsomedial striatum; DLStr, Dorsolateral striatum; NASPM, 1-naphthylacetylspermine; PE, Perseverative error; RE, Regressive error; NE, Non-reinforced error.

The systemic blockade of the GluN2B subunit of *N*-methyl-*D*-aspartate receptors (NMDARs) selectively impairs behavioral flexibility without influencing initial reward-response association learning (Dalton et al., 2011). Subchronic treatment with the noncompetitive NMDAR antagonist ketamine reduces perseverative errors during extradimensional set-shifting but impairs intradimensional reversal learning (Floresco et al., 2009). Set-shifting ability is impaired by subchronic treatment with the NMDAR antagonist phencyclidine (Egerton et al., 2008). The phenotypes induced by these treatments closely resemble heterogeneous symptoms in schizophrenia patients (Jentsch and Roth, 1999), implying that NMDARs in the brain participate in the process of replacing old strategies with new ones. However, the precise brain region where NMDARs play a role in cognitive flexibility requires further elucidation. NMDARs in the striatum may make a large contribution to this type of flexible behavior. NMDARs are widely expressed on striatal neurons that receive glutamate inputs from the frontal cortex. However,

only a few studies have suggested that NMDAR activation in the dorsomedial striatum (DMStr) and dorsolateral striatum (DLStr) differentially contribute to reversal learning (Palencia and Ragozzino, 2004, 2005). In addition to NMDARs, α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors (AMPARs) are another main type of glutamatergic receptor. AMPARs are divided into GluA2-lacking and GluA2-containing receptors, which have different calcium permeability (Plant et al., 2006; Isaac et al., 2007). Although calcium-permeable GluA2-lacking AMPARs comprise a small proportion of the normal central nervous system, their insertion into the synaptic plasma membrane occurs during the early phase of long-term potential (LTP), subsequently triggering GluA2-containing AMPAR insertion into the membrane (Jaafari et al., 2012). NMDAR activation modulates GluA1 subunit phosphorylation and dephosphorylation, which are associated with GluA2-lacking AMPAR trafficking and long-term plastic changes (Toyoda et al., 2009; Opazo et al., 2010; Ai et al., 2011). The infusion of the AMPAR general antagonist LY293558 and NMDA receptor antagonist MK-801 into the mPFC significantly increased the number of trials required to reach criterion performance in a set-shifting task (Stefani et al., 2003). In aged rats with impaired set-shifting ability, higher levels of NMDAR binding in the DMStr are correlated with poorer set-shifting performance, with no changes in AMPAR binding level (Nicolle and Baxter, 2003). Thus, an unresolved issue is whether GluA2-lacking AMPARs in the striatum participate in strategy switching as a molecular event downstream of NMDAR activation.

The present study clarified whether NMDAR and AMPAR activation in the striatum is necessary for cognitive flexibility. We compared the total numbers of trials and errors to reach criterion between control and antagonist-treated groups during set-shifting and reversal learning tasks. Rats were microinjected with the NMDAR-selective antagonist (2*R*)-amino-5-phosphonopentanoate (AP5) or GluA2-lacking AMPAR antagonist 1-naphthylacetylspermine (NASPM) into subregions of the striatum before performing the behavioral tasks. Four subregions of the striatum are the nucleus accumbens (NAc) core, NAc shell, DMStr, and DLStr. Traditionally, the term "striatum" refers to the dorsal striatum. The NAc is referred to as the ventral striatum because it is considered to be the predominant part of the ventral striatum. The shell and core subregions of the NAc have different connectivity, with the shell more closely related to the limbic system and the core more closely related to the dorsal striatum (Berendse and Groenewegen, 1990; Voorn et al., 2004). In the present study, the types of errors were further analyzed to reveal possible behavioral components that were affected by the treatments, including prior memory and the learning and maintenance of new strategies.

MATERIALS AND METHODS

SUBJECTS

Male Sprague-Dawley rats (Vital River Animal Center, Beijing, China), weighing 280–300 g upon arrival, were used in the study. The rats were individually housed in stainless-steel sliding-drawer-type cages (50 cm length × 32 cm width × 22 cm height) with a wire mesh floor and pine wood-shavings below the cages. The rats were maintained on a 12/12 h light/dark cycle (lights on at 7:00 AM), with water and food available *ad libitum*. All of the rats were gently handled daily for 1 week before the behavioral experiment began. The experimental procedures were approved by the International Review Board of the Institute of Psychology, Chinese Academy of Sciences, and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

APPARATUS

The behavioral experiments were conducted in eight operant chambers (29 × 29 × 26 cm; Anilab Software and Instruments Co., Ltd., Ningbo, China) enclosed in sound-attenuating boxes. Each chamber was fitted with two nosepoke operandi, one of which was located on each side of a central liquid receptacle. Two yellow light-emitting-diode cue lights (20 mW) were separately situated inside the nosepoke holes. A white chamber light was located 20 cm above the right nosepoke hole. Solutions were delivered through a metal spout that was attached to a 60-ml syringe pump with tubing that delivered fluid at a rate of 34.450 ml/min. The pumps were calibrated to dispense 0.08 ml (0.139 s) of solution into a liquid receptacle for each reinforcement. A timeout period of 10 s followed each reinforcement, during which further nosepokes produced no effect.

SURGERY

The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) before cannula implantation surgery. After being placed in a stereotaxic frame, a midsagittal incision was made to retract the scalp and expose the cranium. Bilateral 23-gauge stainless-steel guide cannulas (Plastics One, Roanoke, VA, USA) were implanted into the NAc core (flat skull: anterior/posterior [AP], +1.6 mm; medial/lateral [ML], ±1.5 mm from bregma; dorsal/ventral [DV], −7.2 mm from dura), NAc shell (AP, +1.7 mm; ML, ±1.0 mm; DV, −7.8 mm], DMStr (AP, +1.0 mm; ML, ±1.8 mm; DV, −4.5 mm), or DLStr (AP, +1.2 mm; ML, ±3.5 mm; DV, −4.5 mm). The coordinates were based on the Paxinos and Watson (1997) rat brain atlas. Four jeweler's screws and dental acrylic were used to secure the cannulae to the skull. Thirty-gauge obturators were placed on the ends of the guide cannulae until the injections were made. Each rat was given at least 7 days to recover from surgery before behavioral training.

BEHAVIORAL FLEXIBILITY TEST

Habituation

At the beginning of habituation, the rats were placed in the operant chambers. They were allowed to freely explore the chamber for 15 min with the nosepoke light off. Sucrose reinforcement was unavailable during this period. A 60-min session was used to train the rats to learn that the sucrose solution was available in the central liquid receptacle. The chamber was illuminated by two nosepoke lights, and liquid was delivered into the central liquid receptacle on a variable interval (VI) 40-s schedule (Chudasama and Robbins, 2003). One second before the liquid was dispensed, the house light (as the conditioned stimulus) was turned on and remained illuminated for 4 s. During habituation, 20% sucrose (wt/vol) in tap water was used as the liquid reward. To ensure that

the rats drank more sucrose in the operant chambers, water and food were removed from their home cages 2 and 18 h, respectively, before habituation began. Throughout operant training and testing, the rats were allowed access to food for 1 h per day after each daily session to maintain their body weight above 85% of their baseline weight (Dias-Ferreira et al., 2009).

Side preference was assessed during the habituation period. In this session, the rats could nosepoke either the left or right hole, which were not associated with sucrose reward. To balance the effect of side preference, the number of nosepokes was recorded. If the number of left or right nosepokes was two-times higher than the number of nosepokes on the other side, then the former was designated the preferred side.

Visual-cue learning

Twenty-four hours after the habituation session, the rats were required to respond at the nosepoke hole with an illuminated visual-cue stimulus light. During illumination of the active nosepoke hole light, a nosepoke into the correct hole (active hole) turned on the house light and switched on the syringe pump 1 s later. Nosepokes in the other hole (inactive hole) had no scheduled consequences. The acquisition criterion was defined as nine consecutive correct responses. A similar criterion was used in previous studies (Floresco et al., 2008; Haluk and Floresco, 2009; Dalton et al., 2011). The rats were returned to their home cages if they did not successfully achieve the acquisition criterion after 100 trials. Two hours later, the rats were removed to the operant chamber, and a new session began until the criterion of nine consecutive correct responses was reached.

Set-shifting

One day after visual-cue learning, the set-shifting task was conducted using the same group of rats. The acquisition of this discrimination task required the animal to cease the use of the previous visual-cue discrimination strategy and instead use a direction-discrimination strategy to obtain the sucrose reward. A correct response consisted of responding at the nosepoke hole that was opposite to the rat's side bias (left or right), regardless of the location of the stimulus light that was illuminated previously in one of the nosepoke holes. Similar to the initial visual-cue discrimination, in every pair of trials, the left or right stimulus light was illuminated once, and the order was random within the pair of trials. The trials were conducted identically to the initial visual-cue discrimination trials. In each trial, the nosepoke hole that the animal chose and the location of the stimulus light were recorded. The trials continued until either (1) the rat achieved criterion performance of nine consecutive correct responses or (2) 100 trials were completed. In this experiment, all of the rats achieved criterion performance within this allotted number of trials.

Reversal learning

One day after the set-shifting trials, the rats were required to learn a reversal of the response rule. The rats had to nosepoke in the hole opposite to the one they learned during the set-shifting task on the preceding day. Thus, if a rat was required to nosepoke in the left hole during the set-shifting task, then it had to nosepoke in the right hole in the reversal learning task, regardless of the position of the visual cue. The other aspects of the training were identical to the response discrimination training day. One of the visual-cue lights was illuminated prior to the start of each trial and served as a distracting stimulus.

Error analysis

We performed an error analysis of the set-shifting and reversal-learning data as reported previously (Ragozzino and Choi, 2004; Floresco et al., 2008; Ghods-Sharifi et al., 2008). A perseverative error occurred when a rat responded in a nosepoke hole while the stimulus light was illuminated during trials in which the rat was required to nosepoke in the opposite hole. Eight of every 16 consecutive trials required the rat to respond in this manner. These types of trials were separated into consecutive blocks with four trials each. "Perseverative errors" were scored when a rat made such errors in three or more trials per block of four trials that required a nosepoke in the hole that did not have the stimulus light illuminated. When a rat made two or fewer perseverative errors in a block for the first time, all subsequent errors of this type were no longer counted as perseverative errors because at this point the rat was using the original strategy less than 75% of the time (as was used for the operant chamber-based procedures). Instead, these errors were now scored as "regressive errors". "Never-reinforced errors" were scored when a rat nosepoked in the incorrect hole in trials in which the visual-cue light was illuminated at the same hole that the rat was required to nosepoke during the set-shifting task. During the reversal learning session, errors were scored as nosepokes in the hole that was rewarded during the set-shifting task and were subdivided into perseverative and regressive errors using a procedure that was similar to the one used to analyze these types of errors during the set-shifting task. Perseverative errors were scored when a rat made three or more perseverative within a block of four trials, excluding the first block. When a rat made fewer than three errors within a block of four trials, all subsequent errors were scored as regressive errors.

MICROINFUSION PROCEDURE

Before testing set-shifting, reversal learning, or initial visual-cue learning, the rats received a bilateral intra-brain region injection of NASPM (1.25 μg per hemisphere, 5 μg/μl), AP5 (0.25 μg per hemisphere, 0.5 μg/μl), or their corresponding vehicle solution (0.9% NaCl solution). Saline or the antagonist was infused at a rate of 0.25 μl/100 s (NASPM) or 0.5 μl/100 s (AP5) by a microsyringe pump. The injection needles were left in place for an additional 1 min to allow for drug diffusion. Following the infusion, the rats were placed in the operant chamber. Behavioral testing commenced 5 min (NASPM) or 15 min (AP5) later. NASPM and AP5 were purchased from Sigma.

HISTOLOGY

After behavioral testing, the rats were sacrificed in a carbon dioxide chamber. The brains were removed, fixed in a 4% formalin solution, frozen, sliced into 50 μm coronal sections, mounted

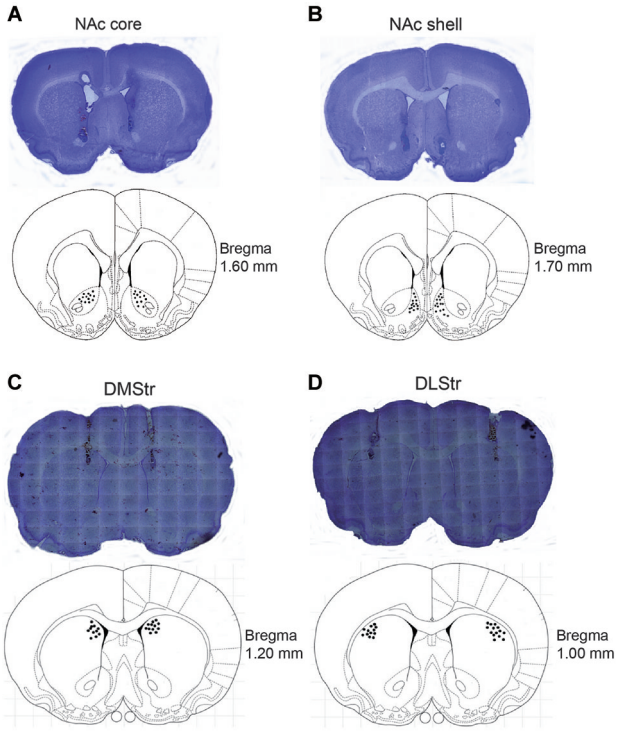

FIGURE 1 | Images showing cannula tracks and needle-tip locations in the NAc core (A), NAc shell (B), DMStr (C), and DLStr (D). After the behavioral tests, the rats were sacrificed. Brains were removed and fixed in a 4% formalin solution. The brain slices were collected in 50 μm coronal sections prior to being mounted and stained with Nissl staining solution. Images are shown on the top of each pattern. The distribution of the infusion sites was plotted on drawings of coronal sections from the Paxinos and Watson (1997) atlas. NAc, nucleus accumbens; DMStr, dorsomedial striatum; DLStr, dorsolateral striatum.

on slides, and stained with Cresyl violet. The needle placements were verified with reference to the neuroanatomical Paxinos and Watson (1997) rat brain atlas. **Figure 1** shows the approximate points of the bilateral infusions into the NAc core, NAc shell, DMStr, and DLStr. Data from rats with improper cannula placements were excluded from the analyses (9% of the total number of animals).

DATA ANALYSIS

The data were analyzed using GraphPad Prism 6.0 software. The numbers of trials and errors to reach criterion were separately analyzed using two-way analysis of variance (ANOVA) for each brain region, with Treatment as the within-subjects factor and Training phase as the between-subjects factor. Significant main effects in the ANOVA were followed by Bonferroni multiple-comparison *post hoc* tests. Subsequent targeted analyses that compared the numbers of each type of error were conducted using two-way ANOVAs. The effects of AP5 on simple learning were analyzed using two-tailed *t*-tests per NAc subregion. The criterion for statistical significance was set to $p < 0.05$.

RESULTS

EFFECTS OF BLOCKING NMDARs IN NAc SHELL AND CORE ON SET-SHIFTING AND REVERSAL LEARNING

Fifty-three rats were trained to obtain 20% sucrose solution by nosepoking the hole associated with the visual cue. Twenty-four hours later, 15 rats were infused with AP5 into the NAc core before the set-shifting task, and 36 rats were infused with the corresponding saline vehicle. Twenty-four hours after the set-shifting task, the rats that received saline treatment during the set-shifting task were divided into an AP5 infusion group (19 rats) and saline infusion group (17 rats) for the reversal learning task. The two-way ANOVA of these data revealed a significant effect of Treatment on both the number of trials to reach criterion ($F_{(1,83)} = 9.441$, $p < 0.01$; **Figure 2A**) and the number of errors before reaching the criterion ($F_{(1,83)} = 14.54$, $p < 0.001$; **Figure 2B**). Multiple-comparison analysis revealed that the rats that received AP5 infusion required significantly more trials ($p < 0.05$) and made more errors ($p < 0.01$) compared with saline infusion during reversal learning. AP5 infusion significantly increased the number of errors to reach criterion ($p < 0.05$) but not the number of trials to reach criterion ($p > 0.05$) during the set-shifting task. Subsequent analysis of the types of errors during the set-shifting task made by the rats that received AP5 revealed a significant effect of Treatment ($F_{(1,147)} = 4.392$, $p < 0.05$; **Figure 2C**). Multiple-comparison analysis indicated that this treatment increased the number of perseverative errors ($p < 0.05$) but not regressive errors or never-reinforced errors ($p > 0.05$). In the reversal learning task, the analysis of the types of errors also showed a significant effect of Treatment ($F_{(1,68)} = 5.624$, $p < 0.05$; **Figure 2D**). However, this difference resulted from an AP5-induced increase in the number of regressive errors ($p < 0.05$) and not perseverative errors ($p > 0.05$). Two subtypes of regressive errors were further analyzed by two-way ANOVA. The results showed a significance increase in the number of regressive errors toward the visual cue ($p < 0.05$) and a trend toward an increase in the number of regressive errors away from the visual cue.

The effects of AP5 infusion in the NAc shell on behavioral flexibility are shown in **Figure 2**. A total of 37 rats were assigned to either the saline or drug treatment group based on their performance during the visual-cue training. AP5 was infused into the NAc shell before the set-shifting task, 24 h after initial cue learning. Twenty-four hours later, half of the rats were infused with saline vehicle during the set-shifting task and infused with AP5 before the reversal learning task. The other half were administered saline. The analysis of these data revealed a significant effect of Treatment on the number of both trials ($F_{(1,55)} = 9.426$, $p < 0.01$; **Figure 2E**) and errors ($F_{(1,55)} = 9.96$, $p < 0.01$; **Figure 2F**) to reach criterion. Multiple-comparison analysis revealed that infusions of the NMDAR antagonist AP5 induced a weak effect on performance during the set-shifting task, whereas AP5 infusion produced a pronounced impairment in reversal learning, leading to a significant increase in the number of trials and errors to reach criterion (both $p < 0.05$). Subsequent targeted analysis of the types of errors made during the set-shifting task after AP5 infusion revealed a significant effect of Treatment ($F_{(1,105)} = 2.494$, $p < 0.05$; **Figure 2G**). Infusions of AP5 into the NAc shell

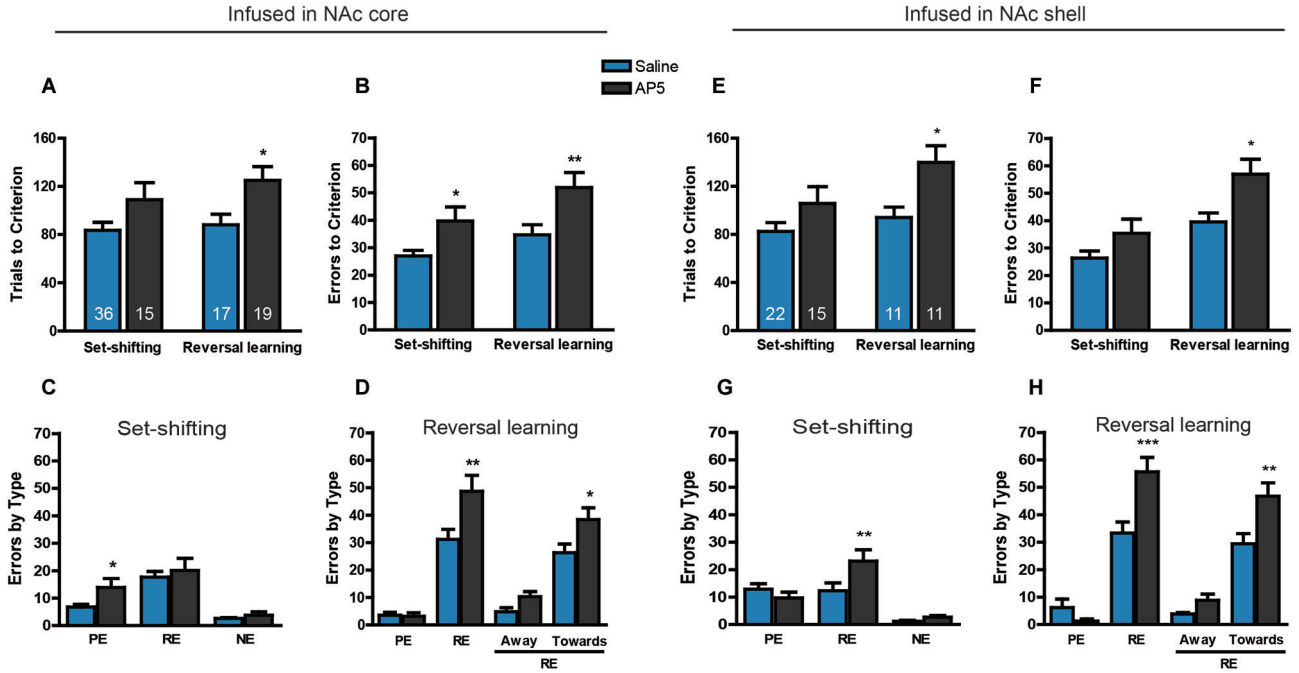

FIGURE 2 | Effects of AP5 infused into the NAc core and NAc shell on behavioral flexibility. The behavioral procedure included three phases: cue learning, set-shifting, and reversal learning. Set-shifting and reversal learning were tested 24 and 48 h, respectively, after cue learning. Each rat was infused with 0.5 μg (0.5 μl) AP5 (dissolved in saline) per side before the test. The sample size in each group is shown in the corresponding column. **(A)** Number of trials and **(B)** number of errors to reach criterion in the set-shifting and reversal learning tasks following intra-NAc core infusions. **(C,D)** Analysis of the types of errors during set-shifting **(C)** and reversal learning **(D)** in rats that received AP5 treatment in the NAc core. **(E)** Number of trials and **(F)** number of errors to reach criterion in the set-shifting and reversal learning tasks following intra-NAc shell infusions. **(G,H)** Analysis of the types of errors during set-shifting **(G)** and reversal learning **(H)** in rats that received AP5 treatment in the NAc shell. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PE, perseverative error; RE, regressive error; NE, non-reinforced error. During the reversal learning task, regressive errors were further divided into "away" and "toward" the visual cue.

increased the number of regressive errors ($p < 0.05$), without affecting perseverative or never-reinforced errors ($p > 0.05$). In the reversal learning task, the analysis of the types of errors also revealed a significant effect of Treatment ($F_{(1,40)} = 5.662$, $p < 0.05$; **Figure 2H**), with a robust increase in the number of regressive errors ($p < 0.001$). Two subtypes of regressive errors were analyzed by two-way ANOVA. The results showed a significance increase in the number of regressive errors toward the visual cue ($p < 0.05$) and a trend toward an increase in the number of regressive errors away from the visual cue.

To assess whether the blockade of NMDARs in the NAc affects the initial learning associated with visual-cue and spatial discrimination, we infused AP5 into the NAc core and shell, respectively, before initial training. The data were analyzed using separate *t*-tests for each kind of learning task based on each brain region. **Figure 3** shows that AP5 infusion in the NAc core or shell did not alter simple learning performance, regardless of cue-associated or spatial discrimination (all $t < 1.1$, no significant differences).

EFFECTS OF BLOCKING NMDARs IN THE DMStr AND DLStr ON SET-SHIFTING AND REVERSAL LEARNING

The effects of infusions of saline and AP5 into the DMStr and DLStr on set-shifting and reversal learning are presented in **Figure 4**. The experimental procedures were the same as those used in the NAc treatment experiments. After initial training, the set-shifting and reversal learning tasks were performed on the second and third days, respectively. AP5 was infused into the corresponding brain region before the behavioral tests. Rats that received infusions of the NMDAR antagonist AP5 in the DMStr required a comparable number of trials ($F_{(1,83)} = 0.3002$, $p > 0.05$; **Figure 4A**) and made a comparable number of errors ($F_{(1,83)} = 2.7956$, $p > 0.05$; **Figure 4B**) to reach criterion compared with controls. A nonsignificant trend toward an increase was observed with AP infusions during the reversal learning task, suggesting that the blockade of NMDARs in the DMStr mildly impaired intradimensional strategy-shifting ability without disrupting extradimentional strategy-switching. The analysis of the types of errors revealed no significant difference between groups in set-shifting ($F_{(1,66)} = 0.0822$, $p > 0.05$; **Figure 4C**) or reversal learning ($F_{(1,66)} = 0.1698$, $p > 0.05$; **Figure 4D**).

Similarly, no difference was found between the AP5-treated and control groups in the number of trials ($F_{(1,86)} = 0.6595$, $p > 0.05$; **Figure 4E**) or errors ($F_{(1,86)} = 0.5259$, $p > 0.05$; **Figure 4F**) to reach criterion. The analysis of the error data indicated that NMDAR blockade induced a statistically significant Treatment × Error type interaction in set-shifting

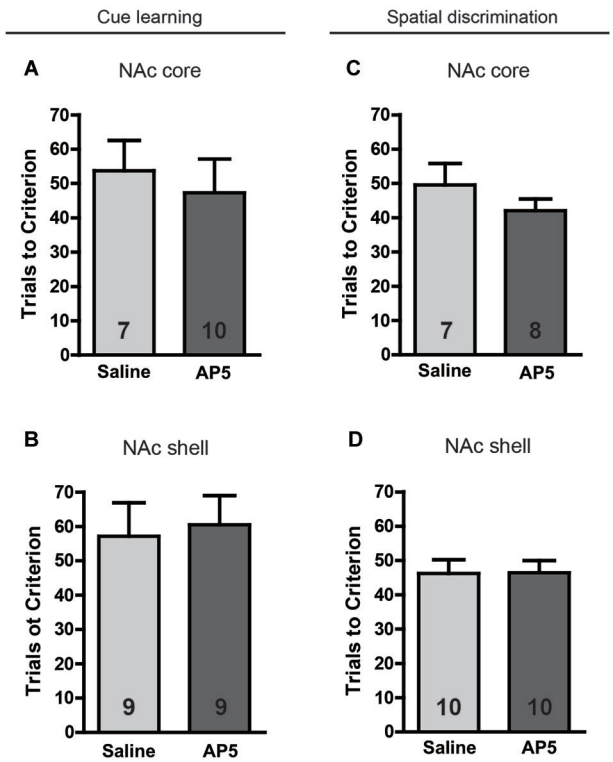

FIGURE 3 | No effect of AP5 infused into the NAc core or shell on initial visual-cue learning (A, NAc core; B, NAc shell) or spatial discrimination (C, NAc core; D, NAc shell). In this experiment, only association learning tasks were conducted. The rats were required to learn an association between a visual cue and reinforcement or between a spatial response and reinforcement. Before the behavioral test, 0.5 μg (0.5 μl) of an AP5 solution or saline vehicle was infused into the NAc core or shell per side. The sample size in each group is shown in the corresponding column.

($F_{(2,156)} = 0.0089$, $p < 0.01$; **Figure 4G**). Infusions of AP5 induced a pronounced increase in the number of perseverative errors ($p < 0.05$), without affecting regressive or never-reinforced errors.

EFFECTS OF BLOCKING GluA2-LACKING AMPARs IN THE STRIATUM ON SET-SHIFTING AND REVERSAL LEARNING

In this experimental session, NASPM was used to determine whether the blockade of GluA2-lacking AMPARs in different striatal subregions induces different strategy switching results. Twenty-four hours after initially learning the association between the visual cue and sucrose reinforcement, the set-shifting task was performed. The rats were required to learn a novel strategy to obtain the reward (i.e., by always nosepoking the left or right hole). The NASPM solution was infused into the NAc core, NAc shell, DMStr, or DLStr 5 min before testing in separate experiments. Twenty-four hours after the set-shifting task, half of the rats, which were infused with saline during the set-shifting task, were infused with NASPM solution before the reversal learning task. The other half of the rats were administered saline as a control. The results are presented in **Figures 5**, **6**, showing the number of trials to reach criterion and the types of errors. Infusions of NASPM in any striatal subregion did not significantly affect the number of trials to reach criterion (NAc core: $F_{(1,78)} = 0.2662$, $p > 0.05$, **Figure 5A**; NAc shell: $F_{(1,68)} = 2.64$, $p > 0.05$, **Figure 5E**; DMStr: $F_{(1,85)} = 0.0064$, $p > 0.05$, **Figure 6A**; DLStr: $F_{(1,86)} = 0.0005$, $p > 0.05$, **Figure 6E**). Similarly, NASPM infusion in any of the striatal subregions did not induce any changes in the number of errors to reach criterion (all $F < 1.9$, no significant differences). A subsequent analysis of the types of errors made during the set-shifting and reversal learning tasks revealed no difference between the NASPM- and saline-infused groups. These data indicate that Ca^{2+}-permeable AMPARs in the striatum do not play a crucial role in behavioral flexibility. The dose of NASPM used in the present study has been shown previously to affect reward-associated behavior (Carr et al., 2009). We may need to test doses of NASPM higher than 2.5 μg in our future studies, but such doses may carry the risk of affecting the reward system.

DISCUSSION

The present study revealed that the blockade of NMDARs in the NAc core impaired set-shifting performance by increasing perseverative errors. The suppression of NMDAR-mediated neurotransmission in the NAc shell induced a deficit in set-shifting by enhancing regressive errors. These findings indicate that the activation of NMDARs in the NAc core alters prior learning or inhibits responses that are no longer appropriate, whereas NMDARs in the NAc shell play an important role in learning and maintaining novel strategies. Reversal learning reflects intradimensional strategy switching and was tested 24 h after the set-shifting task. AP5 infusion in the NAc shell and

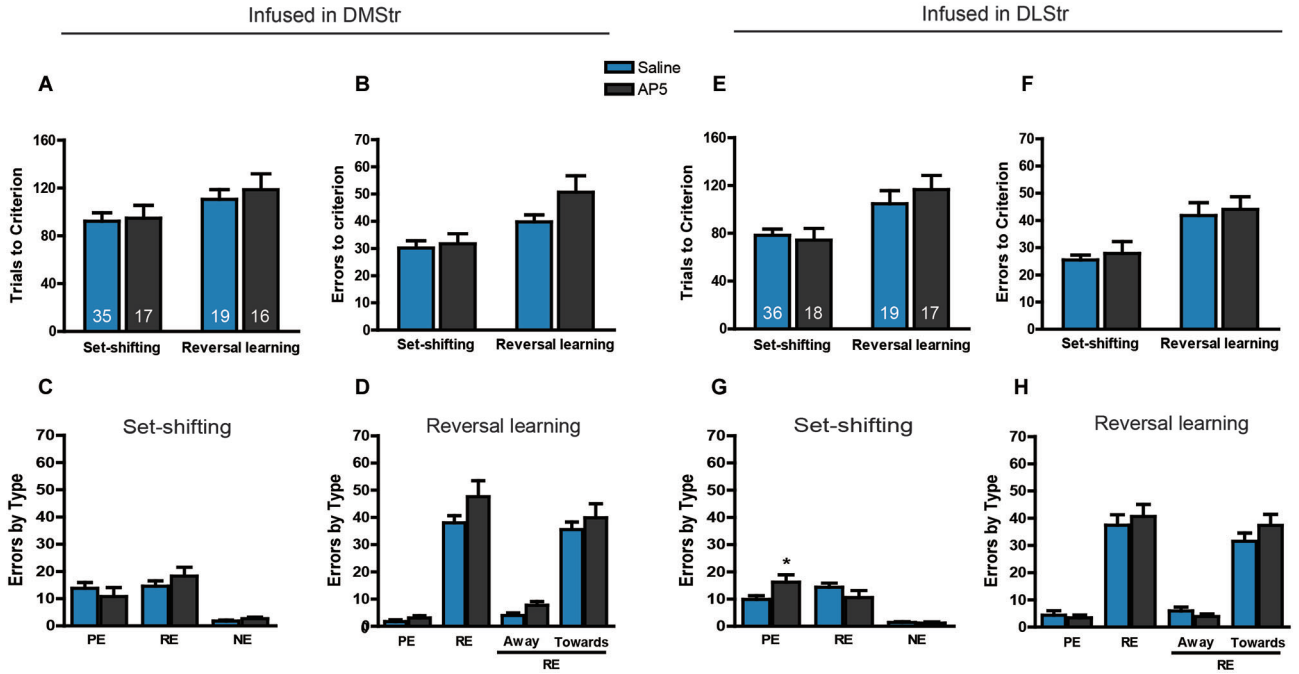

FIGURE 4 | Effects of AP5 infused into dorsomedial striatum and dorsolateral striatum on behavioral flexibility. The behavioral procedure included three phases: cue learning, set-shifting, and reversal learning. Set-shifting and reversal learning were tested 24 and 48 h, respectively, after cue learning. Each rat was infused with 0.5 μg (0.5 μl) AP5 (dissolved in saline) per side before the test. The sample size in each group is shown in the corresponding column. **(A)** Number of trials and **(B)** number of errors to reach criterion in the set-shifting and reversal learning tasks following intra-DMStr infusions. **(C,D)** Analysis of the types of errors during set-shifting **(C)** and reversal learning **(D)** in rats that received AP5 treatment in the DMStr. **(E)** Number of trials and **(F)** number of errors to reach criterion in the set-shifting and reversal learning tasks following intra-DLStr infusions. **(G,H)** Analysis of the types of errors during set-shifting **(G)** and reversal learning **(H)** in rats that received AP5 treatment in the DLStr. * $p < 0.05$. PE, perseverative error; RE, regressive error; NE, non-reinforced error. During the reversal learning task, regressive errors were further divided into "away" and "toward" the visual cue.

core significantly increased the total number of trials to reach criterion and the number of regressive errors, indicating that AP5-treated rats exhibited dysfunction in novel strategy learning or maintenance. In contrast, no significant alteration was induced by AP5 infusion into the DMStr or DLStr in the set-shifting or reversal learning task. We also tested the selective GluA2-lacking AMPAR antagonist NASPM to assess the effects of blocking GluA2-lacking AMPARs in different striatal subregions on behavioral flexibility. No effect was found during the set-shifting or reversal learning task.

Schizophrenia patients exhibit impaired cognitive flexibility measured by the Wisconsin Card Sorting Test (WCST; Everett et al., 2001). In the extradimensional shift stage, both schizophrenia patients and frontal-lesioned subjects exhibit pronounced deficits, whereas only schizophrenia patients make significantly more errors in the intradimensional reversal stage (Pantelis et al., 1999). This suggests that other brain regions, in addition to the frontal cortex, are abnormal in schizophrenia patients. The striatum may be an important target among these regions. Subjects with schizophrenia exhibit striatal dysfunction during reward-related reversal learning (Schlagenhauf et al., 2014), and the striatum receives excitatory projections from the frontal cortex (Reynolds and Zahm, 2005; Xu and Südhof, 2013).

By microinjecting γ-aminobutyric acid receptor agonists to inactivate the NAc, Floresco et al. (2006) demonstrated dissociable contributions made by neurons in the NAc core and shell to behavioral flexibility during the set shifting task. These authors found that set-shifting was impaired after inactivation of the NAc core but not shell, attributable to disruption of the acquisition and maintenance of a new strategy. However, we only blocked NMDAR-mediated transmission, which may be a major reason for the discrepant results between the study by Floresco et al. and the present study. In addition to receptor targets, the discrepancies between these two studies may be attributable to differences in the training protocols. An automated procedure was used in the present study, which decreases the likelihood of experimental bias and increases consistency in timing and stimulus intensity compared with the cross-maze setup that was utilized by Floresco et al. The NAc core receives projections from the prelimbic cortex (PL), whereas the NAc shell receives projections from the infralimbic cortex (IL; Voorn et al., 2004). Lesions of the PL impaired set-shifting performance by inducing deficits in inhibiting the relevant learned strategy. The IL may support behavioral flexibility by maintaining the reliable execution of a new choice pattern (Birrell and Brown, 2000; Ragozzino, 2007). In contrast to the differential roles of NMDAR activation between NAc subregions

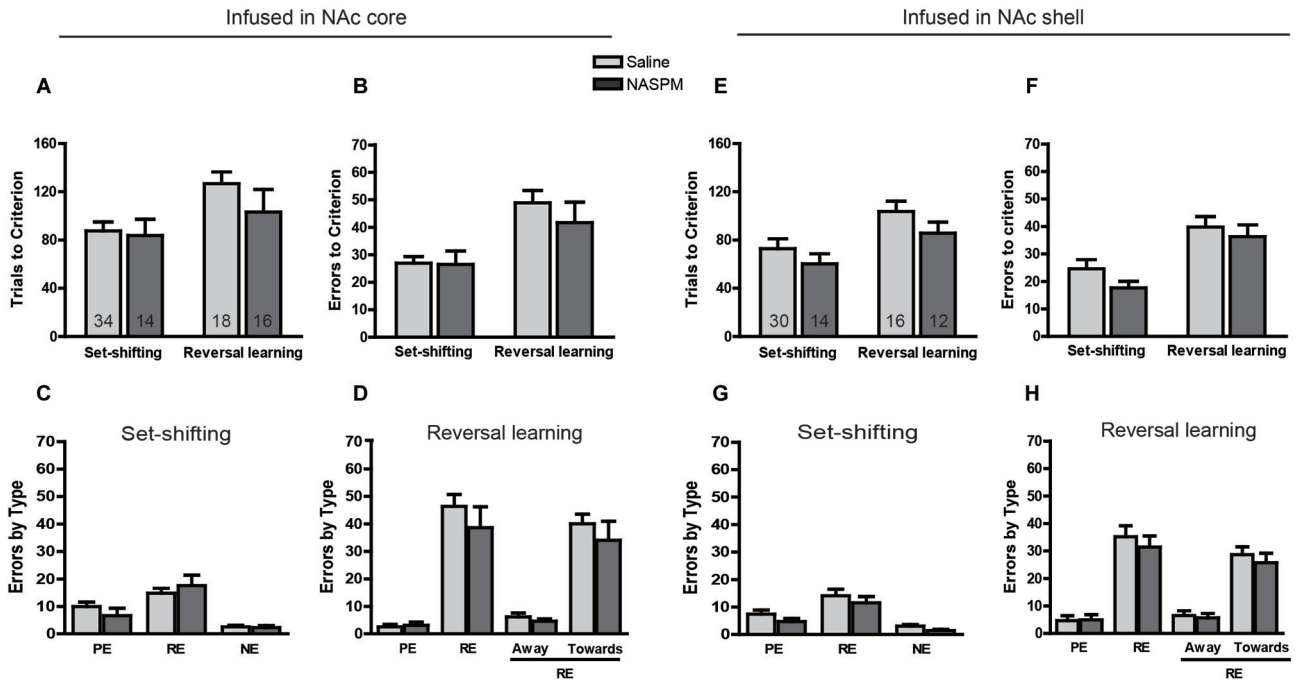

FIGURE 5 | Effects of NASPM infused into the NAc core and NAc shell on behavioral flexibility. The behavioral procedure included three phases: cue learning, set-shifting, and reversal learning. Set-shifting and reversal learning were tested 24 and 48 h, respectively, after cue learning. Each rat was infused with 1.25 μg (0.25 μl) NASPM (dissolved in saline) per side before the test. The sample size in each group is shown in the corresponding column. **(A)** Number of trials and **(B)** number of errors to reach criterion in the set-shifting and reversal learning tasks following intra-NAc core infusions. **(C,D)** Analysis of the types of errors during set-shifting **(C)** and reversal learning **(D)** in rats that received NASPM treatment in the NAc core. **(E)** Number of trials and **(F)** number of errors to reach criterion in the set-shifting and reversal learning tasks following intra-NAc shell infusions. **(G,H)** Analysis of the types of errors during set-shifting **(G)** and reversal learning **(H)** in rats that received NASPM treatment in the NAc shell. No changes were found in the set-shifting or reversal learning phase. PE, perseverative error; RE, regressive error; NE, non-reinforced error. During the reversal learning task, regressive errors were further divided into "away" and "toward" the visual cue.

in extradimensional strategy shifting, NMDARs in both the NAc shell and core make the same contribution to intradimensional strategy switching. The activation of NMDARs in the NAc may facilitate the learning and maintenance of new strategies. In the present study, the rats that underwent reversal learning testing were previously subjected to the set-shifting task. However, the impaired ability in spatial reversal induced by NMDAR blockade did not result from alterations in set-shifting because no interaction was found between "away" and "toward" visual-cue regressive errors. An impaired ability in reversal learning has been previously reported in studies in which the ventral striatum and dorsal striatum were subjected to excitotoxic lesions (Ferry et al., 2000). Similar deficits are also found with lesions and inactivation of the OFC (Chudasama and Robbins, 2003; Boulougouris et al., 2007; Ghods-Sharifi et al., 2008). An interaction has been suggested to exist between striatum and OFC functions in reversal learning (Frank and Claus, 2006). However, the modulation of reversal learning ability by the OFC depends on task difficulty. When the reversal task was performed between two strategies, inactivation or lesions of the OFC produced perseveration (Bohn et al., 2003; Chudasama and Robbins, 2003; Boulougouris et al., 2007). In contrast, OFC inactivation increased both perseverative and regressive errors when four-choice odor discrimination was required (Ragozzino, 2007). In the present study, reversal learning based on an operant task may be more difficult than two-choice patterns. Thus, the blockade of NMDARs in the NAc increased regressive errors in the present study. Although an impaired ability in working memory has been demonstrated in some cases of cognitive inflexibility (Blot et al., 2013; Darvas et al., 2014), working memory performance in the present study did not appear to be altered by blocking NMDARs in the NAc. No significant changes were found in the cue-learning task or spatial response when AP5 was microinjected into the NAc shell or core.

The dorsal striatum contributes less to flexible behaviors compared with the ventral striatum, although impaired reversal learning performance suggests that NMDAR-mediated glutamate transmission in the DMStr plays a role in behavioral flexibility by at least partially supporting a shift in intradimensional strategies. Similar results have been reported in previous studies that used a reversal learning task (Palencia and Ragozzino, 2004, 2005). However, inconsistent results were found in the set-shifting task with DMStr treatment between the present study and previous reports. We did not observe any apparent change in the set-shifting task when NMDAR activation was blocked in the DMStr. Ragozzino et al. inactivated the DMStr based on a cross-maze experimental design and found that this region mediates

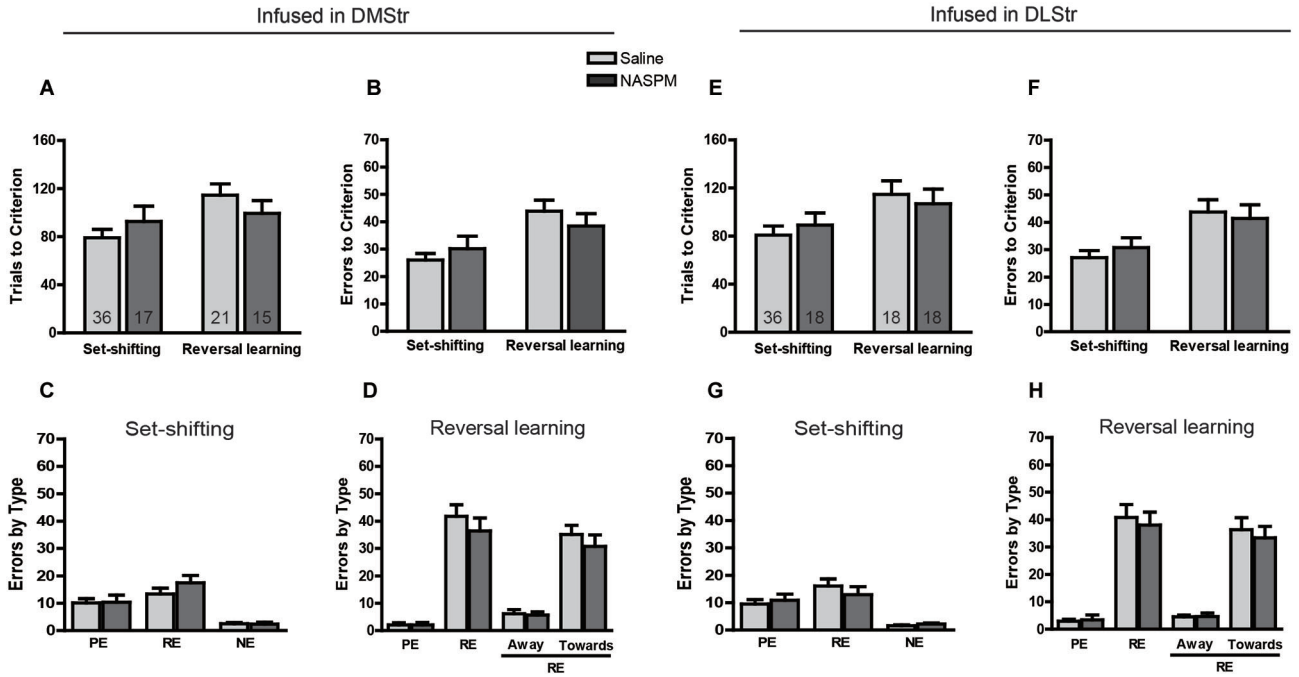

FIGURE 6 | Effects of NASPM infused into the dorsomedial striatum and dorsolateral striatum on behavioral flexibility. The behavioral procedure included three phases: cue learning, set-shifting, and reversal learning. Set-shifting and reversal learning were tested 24 and 48 h, respectively, after cue learning. Each rat was infused with 1.25 μg (0.25 μl) NASPM (dissolved in saline) per side before the test. The sample size in each group is shown in the corresponding column. **(A)** Number of trials and **(B)** number of errors to reach criterion in the set-shifting and reversal learning tasks following intra-DMStr infusions. **(C,D)** Analysis of the types of errors during set-shifting **(C)** and reversal learning **(D)** in rats that received NASPM treatment in the DMStr. **(E)** Number of trials and **(F)** number of errors to reach criterion in the set-shifting and reversal learning tasks following intra-DLStr infusions. **(G,H)** Analysis of the types of errors during set-shifting **(G)** and reversal learning **(H)** in rats that received NASPM treatment in the DLStr. No changes were found in the set-shifting or reversal learning phases. PE, perseverative error; RE, regressive error; NE, non-reinforced error. During the reversal learning task, regressive errors were further divided into "away" and "toward" the visual cue.

the maintenance of novel strategies after perseveration ceases (Ragozzino et al., 2002). In the DLStr, the increase in the number of perseverative errors in the set-shifting task may be attributable to the modulatory role of this region in stimulus-response habits (Jog et al., 1999).

NMDAR-dependent long-term depression (LTD) and synaptic changes can be modulated by endocytosis of GluA2-containing AMPARs (Lim et al., 2012), suggesting that GluA2-lacking AMPARs may replace GluA2-containing AMPARs during learning. However, no deficit was observed after NASPM treatment, indicating that the activation of GluA2-lacking AMPARs is not a downstream event of NMDAR-induced transmission involved in flexible behaviors. AMPAR antagonism with LY293558 in the mPFC induced general cognitive deficits (Stefani et al., 2003), and infusions of the AMPAR antagonist DNQX into the ventral striatum impaired spatial information processing in a nonassociative task (Roullet et al., 2001). These previous studies suggest that AMPARs may be involved in simple learning and general cognition rather than specifically cognitive execution. Under physiological conditions, most AMPARs in the striatum are either GluA1/2 or GluA2/3, whereas <10% are homomeric GluA1 or GluA1/3 (Boudreau et al., 2007). Furthermore, although the endocytosis of GluA2-containing AMPARs is a mechanism associated with synaptic long-term plasticity during learning (Liu and Zukin, 2007), other forms of plasticity may also be involved in this associative task, such as mGluR-dependent patterns (Anwyl, 2009).

In summary, pharmacological tools associated with glutamate transmission may provide a potential clinical treatment for schizophrenia. In addition to the frontal cortex, the ventral striatum may be another important brain region for adjusting behavioral flexibility. These findings suggest that NMDAR-mediated glutamate transmission in the ventral striatum makes more of a contribution to cognitive execution than the dorsal striatum.

AUTHOR CONTRIBUTIONS

Xuekun Ding, Yanhua Qiao, and Chenji Piao performed experiments, analyzed data and wrote the manuscript. Xigeng Zheng and Zhengkui Liu managed the literature searches and analyses. Jing Liang designed the study, wrote the protocol and finalized the manuscript. All authors contributed to and have approved the final manuscript and agreed to be accountable for all aspects of the work.

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Homocysteine levels in schizophrenia and affective disorders—focus on cognition

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Although homocysteine (Hcy) has been widely implicated in the etiology of various physical health impairments, especially cardiovascular diseases, overwhelming evidence indicates that Hcy is also involved in the pathophysiology of schizophrenia and affective disorders. There are several mechanisms linking Hcy to biological underpinnings of psychiatric disorders. It has been found that Hcy interacts with NMDA receptors, initiates oxidative stress, induces apoptosis, triggers mitochondrial dysfunction and leads to vascular damage. Elevated Hcy levels might also contribute to cognitive impairment that is widely observed among patients with affective disorders and schizophrenia. Supplementation of vitamins B and folic acid has been proved to be effective in lowering Hcy levels. There are also studies showing that this supplementation strategy might be beneficial for schizophrenia patients with respect to alleviating negative symptoms. However, there are no studies addressing the influence of add-on therapies with folate and vitamins B on cognitive performance of patients with schizophrenia and affective disorders. In this article, we provide an overview of Hcy metabolism in psychiatric disorders focusing on cognitive correlates and indicating future directions and perspectives.

Keywords: homocysteine, depression, bipolar disorder, schizophrenia, hyperhomocysteinemia, cognition, brain substrates

INTRODUCTION

Homocysteine (Hcy) is one of the non-protein amino acids that is produced in one-carbon metabolism. Two enzymatic pathways are involved in Hcy metabolism—re-mehtylation to methionine and trans-sulfuration to cysteine and taurine. The efficiency of Hcy catabolism depends on the availability of folate, vitamin B12 and vitamin B6. Tans-sulfuration to cysteine, which forms glutathione, is catalyzed by cystathionine beta synthase (CBS) and cystathionase. In turn, conversion from Hcy to methionine is a multistep reaction with a number of enzymes being involved including Hcy methyltransferase, methionine synthase (MS) and methionine synthase reductase (MTRR), as well as the methylenetetrahydrofolate reductase (MTHFR; Scott and Weir, 1998). There are two common polymorphisms located in the *MTHFR* gene—C677T and A1298C that may lower the activity of MTHFR and lead to increased Hcy levels. The most common one—C677T polymorphism, which is present in 10–12% of population (Gilbody et al., 2007), contributes to the expression of a thermolabile variant of MTHFR. Other factors might also increase Hcy level including higher age, male gender, cigarette smoking, alcohol abuse or dependence, low dietary intake of folate and vitamins B, renal dysfunction and certain medications (e.g., sodium valproate and lamotrigine, diuretics, fibrates) (Frankenburg, 2007). In addition, there is an inverse relationship between Hcy and both folate and vitamin B12 levels (Yoshino et al., 2010).

Several lines of evidence indicate that Hcy serves as an important atherosclerotic factor. It has been found that Hcy may induce vascular damage via initiating oxidative stress and reducing the availability of nitric oxide that is a powerful vasodilator (Perna et al., 2003). These mechanisms underlie well-established links between elevated Hcy levels or *MTHFR* polymorphisms and cardiovascular diseases including coronary artery disease, myocardial infarction, cerebrovascular disease and peripheral occlusive disease (Mangoni and Jackson, 2002; Trimmer, 2013).

In the recent years, there is a growing interest in the causative links between Hcy and neuropsychiatric disorders. High Hcy levels are increasingly recognized as a risk factor for age-related cognitive deficits together with various types of dementia (Stanger et al., 2009). Studies in this field have provided several links between Hcy and domains of cognitive functioning (Faux et al., 2011; Kim et al., 2013). However, less attention has been paid to cognitive correlates of elevated Hcy level in psychiatric disorders including schizophrenia and affective disorders. In this article, we review the role of Hcy in the pathophysiology of psychiatric disorders including schizophrenia and affective disorders focusing on cognitive correlates.

MECHANISMS OF HOMOCYSTEINE ACTION—THE RELEVANCE TO PSYCHIATRIC DISORDERS

The exact neural and behavioral mechanism of Hcy action is not known. It seems that the interaction of Hcy with glutamatergic transmission is the most relevant mechanism explaining the association between Hcy and schizophrenia or affective disorders. Both Hcy and its oxidative metabolite—homocysteic acid—serve as agonists within NMDA receptors (Klancnik et al., 1992; Zhang and Lipton, 1992; Lipton et al., 1997). Stimulation of NMDA receptors by Hcy increases calcium influx that exerts neurotoxic effects (Ho et al., 2002). However, in the presence of low concentrations of glycine, Hcy acts as a partial antagonist within the glycine site of NMDA receptors. Thus, in case of low glycine level Hcy manifests its neuroprotective activity (Lipton et al., 1997) and only high Hcy concentrations may be toxic. On the other hand, when glycine levels are high (after head trauma or stroke), low Hcy levels become toxic (Alam et al., 1998). This dual action of Hcy within NMDA receptors may explain why elevated Hcy levels might be implicated in schizophrenia, in which hypofunction of glutamatergic transmission has been reported and depression that is characterized by up-regulated glutamatergic activity.

Also, various studies have suggested that Hcy might regulate the function of other neuromodulators, such as acetylcholine (Chen et al., 2011) and dopamine, and serotonin (Gao et al., 2011). Specifically, Gao et al. (2011) have reported that rats with hyperhomocysteinemia have lower levels of dopamine and serotonin in the cortex than control rats. Other studies suggest that Hcy regulates synaptic plasticity in the hippocampus (Christie et al., 2005; Algaidi et al., 2006). These prior studies suggest that Hcy has multiple functions in the brain; this can likely explain its links to various psychiatric disorders, including schizophrenia and affective disorders.

Animals exposed to Hcy exhibit compromised brain energy metabolism (Streck et al., 2003), altered long-term potentiation, disturbances of synaptic plasticity and cognitive impairment in terms of spatial learning (Algaidi et al., 2006) and memory deficits (Streck et al., 2004). Heterozygous and homozygous *Mthfr* knockout mice are also characterized by neurodevelopmental retardation and altered cerebellar morphology (Chen et al., 2001). Other mechanisms of Hcy toxicity that might be relevant to the pathophysiology of schizophrenia and affective disorders include oxidative stress (Koz et al., 2010; Loureiro et al., 2010; Dietrich-Muszalska et al., 2012), neuronal apoptosis (Wang et al., 2012), vascular damage (Brown et al., 2007) and aberrant DNA methylation (Bromberg et al., 2008, 2009; Kinoshita et al., 2013; **Figure 1**). Neural studies have shown that Hcy acts on various brain regions, including the hippocampus (den Heijer et al., 2003; Matté et al., 2009; Chen et al., 2011), cortex (den Heijer et al., 2003), and the basal ganglia (Genedani et al., 2010). Higher Hcy levels lead to atrophy in the frontal, parietal, and temporal areas (Rajagopalan et al., 2011).

HOMOCYSTEINE AND COGNITION IN HEALTHY INDIVIDUALS

Homocysteine plays an important role in behavioral and cognitive processes as shown in studies measuring Hcy levels in healthy elderly subjects (Prins et al., 2002; Dufouil et al., 2003; Teunissen et al., 2003; Nurk et al., 2005; Feng et al., 2006; Hooshmand et al., 2012). For example, van den Kommer et al. (2010) reported that higher Hcy levels are associated with slow information processing speed in healthy participants. Further, Nurk et al. (2005) found

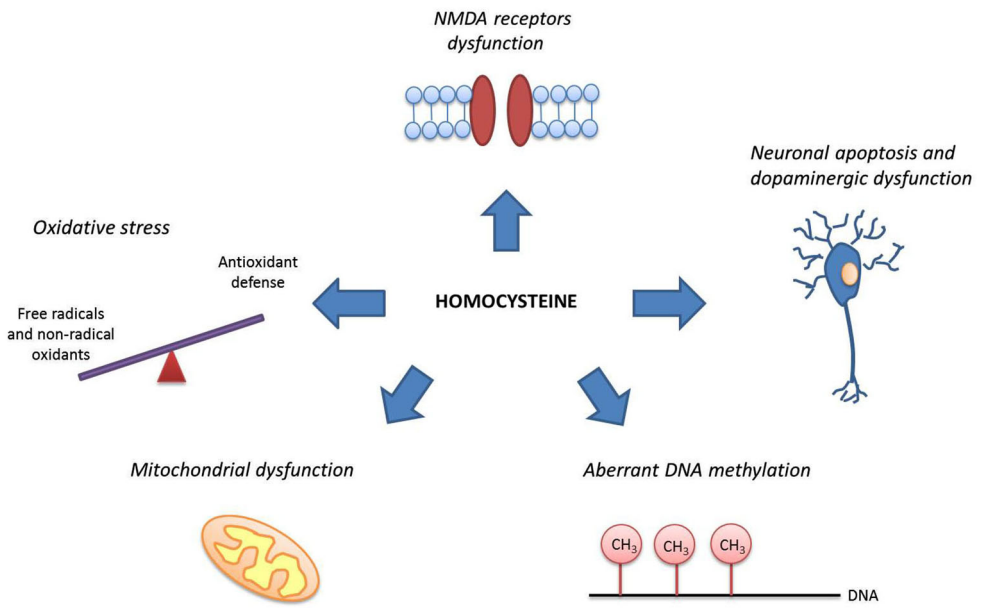

FIGURE 1 | Mechanisms of homocysteine action as relevant to neurological and psychiatric disorders. Homocysteine may interact with NMDA receptors altering glutamatergic transmission, exert toxic effects on dopaminergic neurons, initiate neuronal apoptosis, induce oxidative stress, lead to mitochondrial dysfunction and influence DNA methylation altering gene expression.

that impaired episodic memory performance is associated with increased Hcy levels in healthy individuals. Along the same lines, Garcia et al. (2004) revealed that impaired performance in the Stroop test correlates with higher levels of Hcy. Studies on the role of Hcy in cognitive performance in healthy subjects have shown that Hcy is specifically involved in episodic memory (Faux et al., 2011; Narayan et al., 2011), spatial learning (Pirchl et al., 2010), reversal learning (Christie et al., 2005; Algaidi et al., 2006), and executive function (Narayan et al., 2011). However, it is debatable whether Hcy plays a role in working memory processes, as some studies have found they are not related (Narayan et al., 2011), while other studies found that lowering Hcy levels enhances working memory (Macpherson et al., 2012).

Recently published results reveal associations between total Hcy levels and cognitive functions in healthy subjects. It has been found that lower overall cognitive performance measured by Cambridge Cognitive Examination (CAMCOG) are associated with higher Hcy levels (Budge et al., 2002). This study also revealed an inverse correlation between hippocampal volume and Hcy levels (Budge et al., 2002). Other studies have found a positive correlation between total Hcy levels and ventricle-brain ratios in the anterior and middle ventricular regions in elderly participants (Sachdev et al., 2002). It has also been reported that higher Hcy levels are associated with lower scores in Mini Mental State Examination (MMSE; Kalmijn et al., 1999). It has been demonstrated that impaired cognition in elderly participants correlates with Hcy levels, especially for psychomotor speed and memory functions (Prins et al., 2002).

Recent data show that higher Hcy levels are associated with silent brain infarctions and subcortical white matter lesions in older adults (Vermeer et al., 2002). Higher Hcy levels have been associated with increased prevalence of silent brain infarction and decreased brain volume in comparison with subjects having lower total Hcy (Morris, 2003).

HOMOCYSTEINE IN PSYCHIATRIC DISORDERS

Total Hcy level changes have also been shown to be associated with many psychiatric disorders, including schizophrenia and affective disorders. These observations stimulated further studies on the association between elevated Hcy levels and neuropsychiatric symptoms and disorders.

Patients having cognitive disorders and depression have been reported in many studies to have low vitamin B12 and folate levels. In 1980, an important finding by Shorvon et al. (1980) was published on the neuropsychiatric manifestations in megaloblastic anemia that occurred due to low folate or vitamin B12 levels. Their study revealed that up to 56% of patients with affective disorders have serum folate deficiency (Shorvon et al., 1980). Below, we describe the relationship between changes in Hcy levels and schizophrenia, depression, and bipolar disorder.

SCHIZOPHRENIA

In 1975, Freeman et al. (1975) described a case of homocystinuria, caused by a deficient MTHFR activity, accompanied by psychotic-like behavior that responded to folate treatment. More recently, a new hypothesis for the development of schizophrenia has been proposed—the DNA polymorphism-diet-cofactor-development (DDCD) hypothesis (Johnson, 1999). This hypothesis states that mutations of genes related to folate and vitamins B metabolism potentiated by maternal dietary vitamin deficiencies contribute to the development of schizophrenia. Total Hcy serum levels in schizophrenia were first measured by Regland et al. (1995). In this study, elevated Hcy levels were found in 9 out of 20 schizophrenic patients (Regland et al., 1995).

Subsequently, elevated total Hcy levels have been widely described in various subgroups of schizophrenia patients (Muntjewerff et al., 2006; Nishi et al., 2014) including drug-naïve first-episode psychosis subjects (Kale et al., 2010; Ayesa-Arriola et al., 2012; García-Bueno et al., 2013) and chronic schizophrenia patients (Eren et al., 2010). Total Hcy level has been found to negatively correlate with folate and vitamin B12 levels in this group of patients (Bouaziz et al., 2010). In addition, some authors have found that Hcy levels are higher especially in young male schizophrenia patients (Levine et al., 2002). It has also been estimated that a 5-μmol increase in plasma Hcy level may increase the risk of schizophrenia by 70% (Muntjewerff et al., 2006). Several studies have proved a positive correlation between Hcy levels and the severity of schizophrenia negative symptoms (Goff et al., 2004; Petronijević et al., 2008; Bouaziz et al., 2010; Misiak et al., 2014). These studies are in concordance with the studies showing a negative correlation between duration of untreated psychosis (DUP) and Hcy levels (Ayesa-Arriola et al., 2012; Misiak et al., 2014). The association of increased Hcy levels with schizophrenia psychopathology has provided grounds for add-on therapies with vitamin supplementation (Hill et al., 2011; Roffman et al., 2013). The largest randomized, double-blind and placebo-controlled study of folic acid and vitamin B12 supplementation revealed the improvement of negative symptoms in schizophrenia patients. However, this supplementation strategy was effective only in patients being homozygotes of the 484T > C polymorphism in the *FOLH1* gene that encodes folate hydrolase involved in intestinal folate transport (Roffman et al., 2013).

Elevated Hcy levels found in first-episode psychosis patients suggest that one-carbon metabolism alterations may share common genetic underpinnings with schizophrenia. Another proof for this assumption is that siblings of schizophrenia patients are also characterized by increased plasma Hcy levels (Geller et al., 2013) and schizophrenia patients with positive family history of schizophrenia in first or second degree relatives have significantly higher Hcy levels compared to those with negative family history of schizophrenia (Misiak et al., 2014). Several studies have reported an association of two common polymorphisms in the *MTHFR* gene (C677T and A1298C) with schizophrenia (Lewis et al., 2005; Muntjewerff et al., 2006; Gilbody et al., 2007; Shi et al., 2008). Furthermore, these polymorphisms have been found to predict the development of metabolic syndrome following the treatment with antipsychotics or at least might be associated with increased incidence of metabolic disturbances, such as visceral obesity, impaired metabolism of glucose and lipids (Misiak et al., 2013). Furthermore, schizophrenia patients with the comorbid metabolic syndrome are characterized by higher Hcy levels in comparison with those, who do not meet the criteria of metabolic syndrome (Vuksan-Ćusa et al., 2011, 2013).

Although the *MTHFR* gene polymorphisms are known to influence the risk of metabolic adverse effects of antipsychotics, the influence of antipsychotic treatment on Hcy requires further investigation due to scarcity of well-designed studies. There is only one observational study on drug-naïve first episode schizophrenia patients showing the lack of significant changes in Hcy levels in the course of antipsychotic pharmacotherapy (Bicikova et al., 2011). Another study on acutely relapsed schizophrenia patients has revealed significantly higher Hcy levels during symptomatic exacerbation than during the remission phase (Petronijević et al., 2008). In turn, the cross-sectional study by Eren et al. (2010) on chronic schizophrenia patients revealed significantly lower levels of plasma folate, but not Hcy or vitamin B12, in patients receiving higher doses of typical antipsychotics (chlorpromazine equivalent >400 mg). Another cross-sectional study revealed no significant difference in Hcy level between schizophrenia patients receiving clozapine in monotherapy and healthy controls (Wysokiński and Kłoszewska, 2013). There is also one study showing a positive relationship between Hcy levels and N-desmethyl-olanzapine concentration that is one of the main olanzapine metabolites (Lu et al., 2013). These inconsistent results might be attributed to heterogenous methodology such as the recruitment of different patients defined by illness duration or symptomatic presentation, as well as the lack of adjustment for possible confounders including the *MTHFR* genotype, dietary habits, cigarette smoking or other known factors influencing Hcy metabolism.

Several studies have established direct links between the *MTHFR* gene polymorphisms and cognitive dysfunction in terms of executive function and blunted response to errors in schizophrenia. It has been found that the 677T variant of the *MTHFR* gene induces a dose-dependent blunting of dorsal anterior cingulate cortex activation in response to errors using the antisaccade paradigm (Roffman et al., 2011b), positively correlates with impairments of verbal fluency (Roffman et al., 2007) and interacts with the 108Val allele in the *COMT* gene increasing the number of perseverative errors on the Wisconsin Card Sorting Task (WCST; Roffman et al., 2008b). Although the *MTHFR* gene variants have been reported to influence certain domains of cognitive functioning in schizophrenia patients, Hcy levels have not been found to correlate with cognitive impairment in first-episode schizophrenia spectrum disorders patients (Ayesa-Arriola et al., 2012). These discrepancies suggest that other Hcy-independent consequences of one-carbon metabolism dysfunction due to genetic factors are implicated in the occurrence of cognitive impairment in schizophrenia. Given that the 677T allele in the *MTHFR* gene is associated with lower genomic DNA methylation (Friso et al., 2002), it might be hypothesized that epigenetic phenomena are involved in cognitive impairment in schizophrenia. Furthermore, the 677T variant enhances dopamine metabolism (Roffman et al., 2008a,b), which is linked to schizophrenia pathophysiology and is implicated in the activation of dorsal anterior cingulate cortex in response to errors (Holroyd and Coles, 2002) and influences prefrontally-mediated executive functioning (Tan et al., 2007).

DEPRESSION

Several studies have established that depressive episodes may predict the development of cardiovascular diseases (de Jonge et al., 2014). These findings suggest that depression is linked to co-occurring metabolic deregulation increasing cardiovascular risk. Indeed, elevated Hcy levels have been shown in major depression (Folstein et al., 2007; Yapislar et al., 2012; Delport et al., 2014; Lok et al., 2014). Notably, it has been found that Hcy level negatively correlates with vitamin B12 and folate levels in depressed patients (Ebesunun et al., 2012). There are also studies showing that the *MTHFR* C677T polymorphism may increase the susceptibility to major depression (Wu et al., 2013; Delport et al., 2014; Lok et al., 2014; Shen et al., 2014). Interestingly, it has been found that the *MTHFR* 677T allele may interact with childhood traumatic events influencing the time to recurrence in major depressive disorder (Lok et al., 2013). Indeed, the carriers of the *MTHFR* 677T allele with childhood traumatic events had shorter time to recurrence of major depressive disorder in comparison with those without such events. These findings corroborate emerging evidence indicating that posttraumatic stress disorder (PTSD) patients are also characterized by elevated Hcy levels (Levine et al., 2008; Jendricko et al., 2009).

In the recent study with older adults, it was found that serum folate levels correlate with the severity of depressive symptoms (Ebly et al., 1998). In studies that failed to prove an association between low serum folic acid and depression, there was a negative correlation between folate level and the duration of the depressive episode, or a negative correlation between folate level and length of hospitalization and therefore with treatment outcome. Regarding the severity of depression, patients with lower folate levels were more severely depressed than those with normal folate levels (Alpert et al., 2000). In the Womens Health and Aging Study, low vitamin B12 levels were reported in elderly disabled community participants and significant vitamin B12 deficiency was more common among depressed than healthy participants. Significant vitamin B12 deficiency was associated with a two-fold higher risk of developing severe depression (Penninx et al., 2000). Interestingly, in the study by Gabryelewicz et al. (2007), depression and higher baseline Hcy levels were the strongest predictors of conversion from mild cognitive impairment (MCI) to dementia. Elevated total Hcy levels were also observed in the study of 213 patients with major depression compared with controls (Fava et al., 1997). S-Adenosyl Methionine (SAM), a precursor of Hcy, is used in some countries as an effective adjuvant therapy in the treatment of depression. On the basis of a meta-analysis, Bressa (1994) also suggested that SAM can act as an antidepressive agent. S-Adenosyl Methionine has also been found to be effective in the treatment of depression related to Parkinsons disease (Di Rocco et al., 2000). Studies on patients with geriatric depression have revealed correlations between Hcy and cognitive performance. In this group of patients, Hcy level positively correlated with language processing and processing speed (Alexopoulos et al., 2010).

BIPOLAR DISORDER

Although elevated Hcy levels have been repeatedly reported in bipolar disorder patients (Baek et al., 2013), no significant

differences have been found across various mood states (Chiarani et al., 2013). Studies on bipolar disorder indicate that high Hcy levels are significantly more frequent among males than females with bipolar depressive episode (Permoda-Osip et al., 2013b, 2014b). Similarly to schizophrenia and major depression patients, an inverse relationship between Hcy and both folate vitamin B12 levels has been demonstrated in bipolar disorder subjects (Permoda-Osip et al., 2013b). However, it has been found that Hcy level negatively correlates with the level of endothelial damage markers including E-selectin and intracellular adhesion molecule-1 (ICAM-1) in bipolar depression subjects suggesting that the pathways of cardiovascular risk are not associated with Hcy metabolism in this group of patients (Permoda-Osip et al., 2013b).

As similar to schizophrenia, two common polymorphisms in the *MTHFR* gene (C677T and A1298C) might increase the risk of bipolar disorder and predict the development of comorbid metabolic syndrome suggesting the existence of common genetic underpinnings (Peerbooms et al., 2011; Ellingrod et al., 2012). There is also one study showing an association between the T833C polymorphism in the *CBS* gene and bipolar disorder risk (Permoda-Osip et al., 2014a).

However, in contrast to studies on schizophrenia, evidence for the influence of Hcy on cognition is more convincing. There are studies showing an inverse relationship between plasma Hcy and verbal learning, executive function or immediate memory in euthymic bipolar disorder patients (Dittmann et al., 2007, 2008; Osher et al., 2008). It should be noted that two studies consistently reported the correlation between Hcy levels and executive functioning measured in terms of cognitive flexibility tapped by Trail Making Test subtest B (Osher et al., 2008) and perseverative errors assessed on WCST (Dittmann et al., 2007). Notably, these findings overlap with the influence of *MTHFR* polymorphisms on cognitive performance reported in schizophrenia patients (Roffman et al., 2007, 2008a,b, 2011a,b). As mentioned above, the C677T polymorphism in the *MTHFR* gene has been associated with greater deficits of executive functioning assessed on WCST in schizophrenia subjects (Roffman et al., 2007).

Cognitive deficits due to elevated Hcy level might be particularly prominent among older bipolar disorder patients or those with a delayed onset of the disorder (Dias et al., 2009). However, it should be kept in mind that aging increases Hcy levels and some cognitive deficits due to hyperhomocysteinemia may occur regardless of depression. It has been shown that hyperhomocysteinemia worsens cognitive performance in tests of immediate or delayed memory, as well as global cognitive functioning in older subjects (Ford et al., 2013). It should also be noted that patients with bipolar disorder might exhibit higher Hcy levels due to the treatment with mood stabilizers. Indeed, experimental studies have revealed that sodium valproate inhibits methionine adenosyltransferase, while lamotrigine serves as a weak dihydrofolate reductase inhibitor leading to lower functional folate levels despite of normal blood levels of folate (Baek et al., 2013).

There are two randomized placebo controlled trials investigating the efficacy of folic acid supplementation in bipolar depression. These studies revealed that folic acid may enhance lithium prophylaxis (Coppen et al., 1986) and antidepressant action of fluoxetine in females (Coppen and Bailey, 2000). Furthermore, it has been found that the augmentation of sodium valproate with folic acid might be beneficial in terms of reducing manic symptoms (Behzadi et al., 2009). Inconsistent results also indicate that higher vitamin B12 level may predict favorable response to single ketamine infusion in bipolar depression patients (Permoda-Osip et al., 2013a; Lundin et al., 2014). Ketamine is an NMDA receptor antagonist, emerging as a therapeutic strategy in treatment-resistant depression (Naughton et al., 2014).

FUTURE DIRECTIONS AND CONCLUSION

Undoubtedly, elevated Hcy levels are associated with a wide spectrum of psychiatric disorders including particularly schizophrenia and affective disorders. It might be assumed that the dual action of Hcy (as agonist or antagonist) within NMDA receptors (Lipton et al., 1997) explains why elevated Hcy levels are involved in the pathophysiology of both schizophrenia and affective disorders. This association is probably strengthened by high prevalence of metabolic syndrome and its single components, which is a consequence of antipsychotic treatment. Emerging evidence indicates that high Hcy levels may, to some extent, account for cognitive deficits among these groups of patients. It seems that the influence of Hcy on executive functioning occurs regardless of a psychiatric diagnosis since this correlation has been found both in schizophrenia and bipolar disorder patients. In this regard, it is also recommended to investigate the influence of Hcy on cognition in healthy adults in order to determine the extent of cognitive deficits that are the consequence of elevated Hcy levels. Further, future studies should investigate the relationship between Hcy levels in these patient populations on and off their medications to tease apart the relationship between Hcy, psychiatric disorders, and treatment duration or type of medications.

There is still a scarcity of studies investigating the relationship between Hcy and cognitive deficits in drug-naïve first-episode patients and high-risk populations. These studies are warranted as they may indicate the correlation between Hcy levels and early cognitive deficits that are strictly associated with schizophrenia and affective disorders regardless of medication and disease duration. Irrespective of a diagnostic subgroup, future studies should take into account the confounding effect of such variables as body weight, dietary habits, smoking or alcohol consumption that are less frequently controlled, as previous studies have shown that these variables are correlated with Hcy levels and may thus confound findings in the relationship between psychiatric disorders and Hcy levels. Given the largely known contribution of Hcy to the etiology of various types of dementia, it might be also beneficial to address the role of Hcy in the neuroprogression of cognitive deficits that is widely observed particularly in affective disorders and remains the matter of dispute in schizophrenia. Longitudinal measurements of Hcy along with assessment of cognitive functioning that take into account the effects of age as an confounding factor are required to initiate this vein of research.

It should be noted that supplementation of folic acid and vitamins B may normalize Hcy levels. However, we are not aware of any studies addressing the efficacy of supplementation strategies with respect to alleviating cognitive deficits among patients with schizophrenia or affective disorders. Similarly, a gap exists in addressing the influence of antipsychotic treatment on Hcy metabolism, and its correlations with cognitive processes, which should be the focus in future work. As mentioned above, there is only one cross-sectional study revealing a negative correlation between folate levels and high chlorpromazine equivalents (>400 mg/day) (Eren et al., 2010) in chronic schizophrenia patients and one observational study performed in a small sample of drug-naïve first-episode schizophrenia patients reporting no significant alterations in Hcy levels in the course of antipsychotic treatment (Bicikova et al., 2011). Another study revealed a decrease in Hcy levels during the treatment of acute relapse of schizophrenia (Petronijević et al., 2008). This issue is important due to the known influence of certain antipsychotics on the development of obesity and its metabolic consequences, such as dyslipidemia, diabetes or hypertension that have been found to influence cognitive performance in schizophrenia patients (Lancon et al., 2012; Lindenmayer et al., 2012; Boyer et al., 2013; Li et al., 2014).

Results of studies based on candidate gene approach and investigating genetic variation within the Hcy metabolism enzymes should be interpreted with caution. Previous genome-wide association studies (GWAS) have not confirmed the association between polymorphisms in the *MTHFR* gene or other genes implicated in Hcy metabolism and schizophrenia (Yoshimi et al., 2010; Lencz et al., 2013; Ripke et al., 2013, 2014; Ivorra et al., 2014; Saito et al., 2014) or bipolar disorder (Sklar et al., 2011; Kuo et al., 2014; Mühleisen et al., 2014; Xu et al., 2014) risk. There is only one genome-wide linkage analysis of recurrent depressive disorder providing evidence for linkage on chromosome region 1p36 including the *MTHFR* gene with the LOD score for female-female pairs estimated at 2.73 (McGuffin et al., 2005). In this regard and taking into account the involvement of Hcy pathway in several physical health impairments, it might be hypothesized that discordant results of GWAS and candidate gene approach studies may originate from genetic heterogeneity across studied populations and various clinical phenotypes including distinct somatic comorbidities that have also been attributed to polymorphisms in the *MTHFR* gene.

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GENERAL COMMENTARY
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Corrigendum: Homocysteine levels in schizophrenia and affective disorders—focus on cognition

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Keywords: homocysteine, depression, bipolar disorder, schizophrenia, hyperhomocysteinemia, cognition, brain substrates

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Symptom dimensions are associated with reward processing in unmedicated persons at risk for psychosis

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There is growing evidence that reward processing is disturbed in schizophrenia. However, it is uncertain whether this dysfunction predates or is secondary to the onset of psychosis. Studying 21 unmedicated persons at risk for psychosis plus 24 healthy controls (HCs) we used a incentive delay paradigm with monetary rewards during functional magnetic resonance imaging. During processing of reward information, at-risk individuals performed similarly well to controls and recruited the same brain areas. However, while anticipating rewards, the high-risk sample exhibited additional activation in the posterior cingulate cortex, and the medio- and superior frontal gyrus, whereas no significant group differences were found after rewards were administered. Importantly, symptom dimensions were differentially associated with anticipation and outcome of the reward. Positive symptoms were correlated with the anticipation signal in the ventral striatum (VS) and the right anterior insula (rAI). Negative symptoms were inversely linked to outcome-related signal within the VS, and depressive symptoms to outcome-related signal within the medial orbitofrontal cortex (mOFC). Our findings provide evidence for a reward-associated dysregulation that can be compensated by recruitment of additional prefrontal areas. We propose that stronger activations within VS and rAI when anticipating a reward reflect abnormal processing of potential future rewards. Moreover, according to the aberrant salience theory of psychosis, this may predispose a person to positive symptoms. Additionally, we report evidence that negative and depressive symptoms are differentially associated with the receipt of a reward, which might demonstrate a broader vulnerability to motivational and affective symptoms in persons at-risk for psychosis.

Keywords: reward, salience processing, psychosis, ventral striatum, anterior insula, dopamine, at-risk mental state, functional magnetic resonance imaging (fMRI)

INTRODUCTION

Subcortical dopamine dysregulation is a cornerstone in our understanding of schizophrenia (Howes and Kapur, 2009). There is general agreement on the central role of dopamine in mediating mesostriatal neural activity involved in reward processing, specifically in encoding motivational value and salience (Bromberg-Martin et al., 2010). Accumulating evidence suggests dysregulated dopaminergic transmission as a possible mediator for disturbances associated with altered processing of reward, incentive salience and learning in schizophrenia (Ziauddeen and Murray, 2010).

Both the anticipation and receipt of rewards have distinct neural correlates (Knutson et al., 2001b; Dillon et al., 2008; Berridge et al., 2009). The anticipatory phase involves activation in the ventral striatum (VS), encompassing the nucleus accumbens (NAcc; Knutson et al., 2001b; Schott et al., 2008) and the anterior insula (Volz et al., 2004; Knutson and Greer, 2008; Krebs et al., 2012). This anticipatory signal has been proposed to code the expected value of the predicted reward probability distribution rather than reward prediction error *per se* (Schultz, 2010). It is hypothesized that chaotic firing of dopaminergic neurons projecting to those regions mediates inadequate attribution of salience to irrelevant events, which might contribute to the formation of positive psychotic symptoms (Kapur et al., 2005; Palaniyappan and Liddle, 2012). Nielsen et al. (2012) whose study draws upon the concept of anticipation of reward being associated with salience processing, found a significant correlation between striatal activation during this

stage to positive symptoms, agreeing with the aberrant salience theory.

During reward feedback, VS activation reflects prediction error in response to unexpected rewards (Schultz, 2002) while activity in the ventromedial/medial orbitofrontal cortex (mOFC) signals the updating of reward value (Grabenhorst and Rolls, 2011) and hedonic experience (Kringelbach, 2005). Accordingly, a deficit in the processing of reward receipt on both levels has been associated with anhedonia and depression, although the findings are more consistent for the VS than for the mOFC (McCabe et al., 2009; Pizzagalli et al., 2009; Simon et al., 2010a; Gradin et al., 2011). Dysfunctional activation during both anticipation and outcome in striatal and cortical regions has been associated with negative symptoms (Juckel et al., 2006; Simon et al., 2010a; Waltz et al., 2011). Some groups including our own have suggested that a higher specificity can be reached by investigating subdimensions of negative symptoms, which was not feasible in the context of this high-risk study.

There is consistent evidence that reward processing and associated cortico-striatal interactions are perturbed in schizophrenia (Heinz and Schlagenhauf, 2010; Simon et al., 2010b; Ziauddeen and Murray, 2010). Attenuated striatal responses during the anticipation of rewards have been primarily observed in unmedicated patients with schizophrenia (Juckel et al., 2006; Schlagenhauf et al., 2009), although medicated patients with more severe negative symptoms also seem to show a reduced signal (Simon et al., 2010a; Waltz et al., 2011). However, it is uncertain whether dysregulations of the reward system predate or follow the development of psychosis. Examining reward processing in at-risk individuals may provide further insight into illness susceptibility and its underlying pathophysiological mechanisms. Results from recent studies with positron emission tomography (PET) have suggested that dopaminergic dysregulation begins prior to the first psychotic episode, and importantly appears predictive of conversion to psychotic illness (Howes et al., 2009, 2011). Furthermore, motivational salience processing and associated responses in the VS (Roiser et al., 2013), as well as reduced activation during loss-avoidance anticipation in pre-psychotic individuals has been observed (Juckel et al., 2012).

Therefore, our goal was to explore functional brain correlates during both anticipation and receipt of rewards and to evaluate their association with symptoms in unmedicated persons at risk for psychosis. We compared the neural activation of HCs with an unmedicated at-risk group by administering a modified version of the monetary incentive delay task (Knutson et al., 2001a; Abler et al., 2005; Simon et al., 2010b). Regarding brain-symptom relationships the previous work cited above provides some evidence for differential associations between symptoms reward anticipation and outcome in patients with schizophrenia, although the findings are heterogeneous. Thus, we tested the following hypotheses: (1) positive symptoms are associated with activation of the VS and the anterior insula during reward anticipation, (2) negative symptoms are associated with reduced VS activation during reward anticipation; and (3) depressive symptoms are associated with reduced VS and mOFC activation during processing of rewarding outcomes.

MATERIALS AND METHODS

PARTICIPANTS

This project consisted of 21 medication-free participants at risk for psychosis (Risk) and 24 healthy controls (HC). Participants were recruited in the region of Zurich, Switzerland, within the frame of a larger study on early detection of psychosis,[1] which was approved by the cantonal Ethic Commission Zurich (E-63/2009) and complies with the Declaration of Helsinki.

For the present study, psychopathology (i.e., positive and negative symptoms) was rated with the Structured Interview for Psychosis-Risk Syndrome (SIPS; Miller et al., 2003), the Schizophrenia Proneness Instrument (SPI-A; Schultze-Lutter et al., 2007), and the Calgary Depression Scale for Schizophrenia (CDSS; Addington et al., 1993). All participants in the Risk group fulfilled inclusion criterion for high-risk status as assessed by the SPI-A, which was met when at least one cognitive-perceptive basic symptom or at least two cognitive disturbances were reported. Six individuals in the Risk group reported at least one attenuated psychotic symptom or brief, limited intermittent psychotic symptom as assessed by the SIPS, and thus fulfilled additionally the criterion for UHR status. Imaging of the participants was conducted immediately after entry into the ZInEP study before onset of any treatment.

Persons in the HC group were screened with the Mini-International Neuropsychiatric Interview (Sheehan et al., 1998) to ensure that none had any history of psychiatric illness. Individuals in the Risk and HC groups did not differ significantly in terms of age, gender, handedness (assessed with the Edinburgh Handedness Inventory; Oldfield, 1971), and intelligence (estimated by using tests measuring both verbal (Mehrfachwahl-Wortschatz-Intelligenz Test; MWT-B; Lehrl, 2005) and nonverbal intelligence (Leistungsprüfsystem; LPS-3; Horn, 1983; **Table 1**). Exclusion criteria for both groups were age under 16 or over 35 years, contraindications against MRI, neurological illness, and substance abuse.

EXPERIMENTAL DESIGN AND TASK

We used a modified version of the monetary incentive delay task (**Figure 1**), which has been proven to be a useful probe of neural responses during reward anticipation and receipt. To minimize learning effects during the fMRI, the MID-task was explained carefully by showing each cue and its meaning to the subjects. Participants had to perform a practice version of the task containing 10 trials, for which they did not receive payment. They were also shown the money they could earn by performing the task successfully in the scanner. During functional scan acquisition the test subjects engaged in one session with 50 trials. Two levels of reward were possible: 0 Swiss Francs (CHF) or 4 CHF, with a maximum overall win of 60 CHF. A steady rate of reward vs. non-reward across all participants was accomplished by applying a probabilistic pattern, which entailed no reward being paid in 10 pre-defined trials (out of the 25 trials with a potential reward).

The cue was presented for 750 ms and was followed by a variable delay of 2500–3500 ms (mean 3000 ms). A fixed response

[1] www.zinep.ch

Table 1 | Demographic characteristics and symptom ratings.

	HC	Risk	Statistical evaluation
N	24	21	
Gender (f:m)	11:13	6:15	χ^2 = 1.42, *n.s.**
Handedness (r:l:b)	21:2:1	19:1:1	χ^2 = 0.23, *n.s.**
Age (years)	23.3 ± 5.0	25.1 ± 5.6	$t = -1.8$, *n.s*†
Estimated intelligence	115.8 ± 14.4	111.6 ± 14.4	$t = 1.0$, *n.s*†
SIPS:			
- Positive	—	6.5 ± 3.9	—
- Negative	—	9.8 ± 5.8	—
- General	—	6.6 ± 3.0	—
- Disorganization	—	2.5 ± 2.2	—
GAF	—	58.2 ± 19.0	—
CDSS	—	6.5 ± 2.7	—

*HC, healthy controls; Risk, subjects at risk for psychosis; r:l:b, right, left, both/bimanual; SIPS, symptoms according to Structured Interview for Prodromal Syndromes; GAF, Global Assessment of Functioning Scale Mean; CDSS, Calgary Depression Scale for Schizophrenia. * Pearson's chi-square test; † 2-sample t-test; n.s., not significant ($p > 0.05$), ± SD where appropriate. Estimated intelligence was based upon mean scores from evaluations of verbal (MWT-B; Lehrl, 2005) and nonverbal (LPS-3; Horn, 1983) skills.*

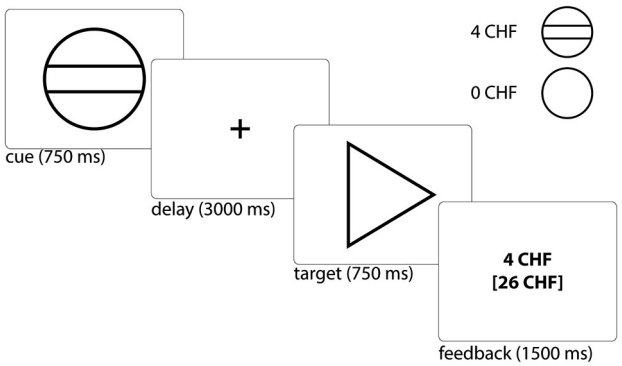

FIGURE 1 | Monetary incentive delay task: Example trial and cues representing possible reward outcomes. Participants first saw a cue stipulating with an unpredictable probability the amount of money (4 CHF or 0 CHF) they could win, if they reacted correctly within 750 ms during the ensuing discrimination task, which involved pressing either a left or right button depending upon the direction of a triangle after an anticipation period (variable delay: 2500–3500 ms, mean of 3000 ms). Immediately after target presentation, subjects were informed about the amount of money they had won during this trial and their cumulative total win so far (feedback) for a total of 1500 ms (Abler et al., 2005). The jittered inter-trial interval (ITI) was between 1000 and 8000 ms with a mean of 4000 ms. Trial types were randomly ordered.

time window of 750 ms was chosen in order to ensure low task difficulty with a very high success rate, i.e., the rate of reward vs. non-reward depended little on the subjects' performance. This procedure was chosen to (i) avoid confounding effects of psychomotor slowing and cognitive impairment (Demjaha et al., 2012; Fusar-Poli et al., 2012; Lin et al., 2012); and (ii) to avoid creating a stressful task environment. Finally, the participant received feedback on the money won in the current trial and the total amount of money won, which was presented for 1500 ms. In order to be sensitive to differences between trials close together in time we used a variable inter-trial interval (ITI), chosen from a Gaussian distribution with range of 1000 to 8000 ms, step of 500 ms and mean of 4000 ms. We measured reaction times (RTs) to cues with potential rewards in order to measure motivation.

IMAGING DATA ACQUISITION

Functional and structural MRI was performed at the Psychiatric Hospital, University of Zurich, Switzerland, using a Philips Achieva TX 3-T whole-body MR unit with an eight-channel head coil. Three-dimensional T1-weighted anatomical images were acquired (160 slices; repetition time (TR) = 1900 ms; TE = 2.2 ms; inversion or echo time (TE) = 900 ms; flip angle $\theta = 78°$; spatial resolution, 1 × 1 × 1 mm). Functional scans included a T2*-weighted echoplanar imaging sequence (265 volumes; TR = 2000 ms; TE = 30 ms; 32 contiguous, inter-leaved slices; spatial resolution, 3 × 3 × 3 mm; $\theta = 80°$). To minimize susceptibility artifacts in the mOFC, we placed the contiguous axial slices at a 20° angle relative to the anterior-posterior commissural plane. Participants viewed visual stimuli with LCD video goggles (Resonance Technologies). Responses were recorded with a Lumitouch response box (Photon Technologies).

fMRI DATA ANALYSIS

Our fMRI data were analyzed using SPM8[2]. The pre-processing steps included realignment, in which fMRI-time series were rigidly registered to a reference image in order to correct for motion artifacts, slice-timing correction, co-registration to a structural T1 scan, spatial normalization to MNI space, and spatial smoothing (8-mm Gaussian kernel). Three of the participants were excluded due to excessive head motion (i.e., linear shift >2 mm, rotation >1°).

A general linear model was constructed for statistical analysis (Friston et al., 1994). Regressors for the two phases of anticipation (expectation of 4 CHF or 0 CHF) and three phases of outcome (receipt of 4 CHF, omission of 4 CHF, or receipt of 0 CHF/neutral outcome) were modeled separately as explanatory variables convolved with the canonical HRF. The six realignment parameters were included together with the onsets of targets and error-trials as regressors of no interest. To examine the anticipation of reward, we contrasted "*anticipation of* 4 *CHF* vs. *anticipation of* 0 *CHF*". The reward outcome was modeled by contrasting "*receipt of reward* vs. *omission of reward*", i.e., we contrasted outcome regressors for which the preceding anticipation was the same (anticipation of 4 CHF). Thus, although the timing of the task did not allow for a definite separation of the trial phases within the temporal resolution of fMRI, our selection of contrasts nevertheless allowed comparisons between the trial-types of interest.

The individual contrast images were then subjected to a second-level random effects analysis. Within-group activation was compared using a one-sample *t*-test. The initial threshold for group-level maps was $p < 0.001$ (uncorrected). Given our strong *a priori* hypothesis regarding involvement of the VS and rAI in the processing of anticipation rewards and mOFC in the feedback of

[2]http://www.fil.ion.ucl.ac.uk/spm

rewards, we employed family-wise error level correction adjusted for small volume (P_{SVC}) across each of our independently derived regions of interest (ROIs) at the voxel level. For the VS, we used anatomical voxel masks for the left and right hemispheres, as retrieved from a publication-based probabilistic MNI-atlas (Nielsen and Hansen, 2002). This method has been used in previous reward-related fMRI studies (Juckel et al., 2006; Schlagenhauf et al., 2009; Simon et al., 2010a). For the mOFC, we used a functional ROI based on an earlier reward-related fMRI investigation with healthy participants using the same paradigm (Simon et al., 2010b). Finally, we selected a rAI ROI, because aberrant activation of this brain region has been previously reported in a high-risk sample (Wotruba et al., 2014) and has been suggested to be relevant for the pathomechanisms underlying the development of positive symptoms (Palaniyappan and Liddle, 2012). Importantly, rAI activation has also been shown during anticipation of a reward (Knutson and Greer, 2008; Krebs et al., 2012). We selected a spherical ROI centered on MNI coordinates ($x = 38$, $y = 22$, $z = -10$; 10 mm radius) (Seeley et al., 2007; Wotruba et al., 2014). The corresponding ROI's are depicted in **Figures 3A**, **4A**.

Individual parameter estimates (beta-values) were extracted using the mean of the data, collapsed across all voxels within each ROI using the REX toolbox[3], and were correlated to symptom scores (SIPS Negative, SIPS Positive, and CDSS) as well as with RT via Spearman's correlation analysis. Significant results are reported at $p < 0.05$. No correction for multiple testing was applied to the correlational analyses.

In addition, we performed a whole-brain analysis using the aforementioned contrasts to identify group differences in brain areas outside the ROIs. The threshold was set to voxelwise $p < 0.001$ and 20 contiguous voxels, corresponding to a false-positive discovery rate of $p < 0.05$ across the whole brain as estimated by Monte Carlo simulation.

[3]http://web.mit.edu/swg/software.htm

All raw data are available from the corresponding author on request.

RESULTS

BEHAVIORAL RESULTS

The average error rate for all subjects was 2.1%. Participants were significantly faster in trials when 4 CHF was promised (mean 379.3 ms, SD 6.7) than when they expected no reward (mean 406.3 ms, SD 5.2; $t = 3.3$, $p = 0.002$). The groups did not differ significantly in either RTs ($p > 0.5$) or error rates ($p > 0.2$).

ANTICIPATION

Within group activations during reward anticipation

We first analyzed within group activations to anticipation of possible rewards (i.e., *anticipation of* 4 *CHF vs. anticipation of* 0 *CHF)* in each of our a priori defined regions of interest. Both HC and Risk groups displayed significant hemodynamic responses within the left VS (HC: $z = 4.81$, $P_{SVC} < 0.000$; Risk: $z = 4.62$, $P_{SVC} = 0.004$), right VS (HC: $z = 5.19$, $P_{SVC} < 0.001$; Risk: $z = 4.79$, $P_{SVC} = 0.003$), and rAI (HC: $z = 4.31$, $P_{SVC} < 0.001$; Risk: $z = 3.48$, $P_{SVC} = 0.004$). Only at-risk persons exhibited activation within the mOFC ($z = 3.32$, $P_{SVC} = 0.03$).

Between group comparisons during reward anticipation

In the a priori defined ROIs (VS, rAI, mOFC) no significant differences between HC and Risk groups were observed. We performed an exploratory whole brain analysis, which revealed significantly increased hemodynamic responses in the Risk vs. HC group within the following regions: posterior cingulate cortex [PCC; Brodmann Area (BA) 31; $x = 3$, $y = -45$, $z = 27$; cluster size = 123 voxels], superior frontal gyrus (SFG; BA 9; $x = 9$, $y = 57$, $z = 30$; cluster size = 41 voxels), bilateral medial frontal gyrus (MFG; BA 8; $x = 30/-24$, $y = 24$, $z = 48/45$; cluster size = 36/50 voxels) (**Figure 2**). Activations were not significantly increased in any brain region for the HC subjects relative to the Risk group.

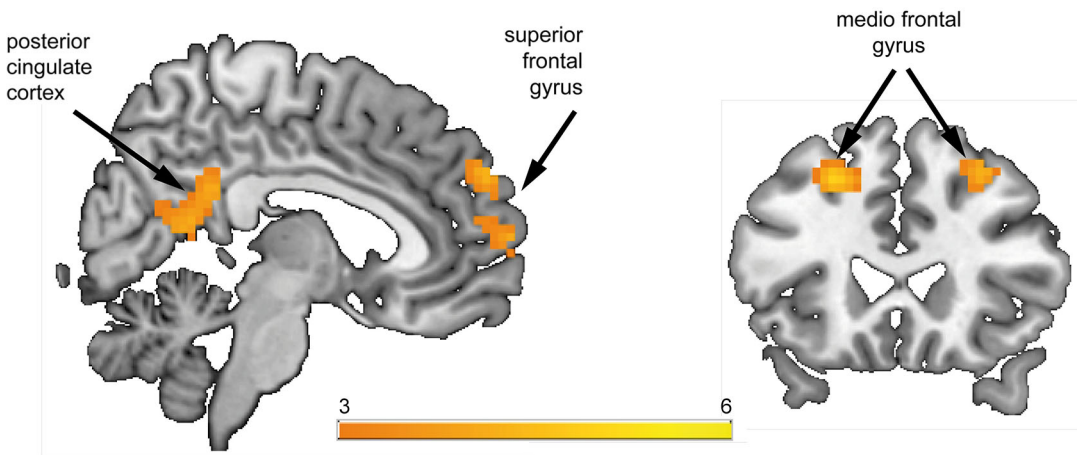

FIGURE 2 | Whole-brain group comparison of the contrast reward anticipation vs. no reward anticipation. Subjects at risk for psychosis showed significantly stronger hemodynamic response compared to healthy controls in the posterior cingulate cortex, superior frontal gyrus, and bilateral medio frontal gyrus (corresponding *t*-values are represented in orange/yellow).

Correlations between ROI activation and psychopathology

The ROI-based analysis revealed significant correlations between the SIPS positive symptom score and hemodynamic response in the left VS ($\rho = 0.54$, $p = 0.012$; **Figure 3A1**) and right VS ($\rho = 0.59$, $p = 0.005$; **Figure 3A2**), as well as in the rAI ($\rho = 0.52$, $p = 0.015$; **Figure 3A3**). No significant association was found between regional brain activation and negative or depressive symptoms during the phase of reward anticipation.

OUTCOME

Within group activations during reward outcome

We first analyzed the contrast *receipt of reward* vs. *omission of reward* in each of our a priori defined regions of interest for each group separately. Both groups showed significant hemodynamic responses within the mOFC (HC: $z = 4.93$, $P_{SVC} < 0.000$; Risk: $z = 3.33$, $P_{SVC} = 0.036$), left VS (HC: $z = 4.80$, $P_{SVC} < 0.000$; Risk: $z = 4.03$, $P_{SVC} = 0.002$), and right VS (HC: $z = 4.87$, $P_{SVC} < 0.000$; Risk: $z = 4.55$, $P_{SVC} < 0.000$), but not within the rAI.

Between group comparison during reward outcome

No significant group differences were found within the a priori defined ROIs. An exploratory whole brain analysis did not reveal any additional regions with significant between group differences.

Correlations between ROI activation and psychopathology

The ROI based correlations for the contrast *receipt of reward* vs. *omission of reward* revealed negative correlations for depressive symptoms with contrast estimates within the mOFC ($\rho = -0.46$, $p = 0.037$; **Figure 4B1**), and for negative symptoms with the left VS ($\rho = -0.44$, $p = 0.045$; **Figure 4B2**). No significant association with positive symptoms could be observed. An additional correlation analysis revealed a significant

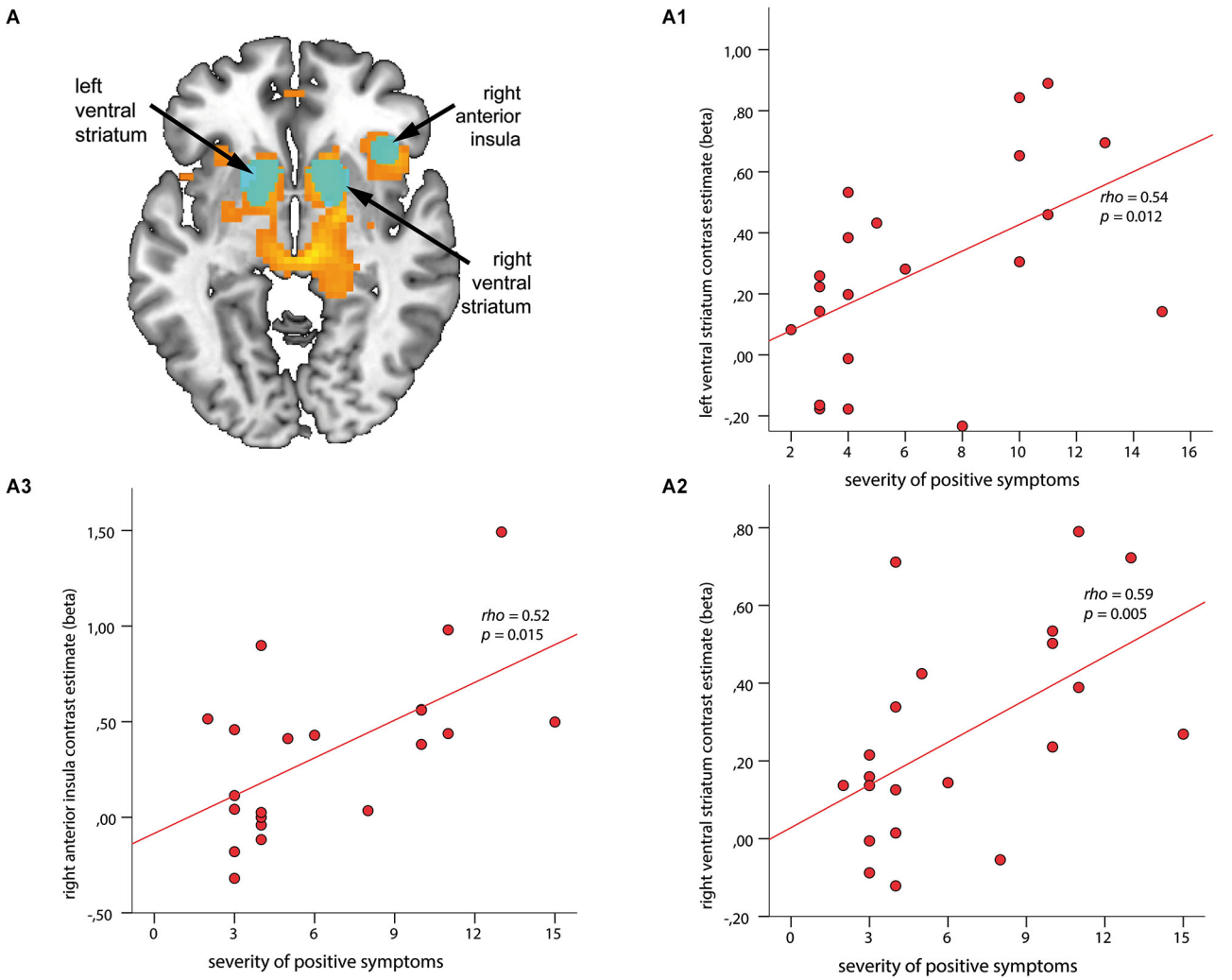

FIGURE 3 | Associations between regions of interest (ROI) and severity of positive symptoms during anticipation of reward. ROIs (depicted in cyan) are overlaid on within-group t-maps for subjects at risk for psychosis **(A)** for the contrast reward anticipation vs. no reward anticipation (shown in orange, both at a voxel-wise threshold $p < 0.001$, with an extent of 20 voxels). ROI-based analysis revealed significant association between contrast estimates of the left and right ventral striatum (VS) **(A1, A2)** and right anterior insula (rAI) **(A3)** with positive symptom scores ($\rho > 0.52$, $p < 0.015$).

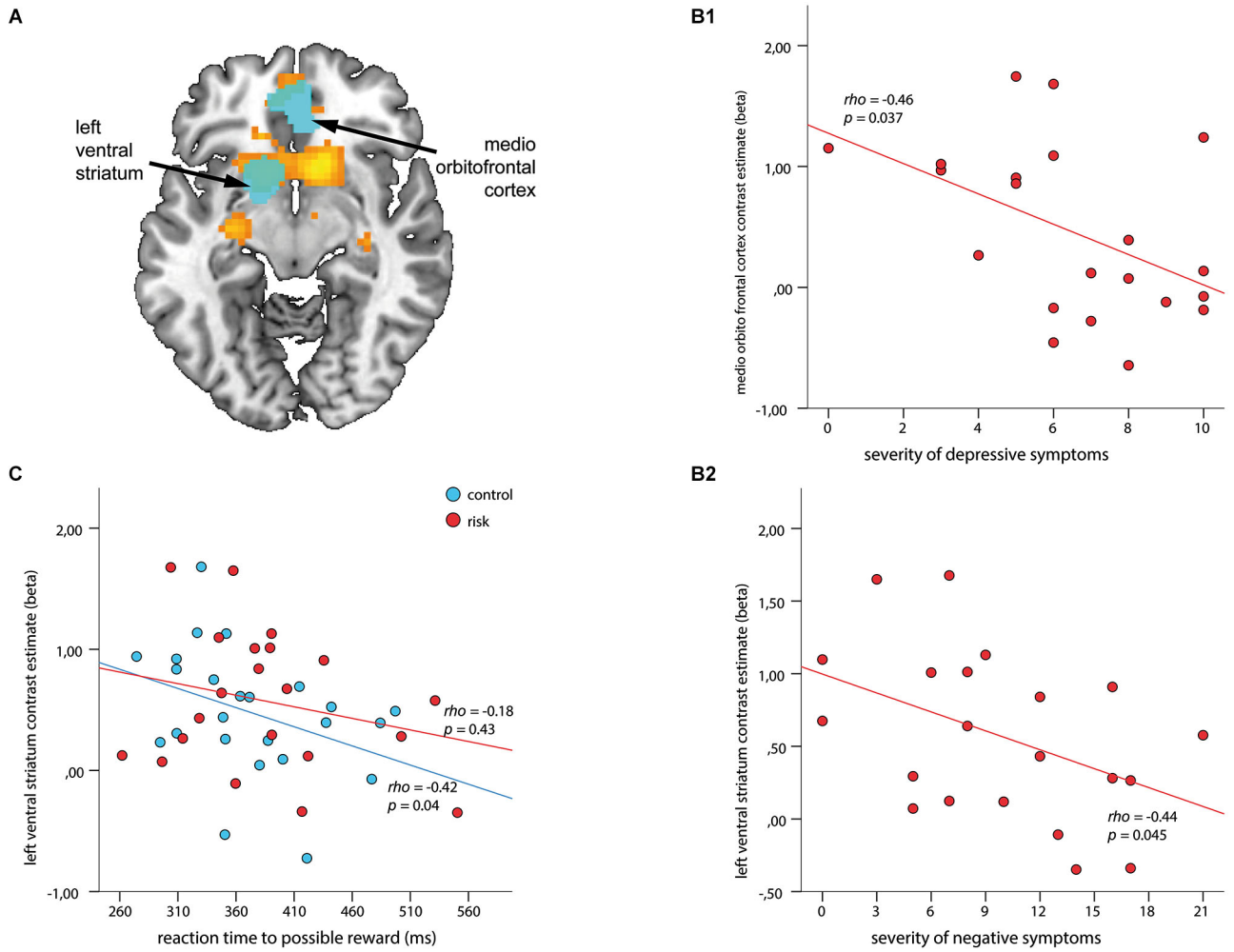

FIGURE 4 | Associations among regions of interest (ROIs), clinical symptoms, and reaction time (RT) to cues with possible reward during outcome. ROIs (depicted in cyan) are overlaid on the within-group t-map for subjects at risk for psychosis **(A)** for the contrast receipt of reward vs. omission of reward (shown in orange, both at a voxel wise threshold of $p < 0.001$, with an extent of 20 voxels). ROI-based analysis revealed a negative association between contrast estimates within the medio orbitofrontal cortex and severity of depressive symptoms **(B1)**, and the left VS and severity of negative symptoms **(B2)** ($\rho > -0.44$, $p < 0.045$). **(C)** Signal in the left VS revealed a significant inverse association with RT in healthy controls (blue; $\rho = -0.42$, $p = 0.04$) but not for subjects at risk for psychosis (red; $\rho = -0.18$, $p = 0.43$).

inverse relationship between RT during the 4 CHF condition and the outcome signal in the left VS ($\rho = -0.42$, $p = 0.04$) for the HC group but not for the Risk group (**Figure 4C**).

DISCUSSION

In our study, unmedicated individuals at risk for psychosis showed similar error rates and RTs as HCs during a monetary incentive delay task. The task was intended to produce low error rates, which lead both groups to perform at ceiling. Both groups recruited similar brain areas when processing reward information. However, during the anticipation phase, those in the Risk group exhibited additional activation in the PCC, MFG, and SFG. During receipt of rewards, the two groups did not differ significantly. Importantly, the neural processing of anticipation and receipt of rewards was differentially related to symptom dimensions. Positive symptoms were associated with the processing of reward anticipation, while negative and depressive symptoms were related to the processing of a rewarding outcome.

The lack of a significant group difference in the VS during reward processing contrasts with earlier findings from unmedicated patients with schizophrenia (Juckel et al., 2006; Schlagenhauf et al., 2009; Esslinger et al., 2012), in which activation in the VS was diminished during the anticipation phase. This may have been due to variations in experimental designs, i.e., the only previous study employing this monetary incentive delay task in a (partially-medicated) high-risk sample (Juckel et al., 2012), found no group differences in the VS during reward anticipation.

During the anticipation period, higher activation in the SFG and MFG was observed in the Risk group. Therefore, the impending action might have required increased effort to maintain task performance, which led to increased frontal

activation. Compensatory hyperactivation of these regions has repeatedly been reported in patients with schizophrenia (Potkin et al., 2009; Deserno et al., 2012). Noteworthy, recent findings (Guitart-Masip et al., 2011) show that anticipatory signals capture some aspects of response preparation, which, in turn, may be related to the frontal hyperactivation shown by subjects in the at-risk group. However, our task does not allow differentiating between response preparation and reward anticipation, which would require future studies. We also found significantly stronger activation in the PCC for the Risk group compared with HC. The PCC is a key node of the default mode network, which, in healthy individuals, activates during rest periods, but deactivates during goal-directed tasks (Fox et al., 2005). Therefore, similar to reports with schizophrenia (Whitfield-Gabrieli and Ford, 2012), our result indicates less task-dependent deactivation of the PCC in the risk state for psychosis.

A central finding of our study is that positive symptoms are correlated with VS and rAI activation during reward anticipation. Dysfunctional activation of VS and rAI has been associated with aberrant assignment of salience to otherwise irrelevant stimuli, which might be part of the neuropathophysiological mechanism leading to psychotic symptoms (Jensen et al., 2008; Palaniyappan and Liddle, 2012). Consistent with this, patients with higher positive symptom scores have been observed to elicit greater hemodynamic responses in the VS to neutral stimuli (Jensen et al., 2008; Romaniuk et al., 2010; Roiser et al., 2013). In contrast, our at-risk participants, with a higher degree of sub-clinical positive symptoms, displayed a stronger signal response to meaningful cues. Thus, individuals with potentially prodromal symptoms might be predisposed to over-attributing salience to *any* event, which might reflect a sign for aberrant salience signaling after the onset of overt psychosis. In contrast to our own results, recent reports with unmedicated schizophrenic patients have pointed to an *inverse* relationship between VS activation and positive symptoms (Esslinger et al., 2012). These different results could be due to the fact, that previous studies employed a salience contrast involving losses while our trials included only reward contrasts.

In addition, a recent report shows that first-degree relatives of patients with schizophrenia show a decrease in VS activation during reward anticipation, which is also influenced by a polymorphism in the neuregulin-1 gene (Grimm et al., 2014). The fact that at-risk participants in our study did not show reduced VS activation in association with reward anticipation might reflect our different method of identifying individuals with increased likelihood of developing psychotic illness (based on subclinical symptoms rather than genotypes).

We observed an inverse relationship between the severity of negative symptoms and VS activation during the receipt of reward. This finding was somewhat unexpected, because in patients with schizophrenia an association of ventral striatal hypoactivation and negative symptoms was mainly reported for the anticipation phase (Juckel et al., 2006; Simon et al., 2010a; Waltz et al., 2010). Nevertheless, dysfunctional outcome processing has also been suggested to be associated with negative symptoms (Waltz et al., 2013), although these findings relate to prefrontal cortical regions. In addition, stronger activation of the VS during the reward phase was associated with faster RTs in HC participants, but not in Risk participants. This implies a dysregulation of the VS in subjects at-risk for psychosis that might affect the positive impact of rewarding actions and, consequently, contribute to the development of negative symptoms.

Furthermore, individuals with higher scores for depressive symptoms exhibit less activation within the mOFC, a region involved in immediate and simple hedonic responses (Kringelbach, 2005). Thus, reduced coding of pleasurable experiences in the mOFC may contribute to the neurobiological origin of depressive symptoms in at-risk persons. In contrast to our previous study in patients with schizophrenia, no association between depressive symptoms and VS activation during outcome was observed (Simon et al., 2010a). For both negative and depressive symptoms, one might speculate that individuals with higher symptom scores show less differentiation between positive and negative outcomes due to unregulated dopamine firing (Schlagenhauf et al., 2009; Heinz and Schlagenhauf, 2010).

A possible shortcoming of the present study is the relatively small sample size. Another constraint is the cross-sectional design, which could limit the relevance of our results. In addition, the correlations between RT and clinical symptom scores with the hemodynamic response in our ROIs were not corrected for multiple comparisons, which warrant caution in interpreting these findings until independently replicated. In addition, we pooled data from participants fulfilling ultra-high-risk and basic symptom criteria. Therefore, this did not allow us to attribute our findings specifically to either of those types of symptoms. Finally, the relationship between salience, value and reward prediction signals is still a matter of intense debate (Morris et al., 2012; Kahnt and Tobler, 2013). Our task is limited in its capacity to specifically attribute activation during reward anticipation and outcome to one of these signals.

In summary, our results provide evidence for a dysregulation of reward-associated processing in subjects at risk for psychosis, which could be compensated by the recruitment of prefrontal regions. Importantly, higher activation in the striatal and insular regions when anticipating reward-relevant cues might reflect abnormal processing of potential rewarding outcomes. This in turn could lead to a higher risk for developing supra-threshold psychotic disorder, which is in line with the aberrant salience theory of psychosis. Finally, we showed that negative and depressive symptoms are differentially related to VS and mOFC during the receipt of reward. Such a relation may reflect a broader vulnerability for motivational and affective symptoms in at-risk persons.

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Functional connectivity density alterations in schizophrenia

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Background: Schizophrenia is characterized by altered resting-state functional connectivity. Most previous studies have focused on changes in connectivity strengths; however, the alterations in connectivity density in schizophrenia remain largely unknown. Here, we aimed to investigate changes in resting-state functional connectivity density (rsFCD) in schizophrenia.

Methods: A total of 95 schizophrenia patients and 93 sex- and age-matched healthy controls (HCs) underwent resting-state functional MRI examinations. The rsFCD, which reflects the total number of functional connections between a given brain voxel and all other voxels in the entire brain, was calculated for each voxel of each subject. Voxel-based comparisons were performed to identify brain regions with significant rsFCD differences between patients and controls ($P < 0.05$, corrected).

Results: Compared with HCs, patients with schizophrenia showed significantly increased rsFCD in the bilateral striatum and hippocampus and significantly decreased rsFCD in the bilateral sensorimotor cortices and right occipital cortex. However, the rsFCD values of these brain regions were not correlated with antipsychotic dosage, illness duration, or clinical symptom severity.

Conclusions: The striatal and hippocampal regions and parietal-occipital regions exhibited completely different changes in rsFCD in schizophrenia, which roughly correspond to dopamine activity in these regions in schizophrenia. These findings support the connectivity disorder hypothesis of schizophrenia and increase our understanding of the neural mechanisms of schizophrenia.

Keywords: schizophrenia, resting state, functional magnetic resonance imaging, functional connectivity density, functional connectivity strength

INTRODUCTION

Schizophrenia is a devastating psychiatric disease that is characterized by hallucinations, delusions, loss of initiative, and cognitive deficits (van Os and Kapur, 2009; Gustavsson et al., 2011). Although there is no accepted theory accounting for schizophrenia (Rubinov and Bullmore, 2013), connectivity disturbance has been considered a hallmark of schizophrenia. Using diffusion tensor imaging (DTI), extensive anatomical connection impairments have been found in schizophrenia (van den Heuvel et al., 2010, 2013; Wang et al., 2012). Using functional magnetic resonance imaging (fMRI), resting-state functional connectivity strength (rsFCS) has been proposed to explore intrinsic association between two brain regions. It measures temporal correlations of spontaneous fluctuations in brain activity between different pairs of brain areas (Biswal et al., 1995). rsFCS may reflect intrinsic functional organization of the brain. Schizophrenia patients have shown rsFCS alterations in multiple specific brain regions and networks (Karbasforoushan and Woodward, 2012; Woodward et al., 2012; Moran et al., 2013; Orliac et al., 2013; Shinn et al., 2013; Argyelan et al., 2014; Klingner et al., 2014; Kraguljac et al., 2014; Liu et al., 2014). For example, schizophrenia patients have decreased inter-regional rsFCS between the amygdala and frontal lobe (Liu et al., 2014) and between the hippocampus and parietal lobe (Kraguljac et al., 2014). They also exhibit decreased intra-network rsFCS in the default-mode network (DMN) and salience network (SN) (Orliac et al., 2013). The patients also have decreased inter-network rsFCS between the SN and central executive network (CEN) and DMN (Moran et al., 2013). Both the decreased (Woodward et al., 2012) and increased (Klingner et al., 2014) prefrontal-thalamic rsFCS have been reported in schizophrenia, although the increased somatosensory-thalamic rsFCS is rather consistent (Woodward et al., 2012; Klingner et al., 2014). However, most previous studies investigating rsFCS abnormalities in schizophrenia used hypothesis-driven methods based on a priori selection of a region of interest (ROI) (Woodward et al., 2012; Shinn et al., 2013; Klingner et al., 2014; Kraguljac et al., 2014; Liu et al., 2014), which cannot provide a full picture of the brain rsFCS changes in schizophrenia. Thus, several studies have investigated rsFCS alterations across the entire brain in schizophrenia using a multiple ROI-based

method (Liang et al., 2006; Venkataraman et al., 2012). Liang et al. found decreased rsFCS between many pairs of brain regions in schizophrenia (Liang et al., 2006). However, Venkataraman et al. reported that patients with schizophrenia exhibited co-existing patterns of increased rsFCS between parietal and frontal regions, and decreased rsFCS between parietal and temporal regions and between the temporal cortices bilaterally (Venkataraman et al., 2012). Almost all previous studies have focused on rsFCS changes in schizophrenia; however, connection number changes of each voxel across the entire brain in schizophrenia are still unclear.

Recently, resting-state functional connectivity density (rsFCD) has been developed to measure the number of resting-state functional connections of a given voxel with all other voxels in the entire brain (Tomasi and Volkow, 2010, 2011a,b). rsFCD mapping is an ultra-fast voxel-wise data-driven method for computing rsFCD with high spatial resolution (3-mm isotropic) (Tomasi and Volkow, 2010, 2011a,b) and has been used to identify age-related connectivity changes, connectivity differences across genetic backgrounds, and connectivity alterations in some disorders (Tomasi and Volkow, 2012a,b, 2014; Tian et al., 2013). rsFCS measures connectivity strength between two voxels or regions or networks, which reflects a one-to-one relationship. However, rsFCD reflects a one-to-many relationship. A greater rsFCD value for a particular voxel indicates that this voxel is functionally connected to a greater number of other brain voxels and suggests that this voxel plays a more important role in the information processing of the brain than those voxels with lower rsFCD values. Thus, rsFCS and rsFCD reflect different connectivity properties. Investigating rsFCD changes in schizophrenia may provide extra information (change in importance of a voxel in information processing) that cannot be obtained from an rsFCS analysis. This information may facilitate our understanding of the neural mechanisms of schizophrenia from a new perspective.

In the present study, we compared rsFCD differences between 95 schizophrenia patients and 93 healthy controls (HCs) to identify brain regions with altered rsFCD in schizophrenia.

MATERIALS AND METHODS

SUBJECTS

A total of 200 right-handed individuals were enrolled in the present study, including 98 schizophrenia patients and 102 HCs. The diagnoses and illness durations of schizophrenia were determined by the consensus of two experienced clinical psychiatrists using the Structured Interview for DSM-IV Axis I Disorders. All HCs were screened using the non-patient edition of the SCID to confirm a lifetime absence of psychiatric illnesses. In addition, all of the HCs were interviewed to exclude individuals with a known history of psychiatric illness in first-degree relatives. Exclusion criteria for all subjects were a history of head trauma with consciousness disturbances lasting more than 5 min, a history of drug or alcohol abuse, pregnancy, and any physical illness such as cardiovascular disease or neurological disorders, as diagnosed by an interview and medical records review. A professional radiologist assessed image quality slice-by-slice, and three patients and 9 HCs with poor image quality were excluded. Consequently, 95 schizophrenia patients and 93 HCs were included in the statistical analysis. Clinical symptoms of psychosis were quantified using the Positive and Negative Syndrome Scale (PANSS). The antipsychotic dosages are reported (**Table 1**) as the chlorpromazine equivalents calculated based on clinically equivalent dosing estimates (Gardner et al., 2010). For each schizophrenia patient, the chlorpromazine equivalent was estimated according to the antipsychotic drugs and dosages used in the latest week before the MRI scan. The Medical Research Ethics Committee of Tianjin Medical University General Hospital approved this study. After a complete description of the study, written informed consent was obtained from each subject.

MRI DATA ACQUISITION

MRI data were acquired using a 3.0-Tesla MR system (Discovery MR750, General Electric, Milwaukee, WI, USA). Tight but comfortable foam padding was used to minimize head motion, and earplugs were used to reduce scanner noise. Sagittal 3D T1-weighted images were acquired using a brain volume sequence with the following parameters: repetition time (TR) = 8.2 ms; echo time (TE) = 3.2 ms; inversion time (TI) = 450 ms; flip angle (FA) = 12°; field of view (FOV) = 256 × 256 mm; matrix = 256 × 256; slice thickness = 1 mm, no gap; and 188 sagittal slices. Resting-state fMRI data were acquired using a gradient-echo single-short echo planar imaging sequence with the following parameters: TR/TE = 2000/45 ms; FOV = 220 × 220 mm; matrix = 64 × 64; FA = 90°; slice thickness = 4 mm; gap = 0.5 mm; 32 interleaved transverse slices; and 180 volumes. All subjects were instructed to keep their eyes closed, relax, move as little as possible, think of nothing in particular, and not fall asleep during the fMRI scans.

fMRI DATA PREPROCESSING

Resting-state fMRI data were preprocessed using SPM8 (http://www.fil.ion.ucl.ac.uk/spm). The first 10 volumes for each subject were discarded to allow the signal to reach equilibrium and the participants to adapt to the scanning noise. The remaining volumes were corrected for the acquisition time delay between slices. Then, realignment was performed to correct the motion between time points. All subjects' fMRI data were within the defined

Table 1 | Demographic and clinical characteristics.

Characteristics	Schizophrenia patients	Comparison subjects	*P*-value
Number of subjects	95	93	
Age (years)	33.6 (7.8)	33.0 (10.2)	0.633
Sex (female/male)	41/54	48/45	0.246
Antipsychotic dosage (mg/d) (chlorpromazine equivalents)	446.5 (341.6)		
Duration of illness (months)	121.4 (92.8)		
PANSS			
Positive score	17.1 (7.9)	–	
Negative score	20.3 (9.1)	–	
General score	34.1 (10.8)	–	
Total score	71.5 (23.2)	–	

Data are shown as the means (SD). Abbreviations: PANSS, The Positive and Negative Syndrome Scale.

motion thresholds (i.e., translational or rotational motion parameters less than 2 mm or 2°). We also calculated frame-wise displacement (FD), which indexes the volume-to-volume changes in head position. There were no significant group differences in FD ($t = 0.56$, $P = 0.58$) between patients (0.117 ± 0.007) and controls (0.113 ± 0.006). Several nuisance covariates (six motion parameters, their first time derivations, and average BOLD signals of the ventricular and white matter) were regressed out from the data. Recent studies have reported that the signal spike caused by head motion significantly contaminated the final resting-state fMRI results even after regressing out the linear motion parameters (Power et al., 2012). Therefore, we further regressed out spike volumes when the FD of the specific volume exceeded 0.5. The datasets were then band-pass filtered in a frequency range of 0.01–0.08 Hz. In the normalization step, individual structural images were linearly co-registered with the mean functional image; the structural images were then linearly co-registered to MNI space. Finally, each filtered functional volume was spatially normalized to MNI space using co-registration parameters and resampled into a 3-mm cubic voxel.

rsFCD CALCULATION

The rsFCD of each voxel was calculated using an in-house script that was written in the Linux platform according to the method described by Tomasi and Volkow (2010, 2011a,b). Pearson's linear correlation evaluated the strength of the functional connectivity between voxels. Two voxels with a correlation coefficient of $R > 0.6$ were considered significantly connected. This threshold was proposed to be the optimal threshold for calculating rsFCD in a previous study (Tomasi and Volkow, 2010). The rsFCD calculation was restricted to a cerebral gray matter (GM) mask. The rsFCD at a given voxel x_0 was computed as the total number of functional connections, $k(x_0)$, between x_0 and all other voxels. This calculation was repeated for all x_0 voxels in the brain. The grand mean scaling of rsFCD was obtained by dividing by the mean value of all brain voxels to increase the normality of the distribution. Finally, the rsFCD maps were spatially smoothed using a $6 \times 6 \times 6$ mm full-width at half maximum (FWHM) Gaussian kernel. To explore the effects of the selection of correlation thresholds on our rsFCD analysis, rsFCD maps were also calculated based on two additional correlation thresholds ($R > 0.2$ and 0.4).

GRAY MATTER VOLUME CALCULATION

The gray matter volume (GMV) of each voxel was calculated using voxel-based morphometry (VBM), as implemented in the VBM8 toolbox (http://dbm.neuro.uni-jena.de/vbm.html). Structural MR images were segmented into GM, white matter and cerebrospinal fluid using the standard segmentation model. After an initial affine registration of the GM concentration map into Montreal Neurological Institute (MNI) space, GM concentration images were nonlinearly warped using diffeomorphic anatomical registration through the exponentiated Lie algebra (DARTEL) technique, and the results were resampled to a voxel size of $3 \times 3 \times 3$ mm. The relative GMV of each voxel was obtained by multiplying the GM concentration map by the non-linear determinants that were derived from the spatial normalization step. Finally, the GMV images were smoothed using a Gaussian kernel of $6 \times 6 \times 6$ mm FWHM. After spatial preprocessing, the smoothed GMV maps were used for statistical analyses.

STATISTICAL ANALYSIS

Group differences in rsFCD were compared in a voxel-wise manner using a general linear model with age and sex as nuisance variables. A permutation-based inference tool for nonparametric statistics in FMRIB's diffusion toolbox (FSL 4.0, http://www.fmrib.ox.ac.uk/fsl) was used to perform this analysis. The number of permutations was set to 5000, and the significance threshold was set at $P < 0.05$ after correcting for family-wise error (FWE) using the threshold-free cluster enhancement (TFCE) option in FSL. To exclude the possible effects of GMV on rsFCD changes, we repeated the group comparisons with the GMV as an additional covariate of no interest at the voxel-wise level. Moreover, the same statistical steps were applied to rsFCD at the thresholds of $R > 0.2$ and 0.4.

The mean rsFCD of each cluster with significant group differences was extracted for each subject. The partial correlation coefficient was used to test the association between rsFCD and the clinical variables, which included antipsychotic agent dosages of chlorpromazine equivalents, illness duration and PANSS scores. Age and gender effects were controlled, and multiple comparisons were corrected using the Bonferroni method ($P < 0.05$).

In addition, correlation analyses between rsFCD and clinical variables were performed in a voxel-wise manner in the whole brain. A linear regression model was used to perform correlation analyses with age and gender as covariates of no interest. Multiple comparisons were corrected using a FWE method ($P < 0.05$). To clarify the relationship between rsFCD and psychosis, we further divided the schizophrenia patients into patients with current psychotic symptoms ($n = 57$) and those without current psychotic symptoms ($n = 38$) and compared the rsFCD differences between the subgroups ($P < 0.05$, FWE correction).

RESULTS

SUBJECT DEMOGRAPHICS AND CLINICAL CHARACTERISTICS

The demographic and clinical characteristics of the subjects are summarized in **Table 1**. There were no significant group differences in sex ($\chi^2 = 1.35$, $P = 0.25$) or age ($t = 0.48$, $P = 0.63$). Eighty-seven patients received medications during the MRI examinations, and the remaining 8 patients had never received any medications. The mean dosage of antipsychotic agents (chlorpromazine equivalents) was 446.5 ± 341.6 mg/d for schizophrenia patients. The mean duration of illness was 121.4 ± 92.8 months. The mean scores of PANSS positive sub-scale, negative sub-scale and general psychopathology sub-scale were 17.1 ± 7.9, 20.3 ± 9.1, and 34.1 ± 10.8.

rsFCD DIFFERENCES BETWEEN SCHIZOPHRENIA PATIENTS AND HEALTHY CONTROLS

Without GMV correction, compared with HCs, schizophrenia patients exhibited increased rsFCD in the bilateral sub-cortical regions (including putamen, pallidum, left caudate body) and hippocampi and decreased rsFCD in the bilateral postcentral gyri, right inferior temporal gyrus and occipital cortex (**Figure 1A** and **Table 2**). After correction for GMV, the distribution of

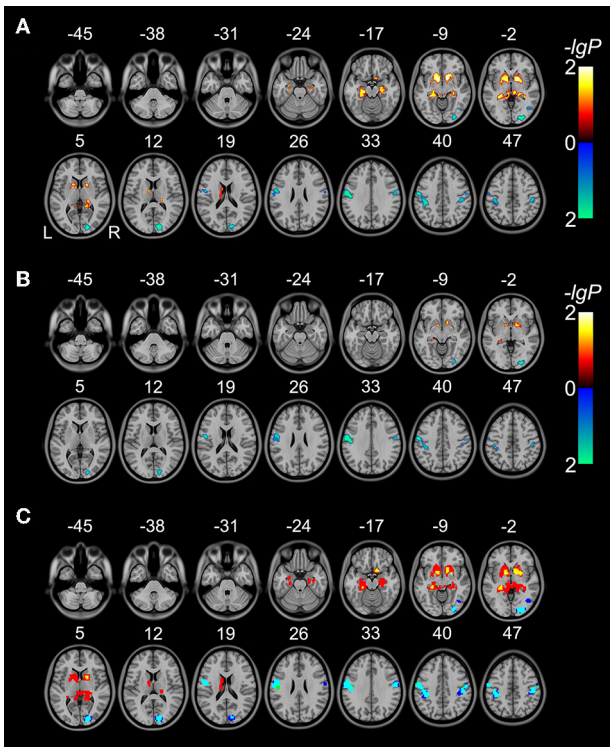

FIGURE 1 | Brain regions with significant differences in rsFCD ($P < 0.05$, FWE corrected) between schizophrenia patients and healthy comparison subjects without (A) and with (B) correction for GMV. The warm color represents increased rsFCD and the cold color denotes decreased rsFCD in patients with schizophrenia. Overlap between significant rsFCD difference maps without and with GMV correction **(C)**. Red denotes increased rsFCD in schizophrenia without GMV correction; yellow represents increased rsFCD both without and with GMV correction. Blue and green denote decreased rsFCD without and with GMV correction, respectively; cyan represents decreased rsFCD both without and with GMV correction.

Table 2 | rsFCD changes in schizophrenia patients relative to healthy controls without GMV correction.

Brain regions	Brodmann areas	Cluster size (voxels)	Peak *t*-values	Coordinates in MNI (*x, y, z*)
SCHIZOPHRENIA PATIENTS > HEALTHY CONTROLS				
Left putamen, pallidum	–	225	4.16	−12, 3, −6
Right putamen, pallidum	–	194	4.27	12, 9, −9
Left caudate body	–	38	2.99	−9,−3, 15
Left hippocampus	36, 35	270	3.81	−27, −36, −3
Right hippocampus	35	237	3.39	27, −30, −6
SCHIZOPHRENIA PATIENTS < HEALTHY CONTROLS				
Right occipital lobe	18	178	−4.80	27, −90, −3
Left postcentral gyrus	2, 3	241	−4.60	−57, −12, 33
Right postcentral gyrus	2, 3	111	−4.35	48, −24, 42
Right inferior temporal gyrus	37	25	−4.83	42, −66, −6

Abbreviations: rsFCD, resting-state functional connectivity density; MNI, Montreal Neurological Institute.

Table 3 | rsFCD changes in schizophrenia patients relative to healthy controls with GMV correction.

Brain regions	Brodmann areas	Cluster size (voxels)	Peak *t*-values	Coordinates in MNI (*x, y, z*)
SCHIZOPHRENIA PATIENTS > HEALTHY CONTROLS				
Left putamen, pallidum	–	24	4.11	−12, 3, −6
Right putamen, pallidum	–	70	4.09	15, 3, −3
Left hippocampus	–	20	3.80	−27, −36, −3
SCHIZOPHRENIA PATIENTS < HEALTHY CONTROLS				
Right occipital lobe	18	107	−4.81	27, −90, −3
Left postcentral gyrus	2, 3	237	−4.67	−54, −12, 36
Right postcentral gyrus	2, 3	63	−4.01	48, −24, 45

Abbreviations: rsFCD, resting-state functional connectivity density; MNI, Montreal Neurological Institute.

brain regions with significant differences in rsFCD was similar to that observed without GMV correction; however, the spatial extent was much smaller in the striatal and hippocampal regions (**Figure 1B** and **Table 3**). Significant rsFCD difference maps with and without GMV correction are overlapped in **Figure 1C**. In addition, brain regions with significant intergroup differences in rsFCD at the correlation thresholds of $R > 0.2$ and 0.4 are shown in Supplementary Figures S1, S2. Almost all of the significant brain regions at the threshold of $R > 0.6$ were included in the maps using thresholds of $R > 0.2$ and 0.4; however, the spatial extent of significant brain regions was much larger at the thresholds of $R > 0.2$ and 0.4 than that at the threshold of $R > 0.6$.

THE ASSOCIATION BETWEEN rsFCD AND CLINICAL VARIABLES

In brain areas demonstrating group differences in rsFCD with correction for GMV, we did not find any statistical correlations between rsFCD and antipsychotic dosages of chlorpromazine equivalents, illness duration, and all PANSS scores (positive, negative, general psychopathology scores) (Supplementary Table S1). Additionally, no brain regions exhibited significant correlations between rsFCD and clinical variables in the voxel-based analyses ($P < 0.05$, FWE corrected). We did not observe any significant differences in rsFCD between patients with and without current psychotic symptoms ($P < 0.05$, FWE corrected).

DISCUSSION

In this study, we used a data-driven voxel-based method to compare rsFCD differences between schizophrenia patients and HCs. We found that schizophrenia patients exhibited increased rsFCD in the striatum and hippocampus and decreased rsFCD in the postcentral gyri and occipital cortex compared with HCs. The rsFCD changes in these regions reflect an alteration of total number of functional connections between these regions and all other

voxels in the whole brain, representing an alteration of communication capacity and functional brain dynamics in these regions. Thus, our findings provide new evidence for the hypothesis that schizophrenia is a disorder of connectivity abnormalities from the perspective of functional connectivity density.

INCREASED FCD IN SCHIZOPHRENIA

In the present study, we found that schizophrenia patients had increased rsFCD in the striatum and hippocampus. The striatum and hippocampus are central components of the brain in the generation of psychosis and other symptom states in schizophrenia (Davis et al., 1991; Epstein et al., 1999; Howes and Kapur, 2009; Perez-Costas et al., 2010; Grace, 2012). Previous studies have demonstrated early striatal hypertrophy in first-episode psychosis (Chua et al., 2009) and significant correlations between GM density in the hippocampus and early psychotic symptoms in adolescents at risk of psychosis (Spencer et al., 2007). Several studies have suggested that the hyper-spontaneous neural activity in the striatum and hippocampus is a pathological feature of schizophrenia. For example, increased regional cerebral blood flow (rCBF) and amplitude of low frequency fluctuation (ALFF) in the striatum and hippocampus have been frequently reported in schizophrenia (Malaspina et al., 2004; Hoptman et al., 2010; Scheef et al., 2010; Pinkham et al., 2011; Liu et al., 2012; Turner et al., 2013). Moreover, previous studies have also reported increased rsFCS of the striatum and hippocampus with other parts of the brain (Salvador et al., 2010; Hoffman et al., 2011). However, Koch et al. found decreased striatal functional connectivity using psychophysiological interactions (PPI), which was closely linked to GMV changes of the striatum (Koch et al., 2014). Different methodologies (PPI vs. FCD) and experimental designs (task-evoked vs. resting-state) may account for the different findings between that study and ours. Although several previous studies suggested that increased functional activity and connectivity in these regions may be an effect of antipsychotic drugs, our non-significant correlations between aberrant rsFCD and antipsychotic dosages do not support this hypothesis. Our findings of increased rsFCD in the striatum and hippocampus may support the connectivity disorder hypothesis of schizophrenia from a new perspective of the number of functional connectivity.

After correcting for GMV, the spatial extent of the striatal and hippocampal regions with significant rsFCD differences between groups was much smaller than without GMV correction. This finding indicates that rsFCD increases in the striatal and hippocampal regions are partially related to GM atrophy. To clarify the specific relationship between rsFCD and GMV, we performed a correlation analysis in patients and controls, respectively. We did not find any significant correlations (Supplementary Table S2). These findings suggest that the relationship between GMV and rsFCD changes in schizophrenia is rather complex. In future studies on schizophrenia, the GMV effect on the functional changes (such as the rsFCD) should be considered.

DECREASED FCD IN SCHIZOPHRENIA

We also found decreased rsFCD in the cortical regions involved in processing of sensorimotor and visual information; this effect is independent of GMV changes in these regions. These findings are consistent with a previous study that reported decreased local FCD in the sensorimotor cortex in schizophrenia (Tomasi and Volkow, 2014). The sensorimotor and visual areas also showed decreased rCBF (Pinkham et al., 2011), ALFF (Hoptman et al., 2010; Turner et al., 2013) and regional homogeneity (ReHo) (Liu et al., 2006; Yu et al., 2013) in schizophrenia. Hypoconnectivity of the visual and sensormotor networks has also been reported in schizophrenia (Collin et al., 2011; Tang et al., 2012; Calderone et al., 2013; Damaraju et al., 2014). Abnormal activity or connectivity of the visual cortex may be associated with deficits in visual processing and object-recognition in schizophrenia (Onitsuka et al., 2007; Wynn et al., 2008). Similarly, abnormal activity or connectivity of the sensorimotor cortex may be related to neurological soft signs that cause neurological abnormalities in sensory integration, motor regulation, and sequencing complex motor acts in schizophrenia patients (Schmauss et al., 1993). Combined with these previous studies, our findings further support the functional disconnectivity hypothesis of schizophrenia from the perspective of connectivity density.

ASSOCIATION BETWEEN FCD CHANGES AND THE DOPAMINE HYPOTHESIS OF SCHIZOPHRENIA

A previous imaging genetics study revealed that FCD can be modulated by the presumed dopamine signaling in healthy subjects, and the modulation pattern is different in different regions (Tian et al., 2013). This study found an association between FCD and dopamine activity. According to the dopamine hypothesis of schizophrenia, hyperdopaninergia in the striatum, and hypodopaminergia in the cortical regions, especially the prefrontal cortex, have been conceived as an important pathological feature of schizophrenia (Howes and Kapur, 2009). Dopamine neuron responsivity is shown to be modulated by the hippocampus, which exhibits hyperactivity in schizophrenia (Grace, 2012). Therefore, hyperdopaninergia in the striatum may reflect an overly responsive state of the dopamine system that is driven by hyperactivity in the hippocampus. This theoretical hypothesis may explain our findings of increased rsFCD in the striatum and hippocampus in schizophrenia. In contrast to the dominant dopamine receptor D2 in the striatum, the dominant dopamine receptor is D1 in the cerebral cortex. D1 dysfunction in the prefrontal cortex has been linked to cognitive impairment and negative symptoms in schizophrenia (Abi-Dargham and Moore, 2003; Goldman-Rakic et al., 2004; Laruelle, 2014). The distribution differences in dopamine receptors between the striatum and cortex may be an explanation for the completely different rsFCD changes in these two kinds of brain regions in schizophrenia. However, most previous studies focused on dopamine activity in the prefrontal cortex; whether the influence of this activity can be applied to the sensory and motor areas need to be further determined. However, there is a lack of direct evidence for an association between FCD changes and dopamine activity; thus, future studies are needed to clarify this potential association.

In this present study, we did not find a correlation between aberrant rsFCD and antipsychotic dose, illness duration, and symptom severity. These results suggest that aberrant rsFCD may be an independent trait characteristic of schizophrenia, which means that all schizophrenia patients have aberrant rsFCD,

regardless of symptom severity. This is not surprising, given that many studies have shown that illness duration, symptom severity, and antipsychotic dose are not linearly related to brain functional activity disturbances. Furthermore, we did not observe any differences in rsFCD between patients with and without current psychotic symptoms. BOLD signal alterations may better track schizophrenia, regardless of the presence or absence of psychosis, rather than psychosis severity or duration and are not linearly related to antipsychotic dose (Woodward et al., 2012; Ren et al., 2013). However, we cannot rule out the possibility that either a lack of statistical power or a lack of data assessing the appropriate dependent clinical variables may account for non-significant correlations between rsFCD and demographic and clinical variables in our study. Therefore, in future studies, appropriate statistical methods and detailed clinical variables may contribute to the solution of this problem.

CONCLUSION

Our study found that increased rsFCD in the striatal and hippocampal regions and decreased rsFCD in the sensorimotor regions are pathological features of schizophrenia, which may support the hypothesis that schizophrenia is a connectivity disorder from the perspective of functional connectivity density. The similar spatial distribution and abnormal patterns between brain regions with rsFCD alterations and those with dopamine alterations are interesting findings and deserve further investigations.

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SUPPLEMENTARY MATERIAL

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Impairments of working memory in schizophrenia and bipolar disorder: the effect of history of psychotic symptoms and different aspects of cognitive task demands

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Comparisons of cognitive impairments between schizophrenia (SZ) and bipolar disorder (BPD) have produced mixed results. We applied different working memory (WM) measures (Digit Span Forward and Backward, Short-delay and Long-delay CPT-AX, N-back) to patients with SZ ($n = 23$), psychotic BPD ($n = 19$) and non-psychotic BPD ($n = 24$), as well as to healthy controls (HC) ($n = 18$) in order to compare the level of WM impairments across the groups. With respect to the less demanding WM measures (Digit Span Forward and Backward, Short-delay CPT-AX), there were no between group differences in cognitive performance; however, with respect to the more demanding WM measures (Long-delay CPT-AX, N-back), we observed that the groups with psychosis (SZ, psychotic BPD) did not differ from one another, but performed poorer than the group without a history of psychosis (non-psychotic BPD). A history of psychotic symptoms may influence cognitive performance with respect to WM delay and load effects as measured by Long-delay CPT-AX and N-back tests, respectively. We observed a positive correlation of WM performance with antipsychotic treatment and a negative correlation with depressive symptoms in BPD and with negative symptoms in SZ subgroup. Our study suggests that WM dysfunctions are more closely related to a history of psychosis than to the diagnostic categories of SZ and BPD described by psychiatric classification systems.

Keywords: schizophrenia, psychotic vs. non-psychotic bipolar disorder, working memory, history of psychosis

INTRODUCTION

The dichotomy between mood disorders and schizophrenia has been described by Kraepelin in the nineteenth century, mainly based on their different illness course and prognosis (Kraepelin, 1921). However, until today the nosological and clinical implications of psychotic features in the course of mood disorders (Henry and Etain, 2010) and the concept of unitary psychosis including affective and non-affective psychotic disorders are widely debated (van Os et al., 2000; Angst, 2002; Peralta and Cuesta, 2005; Reininghaus et al., 2013). Current diagnostic systems make a categorical distinction between the disorders that are primarily affective and the disorders that are primarily psychotic in nature. However, psychotic and affective disorders are increasingly perceived as dimensionally different rather than categorically separate entities (Kane and Engle, 2002). This concept is supported by research showing that neuropsychological dysfunctions among psychotic and affective disorders depend on a history of psychosis rather than a diagnostic group itself.

Neurocognitive dysfunctions are core characteristics of schizophrenia (SZ) and are increasingly recognized as an important feature of bipolar disorder (BPD) (Vohringer et al., 2013). However, comparisons of cognitive impairments between BPD and SZ have produced mixed results. Some authors indicate that patients with BPD exhibit a similar pattern of deficits to patients with SZ, but the level of impairment can be placed between SZ patients and healthy controls (HC) (Krabbendam et al., 2005; Daban et al., 2006; Schretlen et al., 2007; Hamilton et al., 2009; Reichenberg et al., 2009), while others show no qualitative or quantitative neurocognitive differences between patients with SZ and BPD (Mojtabai et al., 2000; McClellan et al., 2004; Simonsen et al., 2011).

It has been suggested that the discrepancy in these findings may be explained by the fact that neurocognitive dysfunction in BPD and SZ spectrum disorders depend on a history of psychosis rather than a diagnostic group (Simonsen et al., 2011; Ivleva et al., 2012). (Pearlson et al., 1995; Strasser et al., 2005; Glahn et al., 2007; Cui et al., 2011; Anticevic et al., 2014). This hypothesis has been supported by findings showing that BPD patients with psychotic symptoms [BPD(+)] perform worse than BPD patients without psychotic symptoms [BPD(−)] on a variety of

neurocognitive measures (Glahn et al., 2006, 2007; Bora et al., 2007; Martinez-Aran et al., 2008; Allen et al., 2010; Simonsen et al., 2011; Ivleva et al., 2012). In the recent meta-analytic study, a history of psychotic symptoms in BPD was found to be associated with moderately greater impairment in planning and reasoning, working memory, verbal memory and processing speed (Bora et al., 2010). Interestingly, it has been suggested that unlike BPD(−), BPD(+) is associated with biological correlates typical for SZ (Glahn et al., 2007), such as significantly enlarged lateral and third ventricles (Strasser et al., 2005), increased D2 dopamine receptor density (Pearlson et al., 1995), reduced neural connectivity (Anticevic et al., 2014) and gray matter deficits (Cui et al., 2011).

Working memory (WM) is the system for the temporary storage of information on a moment-to-moment basis and is a critical function that underlies various complex cognitive tasks such as language, comprehension, learning and reasoning (Baddeley, 1992). WM is a complex and multifaceted construct. It is able to encode, retrieve, store, update, and manipulate information in the mental workspace. Strong evidence has accrued that these processes are relatively separate (Cornoldi et al., 2000; Woodman and Vogel, 2005) and that there is high individual variability in their capacity (Kane and Engle, 2002). Neuroimaging studies provide evidence that these processes may also involve different neural networks (Jolles et al., 2011; Marvel and Desmond, 2012; Liao et al., 2014) and are associated with different neural network activity level (Veltman et al., 2003; Jolles et al., 2011). The capacity of different WM processes can be assessed by tasks that manipulate either the load of data to be stored (load effect), the duration of the maintenance period (delay effect) or the number of items to be manipulated within WM (manipulation effect) (Jolles et al., 2011; Pinal et al., 2014).

Although WM impairments were previously probed in SZ and BPD, comparisons across disorders have yielded inconsistent findings and the relationships between cognitive and clinical symptom domains across the psychotic variations of the disorders remain unclear (Hamilton et al., 2009; Mayer and Park, 2012; Milanovic and Vangel, 2012; Zhang et al., 2012). Discrepancy in previous studies may be due to a heterogeneous patient group and/or differences in specific task demands of the cognitive measures employed. Among other cognitive domains, WM has been shown to be associated with a history of psychosis rather than affective or psychotic diagnostic category by numerous studies (Bora et al., 2007; Glahn et al., 2007; Martinez-Aran et al., 2008; Savitz et al., 2009; Simonsen et al., 2011). However, WM tasks employed in the previous studies measure different components of WM, not allowing for comparisons across different levels of task demands (load of information, duration, or maintenance, number of items to be manipulated within WM). The lack of capacity measurements for the different WM domains make it impossible to precisely and reliably compare cognitive performance between different psychiatric subpopulations.

Taking into account the limitations of previous studies, the aim of our research is to examine different WM aspects in a cross diagnostic sample to determine as to whether the symptomatology dimension in terms of psychotic, affective and both psychotic and affective symptoms is a common denominator of different WM impairments in SZ and BPD patients. On the basis of the psychosis dimension concept, we hypothesize that BPD(+) and SZ patients share common WM deficits that are different than those observed in BPD(−) patients and that these groups of patients can be classified on the basis of WM performance. We assess three components of WM: the delay effect, measured with Short-delay and Long-delay CPT-AX tasks; the load effect, as measured using the Forward Digit Span (FDS) task and N-back task; and manipulation of information capacity was measured with the Backward Digit Span (BDS). Although there are many studies of WM in SZ and BPD, this is the only study to assess different aspects of WM with respect to psychosis dimension in these patient populations.

MATERIALS AND METHODS

PARTICIPANTS

We included 66 patients [24 BPD(−), 19 BPD(+) and 23 SZ] and 18 healthy controls (HC) in the study (for raw data see Supplementary Material 1). All HC were recruited from the community via ads, word of mouth, or others who participated in previous studies. Patients were recruited from the Psychogeriatric Research Center, Institute of Psychiatry, Faculty of Medicine, Ain Shams University, Cairo, Egypt. Diagnosis of BPD and SZ was based on Structured Clinical Interview for DSM-IV Axis I disorders (SCID-I) performed by the same trained clinician. Clinical data in SCID-I was gathered from personal interviews, clinical observation, medical records (hospital and outpatient clinic case notes) and family interviews (if the patient was unable to provide required information). Any patients with other schizophrenia spectrum disorders including: schizophreniform disorder, schizoaffective disorder, delusional disorder, brief psychotic disorder, substance-induced psychosis, and psychotic disorders due to general medical conditions, as well as first-episode psychosis of any type were not included in the study. None of the diagnoses were changed from those originally given by the treating psychiatrist. Patients diagnosed with BPD and had experienced delusions or hallucinations during a mood episode were included in the BPD(+) group. While BPD patients who had not experienced hallucinations or delusions during any of the mood episodes were included in the BPD(−) group. Psychotic symptoms were coded based on module B of SCID-I.

All patients were outpatients and had been on a stable medication regimen for at least 4 weeks prior to testing. All participants had no history of significant neurological injury and reported no serious medical illness or substance use disorders. None of the participants had any first-degree relatives with psychiatric illness. The demographic and clinical characteristics are shown in **Tables 1, 2**. All participants provided informed consent for a protocol approved by the Bioethical Committee of Ain Shams University and its associated clinics.

MATERIALS

The participants of the study were assessed using a diagnostic interview, clinical scales and neuropsychological tasks. SZ patients were evaluated on Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987), BPD patients were assessed for depressive symptoms on Hamilton Depression Rating Scale (HDRS) (Hamilton, 1960) and for manic symptoms on Young

Table 1 | Demographic and clinical characteristics for the participants.

	HC	BPD(−)	BPD(+)	SZ	*P*-value*
Number of participants	18	24	19	23	
Age (mean ± *SD*)	44.34 ± 6.99	43.01 ± 5.11	44.63 ± 4.02	42.09 ± 6.02	0.41
%Male	33.4	41.7	36.8	39.1	0.95
Education[a] (mean ± *SD*)	11.94 ± 2.88	11.9 ± 2.96	11.63 ± 3.32	12.00 ± 3.00	0.97
NAART[b] (mean ± *SD*)	105.76 ± 7.25	100.88 ± 9.14	104.72 ± 10.35	104.94 ± 8.55	0.98
CPZ[c] equivalent dosage (mean ± *SD*)	–	403.54 ± 187.67	396.00 ± 127.6	373.00 ± 111.77	0.59
Duration of illness (mean ± *SD*)	–	18.29 ± 4.07	19.37 ± 4.64	17.52 ± 4.86	0.37
PANSS[d] (mean ± *SD*)					
PANSS Negative Symptoms	–	–	–	19.35 ± 4.69	
PANSS Positive Symptoms	–	–	–	16.70 ± 3.98	
PANSS General Symptoms	–	–	–	24.48 ± 5.13	
YMRS[e] (mean ± *SD*)	–	18.42 ± 3.97	24.00 ± 4.55	–	0.00
HDRS[f] (mean ± *SD*)	–	23.17 ± 3.96	23.74 ± 2.99	–	0.30
AES[g] (mean ± *SD*)	39.94 ± 5.33	36.20 ± 7.85	36.31 ± 7.61	35.88 ± 8.76	0.34

HC, healthy controls; BPD(−), BPD patients without history of psychosis; BPD(+), BPD patients with history of psychosis; SZ, patients with schizophrenia.

**p-value for Kruskal-Wallis test for continuous variables and for chi-square test for categorical variables.*

[a] Number of years of completed education.

[b] Estimate of premorbid IQ measured by North American Adult Reading Test (NAART).

[c] CPZ, chlorpromazine.

[d] PANSS, Positive and Negative Symptoms Scale.

[e] YMRS, Young Mania Rating Scale.

[f] HDRS, Hamilton Depression Rating Scale.

[g] AES, Apathy Evaluation Scale.

Table 2 | Detailed information about treatment regime with respect to different patient groups.

Antipsychotic medication	BPD(−)	BPD(+)	SZ
%Chlorpromazine only	12.5	26.3	13.0
%Clozapine only	8.3	52.6	47.8
%Haloperidol only	16.7	5.3	8.7
%Risperidone only	33.3	10.5	8.7
%Chlorpromazine plus another antipsychotic	8.3	5.3	8.7
%Clozapine plus another antipsychotic	12.5	–	4.3
%Haloperidol plus another antipsychotic	4.2	–	8.7
%Risperidone plus another antipsychotic	4.2	–	–

HC, healthy controls; SZ, schizophrenia; BPD(−), bipolar disorder without history of psychosis; BPD (+), bipolar disorder with history of psychosis.

Mania Rating Scale (YMRS) (Young et al., 1978). All participants were evaluated on Apathy Evaluation Scale (Marin et al., 1991). Premorbid IQ was estimated based on the performance on the North American Adult Reading Test (NAART) (Blair and Spreen, 1989). Neuropsychological tasks included Forward Digit Span (FDS) and Backward Digit Span (BDS) (Wechsler, 1997), as well as Short-delay CPT-AX, Long-delay CPT-AX and N-back task.

TASKS

The experimenters were present during the entire testing session to ensure participants responded to all stimuli presentations.

Forward digit-span (FDS) and backward digit-span (BDS)

Digit Span tasks are used to measure WM's load capacity. Participants are presented with a series of digits and must repeat them back. If they do this successfully, they are given a longer list. The length of the longest list is the person's digit span. We used the Digit Span subtests of the Wechsler Adult Intelligence Scale III (WAIS-R-III) (Wechsler, 1997). Participants are required to recall the digit lists both in forward chronological order (FDS), and in reverse order (BDS).

Short-delay CPT-AX and Long-delay CPT-AX

The CPT-AX task used here is a variation of delayed-response task. For similar designs see (Barch et al., 1997; Cohen et al., 1999; Moustafa et al., 2008; Paxton et al., 2008). Participants were presented with a sequence of four-letter stimuli, one at a time (H, K, Z, P). Participants were presented with H before Z, H before P, K before Z and K before P. Participants were instructed to figure out the target sequence by trial and error based on correct or incorrect feedback given to them after each stimulus. In this task, a correct response to a given letter depends on the letter preceding it. In the task, HZ is a target sequence and all other sequences (HP, KZ, KP) are incorrect.

Participants were to press a key on the left side of the keyboard ("z") in case of non-target sequence, and a key on the right side of the keyboard ("m") in case of target sequence. Correct and incorrect feedback were shown on the screen as green "CORRECT" or red "INCORRECT," following participants' responses on each trial.

The instructions here were as follows: "In this task, you will have to figure out the target sequence by trial and error. At the end of each trial, you will get feedback to see if you were CORRECT or INCORRECT. You will use this feedback to figure out the right sequence. You will see the letters H, K, Z, P. Try to keep track of the letters. Press "z" button for every letter, EXCEPT when you think you have seen the target sequence, press "m" button. At first you will have to guess. You will figure out what the target sequence is as you get CORRECT/INCORRECT feedback to your button presses."

The measure reported in this task is accuracy (percentage of correct responses) across all four trial types (HZ, HP, KZ, KP). There were 150 trials in total in the task. In Short-delay CPT-AX task, delay interval between each letter presentation was 1 s, while in Long-delay CPT-AX task, delay interval was 5 s and different set of letters was used (M, T, R, S).

N-back task

The N-back task tests the effects of WM load on performance (Cohen et al., 1997; Owen et al., 2005; Wallwork et al., 2012). In this task, a sequence of letters was presented to the participants, one at a time. The task involves the presentation of the sequences made up of the following distinct letters: BFKHMQRX. Participants were instructed to indicate when the current stimulus matches the one from *n* steps earlier in the sequence. The load factor *n* was 2 for easier version of the task and 3 for the more difficult version. For each of 2- and 3-back conditions, we presented participants with four blocks of 48 stimulus presentations. As in the CPT-AX task, we reported the percentage of correct trials across 2- and 3-back conditions. Stimulus encoding and response demands were constant across conditions; only requirements to maintain and update increasingly greater amounts of information at higher loads differed. Pseudorandom sequences of single consonants were presented, and participants responded to each stimulus, pressing one button to targets and another one to non-targets. Order of task conditions was randomized across participants. Due to the fact that a significant percentage of patients and controls were not successful at 3-back condition, in the further analysis we include only results based on the performance on 2-back condition of N-back task.

STATISTICAL ANALYSIS

For statistical analysis, we used Statistical Package for Social Sciences (SPSS) computer program version 19.0. Neuropsychological variables were tested for the assumptions of normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test). Due to the small number of the participants, lack of normal distribution and homogeneity of variances of some variables, non-parametric analyses of data were performed. Differences in socio-demographic and clinical data between the groups were compared with chi-square test for categorical data and nonparametric tests for continuous data (Kruskal-Wallis and Mann-Whitney tests). Relations between neuropsychological tests' scores with respect to demographic and clinical variables were tested with Spearman's rank correlations. To determine the degree of association in cognitive performance on WM tests between diagnostic groups, we calculated effect size as follows: $r = Z/\sqrt{N}$, where r is effect size, Z is z-score and N is the number of observations (Corder and Foreman, 2009). In order to control for co-varying variables, we have used the non-parametric method—rank analysis of covariance (Quade, 1967) (Supplementary Material 2). All tests were two-tailed using a 0.05 level of significance.

RESULTS

IMPACT OF DEMOGRAPHIC VARIABLES ON COGNITIVE PERFORMANCE

The demographic characteristics, clinical variables and pharmacological status of HC, BPD(−), BPD(+), SZ at the time of neuropsychological testing are summarized in **Tables 1, 2**. The groups were homogenous with respect to age, number of years of completed education, estimated premorbid IQ, duration of illness and chlorpromazine (CPZ) equivalent dosage. There were no differences across groups with respect to the severity of apathy or depressive symptoms; however, BPD(+) had more pronounced manic symptoms in comparison with BPD(−). It is worth noting that the premorbid IQ scores and number of years of completed education did not differ across groups. This means that our SZ sample may be less representative to the general SZ population, yet suitable for contrasting cognitive characteristics between groups.

Age, number of years of completed education, premorbid IQ and disease duration were not significantly related to task performance (**Table 3**). However, the CPZ dosage equivalent was correlated with performance on the more demanding WM tasks: Long-delay CPT-AX ($r = 0.561$, $p < 0.001$) and N-back ($r = 0.458$, $p < 0.001$) tests. Additionally, CPZ dosage equivalent was correlated with negative symptoms (PANSS) ($r = -0.55$, $p = 0.006$) and depressive symptoms (HDRS) ($r = -0.46$, $p = 0.002$).

IMPACT OF DIAGNOSIS ON COGNITIVE PERFORMANCE

The results of between group comparisons in performance on all WM measures are presented on **Figure 1** and in Supplementary Material 3.

Differences in cognitive performance with respect to WM across diagnostic groups depended on the neuropsychological tests applied. Firstly, we analyzed the performance between HC, the combined BPD groups and SZ patients. We found that there were no statistically significant differences in performance on FDS, BDS and Short-delay CPT-AX ($p > 0.05$); however, with respect to Long-delay CPT-AX and N-back tests, SZ patients performed significantly worse than BPD patients ($p = 0.007$ and $p < 0.001$ respectively), and BPD patients performed significantly worse than HC ($p = 0.002$ and $p = 0.006$, respectively).

Further, we compared the cognitive performance between groups by subdividing BPD patients into BPD(−) and BPD(+).There were no significant differences with respect to FDS, BDS and Short-delay CPT-AX between SZ, BPD(+), BPD(−) and HC ($p > 0.05$). However, with respect to Long-delay CPT-AX test, BPD(−) performed equally well as HC ($p > 0.05$). Additionally, BPD(+) and SZ patients performed significantly worse in comparison to HC ($p < 0.001$). With respect to N-back test, BPD(−) patients performed significantly worse than HC ($p = 0.001$), but at the same time significantly better than BPD(+) and SZ patients ($p < 0.001$).

Table 3 | Correlations between demographic and clinical variables with WM tests (Digit span forward, Digits span backward, Short-delay CPT-AX test, Long-delay CPT-AX test and N-back test).

	Working memory tests				
	Digit Span Forward	**Digit Span Backward**	**Short-delay CPT-AX**	**Long-delay CPT-AX**	**N-back**
Age	$r = 0.04$ $p = 0.56$	$r = -0.03$ $p = 0.80$	$r = 0.06$ $p = 0.59$	$r = 0.01$ $p = 0.91$	$r = -0.05$ $p = 0.66$
Number of completed years of education	$r = -0.08$ $p = 0.45$	$r = 0.06$ $p = 0.45$	$r = 0.04$ $p = 0.56$	$r = 0.02$ $p = 0.82$	$r = -0.01$ $p = 0.91$
Premorbid IQ	$r = 0.03$ $p = 0.79$	$r = 0.05$ $p = 0.64$	$r = 0.02$ $p = 0.82$	$r = -0.09$ $p = 0.41$	$r = -0.07$ $p = 0.54$
Disease duration	$r = 0.04$ $p = 0.75$	$r = -0.08$ $p = 0.53$	$r = -0.06$ $p = 0.61$	$r = -0.14$ $p = 0.27$	$r = 0.17$ $p = 0.17$
CPZ equivalent dosage	$r = -0.131$ $p = 0.29$	$r = -0.125$ $p = 0.32$	$r = 0.091$ $p = 0.46$	**$r = 0.561$ $p < 0.0001$**	**$r = 0.458$ $p < 0.0001$**

r, Spearman's correlation coefficient, p-value- two-tailed.
Significant correlations ($p < 0.05$) were marked in bold.

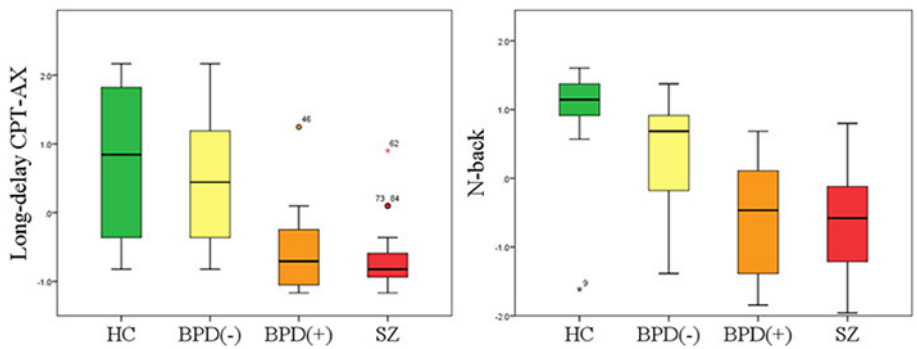

FIGURE 1 | Neurocognitive performance in HC, BPD(−), BPD(+) and SZ patients on D Long-delay CPT-AX and N-back tasks. Results are converted to Z-scores for better visualization. Box plots are shown with 95% CI as error bars. Abbreviations: HC, healthy controls; SZ, schizophrenia; BPD(−), bipolar disorder without history of psychosis; BPD(+), bipolar disorder with history of psychosis.

To determine whether performance differences between BPD(+) and BPD(−) in Long-delay CPT-AX and N-back tests were secondary to current symptomatology and/or differences in medication usage, non-parametric analyses of covariance were performed including the following variables: age, gender, number of years of completed education, estimated premorbid IQ, duration of illness, depressive symptoms (HDRS), manic symptoms (YMRS), apathy level (AES) and CPZ equivalent dosage. A significant between-group differences on Long-delay CPT-AX ($p < 0.0056$ after Bonferroni correction) and N-back tests ($p < 0.0056$ after Bonferroni correction) indicated that even with these additional covariates, a history of psychotic symptoms may influence cognitive performance (Supplementary Material 4).

IMPACT OF CLINICAL RATINGS ON COGNITIVE PERFORMANCE

Correlations between clinical ratings and WM measures indicated that apathy (AES), mania (YMRS), positive and general symptoms (PANSS) were not associated with any neurocognitive outcome on our WM tests ($p > 0.05$). Depression severity (HDRS) was correlated with lower performance on BDS ($r = -0.463$, $p = 0.023$), Long-delay CPT-AX ($r = -0.535$, $p = 0.007$) and N-back ($r = -0528$, $p = 0.008$) among BPD(−) patients. In BPD(+) patients depressive symptoms (HDRS) correlated significantly with Long-delay CPT-AX ($r = -0.758$, $p < 0.001$) and N-back ($r = -0.584$, $p = 0.009$) (**Table 4**). The severity of negative symptoms (PANSS) was significantly correlated with lower performance on Long-delay CPT-AX ($r = -0.454$, $p = 0.029$) and N-back ($r = -0.633$, $p = 0.009$) among patients with SZ (**Table 4**).

DISCUSSION

DIFFERENT DOMAINS OF WM WITH RESPECT TO PSYCHOSIS DIMENSION

FDS and BDS are among the most frequently used measures to assess WM in clinical studies of patients with BPD and SZ. In

Table 4 | Correlations between clinical ratings and WM tests (Digit span forward, Digits span backward, Short-delay CPT-AX test, Long-delay CPT-AX test and N-back test) with respect to diagnostic groups.

		Working memory tests				
		Digit span forward	**Digit span backward**	**Short-delay CPT-AX**	**Long-delay CPT-AX**	**N-back**
BPD(−)	Depressive symptoms (HDRS)	$r = 0.015$ $p = 0.944$	$\mathbf{r = -0.463}$ $\mathbf{p = 0.023}$	$r = -0.536$ $p = 0.088$	$\mathbf{r = -0.535}$ $\mathbf{p = 0.007}$	$\mathbf{r = -0.528}$ $\mathbf{p = 0.008}$
	Manic symptoms (YMRS)	$r = -0.232$ $p = 0.276$	$r = -0.144$ $p = 0.501$	$r = -0.053$ $p = 0.807$	$r = -0.244$ $p = 0.251$	$r = 0.370$ $p = 0.076$
BPD(+)	Depressive symptoms (HDRS)	$r = 0.273$ $p = 0.258$	$r = 0.134$ $p = 0.583$	$r = 0.183$ $p = 0.453$	$\mathbf{r = -0.758}$ $\mathbf{p<0.001}$	$\mathbf{r = -0.584}$ $\mathbf{p = 0.009}$
	Manic symptoms (YMRS)	$r = 0.105$ $p = 0.668$	$r = 0$–0.356 $p = 0.134$	$r = 0.17$ $p = 0.944$	$r = 0.312$ $p = 0.251$	$r = 0.117$ $p = 0.632$
SZ	Negative symptoms (PANSS)	$r = 0.012$ $p = 0.958$	$r = 0.158$ $p = 0.472$	$r = -0.076$ $p = 0.732$	$\mathbf{r = -0.454}$ $\mathbf{p = 0.029}$	$\mathbf{r = -0.633}$ $\mathbf{p = 0.001}$

SZ, schizophrenia; BPD(−), bipolar disorder without history of psychosis; BPD(+), bipolar disorder with history of psychosis; PANSS, Positive and Negative Symptoms Scale; YMRS, Young Mania Rating Scale; HDRS, Hamilton Depression Rating Scale; r, Spearman's correlation coefficient, p-value- two-tailed.
Significant correlations ($p < 0.05$) were marked in bold.

our study, we have shown that FDS and BDS tests did not differentiate HC, BPD(−), BPD(+) and SZ patients, while more demanding tasks such as Long-delay CPT-AX or N-back did. It is in accordance with previous reports showing that FDS and BDS are less sensitive in assessing WM deficits than other WM measures (Perry et al., 2001; Egeland et al., 2003; Haatveit et al., 2010). Additionally, it has been shown that different WM tests require varying amounts of executive function and research using factor analytic approach demonstrated that FDS and BDS tasks load together to form a factor separate from other tests with higher central executive WM demands, such as N-back task (Allen et al., 2010). Farkas et al. (2008) found that schizophrenia patients with severe negative symptoms are more impaired at rule learning tasks than schizophrenia patients with milder negative symptoms.

To the best of our knowledge, this is the first study using tasks measuring the performance on different WM domains such as: the maintenance of information over a variable delay (delay effect), the number of items maintained in WM (load effect), and manipulation of items in WM (manipulation effect). Based on our results, it seems that the effect of psychosis may not be related to manipulation of information in WM, since there was no difference in performance on FDS and BDS tasks. However, a history of psychotic symptoms may influence cognitive performance with respect to WM delay and load effects as measured by Long-delay CPT-AX and N-back tests, respectively.

COMPARISON OF COGNITIVE PERFORMANCE ON SHORT-DELAY CPT-AX AND LONG-DELAY CPT-AX TESTS

In our study, the difficulty level of CPT-AX task was manipulated by prolonging delays between stimuli, and thus increasing the WM load. Longer delays resulted in greater WM deficits in SZ and BPD(+) in comparison with BPD(−) and HC. This may imply that the likelihood of disrupting mental representations increases with time among persons vulnerable to such interference. Our results are in accordance with previous reports showing that an increase in cue-target delay enables researchers to diagnose the selective WM deficit, namely "maintenance difficulty." This difficulty in maintaining information in WM and using it to produce appropriate response has so far been described in SZ patients (Servan-Schreiber et al., 1996; Lee and Park, 2006) However, the results of our study suggest that it may also be specific to BPD(+) or other groups of patients from psychosis spectrum as WM deficits have been reported in first-episode psychosis patients (Gooding and Tallent, 2004), individuals genetically at risk for schizophrenia (Horan et al., 2008), prodromal and high-risk subjects (Smith et al., 2006; Kelleher et al., 2013), as well as individuals reporting psychotic-like experiences (Laurens et al., 2008).

COMPARISON OF COGNITIVE PERFORMANCE BETWEEN BPD(+) AND BPD(−) PATIENTS

In our study, we have found that there are no differences in performance between BPD(+) and BPD(−) patients with respect to some WM tests (FDS, BDS, Short-delay CPT-AX). With regard to more demanding WM tests (Long-delay CPT-AX and N-back), we were able to observe the difference in the level of cognitive functioning between BPD(+) and BPD(−). This difference prevailed even after controlling for age, gender, number of years of completed education, estimated premorbid IQ, duration of illness, clinical symptoms and CPZ equivalent dosage.

Our results are in line with previous reports showing no significant differences in performance between BPD(+) and BPD(−) on FDS (Glahn et al., 2007; Selva et al., 2007; Martinez-Aran et al., 2008; Savitz et al., 2009; Allen et al., 2010; Brissos et al., 2011) and BDS (Glahn et al., 2007; Selva et al., 2007; Martinez-Aran et al., 2008; Savitz et al., 2009; Allen et al., 2010; Brissos et al., 2011). However, some authors were able to observe differences in cognitive performance between those two groups of patients on these tests (Glahn et al., 2006; Simonsen et al., 2011).

There are few studies showing that WM deficits are indeed associated with the history of psychosis in BPD patients. It has been found that there are significant differences in performance between BPD(+) and BPD(−) with regard to Spatial Delayed Response Task (SDRT) (Glahn et al., 2006, 2007) and Working Memory-Mental Arithmetic Test 2-back (WM-MA) (Simonsen et al., 2011). To the best of our knowledge, our study is the first report showing differences in performance between BPD(+) and BPD(−) with respect to Long-delay CPT-AX and N-back tests, while additionally controlling for demographic and clinical parameters.

Our findings support the notion that the expression of psychotic symptoms may represent a meaningful distinction within the BPD construct. It has also been shown that patients with a history of psychosis, regardless of a diagnosis, are impaired on a variety of cognitive measures, such as executive functioning, verbal learning and memory, verbal fluency, control inference (Glahn et al., 2007; Martinez-Aran et al., 2008; Simonsen et al., 2011; Udal et al., 2012) and additionally, psychotic features in mood disorders have validity in terms of prognosis, treatment response and family history for psychiatric illness (Mazzarini et al., 2010; Souery et al., 2011; Schultze-Lutter et al., 2012). It has also been shown that psychotic symptoms in mood disorders are associated with higher number of hospitalizations (Jager et al., 2005), poorer response to medications (Coryell et al., 1984), increased recurrence (Tohen et al., 2003), greater symptom severity worse short- and long-term outcome (Coryell et al., 2001), longer duration of recovery (Geller et al., 2002) and overall greater functional impairment (Haro et al., 2006). Moreover, executive dysfunction in BPD patients was reported to be related to a history of psychosis in their families (Tabares-Seisdedos et al., 2003).

Future longitudinal research should distinguish whether psychosis is a marker of a more serious developmental subtype of BPD with more pronounced cognitive deficits or a consequence of psychotic episodes in BPD (Udal et al., 2012). Reports of normal pre-morbid cognitive development in BPD support the latter suggestion (Quackenbush et al., 1996; Lewandowski et al., 2011), whereas the results of studies investigating cognitive deficits in two offspring studies indicate the presence of a more serious neurodevelopmental subtype of BPD (Meyer et al., 2004; Maziade et al., 2009). If cognitive deficits are the consequence of psychotic episodes, it emphasizes the importance of identification and treatment of early-onset BPD, in light of the poor prognosis (Bonnín et al., 2010; Torres et al., 2010).

COMPARISON OF COGNITIVE PERFORMANCE BETWEEN SZ AND BPD(+) PATIENTS

In our study, we observed the same level of functioning among SZ and BPD(+) across all cognitive tasks that we applied (FDS, BDS, Short-delay CPT-AX, Long-delay CPT-AX and N-back). Some studies show that SZ patients exhibit more severe WM-related dysfunctions than BPD participants (Hamilton et al., 2009); however, this result might be due to the fact that BPD patients have not been divided into BPD(+) and BPD(−) subgroups. In studies testing performance in BPD(+) and BPD(−) groups separately, it has been shown that BPD(+) group is cognitively closer to the SZ spectrum disorders, than to the BPD(−) group (Simonsen et al., 2011; Ivleva et al., 2012).

Recent evidence indicates common genetic, neurobiological, psychopharmacological and neurocognitive aspects of schizophrenia and psychotic affective disorders. Existing research supports the conceptualization of SZ and BPD as a "psychosis continuum," that suggests that these two psychotic disorders arise from common neurobiological processes (Ivleva et al., 2010). It has been suggested that BPD(+) sub-phenotype may be a clinical manifestation of gene expression pattern that is common for BPD and SZ. It has been shown that the relation between dopaminergic neurotransmission and psychosis is not unique to SZ but is also observed in BPD(+) (Benedetti et al., 2010). This implies the existence of biological markers associated with the propensity to develop psychosis regardless of a diagnosis of SZ or BPD.

COGNITIVE PERFORMANCE OF BPD(−) AND BPD(+) PATIENTS WITH RESPECT TO AFFECTIVE SYMPTOMS

In our study, we have shown that depressive symptoms have influence on WM performance in BPD patients. In BPD(+) patients, affective symptoms were associated with performance on Long-delay CPT-AX and N-back tests, while in BPD(+) affective symptoms were also correlated with BDS performance. Our study confirms, that in BPD patients there are state-dependent differences in WM performance that are salient in more demanding WM tasks, which is in line with some studies comparing cognitive performance between manic, depressed and euthymic states in BPD across different cognitive tasks (Martinez-Aran et al., 2004; Rosa et al., 2010). BPD patients have also been found to demonstrate a significant association between WM performance and mood scores (Thermenos et al., 2011), as well as with functional activity changes and morphometric abnormalities of fronto-limbic-striatal gray matter regions implicated in mood regulation (Zanetti et al., 2009; Thermenos et al., 2011). It has been suggested that the activity in WM circuits is affected by the activity in mood regulation circuits, even in euthymic BPD patients, as well as in their first-degree relatives (Thermenos et al., 2011). Neuroimaging studies that are in accordance with our results show that bipolar depression is associated with changes in prefrontal cortex activity during performance of cognitive tasks, especially WM tasks (Townsend et al., 2010). Additionally, reduced activation in the prefrontal cortex has been shown to be inversely correlated with the severity of depressive symptoms (Fernandez-Corcuera et al., 2013).

COGNITIVE PERFORMANCE OF SZ PATIENTS WITH RESPECT TO CLINICAL SYMPTOMS

In our study, we observed no association between WM performance with positive symptoms, general symptoms, or apathy in SZ patients. However, there was an inverse correlation between negative symptoms and cognitive performance observed only in more demanding WM tasks with higher central executive WM demands (Long-delay CPT-AX and N-back), but not in less sensitive WM measures (FDS, BDS and Short-delay CPT-AX). This inverse correlation is in accordance with the vast majority of studies repeatedly showing that SZ patients with the most prominent negative symptoms present the greatest cognitive impairment

(Brazo et al., 2002; Rodriguez-Sanchez et al., 2008; Dominguez Mde et al., 2009; Sanz et al., 2012), and more specifically also WM deficits (Schmidt-Hansen and Honey, 2009; Barr et al., 2010; Chan et al., 2010). Similarly, studies investigating the relationship between clinical symptoms and cognitive profiles in first-degree relatives of patients with SZ show that neuropsychological deficits partially mediate the increase in negative symptoms (Delawalla et al., 2006; Scala et al., 2012). The relationship between negative symptoms and impaired WM performance converge with brain imaging studies showing that negative symptoms are associated with structural abnormalities of the frontal cortex (Wible et al., 2001).

In our study, we observed the lack of association between WM performance in SZ patients with positive and general symptoms as well as apathy in the presence of a correlation with negative symptoms. These findings may be attributed to the fact that while negative symptomatology is believed to result from decreased dopaminergic activity in the prefrontal cortex directly involved in WM (Okubo et al., 1997; Heckers et al., 1999; Monteleone et al., 2002). Positive and general symptoms as well as apathy are associated with possibly altered activity in the areas less crucial to WM performance, such as the hippocampus (Krieckhaus et al., 1992; Tamminga et al., 2010) and the basal ganglia (Pankow et al., 2012; Khadka et al., 2013; Sorg et al., 2013).

Our study has some limitations that should be discussed. Due to time constraints and the use of many WM tasks, we could not test the effects of SZ and BPD on other cognitive domains, such as executive functions, attention, or learning. Future research should investigate how these disorders affect executive functioning as well as how they influence WM performance. Moreover, in our study we included BPD patients, who were not euthymic and some extent of cognitive dysfunction can be attributed to affective symptoms. However, manic symptoms did not correlate with WM performance and our overall findings are in agreement with previous studies performed on euthymic BPD patients with and without a history of psychosis (Glahn et al., 2006; Simonsen et al., 2011). Additionally, it should also be noted that the severity of depressive symptoms assessed in HDRS correlated significantly with the performance on Long-delay CPT-AX and N-back tasks both in BPD(+) and BPD(−) patients. However, these subgroups of patients differed significantly with respect to the severity of WM impairments evaluated using these tasks. Therefore, between group differences in WM performance can be attributed to a history of psychosis in BPD patients. Other limitations include small sample size of the groups and the lack of assessment with all psychopathology scales across all patients' groups. It has to be noted that we did not apply PANSS scale to assess current symptomatology of BPD(+) patients. This would allow us to analyze the influence of the severity of current symptoms on cognitive functions in this subgroup of patients. However, it should be noted that PANSS scale was primarily developed for the examination of SZ patients. Moreover, the lack of inclusion of patients with schizoaffective disorder might be also perceived as a limitation since such subgroup of subjects would enable more comprehensive insight into the psychosis continuum concept. Additionally, applying a single measure of psychotic symptoms severity to all participants, or at least all patients, would allow more detailed exploration of psychosis with respect to a categorical diagnostic system.

In summary, measuring different aspects of WM allows observation of between group differences in cognitive performance among BPD and SZ patients. These differences may depend on the patients' history of psychosis rather than a categorical diagnostic group defined by current diagnostic systems.

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SUPPLEMENTARY MATERIAL

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Relationships between brain-derived neurotrophic factor, clinical symptoms, and decision-making in chronic schizophrenia: data from the Iowa Gambling Task

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The levels of brain-derived neurotrophic factor (BDNF) are significantly decreased in patients with schizophrenia and correlate with impairments in cognitive function. However, no study has investigated the relationship between the serum BDNF levels and decision-making. We compared patients with schizophrenia to healthy controls with respect to their decision-making ability and serum BDNF levels. Eighty-six chronic schizophrenia patients and 51 healthy controls participated in this study. We controlled for gender, age, and estimated intelligence quotient (IQ), and we investigated the differences in decision-making performance on the Iowa Gambling Task (IGT) between the schizophrenia patient and control groups. We also compared the IGT scores, the serum BDNF levels, and the clinical symptoms between the groups. The IGT scores of the schizophrenia patients were lower than those of the controls. A negative correlation was detected between the mean net scores on the trials in the final two blocks and the serum BDNF levels ($p < 0.05$). Multiple regression analysis revealed that depressive symptoms and the serum BDNF levels were significantly associated with the mean net scores on the trials in the final two blocks. Based on these results, impaired sensitivity to both reward and punishment is associated with depressive symptoms and reduced serum BDNF levels in chronic schizophrenia patients and may be related to their poor performance on the IGT.

Keywords: brain-derived neurotrophic factor, decision-making, schizophrenia, gambling task, cognition, depression

INTRODUCTION

Brain-derived neurotrophic factor (BDNF), is a member of neurotrophins involved in growth, differentiation, maturation, and survival in immature neurons. In mature neurons, it plays an important role in synaptic plasticity, augmentation of neurotransmission and regulation of receptor sensitivity (Numakawa et al., 2010). BDNF and its high affinity receptor TrkB are widely expressed in developing and adult nervous system, and BDNF is the most abundantly expressed neurotrophic factor in the central nervous system (Balaratnasingam and Janca, 2012). Recent research has provided evidence for the contribution of BDNF to the pathophysiology of schizophrenia. Studies of the BDNF Val66Met (rs6265) showed that the Met allele is associated with lower levels of BDNF secretion and with abnormal hippocampal structure and function, providing evidence for the direct involvement of BDNF in schizophrenia (Egan et al., 2003).

Recent advances in clinical neuroscience indicate that the hippocampus and the orbitofrontal cortex (OFC) play a critical role in complex decision-making processes (Rolls, 1999; Krawczyk, 2002; Johnson et al., 2007; Yu and Fank, 2014). Patients experiencing damage to the hippocampus and the OFC exhibit striking deficits in real-life decision-making, especially social or emotional decision-making, in the context of generally well-preserved intellectual functioning. In addition, growing evidence demonstrates that schizophrenia patients exhibit emotional disturbances and social dysfunction (Mandal et al., 1999; Kohler et al., 2000; Chemerinski et al., 2002), which could be partially explained by impaired decision-making. This impairment in decision-making may occur during interpersonal interactions and social situations (Damasio, 1994). In general, decisions are made based on the assessment of reward and punishment outcomes using both cognitive and affective information (Solms and Turnbull, 2004).

The Iowa Gambling Task (IGT) was developed to assess the role of affective information in decision-making (Bechara et al., 1994). In this task, subjects are presented with four decks of cards and are asked to select any deck in any sequence, and then to take a card from it. The subjects win or lose money with each turn of a card. The participants do not appear to understand the contingencies of the game at the onset. Nevertheless, they can quite rapidly develop a "feeling," or "hunch" in the absence of conceptual awareness.

Because cognitive dysfunction is associated with schizophrenia, we hypothesized that the serum BDNF levels are associated with the IGT scores of the chronic schizophrenia patients.

MATERIALS AND METHODS

SUBJECTS

Eighty-six chronic schizophrenia outpatients recruited from the University of Occupational and Environmental Health participated in the present study and met the following inclusion

criteria: (1) aged 20–60 years; (2) chronic illness without acute exacerbation; and (3) continuously receiving a stable dose of antipsychotics for at least 3 months. The exclusion criteria were: (1) any comorbid central nervous system disorder; (2) severe psychotic symptoms; (3) meeting the DSM-IV criteria for alcohol or other substance dependence; (4) meeting the DSM-IV criteria for mental retardation; (5) receiving antidepressants; (6) treatment with electroconvulsive therapy in the 6 months preceding the study; (7) receiving clozapine; and (8) inability to understand the study protocol. The diagnosis of schizophrenia was established based on the Structured Clinical Interview for DMS-IV (SCID) (First et al., 1996) and a comprehensive review of the patients' medical records. All patients met the criteria for schizophrenia. None were comorbid with any other psychiatric disorders. Seventy-eight of the schizophrenia patients were receiving stable dose of one antipsychotic drug (risperidone, olanzapine, quetiapine, aripiprazole, blonanserin, or perospirone). The remaining schizophrenia patients were receiving at least two antipsychotic drugs. Regarding other medications, nine patients were taking stable dosages of mood stabilizer (lithium, valproic acid, or carbamazepine), five were taking stable dosages of anticholinergic drugs and two were taking antidepressant (paroxetine or mirtazapine). Schizophrenic symptoms were rated using the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987).

Additionally, we recruited 51 healthy volunteers (26 females and 25 males) for the healthy control group. The healthy controls consisted of individuals matched to the patients with respect to age, gender, and estimated intelligence quotient (IQ). The healthy controls had not experienced a head injury and did not suffer from any neurologic, psychotic, mood, or substance use disorder as evaluated by the SCID.

INTELLIGENCE TEST

The IQ of the participants was estimated using the Japanese Adult Reading Test (Matsuoka et al., 2002; Hori et al., 2008), a Japanese version of the National Adult Reading Test (Nelson and Wilson, 1991), and those individuals exhibiting estimated IQ scores of less than 80 were excluded from this study.

IGT

Decision-making ability was assessed using the computerized version of the IGT in Japanese (Bechara et al., 1994, 1999, 2005). Decks of cards labeled "A," "B," "C," or "D" were placed in front of the subjects from left to right. Initially, 200,000 yen were given to each subject. The subjects were told that (1) they are to draw one playing card from one of the four decks on each turn, (2) this game involves betting across multiple turns, (3) they receive money every time that they draw a card but that a penalty is occasionally applied, and (4) the objective of this game is to maximize the amount of money that they have. When selecting a card, the subjects can draw a card from any of the decks and can change their selection any time as many times as they choose. The game ended at the 100th draw of a card by a subject, but the subjects were not informed about this rule beforehand. The subjects received a reward each time they drew a card; if they selected a card from the deck A or B, a reward of 10,000 yen was applied, and if they selected a card from the deck C or D, a reward of 5000 yen was applied. Simultaneously, a penalty is applied; decks A and B are referred to as "bad decks" because the immediate reward at the time of the draw is high but the penalty is also high and frequent; therefore, the player ultimately loses money as cards are drawn from these decks. Alternatively, decks C and D are referred to as the "good decks" because the immediate reward is low but the frequency and amount of the penalty is low; therefore, players who draw cards from these decks ultimately earn money. Additionally, decks A and C are categorized as "low magnitude decks" in which a low penalty is applied at a relatively high frequency, whereas decks B and D are categorized as "high magnitude decks" in which a high penalty is applied at a relatively low frequency. The task ended after 100 selections. Neither the risks of rewards or penalties for each deck nor the number of selections allowed was disclosed to the subjects. The composition of the final score and the total amount of money held by each subject at the end of the task was not disclosed to the subjects. This score represented the extent to which socially valuable resources had been increased and may also indicate the amount of risk that the subject was willing to accept given that they may have continued to lose. The frequency of shifting between advantageous (C and D) and disadvantageous (A and B) decks by the subject was computed for every 20 cards, for a total of 5 blocks.

This examination is an exercise in which the subjects are rewarded as well as occasionally penalized with each draw of a card. The subjects can learn the types of rewards and penalties that are applied and can evaluate and change their selections during the process of the game. In this examination, the subjects must use cognitive processing to predict outcomes associated with their selection of cards and generate future predictions using a complex set of results and repeated decisions.

BDNF MEASUREMENT

All blood samples were obtained between 7:00 and 10:00 a.m. in the morning fasting. Fifteen milliliters of venous blood was drawn with subjects in the supine position, after the subjects had been lying at rest overnight.

The serum BDNF levels were measured using a BDNF Emax Immunoassay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. In short, 96-well microplates were coated with an anti-BDNF monoclonal antibody and incubated at 4°C for 18 h. The plates were incubated in a blocking buffer for 1 h at room temperature. The samples were diluted 100 times with assay buffer, and BDNF standards were maintained at room temperature under horizontal shaking for 2 h, followed by washing with the appropriate washing buffer. The plates were incubated with anti-human BDNF polyclonal antibody at room temperature for 2 h and washed with the washing buffer. Then, the plates were incubated in an anti-IgY antibody conjugated to horseradish peroxidase for 1 h at room temperature, followed by incubation in peroxidase substrate and a tetramethylbenzidine solution to induce a colorized reaction. This reaction was stopped using 1 mol/L hydrochloric acid. The absorbance at 450 nm was measured using an Emax automated microplate reader. The measurements were performed in duplicate. The standard curve was linear from 5 to 5000 pg/mL, and the detection limit was 10 pg/mL. The intra- and inter-assay coefficients of variation were 5 and 7%,

respectively. The recovery rate of exogenously added BDNF to the measured plasma samples was more than 95%.

Written informed consent was obtained from all of the subjects who participated in this study. The study protocols were approved by the Ethics Committee of the University of Occupational and Environmental Health and included standard procedures for clinical research involving vulnerable participants in Japan. This study was performed according to the ethical standards of the Declaration of Helsinki. If a participant exhibited a compromised ability to consent, we excluded this individual from this study. All participants who declined to participate or otherwise did not participate were eligible for treatment and were not disadvantaged in any other manner because of their lack of participation in this study.

STATISTICAL ANALYSIS

The differences in the clinical variables between the groups were assessed using the *t*-test and the χ^2 test for parametric data or using the Mann–Whitney *U*-test and the Fisher exact test for non-parametric variables. Data analysis of the IGT outcome variables was conducted using *t*-tests and repeated-measures analysis of variance. A multiple linear regression was employed to analyze the effect of the serum BDNF levels on the IGT scores while adjusting for confounding factors [depression score on the PANSS, age, estimated IQ, Chlorpromazine-equivalent (CPZ-eq), and PANSS-T score]. The correlations between the PANSS scores, the IGT scores, and the serum BDNF levels were evaluated using Pearson's correlation analysis. *P*-values of <0.05 were considered to be significant. The data were analyzed using stata13.1 software for Windows.

RESULTS

The demographic characteristics of the subjects are summarized in **Table 1**. No significant differences in age, gender, estimated IQ, or the serum BDNF levels were detected between the two groups.

GAMBLING TASK PERFORMANCE

Descriptive data for the performance on the IGT are presented in **Table 2**. The schizophrenia patients displayed significantly smaller difference scores on the advantageous minus disadvantageous deck selection index and earned significantly less money than the controls.

The learning curves of each group are shown in **Figure 1**. There were significant main effects of the group ($p < 0.001$) and the block ($p < 0.001$) and a significant group × block interaction ($p < 0.001$). A follow-up independent *t*-test indicated that the controls performed better than the schizophrenia patients during the final three blocks but not during the first two blocks. Even after correction for multiple comparisons, this between-group difference remained statistically significant for the final three blocks.

The between-group differences in selections from each deck were examined using a 2 (group) × 4 (deck) repeated-measures ANOVA. There was a significant main effect of the deck ($p < 0.001$) and a significant group × deck interaction ($p < 0.001$). Follow-up *t*-tests evaluated the between-group differences in the selection from each deck. As shown in **Figure 2**, the schizophrenia patients selected deck B more frequently and deck C less frequently than the controls, whereas the two groups did not differ in the selection of decks A and D ($p < 0.05$, even after the Bonferroni correction). Within-group comparisons were used to clarify the pattern of performance; the controls selected the advantageous decks more frequently [(C + D) − (A + B)], ($t = 3.95$, $p < 0.001$) than the patients with schizophrenia.

Table 1 | Demographic and clinical information of the schizophrenia patients and the healthy control group.

	Healthy control	Schizophrenia group	*p*-value
Age (year)	36.7 ± 9.9	35.1 ± 12.1	0.43
Gender (M/F)	25/26	43/43	0.91
Education (years)	13.4 ± 2.2	12.7 ± 2.7	0.15
Estimated IQ	101.8 ± 7.6	99.4 ± 8.2	0.09
Duration of the illness (years)		11.4 ± 13.2	
SCHIZOPHRENIA DIAGNOSIS			
Paranoid type		47	
Disorganized type		27	
Catatonic type		5	
Indifferenciated type		7	
PANSS-P		17.2 ± 4.7	
PANSS-N		20.4 ± 4.6	
PANSS-G		33.2 ± 7.0	
PANSS-T		70.7 ± 11.7	
CPZeq of total antipsychotic drugs(mg/day)		470.1 ± 293.0	
Serum BDNF(ng/ml)	14.1 ± 7.3	11.8 ± 7.0	0.06

PANSS-P, PANSS positive score; PANSS-N, PANSS negative score; PANSS-G, PANSS general psychopathological score; PANSS-T, PANSS total score; CPZ-eq, chlorpromazine-equivalent.

GAMBLING TASK PERFORMANCE, CLINICAL SYMPTOMS, AND SERUM BDNF LEVELS

No correlation was found between the total scores and mean net scores on the IGT and the PANSS-P, PANSS-N, or PANSS-T (**Table 3**). A significant negative correlation was detected between the mean net scores on trials 61–100 and the PANSS-G scores. A significant negative correlation was observed between the mean net scores on the trials in the final two blocks (61–80 and 81–100) and the serum BDNF levels (**Table 3**). **Table 4** shows the multiple regression analysis results for the mean net scores on each block of the trials. Depressive symptoms and the serum BDNF levels were significantly associated with the mean net scores on the trials in the final two blocks.

DISCUSSION

A significant finding in the present study was that the serum BDNF levels and depressive symptoms correlated with decision-making. Several previous studies reported that schizophrenia patients exhibited diminished decision-making abilities compared to healthy individuals (Beninger et al., 2003; Ritter et al., 2004; Lee et al., 2007; Kim et al., 2009; Struglia et al., 2011). To the best of our knowledge, this is the first report to provide

Table 2 | Iowa Gambling Task performance of the schizophrenia patients and the healthy control group.

	Healthy control	Schizophrenia	*t*	*df*	*p*
Mean amount of money earned (yen)	219902.0 ± 72770.1	162348.8 ± 72369.9	4.48	135	< 0.001
No. of cards chosen from deck A	17.7 ± 6.9	18.6 ± 6.0	−0.75	135	0.44
No. of cards chosen from deck B	26.0 ± 12.2	34.5 ± 11.1	−4.1	135	< 0.001
No. of cards chosen from deck C	30.7 ± 12.6	19.6 ± 6.1	5.89	135	< 0.001
No. of cards chosen from deck D	25.2 ± 8.9	27.2 ± 9.0	−1.21	135	0.23
Choice advantageous minus disadvantageous decks	12.2 ± 32.6	−6.3 ± 22.3	−3.6	135	< 0.001

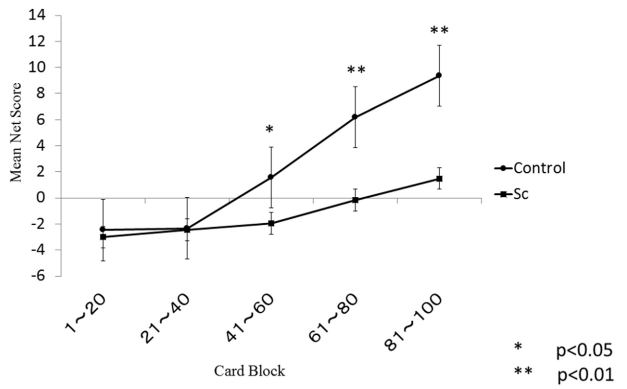

FIGURE 1 | Iowa Gambling Task in schizophrenia and healthy subjects.

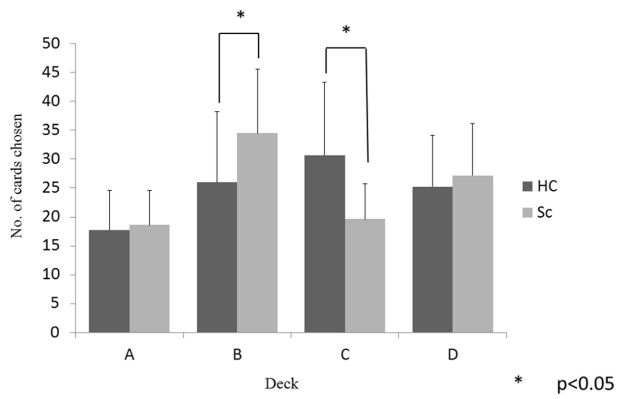

FIGURE 2 | Number of card choice selected from each deck during the 100-card task.

evidence suggesting that the serum BDNF levels reflect decision-making ability in patients with chronic schizophrenia. A recent meta-analysis revealed that the serum BDNF levels in schizophrenia patients are lower than those in healthy individuals (Green et al., 2011). In addition, recent studies have reported an association between poor performance on the IGT and the volume of several lesions in the hippocampus (Bonatti et al., 2009; Labudda et al., 2009; Yamano et al., 2011). The expression level of BDNF is normally high in the hippocampus. Taking these findings into account, diminished serum BDNF level in schizophrenia patients may reflect their poor decision-making performance. In short, serum BDNF may serve as a biomarker of decision-making ability in schizophrenia patients.

Wilder et al. reported that schizophrenia patients often selected the decks corresponding to infrequent and high-magnitude punishments (Wilder et al., 1998). Another study confirmed the finding that schizophrenia patients performed poorly compared to healthy individuals and often selected cards from the deck corresponding to high-magnitude punishments (Shurman et al., 2005). In the present study, the schizophrenia patients selected from good decks during the latter half of the task; however, optimizing the selection pattern appeared to be more difficult for the schizophrenia patients compared to the healthy individuals. Therefore, schizophrenia patients may tend to perform the same pattern of changes in the selection of the decks on the IGT as the control subjects. The magnitude of the occasional penalties has been reported to have little impact on the pattern of card selection.

The results in the present study are in accordance with those of previous publications (Beninger et al., 2003; Ritter et al., 2004; Shurman et al., 2005; Lee et al., 2007; Kim et al., 2009; Struglia et al., 2011). In addition, the present study revealed a significant correlation between decision-making performance and certain psychiatric symptoms. Previous studies have reported inconsistent results regarding the relationship between the IGT score and symptoms of schizophrenia, including a significant correlation to not only negative symptoms of schizophrenia (Shurman et al., 2005) but also positive symptoms of schizophrenia (Struglia et al., 2011). Several studies demonstrated an association between the serum BDNF levels and severity of major depressive disorder (Shimizu et al., 2003; Dell'Osso et al., 2010; Kurita et al., 2012; Yoshiumra et al., 2012). In contrast, no correlations existed between the serum BDNF levels and severity of depression (Karege et al., 2002b; Piccinni et al., 2008; Park et al., 2014). Taken together, it remains controversial that severity of a depressive state influences decision-making in schizophrenia patients. In short, it is not elucidated whether serum BDNF levels reflect or not depressive factors in schizophrenia. One study reported that depressive symptoms are associated with QOL in schizophrenia (Ueoka et al., 2011). Therefore, treatment targeting depressive symptoms may improve the QOL in schizophrenia patients.

The present study contained several limitations. First, the patients with schizophrenia were not classified into subtypes. Second, the schizophrenia patients were receiving various antipsychotic medications when the IGT was performed. Third, the number of subjects in the control group was small. Fourth, the schizophrenia patient group was receiving antipsychotics, a

Table 3 | Correlation between the Iowa Gambling Task performance and serum BDNF, estimated IQ, and clinical symptoms.

	Mean amount of money earned (yen)	1∼20	21∼40	41∼60	61∼80	81∼100
Serum BDNF concentration (ng/ml)	0.06	0.02	0.03	0.09	0.24*	0.24*
CPZeq of total antipsychotic drugs(mg/day)	0.06	−0.05	−0.09	0.00	−0.1	−0.07
Estimate IQ	0.02	−0.06	−0.04	−0.01	−0.03	−0.06
PANSS-P	0.08	0.02	−0.08	0.00	0.02	0.02
PANSS-N	0.07	−0.03	−0.02	−0.1	−0.08	−0.02
PANSS-G	0.04	−0.03	−0.07	−0.01	−0.25*	−0.23*
PANSS-T	0.08	−0.01	−0.08	−0.04	−0.17	−0.13

CPZ-eq, chlorpromazine-equivalent.
*$p < 0.05$.

Table 4 | Multiple regression analysis results for the mean net scores on each block of the trials.

	Independent variables	Multiple regression statistic			
		β	***SE***	***t*-value**	***p*-value**
IGT-1	Age	0.019	0.045	0.43	0.667
	CPZ-eq	−0.001	0.001	−0.62	0.535
	Depression	1.252	0.869	1.44	0.153
	PANSS-T	−0.459	0.051	−0.91	0.367
	Estimated-IQ	−0.399	0.060	−0.67	0.506
	Serum BDNF levels	0.009	0.070	0.13	0.894
IGT-2	Age	−0.050	0.054	−0.93	0.354
	CPZ-eq	−0.000	0.002	−0.21	0.831
	Depression	0.518	1.041	0.50	0.620
	PANSS-T	−0.049	0.06	−0.80	0.424
	Estimated-IQ	−0.039	0.071	−0.55	0.584
	Serum BDNF levels	0.019	0.084	0.23	0.819
IGT-3	Age	−0.117	0.056	−2.07	0.042*
	CPZ-eq	0.002	0.002	1.02	0.313
	Depression	0.834	1.084	0.77	0.444
	PANSS-T	−0.040	0.063	−0.63	0.532
	Estimated-IQ	−0.028	0.075	−0.37	0.710
	Serum BDNF levels	0.078	0.088	0.89	0.378
IGT-4	Age	−0.054	0.071	−0.75	0.453
	CPZ-eq	−0.000	0.002	−0.10	0.919
	Depression	−3.502	1.376	−2.54	0.013*
	PANSS-T	0.034	0.080	0.42	0.673
	Estimated-IQ	−0.017	0.095	−0.18	0.857
	Serum BDNF levels	0.23	0.112	2.06	0.043*
IGT-5	Age	−0.674	0.084	−0.81	0.423
	CPZ-eq	0.000	0.003	0.09	0.929
	Depression	−4.686	1.619	−2.89	0.005*
	PANSS-T	0.077	0.094	0.82	0.414
	Estimated-IQ	−0.458	0.111	−0.41	0.682
	Serum BDNF levels	0.269	0.131	2.05	0.044*

PANSS-T, PANSS total score; CPZ-eq, chlorpromazine-equivalent; IGT-1: Card block 1-20; IGT-2: Card block 21-40; IGT-3: Card block 41-60; IGT-4: Card block 61-80; IGT-5: Card block 81-100.
$p < 0.05$.

relevant problem because of the influence of the drugs on the BDNF levels. Fifth, since we evaluated the depressive symptoms in schizophrenia with only PANSS depressive items, we were poorly assessed and insufficient. Last, there is increasing evidence that sampling characteristics, several sociodemographic variables (such as age, sex, urbanicity, BMI), life-style factors (such as food, alcohol intake, and smoking status), somatic disease, and even self-reported depressive symptoms are relevant determinants of serum BDNF levels (Radka et al., 1996; Karege et al., 2002a; Bus et al., 2011). In conclusion, both depressive symptoms and the serum BDNF levels may be associated with the impairment of decision-making in schizophrenia patients.

AUTHOR CONTRIBUTIONS

Dr. Hikaru Hori designed the study, performed the cognitive battery, collected the clinical data, performed the statistical analyses, wrote the first draft of the manuscript, and performed literature searches. Dr. Reiji Yoshimura and Dr. Jun Nakamura developed the study protocol and wrote the final manuscript. Dr. Asuka Katsuki performed the cognitive battery. Dr. Kiyokazu Atake collected the clinical data. All authors contributed to and approved the final manuscript.

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Exposure to kynurenic acid during adolescence increases sign-tracking and impairs long-term potentiation in adulthood

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Changes in brain reward systems are thought to contribute significantly to the cognitive and behavioral impairments of schizophrenia, as well as the propensity to develop co-occurring substance abuse disorders. Presently, there are few treatments for persons with a dual diagnosis and little is known about the neural substrates that underlie co-occurring schizophrenia and substance abuse. One goal of the present study was to determine if a change in the concentration of kynurenic acid (KYNA), a tryptophan metabolite that is increased in the brains of people with schizophrenia, affects reward-related behavior. KYNA is an endogenous antagonist of NMDA glutamate receptors and α7 nicotinic acetylcholine receptors, both of which are critically involved in neurodevelopment, plasticity, and behavior. In Experiment 1, rats were treated throughout adolescence with L-kynurenine (L-KYN), the precursor of KYNA. As adults, the rats were tested drug-free in an autoshaping procedure in which a lever was paired with food. Rats treated with L-KYN during adolescence exhibited increased sign-tracking behavior (lever pressing) when they were tested as adults. Sign-tracking is thought to reflect the lever acquiring incentive salience (motivational value) as a result of its pairing with reward. Thus, KYNA exposure may increase the incentive salience of cues associated with reward, perhaps contributing to an increase in sensitivity to drug-related cues in persons with schizophrenia. In Experiment 2, we tested the effects of exposure to KYNA during adolescence on hippocampal long-term potentiation (LTP). Rats treated with L-KYN exhibited no LTP after a burst of high-frequency stimulation that was sufficient to produce robust LTP in vehicle-treated rats. This finding represents the first demonstrated consequence of elevated KYNA concentration during development and provides insight into the basis for cognitive and behavioral deficits that result from exposure to KYNA during adolescence.

Keywords: autoshaping, schizophrenia, NMDA, nicotinic, kynurenine, addiction, incentive salience, motivation

INTRODUCTION

Kynurenic acid (KYNA) is a final product of tryptophan metabolism that is synthesized and released in the central nervous system by astroglia (Schwarcz and Pellicciari, 2002). The concentrations of KYNA and its precursor, kynurenine, are significantly increased in the brains of persons with schizophrenia (Erhardt et al., 2001; Schwarcz et al., 2001; Linderholm et al., 2012) and growing evidence indicates that KYNA may contribute to the cognitive deficits that are associated with the disorder. Indeed, KYNA is an endogenous antagonist of NMDA glutamate receptors (NMDA-Rs) and α7 nicotinic acetylcholine receptors (α7-nAChRs; Hilmas et al., 2001; Parsons et al., 1997; Pereira et al., 2002; Stone, 1993), thus an increase in KYNA concentration is likely to impair functions that depend on these receptors, such as attention, learning, and memory (Bast et al., 2003; Gould and Higgins, 2003; Bloem et al., 2014). Consistent with this, a growing number of studies provide evidence of a causal role for KYNA in cognitive dysfunction by showing that an acute increase in KYNA in adult rats impairs cognitive domains that are affected in schizophrenia, including attention (Chess and Bucci, 2006; Alexander et al., 2012), sensory gating (Shepard et al., 2003; Erhardt et al., 2004), context memory (Chess et al., 2009), and spatial memory (Chess et al., 2007).

More recent studies have focused on the effects of KYNA exposure earlier in life, since KYNA levels are likely increased for much longer periods of time in persons with schizophrenia and become elevated earlier than adulthood (Miller et al., 2004, 2006, 2008; Holtze et al., 2008; Asp et al., 2010). This has important ramifications because NMDA-Rs and α7-nAChRs are also essential for neural plasticity and brain development (Komuro and Rakic, 1993; Broide and Leslie, 1999), thus exposure to high levels of KYNA early in life may lead to lasting cognitive and behavioral impairments later in adulthood. In line with this, it has been shown that an increase in KYNA throughout adolescence impaired contextual memory (Akagbosu et al., 2012) and social behavior (Trecartin

and Bucci, 2011) when rats were subsequently tested drug free in adulthood. Similarly, treating rat dams with food enriched with L-kynurenine (L-KYN) from gestational day 15 to postnatal day (PND) 21 increased KYNA concentration in the offspring and impaired cognition (Pocivavsek et al., 2012; Alexander et al., 2013).

The present study extended this work in two important directions. Schizophrenia is associated with dysfunctional brain reward circuitry, particularly the midbrain dopamine system. Patients have higher levels of dopamine synthesis and release compared to age-matched controls (Carlsson et al., 2001) and elevated levels of KYNA may contribute to this abnormal pattern of dopamine activity. In rodents, pharmacologically elevated levels of endogenous KYNA increase the firing rate and bursting activity of dopamine neurons in the ventral tegmental area (VTA), an effect mediated by NMDA-Rs (Erhardt and Engberg, 2002). In addition, manipulating KYNA levels with cyclooxygenase inhibitors similarly alters the activity of VTA dopamine neurons (Schwieler et al., 2006). Furthermore, elevation of KYNA levels increases dopamine release in the nucleus accumbens (NAc; Nilsson-Todd et al., 2007), a structure that is involved in reward learning and addiction (e.g., Robinson and Berridge, 1993; Di Chiara, 2002; Kelley, 2004). Thus, Experiment 1 tested whether an increase in KYNA concentration during adolescence impacts reward-related behavior and motivation. Rats were treated with 100 mg/kg of L-KYN throughout adolescence, which results in a three- to fourfold increase in KYNA levels (Erhardt et al., 2004; Akagbosu et al., 2012) and is consistent with the increases observed in persons with schizophrenia (Erhardt et al., 2001; Schwarcz et al., 2001). As adults, the rats were tested drug-free in an autoshaping procedure in which one lever conditioned stimulus (CS) was presented for a short period of time and followed immediately by delivery of a food unconditioned stimulus (US) upon lever retraction, while a second lever was not paired with food (Chang et al., 2012). Although food delivery is not contingent on the rat's behavior, rats will typically approach, contact, and bite the lever CS that is paired with the US (Davey and Cleland, 1982). These CS-directed behaviors (also known as sign-tracking, e.g., Flagel et al., 2011) are thought to reflect the lever acquiring incentive salience (motivational value) as a result of its pairing with the US (e.g., Berridge, 2004). Importantly, sign-tracking has been shown to be mediated by the NAc and its dopaminergic inputs from the VTA (Flagel et al., 2011; Chang et al., 2012; Saunders and Robinson, 2012). Substance abuse and addiction are associated with increased sensitivity to drug-related cues, perhaps resulting from sensitization of midbrain dopamine neurons in response to drugs that imbues the drug-related stimuli with excessive incentive salience (Robinson and Berridge, 1993, 2001). Since KYNA enhances dopaminergic neurotransmission in midbrain reward circuits (Erhardt and Engberg, 2002), we predicted that rats exposed to KYNA during adolescence would exhibit excessive sign-tracking (lever pressing) as adults. Importantly, behavioral testing took place beginning on PND 100, which is well into adulthood and when KYNA levels are no longer elevated following this treatment regimen (Akagbosu et al., 2012). Thus, any behavioral differences observed between L-KYN-treated rats and vehicle-treated rats could not be attributed to differences in KYNA concentration at the time of testing.

The second goal of the current study was to determine how exposure to KYNA during adolescence alters neural function in adulthood. Indeed, despite the demonstration that cognitive deficits result from increasing KYNA levels during adolescence (Trecartin and Bucci, 2011; Akagbosu et al., 2012), no study has investigated the neural substrates underlying those deficits. Thus, we examined the effects of L-KYN treatment during adolescence on the ability of adult hippocampal neurons to undergo long-term potentiation (LTP). We chose to study hippocampal LTP because of the recent interest in hippocampal involvement in autoshaping and because it has been shown previously that exposure to KYNA during adolescence impairs hippocampal-dependent behavior (Akagbosu et al., 2012). We predicted that LTP would be reduced in rats treated with L-KYN during adolescence compared to control rats.

MATERIALS AND METHODS

EXPERIMENT 1

Subjects

Sixteen male Long–Evans rats were obtained from Harlan Laboratories (Indianapolis, IN, USA) at 21 days of age. Rats were housed in groups of 4 upon arrival with food and tap water available *ad libitum* (Purina standard rat chow; Nestle Purina). Rats were allowed 6 days to acclimate to the vivarium before drug treatment began. When they reached 63 days old, the rats were separated into individual cages and gradually food restricted to 85% of their baseline free-feeding body weights over the next 7 days. Weights were measured daily and maintained by supplementing with rat chow. Rats were maintained on a 14:10 light-dark schedule (lights on at 7:00 a.m., off at 9:00 p.m.) throughout the study and monitored and cared for in compliance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines and the Dartmouth College Institutional Animal Care and Use Committee.

Drug preparation

L-KYN (SAI Chemicals, India) was prepared fresh daily by dissolving in 2N sodium hydroxide and adding 0.1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer to bring it to a final concentration of 30 mg/ml. The solution was then brought to a neutral pH by adding drops of 1N hydrochloric acid.

Treatment regimen

On PND 27, rats were quasi-randomly assigned to either the L-KYN-treated group or the vehicle-treated control group ($n = 8$/group) with two of each set of four group-housed rats assigned to each condition. On PND 27-29, each rat received a daily intraperitoneal injection of either the L-KYN solution (100 mg/kg) or a comparable volume of 0.1M HEPES buffer (vehicle). Injections were administered on alternate sides of the abdomen to reduce discomfort at the injection site. On PND 30–32, no injections were administered and the rats were not handled. This 3-day-on/3-day-off drug treatment regimen was repeated another 4 times resulting in a total of 15 injections of L-KYN (or vehicle), the last of which occurred on PND 53. As shown previously, this procedure increases KYNA concentration fourfold on days when rats are treated with L-KYN (Akagbosu et al., 2012). Compared

to injecting rats every day from PND 27–53, the 3-day injection cycle reduces distress and sensitivity at the injection site and also minimizes the potential for metabolic adaptations following chronic systemic L-KYN treatment for more than 3 consecutive days (Vecsei et al., 1992).

Behavioral apparatus

The behavioral procedures were carried out in eight standard conditioning chambers (Med Associates, Georgia, VT, USA) enclosed in sound-attenuating cubicles (62 × 56 × 56 cm) outfitted with exhaust fans to provide airflow and background noise (~68 dB). The chambers consisted of aluminum front and back walls, clear acrylic sides and top, and grid floors composed of stainless steel rods (5 mm diameter) spaced 1.5 cm apart (center to center). Each chamber was outfitted with a food cup that was recessed in the center of the front wall. Retractable levers were positioned to the left and right of the food cup. The levers were 4.8 cm long, positioned 6.2 cm above the grid floor, and protruded 1.9 cm when extended. A 2.8-W house light was mounted 15 cm above the grid floor on the back wall of the chamber and provided background illumination. Four panel lights were also present in the chamber but were not used in this experiment. Three of the lights were located 10.8 cm above the floor, two of them were positioned 6.4 cm to the left or right of the food cup, and one was located directly above the food cup. A fourth panel light was located 15 cm above the floor. The reinforcer was a 45-mg grain-based rodent food pellet (Bioserv, Flemington, NJ, USA). A pair of photobeam sensors was located across the front of the food cup. The levers and food delivery were controlled by a PC computer that also monitored breaks in the photobeam.

Behavioral procedure

During PND 70–100, the rats participated in a prior experiment with different visual, tactile, and olfactory stimuli in the test chamber. In that experiment, rats were trained to press the right lever and received several presentations of an auditory cue as well as six mild footshocks (0.5 mA). Because of this previous lever-pressing experience, the following procedure was used to extinguish lever pressing prior to the start of the present experiment. Rats were exposed to the conditioning chambers for 1 h on each of 4 consecutive days. During these 1-h sessions, both levers were continuously extended but responses had no consequence. By the fourth session, the mean rate of responding on the left lever was 0.46 presses/min for rats in the L-KYN group and 0.39 presses/min for rats in the saline group. The mean rate of responding on the right lever was 0.98 presses/min for the L-KYN group and 1.27 presses/min for the saline group.

On the first day of the present experiment, all rats received one 30-min magazine training session in which food pellets were delivered freely on a random time (RT) 30-s schedule, resulting in approximately 60 pellets being delivered. On each of the next 7 days, all rats received a single 60-min session of training. Each session consisted of 25 CS+ and 25 CS− trials with an average intertrial interval (ITI) of 1 min. The CS+ trials consisted of a 10-s extension of one lever and delivery of two food pellets upon retraction of the lever. The CS− trials consisted of a 10-s extension of the other lever with no delivery of food pellets. Trial order was random with the exception that no more than two trials of the same type were allowed to occur consecutively. Levers were counterbalanced so that for half of the rats within each group the CS+ lever was the right lever, and for the other half of the rats the CS+ lever was the left lever.

Data analysis

The rate of lever pressing and the percentage of trials in which at least one lever press occurred were recorded, as well as the number of head entries into the food cup and percent of time spent in the food cup. Data were analyzed using a 2 (Group: Vehicle vs. L-KYN) × 2 (Cue: CS+ vs. CS−) × 7 (Session) analyses of variance.

EXPERIMENT 2

Subjects and drug treatment

Six male Long–Evans rats were obtained from Harlan Laboratories on PND 21 and maintained as described in Experiment 1. L-KYN or vehicle was prepared and administered as described in Experiment 1 ($n = 3$ rats/group).

Preparation of hippocampal slices

On ~PND 70, rats were decapitated and the brains were quickly removed and immersed in ice-cold artificial cerebral spinal fluid (aCSF) saturated with 95% O_2 and 5% CO_2. The aCSF contained (in mM): 125 NaCl, 2.5 KCl, 2.5 CaCl, 2.2 H_2O, 1.3 $MgCl_2$, 1.25 NaH_2PO_4, 25 Glucose, and 25 $NaHCO_3$. Sagittal slices of hippocampus (350 μm) were cut from each brain (3–5 sections/brain) using a vibroslicer (Electron Microscopy Sciences, Hatfield, PA, USA) and incubated for at least 1 h in a recovery chamber prior to recording. Individual slices were then transferred to a recording chamber, perfused continuously with oxygenated aCSF at a flow rate of 3–4 ml/min, and maintained at a temperature of 32 ± 1°C.

Electrophysiological recordings

Excitatory postsynaptic field potentials (fEPSP) were recorded in CA1 stratum radiatum (dendritic region) with a glass microelectrode pulled from 1.5-mm fiber-filled capillary tubing using a Brown-Flaming electrode puller (P97, Sutter Instruments, Novato, CA, USA) and filled with 150 mM NaCl. A platinum/iridium concentric bipolar electrode (FHC Inc., Bowdoinham, ME, USa) was placed at the path of the Schaffer collaterals to evoke fEPSPs. Electrical signals were amplified by an Axopatch-1D amplifier (Axon Instruments, Foster, CA, USA). An Intel Pentium-based computer with pCLAMP version 9.2 (Molecular Devices, Sunnyvale, CA, USA) was used for on-line acquisition and off-line analysis of data. LTP was induced at 25 or 50% of maximal amplitude by high-frequency stimulation (HFS; 2 trains at 10-s intervals with each train consisting of pulses delivered at 100 Hz).

Statistical analysis

Baseline fEPSPs were collected each min for the 10-min period prior to the induction of LTP. Normalized values were calculated from the average response from all rats. After induction, the peak fEPSP amplitude was measured with respect to the baseline every minute for a total of 42 min. Mean baseline data and mean post-induction data were analyzed using a repeated measures ANOVA with Group (vehicle, L-KYN) as the between-subjects variable and

Time (pre, post) as the within-subjects variable. Interactions were decomposed using pairwise *t*-tests. An alpha level of 0.05 was used for all analyses.

RESULTS

EXPERIMENT 1

One L-KYN-treated rat was excluded from the data analysis because responding was 3 standard deviations lower than the group mean (a response was made on only 6.9% of the CS+ presentations; the next lowest in the L-KYN group was 83.4%).

Lever pressing

Response rate. As shown in **Figure 1**, the L-KYN-treated rats exhibited higher rates of sign-tracking to the CS+ (as measured by lever presses per minute) than control rats, while no differences were observed in responding to the CS−. A three-way ANOVA revealed a main effect of Group [$F(1,13) = 10.4, p < 0.01$] and a significant main effect of Cue [$F(1, 13) = 63.5, p < 0.001$]. The ANOVA also revealed significant interactions between Group and Cue [$F(1, 13) = 8.5, p < 0.02$] and Cue and Session [$F(6, 78) = 4.5, p < 0.001$]. The main effect of Session was not statistically significant ($p > 0.9$), nor were the Group × Session ($p > 0.9$) or Group × Cue × Session ($p > 0.9$) interactions.

To assess the source of the critical Group × Cue interaction, a two-way ANOVA for the CS+ data revealed a significant main effect of Group [$F(1, 13) = 9.9, p < 0.01$]. There was neither significant main effect of Session ($p > 0.1$) nor a significant Group × Session interaction ($p > 0.9$). A two-way ANOVA for the CS− cue revealed a significant main effect of Session [$F(6, 78) = 7.1, p < 0.001$], but no main effect of Group ($p > 0.2$) nor a Group × Session interaction ($p > 0.6$). Thus, L-KYN treated rats responded more to the CS+ than vehicle-treated rats, but responding to the CS− was not different between groups.

Percentage of trials with a response. **Figure 2** illustrates the percentage of trials with at least one lever press during the presentation of the CS+ or the CS−. L-KYN-treated rats initially responded on a greater percentage of CS+ trials than the vehicle-treated rats, but the vehicle-treated rats eventually reached a similar level of responding by day 7. A three-way ANOVA revealed a main effect of Group [$F(1, 13) = 8.3, p < 0.02$]. There was also a significant effect of Cue [$F(6, 78) = 59.9, p < 0.001$] and a significant interaction between Session and Cue [$F(6, 78) = 10.5, p < 0.001$], indicating a change in responding to each cue across time. The main effect of Session was not significant ($p > 0.8$) and neither were any of the interactions (Group × Cue, $p > 0.5$; Group × Session, $p > 0.1$; Group × Cue × Session, $p > 0.6$).

A two-way ANOVA used to further investigate the effect of chronic adolescent L-KYN administration on responding to the CS+ revealed a significant main effect of Group [$F(1, 13) = 6.1, p < 0.03$] and a significant main effect of Session [$F(6, 78) = 5.8, p < 0.001$]. A significant Group × Session interaction was also detected [$F(6, 78) = 2.3, p < 0.05$], indicating that the L-KYN-treated rats and vehicle-treated rats responded differently to the CS+ across the seven sessions. One-way ANOVAs for each session revealed significant differences between the vehicle and L-KYN

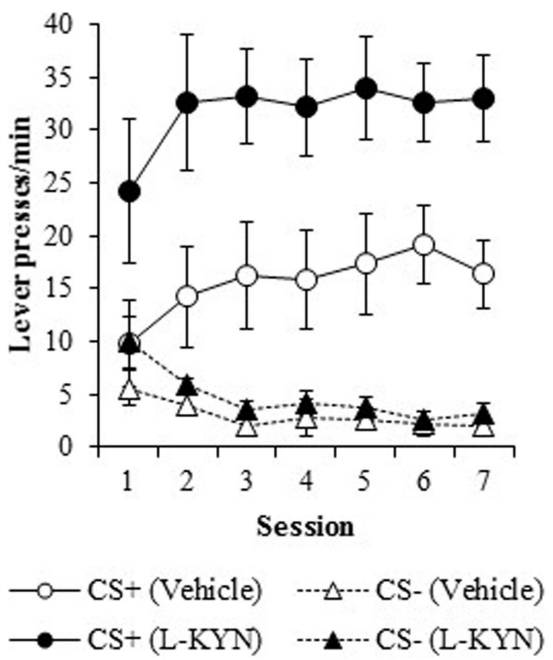

FIGURE 1 | Lever-pressing behavior (responses/min) during the autoshaping procedure in Experiment 1. Compared to vehicle-treated rats, those that were treated with L-KYN throughout adolescence exhibited an increase in sign-tracking as evidenced by significantly higher rates of responding when they were tested as adults. Data are means ± SEM.

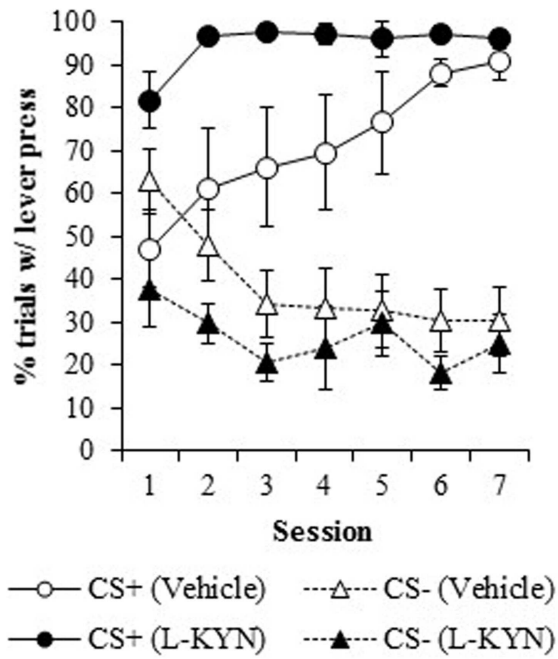

FIGURE 2 | The percentage of trials in which a lever press occurred in Experiment 1. Compared to vehicle-treated rats, those that were treated with L-KYN throughout adolescence exhibited an increase in sign-tracking as evidenced by a significantly higher percentage of trials with a lever press when they were tested as adults. Data are means ± SEM.

groups on day 1 [$F(1, 13) = 9.2$, $p < 0.01$], day 2 [$F(1, 13) = 5.5$, $p < 0.03$], and day 6 [$F(1, 13) = 5.6$, $p < 0.03$], a marginally significant difference on day 3 [$F(1, 13) = 4.5$, $p = 0.05$], and no significant differences between the groups on day 4 ($p > 0.1$), day 5 ($p > 0.1$), or day 7 ($p > 0.4$). For the CS−, only the main effect of Session was significant [$F(6, 78) = 7.1$, $p < 0.001$], indicating that there were no differences between the groups in responding to the CS−. Thus, L-KYN-treated and vehicle-treated rats differed in CS+ responding early but not late in training.

On both measures (response rate and percentage of trials with a response), there was a significant difference between groups beginning on the first session, as described above. To determine if L-KYN-treatment simply resulted in higher levels of unconditioned lever pressing, we analyzed responding during the first 5-trial block in Session 1. There was no group difference in responding on either measure during the first block of trials (ps > 0.1), indicating that levels of unconditioned lever pressing did not differ between groups. Instead, the group difference emerged over the course of training.

Food cup behavior

A three-way ANOVA of the number of food cup entries during the CS+ and CS− revealed a significant main effect of Cue [$F(1, 13) = 6.6$, $p < 0.02$] and a significant main effect of Session [$F(6, 78) = 3.1$, $p < 0.01$]. Neither the main effect of Group ($p > 0.2$) nor any interactions (Cue × Group, $p > 0.1$; Session × Group, $p > 0.8$; Session × Cue, $p > 0.2$; Cue × Session × group, $p > 0.2$) were statistically significant. Overall, food cup behavior was lower during the CS+ than the CS−, likely due to sign-tracking to the CS+ since lever pressing and food cup behavior are competing responses. For time spent in the food cup, a three-way ANOVA revealed a main effect of Session [$F(6, 78) = 4.3$, $p < 0.001$] and a significant Group × Session interaction [$F(6, 78) = 2.4$, $p < 0.03$]. Analysis of the interaction indicated that during Session 1, the L-KYN and vehicle groups did not discriminate between CS+ and CS− trials. During Session 7, however, L-KYN-treated rats spent more time in the food cup during CS− trials than CS+ trials, while vehicle-treated rats spent comparable amounts of time in the food cup during CS+ and CS− trials (see **Table 1**). This was likely due to the increased lever pressing during CS+ trials in L-KYN-treated rats. There were no other significant main effects (Cue, $p > 0.3$; Group, $p > 0.2$) or interactions (Cue × Group, $p > 0.3$; Session × Cue, $p > 0.6$; Session × Cue × group, $p > 0.2$) detected.

EXPERIMENT 2

The LTP data are shown in **Figure 3** and represent the average values obtained from 11 sections from vehicle-treated rats and 15 sections from L-KYN-treated rats. A repeated measures

Table 1 | Food cup behavior.

	L-KYN		Vehicle	
	CS+	CS−	CS+	CS−
Entries/min: Session 1	20.85 ± 4.68	27.63 ± 3.06	30.99 ± 5.04	33.51 ± 4.14
Session 7	7.44 ± 4.59	23.83 ± 6.58	17.52 ± 5.34	25.29 ± 3.72
% Time in food cup: Session 1	20.01 ± 5.40	28.68 ± 5.46	39.64 ± 8.75	38.91 ± 7.17
Session 7	7.33 ± 3.51	24.84 ± 9.39	26.59 ± 11.91	25.61 ± 7.79

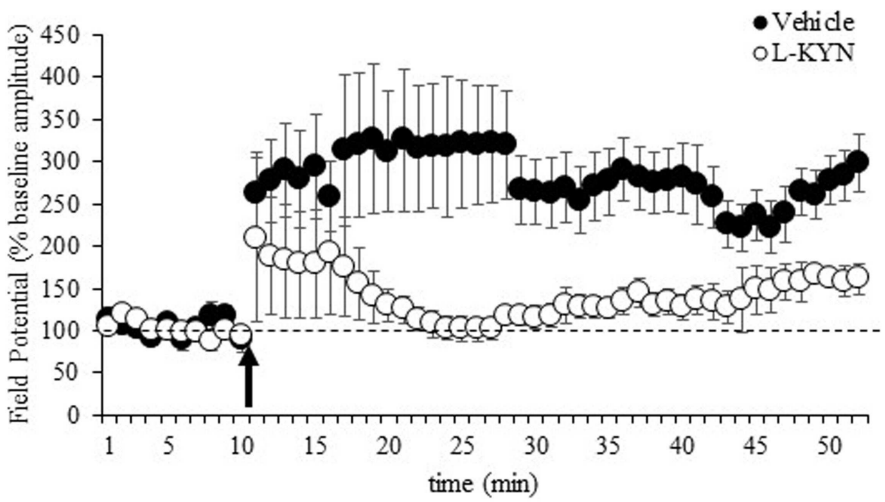

FIGURE 3 | fEPSPs recorded in Experiment 2 from hippocampal slices from adult rats before and after a burst of high-frequency stimulation (HFS, 100 Hz; arrow). Values after HFS are expressed relative to the baseline (average of the responses during the 10-min period prior to LTP induction; dotted line). The burst of HFS induced LTP in vehicle-treated rats but not in rats treated with L-KYN during adolescence. Data are means ± SEM (11 slices from vehicle-treated rats and 15 slices from L-KYN-treated rats).

ANOVA revealed significant main effects of Group [$F(1, 24) = 5.7$, $p < 0.03$] and Time [$F(1, 24) = 14.4$, $p < 0.001$] and a significant Group × Time interaction [$F(1, 24) = 5.4$, $p < 0.03$]. In the vehicle-treated group, there was a 305 ± 58% increase above baseline in the amplitude of the fEPSP, which was statistically significant [$t(10) = 3.5$, $p < 0.01$]. For L-KYN-treated rats, the post-induction response was only 150 ± 37% above baseline and did not reach statistical significance [$t(14) = 1.3$, $p > 0.2$], indicating that LTP was not induced. In addition, the post-induction response was significantly greater in the controls compared to the L-KYN-treated rats [$t(24) = 2.6$, $p < 0.03$], while there was no group difference in the pre-induction baseline responses [$t(24) = 1.0$, $p > 0.3$].

DISCUSSION

The present study examined the effects of increased KYNA concentration during adolescence on the brain and behavior in adulthood. Experiment 1 tested the hypothesis that rats treated with L-KYN throughout adolescence, which increases KYNA levels three- to fourfold (Akagbosu et al., 2012), would exhibit increased sensitivity to reward-related cues later in adulthood. Consistent with this hypothesis, we found that L-KYN-treated rats exhibited more sign-tracking behavior compared to vehicle-treated rats when they were tested drug-free as adults. This was manifest as higher rates of lever pressing to the CS+ (a lever paired with food reward) and a greater percentage of trials with a lever press. In contrast, treatment with L-KYN did not increase responding to the CS− (the lever that was not reinforced). These findings indicate that exposure to elevated levels of KYNA during adolescence, a critical time in development, results in an increased sensitivity to reward-related cues later in life.

A similar increase in sensitivity occurs to drug-related cues and is thought to arise from the sensitization of dopamine neurons in response to drugs that imbue the drug-related stimuli with excessive incentive salience (Robinson and Berridge, 1993, 2001). Interestingly, schizophrenia often co-occurs with a variety of substance abuse disorders, particularly alcohol (Green et al., 2008; Koskinen et al., 2009) and nicotine addiction (Lohr and Flynn, 1992; Kumari and Postma, 2005; Brown et al., 2012) and is thought to result from dysfunctional reward circuitry (Green et al., 1999; Chambers et al., 2001). Rats treated subchronically with L-KYN show elevated levels of dopamine release in NAc compared to controls in response to amphetamine injections (Olsson et al., 2009), suggesting that prior treatment with L-KYN may sensitize the midbrain dopamine system and thus enhance the incentive value attributed to rewards and their associated cues. The present finding that exposure to KYNA increases sign-tracking behavior suggests that elevated levels of KYNA in persons with schizophrenia may contribute to the propensity to engage in drug use. Indeed, sign-tracking is mediated by the NAc and its dopaminergic inputs from the VTA (Flagel et al., 2011; Chang et al., 2012; Saunders and Robinson, 2012) and KYNA enhances dopaminergic neurotransmission in midbrain reward circuits (Erhardt and Engberg, 2002). Moreover, dopamine receptor antagonists such as apomorphine, as well as commonly used antipsychotic drugs like clozapine and haloperidol, reduce sign-tracking behavior (Dalley et al., 2002; Danna and Elmer, 2010) while goal-tracking behavior (approaching the reward itself) is unaffected.

In Experiment 2, we found that hippocampal LTP was deficient in adult rats that had been treated with L-KYN during adolescence compared to vehicle-treated controls, indicating that elevated levels of KYNA impair LTP. Conversely, Potter et al. (2010) demonstrated that LTP was enhanced in mice that lacked kynurenine aminotransferase II, the major biosynthetic enzyme of brain KYNA. These mice had significantly lower levels of KYNA compared to wild-type controls. Together with the present findings, this suggests that changes in the concentration of KYNA can bi-directionally modulate the ability of hippocampal neurons to undergo LTP, a process that is critical for normal brain development and cognitive function.

The observed decrease in the ability to undergo LTP is the first identified consequence of exposure to high levels of KYNA during adolescence and provides new insight into the basis of previously reported behavioral deficits. For example, it has been shown that contextual fear conditioning (Akagbosu et al., 2012) and social behavior (Trecartin and Bucci, 2011) are impaired in adult rats that have been exposed to KYNA during adolescence. It is well established that contextual fear memory is dependent on the hippocampus (Maren et al., 1997) and likewise, social behavior is altered by manipulations of hippocampus (Flores et al., 2005). Interestingly, lesions of the hippocampus also affect sign-tracking. Ito et al. (2005) found that hippocampal damage increased sign-tracking behavior, similar to the effects of adolescent L-KYN exposure in the present study. It is important to note, however, that Ito et al. (2005) used a different CS+ and CS− modality (a touch screen) than the one used here, which may involve different brain areas and systems (Chang and Holland, 2013). In contrast, other studies have found that hippocampal damage attenuates sign-tracking (Good and Honey, 1991; Fitzpatrick and Morrow, 2014). In those studies, the lack of a CS− lever may have contributed to the contrasting results. Indeed, it has been shown that different results are sometimes obtained depending on whether a CS− lever is included in the experimental design (Chang et al., 2012). Additionally, only the ventral hippocampus was damaged in the study by Fitzpatrick and Morrow (2014), whereas L-KYN treatment, and the resulting increase in KYNA, likely affected the entire hippocampus. This is important to consider because of the behavioral and anatomical differences between dorsal and ventral subregions of hippocampus (Fanselow and Dong, 2010). Regardless, the present findings suggest that changes in the potential for hippocampal neurons to undergo synaptic plasticity, induced by exposure to elevated levels of KYNA during adolescence, may contribute significantly to the cognitive and behavioral deficits observed later in adulthood. Future studies are needed to expand on this by identifying the changes in hippocampal morphology and/or connectivity that may result from exposure to high levels of KYNA during development. In addition, there are likely to be changes in other brain structures following KYNA exposure and further studies will be needed to determine which behavioral impairments are due to KYNA-induced changes in hippocampus *per se*.

Nonetheless, these findings have several potential implications for understanding the neural substrates of schizophrenia. The cognitive deficits in schizophrenia include similar impairments

in hippocampal-dependent processes, including contextual and spatial memory (Cohen et al., 1999; Waters et al., 2004; McClure et al., 2008), sensory gating (Tregellas et al., 2007), and social withdrawal (Sams-Dodd, 1999). Moreover, schizophrenia is thought to involve alterations in hippocampal structure and function (Harrison, 2004), and neonatal ventral hippocampal lesions in rats result in various symptoms of schizophrenia (Tseng et al., 2009; Peleg-Raibstein et al., 2012). KYNA-induced changes in hippocampal function may thus contribute significantly to the cognitive deficits that are often associated with schizophrenia. In addition, it has recently been proposed that the alterations in brain reward systems and dopamine function thought to underlie schizophrenia may be the result of dysfunctional modulatory control by the hippocampus (Grace, 2012). In this way, changes in hippocampal function resulting from high levels of KYNA may lead to dys-regulated reward-related behavior.

The L-KYN injection regimen used here increases KYNA concentration during treatment, but levels return to normal at the time of testing as adults (Akagbosu et al., 2012). Thus, the effects we observed on sign-tracking and on hippocampal LTP cannot be attributed to elevations in KYNA concentration at the time of testing. It is also unlikely that the increase in lever pressing to the CS+ merely reflects a change in baseline activity, since we have previously shown that locomotor activity is unchanged by adolescent exposure to KYNA (Akagbosu et al., 2012). Moreover, there were no group differences in responding to the CS− in the present study. It is possible that the effects of L-KYN administration could be due to changes in the concentration of kynurenine metabolites other than KYNA. However, previous data indicate that a higher dose of L-KYN (150 mg/kg compared to 100 mg/kg in our study) failed to significantly increase levels of quinolinic acid, for example (Shepard et al., 2003). Although the possibility of increasing the concentration of other metabolites could be mitigated by directly injecting synthetic KYNA into the cerebral ventricles, there are several important advantages to elevating KYNA levels by injecting L-KYN. For example, increasing KYNA concentration by administering its precursor allows KYNA to be synthesized only in brain regions that have the neural machinery to produce KYNA. This is important because KYNA acts at receptors that are distributed ubiquitously in the brain, yet the distribution of KYNA-synthesizing astroglia is not uniform and the increase in KYNA concentration in schizophrenia is region-dependent (Schwarcz et al., 2001). In addition, the increase in KYNA observed in schizophrenia is due to upregulation of enzymes such as TDO2, which increases the availability of kynurenine (Miller et al., 2006). Thus, increasing KYNA levels by administering L-KYN lends a high degree of physiological relevance.

Schizophrenia is characterized by a constellation of positive symptoms (e.g., hallucinations), negative symptoms (e.g., social withdrawal), and cognitive deficits (e.g., attention, memory, inhibition). While standard-of-care dopaminergic compounds are often effective in alleviating positive symptoms, the cognitive deficits are notoriously unresponsive to treatment (Blin, 1999) and their cause has remained unclear. Similarly, although the majority of persons diagnosed with schizophrenia also suffer from substance abuse disorders (Green and Brown, 2006; Hartz et al., 2014), few treatment options exist and little is known about the biological link between schizophrenia and substance abuse. The present findings indicate that reward-related behavior and hippocampal synaptic plasticity are compromised in adult rats that had been exposed to high levels of KYNA during adolescence, a critical stage in neural development. Consistent with the notion that KYNA levels may be particularly impactful during development, it has been shown previously that chronic L-KYN treatment during adolescence, but not during adulthood, reduces social behavior (Trecartin and Bucci, 2011). Moreover, the increase in KYNA concentration observed in persons with schizophrenia likely begins early in life (Miller et al., 2004, 2006, 2008; Holtze et al., 2008; Asp et al., 2010), and the symptoms of schizophrenia typically emerge during late adolescence (Harrop and Trower, 2001). However, additional studies are needed to fully differentiate between the behavioral, physiological, and morphological changes induced by exposure to KYNA during adolescence compared to adulthood. Nonetheless, the findings add to a growing literature implicating an elevation in KYNA concentration as a causal factor leading to the cognitive and behavioral symptoms of schizophrenia. The data also support the notion that the development of anti-kynurenergic compounds may provide a new therapeutic avenue for treating schizophrenia (Erhardt et al., 2009; Wonodi and Schwarcz, 2010; Schwarcz et al., 2012; Wu et al., 2014).

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Nicotine receptors mediating sensorimotor gating and its enhancement by systemic nicotine

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Prepulse inhibition (PPI) of startle occurs when intensity stimuli precede stronger startle-inducing stimuli by 10–1000 ms. PPI deficits are found in individuals with schizophrenia and other psychiatric disorders, and they correlate with other cognitive impairments. Animal research and clinical studies have demonstrated that both PPI and cognitive function can be enhanced by nicotine. PPI has been shown to be mediated, at least in part, by mesopontine cholinergic neurons that project to pontine startle neurons and activate muscarinic and potentially nicotine receptors (nAChRs). The subtypes and anatomical location of nAChRs involved in mediating and modulating PPI remain unresolved. We tested the hypothesis that nAChRs that are expressed by pontine startle neurons contribute to PPI. We also explored whether or not these pontine receptors are responsible for the nicotine enhancement of PPI. While systemic administration of nAChR antagonists had limited effects on PPI, PnC microinfusions of the non-α7nAChR preferring antagonist TMPH, but not of the α7nAChR antagonist MLA, into the PnC significantly reduced PPI. Electrophysiological recordings from startle-mediating PnC neurons confirmed that nicotine affects excitability of PnC neurons, which could be antagonized by TMPH, but not by MLA, indicating the expression of non-α7nAChR. In contrast, systemic nicotine enhancement of PPI was only reversed by systemic MLA and not by TMPH or local microinfusions of MLA into the PnC. In summary, our data indicate that non-α7nAChRs in the PnC contribute to PPI at stimulus intervals of 100 ms or less, whereas activation of α7nAChRs in other brain areas is responsible for the systemic nicotine enhancement of PPI. This is important knowledge for the correct interpretation of behavioral, preclinical, and clinical data as well as for developing drugs for the amelioration of PPI deficits and the enhancement of cognitive function.

Keywords: prepulse inhibition, sensory gating, sensorimotor gating, startle, rat, schizophrenia, nicotine receptor, alpha-7 nicotine receptor

INTRODUCTION

Prepulse inhibition (PPI) describes the attenuation of a reflexive motor response due to the presentation of a sensory stimulus prior to the response eliciting stimulus, and can be easily measured in humans and animals using the acoustic startle response. Sensory processing of e.g., the prepulse is facilitated when colliculus-mediated orienting responses occur, leading to inhibition of the startle pathway at the same time. In particular, mesopontine cholinergic arousal systems provide diffuse thalamocortical activation while inhibiting startle (Fendt et al., 2001; Yeomans, 2012). PPI disruptions are a hallmark of schizophrenia, but PPI is also disrupted in several other neurological disorders (for review see Powell et al., 2012; Kohl et al., 2013; Swerdlow, 2013), and PPI disruptions are correlated with other cognitive dysfunctions, such as disorganized thought, attention deficits and working memory disruptions (Braff et al., 1978; Geyer and Braff, 1987; Young et al., 2009; Singer et al., 2013).

The mesopontine cholinergic projection from the pedunculopontine and laterodorsal tegmentum (PPT/LDT) to the startle mediating giant neurons in the caudal pontine reticular nucleus (PnC) has been identified as a crucial system for inhibiting startle during PPI (for review see Koch, 1999; Fendt et al., 2001). Lesions to the PPT/LTD severely attenuate PPI in rats (Kodsi and Swerdlow, 1997; Jones and Shannon, 2004; but see MacLaren et al., 2014) and stimulation of this projection activates muscarinic receptors inhibiting startle-mediating giant neurons in the PnC (Bosch and Schmid, 2006). In addition, there is evidence that ionotropic nicotine receptors (nAChRs) contribute to this cholinergic effect (Bosch and Schmid, 2008). In fact, the activation of both ionotropic and metabotropic receptors have been suggested to mediate the fast (10–100 ms) and long-lasting (100–1000 ms) effects of PPI, respectively (Jones and Shannon, 2000a; Yeomans et al., 2010). Thus, pontine nAChRs might play a role in mediating PPI

especially at shorter intervals between prepulses and startle stimuli.

Systemic administration of nicotine has also been shown to enhance PPI in healthy human participants (Warburton, 1992; Warburton et al., 1992; Kumari et al., 1996; Della Casa et al., 1998; Levin et al., 1998b; Mancuso et al., 1999; Levin and Rezvani, 2006), in individuals with schizophrenia (Kumari et al., 2001; Postma et al., 2006; Hong et al., 2008), and in rodents (Acri et al., 1994; Curzon et al., 1994; Levin et al., 1998a; Faraday et al., 1999; Azzopardi et al., 2013). It is not known if nicotine affects PPI directly within the PnC, or indirectly by modulating neuronal activity in higher brain regions that exert modulatory control over the startle and PPI circuitry, such as the hippocampus, striatum, medial prefrontal cortex, or amygdala (for review see Koch, 1999).

In this study we tested the hypothesis that the activation of nAChRs located in the PnC contributes to PPI, particularly at short interstimulus intervals, using systemic and local PnC microinfusions of nAChR antagonists and agonists in rodents. Subsequently, following the identification of the nAChR subtype(s) involved, using behavioral and electrophysiological approaches, we tested whether or not the same nAChRs are responsible for the systemic nicotine enhancement of PPI.

MATERIALS AND METHODS

BEHAVIORAL EXPERIMENTS

Animals

Male adult Sprague Dawley rats, weighing 250–350 g, were obtained from Charles River Canada (St. Constant, QC, Canada). Adult rats were housed in groups of two or three (except during recovery period after surgery) at a temperature of 21 ± 1°C in individual cages on a 12 h light/dark cycle with lights on at 7 a.m., and food and water available *ad libitum*. All animal procedures were approved by the University of Western Ontario Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care. All efforts were made to minimize the number of animals used and any discomfort resulting from surgical or behavioral procedures. Testing occurred during the light part of the light/dark cycle to minimize movement during testing in these nocturnal animals.

Surgery

For local microinfusions into the PnC, rats were anaesthetized, using a mixture of 2% isoflurane and 98% oxygen. A subcutaneous injection of 0.05 mg/kg of buprenorphine and 5 mg/kg of ketoprofen was given during surgery for analgesia. Blunt-ended ear bars and a snout mask were used to secure the head in the stereotaxic device. Two 23-gauge stainless steel guide cannulae (Plastics One, Roanoke, VA, USA) were stereotaxically aimed bilaterally at the ventrocaudal segment of the PnC based on coordinates derived from the Paxinos and Watson rat brain atlas (Paxinos and Watson, 2005). Coordinates relative to Lambda were: +2.5 mm in the medial/lateral plane; −8.80 mm in the ventral/dorsal plane; −2.1 mm in the rostral/caudal plane, with a 10 degree mediolateral angle. The cannulae were mounted on the skull with dental cement casted around four jeweler's screws implanted bilaterally into the parietal skull plates. Stainless steel stylets were inserted into cannula to prevent clogging (PlasticsOne, Roanoke, VA, USA). Silk suture was used to close the wound, and rats were allowed a 7-day recovery period in the animal care facility.

Handling

Approximately 3 days before testing, rats were handled to ensure familiarity with the handler and the startle boxes. Rats were socialized for approximately 10 min each day for 3 days. On the second and third handling days, rats were afterwards placed in the startle apparatus for 5–10 min while the constant sound of a 65 dB white noise played in the background.

Injections/microinfusions

Each rat received two systemic injections or local microinfusions unless otherwise noted: one drug and one vehicle, in a pseudo-randomized order and at least 5 days apart. Stereotaxic microinfusions were accomplished with 30 G Infusion cannulae that were inserted into the guide cannulae and extended 1 mm beyond the tip of the guide cannulae. 0.5 μL of drug or vehicle was injected bilaterally in awake rats over a 4-min period, using a mechanical syringe pump (World Precision Instruments, Sarasota, FL). Infusion cannulae remained inside the guide cannulae for an extra minute to ensure complete diffusion of drug.

Startle testing

After injections or microinfusions, rats went back to their home cage and were placed in startle chambers (Med Associates, Vermont, USA) 5–20 min after injection, unless otherwise noted. The startle reflex software SOF-825, version 5.95 (Med Associates, Vermont, USA) was used to perform experiments and analyze data. Rats were first subjected to a 5 min acclimation period with a 65 dB white noise background without further stimulation. They were then exposed to 30 pulse-alone trials (Block 1) for habituation. Immediately following Block 1, they were subjected to up to 60 pseudo-randomized trials consisting of different stimulation trials, each administered 10 times (Block 2): a pulse-alone trial, and up to five different prepulse-pulse trials with the following Interstimulus intervals (ISI) between prepulse and startle pulses: 12, 20, 50, 100, and/or 250 ms. The startle evoking pulse always consisted of a 20 ms long burst of white noise of 105 dB. Two different prepulse intensities, either 75 or 85 dB, were used as indicated. All prepulses consisted of a 4 ms long burst of white noise. Trials were 20 s apart. For a more detailed description, see (Valsamis and Schmid, 2011). Protocols were shortened by omitting trial types in experiments involving injections of nicotine, since nicotine has a short half-life.

Histology

Rats that had received microinfusion through cannula implants were injected with a lethal dose of pentobarbital and received a microinfusion of a small amount of 3% thionine dye through the implanted cannulae in order to mark placements. Rats were then perfused transcardially. Brains were harvested and post-fixed by immersion in 4% paraformaldehyde (PFA) for at least 1 h, and then transferred to 15% sucrose (in buffer) for another 24 h. Brains were sliced into 50 μm-thick sections by a freezing microtome. Sections were mounted, dried, and stained using the Haematoxylin and Eosin counterstaining procedure. Cannulae

coordinate determination was made using a rat brain atlas by Paxinos and Watson (2005). Microinfusion tips that reached or penetrated the borders of the PnC were deemed as successful hits. All other placements were deemed as misses and their data were discarded.

Data analysis

The 10 startle responses that were preceded by a specific prepulse were averaged and divided by the average startle responses that had no preceding prepulse in block 2. These numbers were then subtracted from 1 and multiplied by 100 to yield a "Percent PPI" score:

$$\%\ prepulse\ inhibition = \left(1 - \frac{(prepulse - pulse\ trial\ amplitude)}{(pulse - alone\ trial\ amplitude)}\right) * 100$$

Averages of both PPI intensities were taken for every ISI, and Two-Way, repeated measures ANOVAs were conducted using GB Stat software (GB Stat®). If warranted, a Fischer's Least Significance Difference (LSD) *post-hoc* test was used to assess points of significance. Baseline startle measurements were calculated by averaging, for each rat, the first 30 startle alone trial responses in block 1. These individual baseline startle scores were analyzed for different drug conditions using a two-tailed, paired Student's *t*-test. In both the ANOVAs and the Student's *t*-tests, differences in the data were deemed significant if *p*-values were less than 0.05 ($\alpha = 0.05$).

ELECTROPHYSIOLOGY

Slice preparation

A more detailed description of the slice preparation containing the PnC giant neurons and their afferent projections within the startle pathway has been published previously (Weber et al., 2002; Simons-Weidenmaier et al., 2006; Schmid et al., 2010, see also Supplementary Figures S1, S2). In brief, juvenile Sprague-Dawley rats (P9-14, with P1 defined as the day of birth) were anaesthetized with isoflurane and their brains rapidly removed and transferred into ice-cold preparation solution containing (in mM): KCl, 2; $MgCl_2$, 2; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $NaHCO_3$, 26; glucose, 10; saccharose, 210; $CaCl_2$, 2; myoinositol, 3; sodium pyruvate, 2; ascorbic acid, 0.4; equilibrated with 95% O2/5% CO_2. Coronal slices of 350–400 μm thickness were cut with a vibratome (Microm, Walldorf, Germany) in a submerged chamber filled with ice-cold preparation solution and transferred into a holding chamber filled with artificial cerebrospinal fluid (ACSF) containing (in mM): KCl, 2; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $NaHCO_3$, 26; NaCl, 124; glucose, 10. $CaCl_2$ (2 mM) was added a few minutes after slices had been transferred. In order to improve later patch success the holding chamber was heated for 30 min to 32–35°C. Slices were then kept at room temperature for at least an additional 30 min.

Electrophysiological recordings

For recording, slices were transferred into a superfusion recording chamber mounted on an upright microscope (Zeiss, Oberkochen, Germany) with an infrared sensitive camera (Kappa, Germany). Superfusion rate was 2–3 ml ACSF/min at room temperature and patch-clamp recordings have been carried out under visual guidance. Patch electrodes were pulled out of borosilicate capillaries (Science Products, Hofheim, Germany) and filled with a solution containing (in mM): K-gluconate, 130; EGTA, 0.5; $MgCl_2$, 2; KCl, 5; HEPES, 10; pH 7.2 (KOH), 270–290 mosm. Electrodes had a resistance of 2.3–3.5 MΩ. The calculated junction potential using pClamp 10 is 11.8 mV and data was not corrected for it.

Giant neurons in the PnC were identified by a soma diameter greater than 35 μm and all recordings were made in voltage-clamp mode at a holding potential of −70 mV unless otherwise noted. Resting membrane potentials were measured by briefly switching into current clamp mode ($I = 0$). Presynaptic stimuli were applied by bipolar tungsten electrodes (Science Products). One stimulation electrode was positioned medial to the principal sensory trigeminal nucleus (Pr5) and the seventh nerve in order to stimulate trigeminal afferents. The second electrode was positioned ventral to the lateral superior olive for auditory afferent fiber stimulation. Both electrodes were connected via isolators to a pulse generator (Master-8, Science Products). Stimulus pulse duration was always 150 μs. Stimulus intensities were kept low to avoid spiking of the postsynaptic neurons. Recordings were made using an Axopatch 200B amplifier and digitized by Digidata 1200 (both Axon Instruments, Union City, USA). The data was filtered with a 5 kHz low-pass filter, the sampling rate was 20 kHz. The pClamp 8.2.0 software (Axon Instruments) was used for data acquisition and analysis. Series resistance and seal quality were monitored at the beginning and several times throughout the recordings. Only one cell was recorded per slice, and there was only one slice per rat in most cases. Statistical analysis was performed using SPSS. When only one drug/dose was tested, a Student's *t*-test was perfomed, and the *p*-value is reported. When several drugs or doses were tested, a repeated measurement mixed design ANOVA was used and both *F*- and *p*-values are reported. A LSD test was used for *post-hoc* analysis. In both the ANOVAs and the Student's *t*-tests, differences in the data were deemed significant if *p*-values were less than 0.05 ($\alpha = 0.05$).

DRUGS

The following drugs were used in behavioral and electrophysiological experiment in concentrations as indicated in the Results section: Liquid (−)-Nicotine (Sigma Chemical Co. Ltd., USA, concentration reflects the free base), the cholinergic receptor agonist carbachol (Sigma Chemical Co. Ltd., USA), the natural alkaloid and nicotine receptor blocker (+)-tubocurarine chloride (Tocris Bioscience, USA), the natural alkaloid and specific alpha-7 nicotine receptor blocker methyllycaconitine (MLA; Tocris Bioscience, USA), the highly alpha-7 specific agonist N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide hydrochloride (PHA 543 613; Tocris Bioscience, USA) and the non-α7 preferring non-competitive nAChR antagonist, 2,2,6,6-tetramethylpiperidin-4-yl heptanoate (TMPH) hydrochloride (Tocris Bioscience, USA). Drugs were dissolved in double distilled water in a 100X stock solution and kept at −18°C until used in behavioral experiments. Before usage,

they were diluted in physiological saline. Drug concentrations were selected based on previously published studies (nicotine: Hamann and Martin, 1992; Acri et al., 1994; Schreiber et al., 2002; MLA: Panagis et al., 2000; Chilton et al., 2004; TMPH: Damaj et al., 2005). For electrophysiological experiments, drugs were dissolved in a 1000* stock solution in double distilled water and they were added to the bath solution (1:1000) during experiments. Cadmium (100 μM; Sigma-Aldrich, Canada) was prepared as a 50 mM $CdCl_2$ stock solution and added to the perfusing (oxygenated) ACSF to block synaptic transmission by blocking voltage-gated calcium channels.

RESULTS

SYSTEMIC NICOTINE ANTAGONISTS

In order to test the role of nAChRs in prepulse inhibition, we systemically injected a group of 8 rats with the non-α7nAChR preferring antagonist TMPH and the α7nAChR antagonist MLA, at a dose of 10 mg/kg which has been shown by others to be highly effective (Damaj et al., 2005; Gao et al., 2010). Each rat received both drugs and a vehicle injection in a pseudorandomized order, and at least 5 days apart, for within subject comparison. Systemic administration of nAChR antagonists did not alter PPI [$F_{(2, 34)} = 1.896$; $p = 0.200$; **Figure 1**, *top*]. There was also no effect on baseline startle amplitudes [$F_{(2, 34)} = 0.108$; $p = 0.200$; **Figure 1**, *bottom*].

NICOTINE AND nAChR ANTAGONISTS IN THE PnC

nAChRs are abundantly expressed and might mediate opposing effects on PPI when targeted systemically. We therefore, targeted nAChRs directly in the PnC where the mesopontine cholinergic neurons supposedly synapse on startle mediating giant neurons, using microinfusions through chronically implanted bilateral cannulas prior to behavioral testing. Post-mortem histology confirmed PnC placement of cannulas for a group of 9 rats. Data of 3 rats were discarded since one or both cannulas missed the PnC. Intracranial (i.c.) microinfusions of 10 mM nicotine (0.5 μl) revealed a main group effect of nicotine in these 9 rats [$F_{(1, 17)} = 67.06$; $p < 0.0001$, **Figure 2**]. A LSD *post-hoc* analysis showed that 5 nmol nicotine severely disrupted PPI at all tested ISIs (20, 50, and 100 ms). Furthermore, the *post-hoc* analysis showed that PPI disruption was significantly stronger at a short ISI of 20 ms compared to a longer ISI of 100 ms. Baseline startle was not affected by microinfusions of nicotine into the PnC ($p = 0.34$, **Figure 2**).

Subsequently, we infused the α7nAChRs antagonist MLA and the non-α7nAChRs preferring antagonist TMPH locally into the PnC. At doses of 0.1, 1, and 8 mM, MLA did not have any significant effect on PPI at any ISI [$F_{(3, 20)} = 1.90$; $p = 0.15$; $n = 6$ animals]. Baseline startle amplitudes were also not affected by PnC microinfusions of MLA ($p = 0.08$; $n = 6$; **Figure 3A**). In contrast, PnC microinfusions of TMPH hydrochloride at doses of 0.1, 1, and 10 mM showed significant main group effects of drug on PPI [$F_{(3, 36)} = 115.89$; $p < 0.0001$; $n = 10$ animals] with *post-hoc* analysis revealing significant attenuation of PPI for the 10 mM dose of TMPH at short ISIs of 20 and 50 ms, and no significant effect at ISIs of 100 and 250 ms. Baseline startle was not affected by PnC application of TMPH ($p = 0.11$; $n = 10$; **Figure 3B**). In summary, microinfusions of nicotine antagonists into the PnC indicate a role of non-α7nAChRs in mediating a part of total PPI, specifically at short intervals between prepulse and startle stimuli.

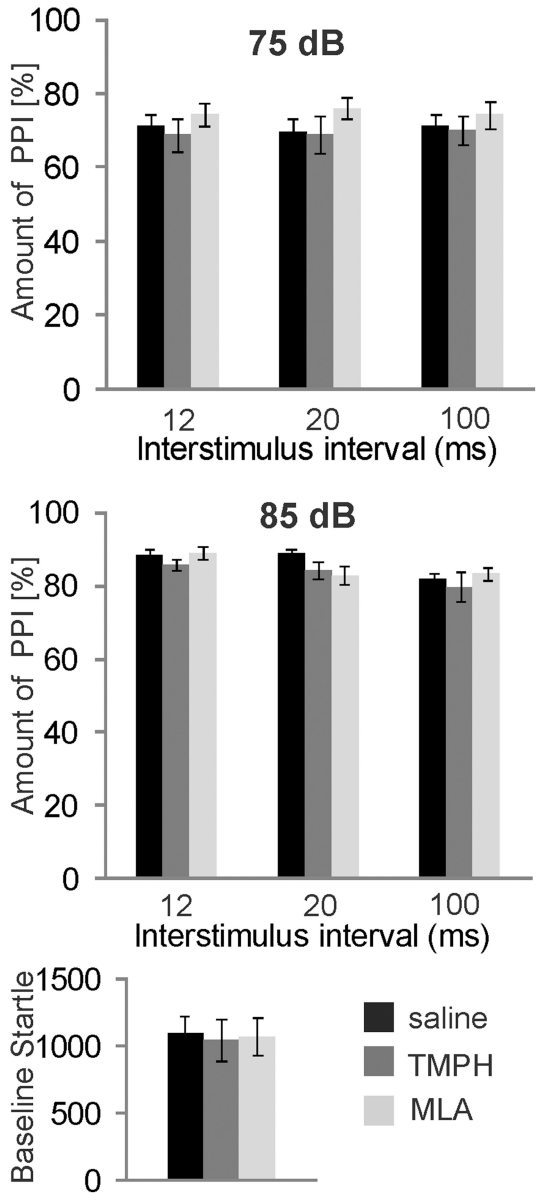

FIGURE 1 | Systemically injected nicotine antagonists do not affect PPI. Prepulse inhibition was measured using a 75 or 85 dB white noise prepulse preceding the startle pulse by different intervals, as indicated. The amount of PPI was calculated using the intermingled trials without prepulse as baseline startle (0% PPI). Rats were systemically injected with saline, the α7nAChR preferring antagonist MLA, or the non-α7nAChR antagonist TMPH at doses previously shown to be effective. There was no effect of drug on PPI at prepulse levels of 75 dB (*top*), or 85 dB (*middle*). Baseline startle amplitudes were analyzed during the habituation block (see Materials and Methods; $n = 8$ rats per group).There was also no effect of drug on the baseline startle response amplitude (*bottom*).

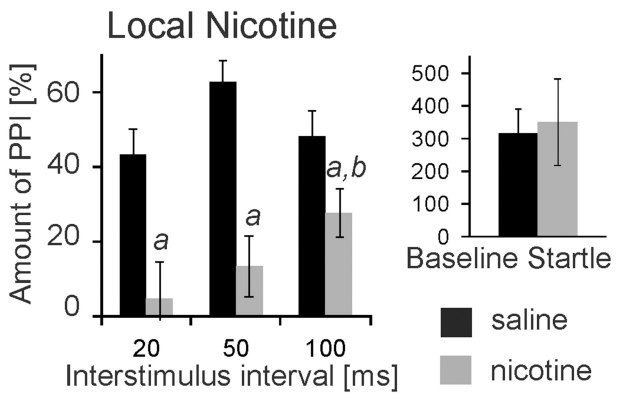

FIGURE 2 | Local PnC microinfusions of 0.5 μl nicotine (10 mM) impair PPI. Rats were bilaterally injected with 10 mM nicotine (in 0.5 μl saline) or vehicle through chronically implanted cannulae targeting the PnC. They were immediately tested for startle and PPI using a 75 db white noise prepulse. The amount of PPI was significantly decreased in rats injected with nicotine when compared to rats injected with saline (labeled by "*a*"). The decrease was significantly stronger at 20 ms interstimulus intervals compared with 100 ms intervals (labeled by "*b*"). Baseline startle amplitudes did not significantly differ between groups ($n = 9$ rats).

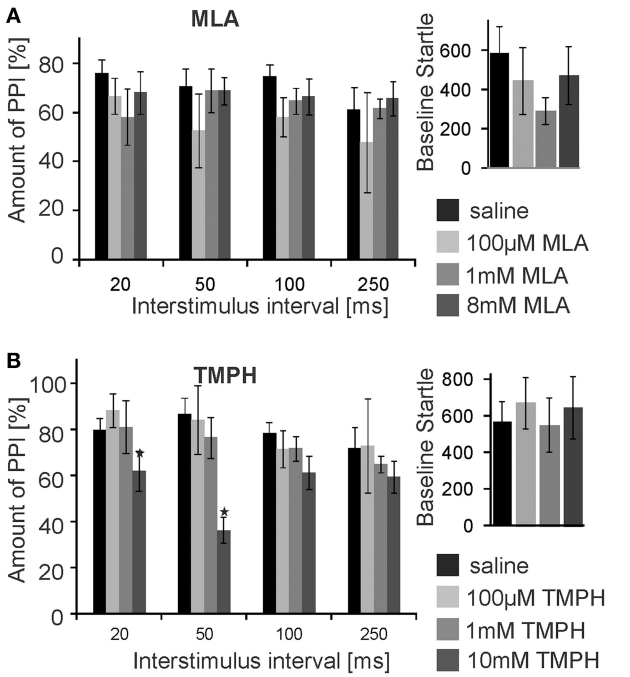

FIGURE 3 | PnC microinfusion of TMPH, but not of MLA, into the PnC disrupts PPI. (A) Intracranial microinfusions of the α7 preferring nicotinic antagonist MLA into the PnC in doses as indicated does not significantly affect PPI at any ISI. Local MLA infusion has also no significant effect on the baseline startle amplitude ($n = 6$ rats). **(B)** Intracranial microinfusions of TMPH significantly disrupted PPI at the highest dose tested. The effect was restricted to short ISIs of 20 and 50 ms. TMPH did not affect the baseline startle ($n = 10$ rats).

EFFECTS OF NICOTINE ON PnC GIANT NEURONS IN BRAIN SLICES

In addition to PnC microinfusions of nicotine *in vivo,* we applied nicotine in acute brain slices and monitored glutamatergic synaptic transmission of sensory signaling from afferent startle pathways as well as membrane properties in visually identified startle-mediating PnC giant neurons (see Materials and Methods, Supplementary Figures S1, S2, and Weber et al., 2002). As shown in **Figure 4A**, bath application of 10 μM nicotine significantly reduced the amplitude of both presynaptically evoked trigeminal and auditory synaptic currents to 79.4 ± 3.5% of control and 72.0 ± 4.2% of control, respectively ($p < 0.001$ and $p < 0.001$, respectively; $n = 14$ cells). After subsequent washout of nicotine EPSC amplitudes recovered to close to control levels (**Figure 4C**). Paired-pulse ratios with 100 ms IS were unchanged (1.52 ± 0.14 control, and 1.51 ± 0.08 during nicotine). Additionally, membrane resistance was calculated by applying hyper- and depolarizing 10 mV voltage steps and measuring the resulting change in whole cell (leak-) current according to Ohm's law. Membrane resistance of the analyzed PnC giant neurons was significantly reduced during the application of nicotine to 61 ± 5.9% MΩ from an average of 245.14 ± 20.95 MΩ under control condition to an average of 124.92 ± 10.96 MΩ ($p = 0.002$; $n = 14$; **Figure 4B**). This effect was also reversed by subsequent wash out (**Figure 4C**). The holding current significantly increased from −143 ± 19.9 pA to −269 ± 24.6 pA ($p < 0.0001$, $n = 14$) during nicotine application and the resting membrane potential (measured during a brief switch into current clamp mode) shifted to less negative values by an average of 7.07 ± 0.89 mV ($p < 0.0001$, $n = 14$) to an average of 48.0 ± 1.98 mV.

We further tested whether nicotine affects PnC giant neurons directly or through the activation of interneurons. Thus, we blocked voltage-gated calcium channels required for synaptic transmission by bath application of 100 μM cadmium. Auditory and trigeminal stimulation was used to verify the block of synaptic transmission. There was no significant effect of cadmium on the membrane resistance, as shown in **Figure 5** ($p = 0.75$; $n = 9$). However, even during the blockage of synaptic transmission with cadmium, nicotine significantly reduced the membrane resistance of PnC giant neurons to 75 ± 5.9% of its original value to an average of 126 ± 10.47 MΩ ($p = 0.03$; $n = 9$), indicating that nicotine either activates receptors that are directly located on the recorded neurons, or it activates the presynaptic release of inhibitory neurotransmitter independently from the activation of voltage-gated calcium channels.

In the following experiments we aimed to confirm that the observed nicotine effect is due to specific activation of nAChR in the PnC. We have previously shown that the non-specific cholinergic agonist carbachol inhibits PnC giant neurons and that this inhibition is only partly reversed by specific muscarinic blockers (Bosch and Schmid, 2006). We therefore tested, whether the specific nicotinic blocker tubocurarine reverses the inhibitory action of carbachol on PnC giant neurons. We added carbachol (10 μM) and tubocurarine (50 μM) to the bath solution. Tubocurarine is known to be effective specifically on neuronal nAChR at concentrations used in this study (Jensen et al., 2005). Carbachol significantly decreased trigeminally evoked EPSC amplitudes to 42.60 ± 3.84% of the control amplitude and auditory evoked EPSCs to 39.23 ± 4.02% of the control amplitude ($p < 0.001$ in

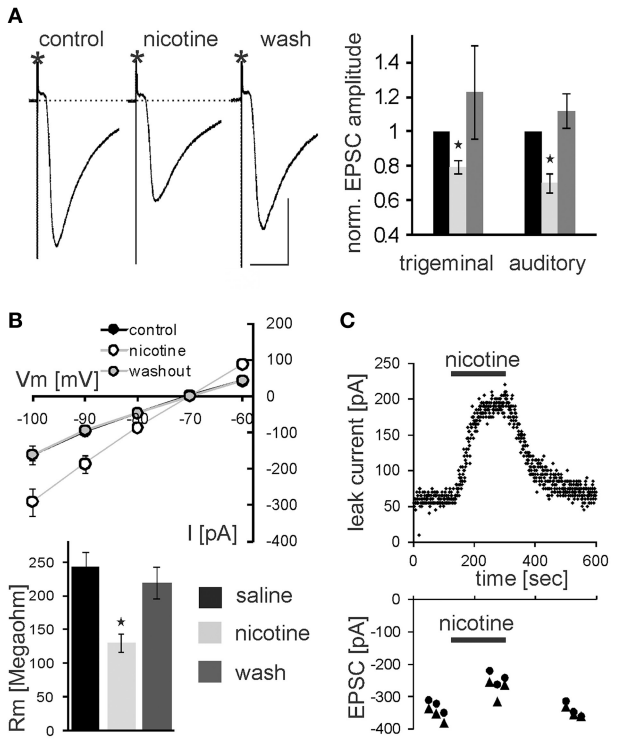

FIGURE 4 | Nicotine affects synaptic transmission and membrane resistance in PnC giant neurons. (A) Trigeminally evoked excitatory synaptic currents in control conditions, during perfusion with nicotine and after wash out. The asterisks mark the stimulus artifacts. Bars indicate 25 ms (horizontal) and 100 pA (vertical). 10 μM Nicotine significantly reduced EPSC amplitudes following trigeminal and auditory stimulation. This effect was reversed after wash out ($n = 14$ cells). **(B)** Whole-cell membrane leak current amplitudes in response to 10 mV step hyper- and depolarisations from −70 mV to the indicated potentials. Current amplitudes were increased during nicotine perfusion, indicating a lower membrane resistance *(top)*. Calculated membrane resistances ($Rm = \Delta Vm/\Delta I$) show that nicotine significantly reduces membrane resistance in PnC giant neurons *(bottom)*. **(C)** Time course showing whole-cell leak current amplitudes in response to a depolarizing 10 mV voltage step from −70 mV in an exemplary cell before, during, and after nicotine bath application *(top)*, and the corresponding EPSC amplitudes in the same cell *(bottom)*.

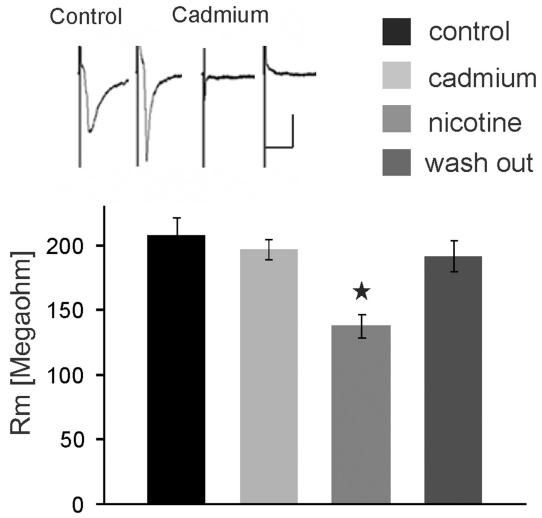

FIGURE 5 | The nicotine effect persists in presence of cadmium. Auditory and trigeminally evoked EPSCs were completely blocked by bath application of 100 μM cadmium, see insert (horizontal bar indicates 25 ms and vertical bar100 pA.) The effect of nicotine on the membrane resistance in PnC giant neurons persists in presence of 100 μM cadmium, indicating that it does not require synaptic transmission. Asterisk marks statistical significance with $p < 0.05$ ($n = 9$ cells).

both cases; $n = 14$; **Figures 6A,B**). Please note that the inhibition of EPSCs by carbachol was much stronger than the inhibition of EPSCs by nicotine shown above in **Figure 3**. Tubocurarine had no significant effect on EPSCs when given alone ($p = 0.9$ for both trigeminal and auditory stimulation; $n = 10$; **Figure 5B**). Perfusion of slices with tubocurarine, however, significantly reduced the inhibitory effect of carbachol. EPSC amplitudes were only reduced to 67.15 ± 4.86% of control amplitudes following trigeminal stimulation, and 56.19 ± 3.49% of control amplitudes following auditory stimulation ($p < 0.001$ in both cases; $n = 10$, **Figure 6A**). Furthermore, carbachol significantly decreased the membrane resistance to 69.58 ± 4.50% of control. The effect on membrane resistance was completely reversed by the additional application of tubocurarine [$F_{(2, 31)} = 12.996$; $p = 0.001$; **Figure 6B**]. *Post-hoc* Tukey adjustment revealed a significant difference between tubocurarine alone (98.91 ± 2.71% of control) and carbachol alone condition ($p = 0.001$); and also between carbachol and carbachol plus tubocurarine condition ($p = 0.011$). In summary, tubocurarine only partly reversed the carbachol effect on EPSC amplitude, while it completely blocked the carbachol effect on membrane resistance. This indicates that carbachol activates both muscarinic and nicotinic receptors, but that its effect on the membrane resistance of PnC giant neuron is exclusively mediated by nicotinic receptors.

In order to determine the nAChR subtype responsible for the nicotine effect on PnC giant neurons we recorded under control conditions, and then added the α7nAChR preferring antagonist MLA (100 nM). After recording in presence of MLA, we added nicotine (10 μM) in order to see whether the nicotine effect is blocked in presence of the antagonist. There was a significant effect of drug on membrane resistance [$F_{(2, 18)} = 4.45$, $p = 0.02$; $n = 4$] and the *post-hoc* analysis revealed no effect of MLA alone (101 ± 3.9%; $p = 0.13$), and a significant effect of nicotine in the presence of 100 nM MLA (60 ± 5%; $p = 0.001$; **Figure 7A**). There was also a significant drug effect on EPSC amplitudes [$F_{(2, 18)} = 4.5$; $p = 0.03$; $n = 4$]. MLA alone did not change EPSCs (99.8 ± 2.7%; $p = 0.13$), but the nicotine effect persisted in presence of MLA, such that EPSC amplitudes decreased to 70 ±5% ($p = 0.001$; **Figure 7A**).

We also applied the highly specific α7 agonist PHA 543-613 (30 μm). The membrane resistance was unaffected by the perfusion of PHA with the resistance changing to 98 ± 2.1% from 192 to 189 MΩ ($p = 0.69$; $n = 6$). EPSC amplitudes were also unaffected by PHA ($p = 0.21$; $n = 4$; **Figure 7B**). We then tested the non-α7 preferring nAChR antagonist 2,2,6,6-Tetramethylpiperidin-4-yl heptanoate (TMPH hydrochloride, 100 nM) using the same experimental procedure

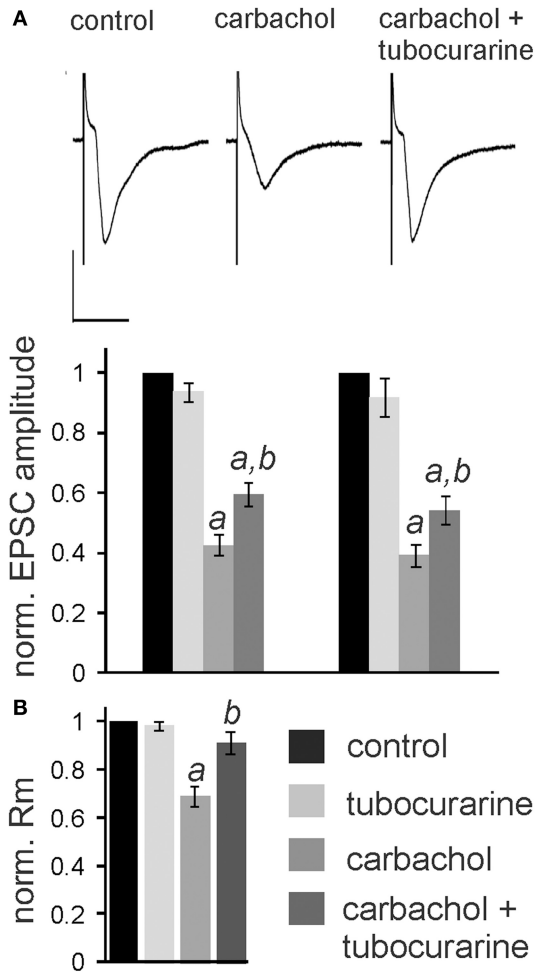

FIGURE 6 | Tubocurarine partly reverses the effect of carbachol on PnC giant neurons. (A), *top*: Exemplary traces of trigeminally evoked synaptic currents during perfusion of tubocurarine, carbachol, and the two drugs combined. Bars indicate 25 ms (horizontal) and 100 pA (vertical). *Bottom*: The perfusion of 50 μM tubocurarine had no effect on synaptic currents, whereas 10 μM carbachol significantly reduced synaptic current amplitudes. When tubocurarine application was combined with carbachol, the reduction of synaptic currents was significantly smaller than with carbachol alone ($n = 10$ cells for tubocurarine and tubocurarine plus carbachol, $n = 14$ cells for carbachol alone). Significant differences to control are labeled by "*a*," significant differences to the carbachol condition are labeled by "*b*." **(B)** Membrane resistance (Rm) normalized to the resistance of each cell under control condition. Tubocurarine had no effect on Rm, whereas the application of carbachol significantly decreased Rm. The additional application of tubocurarine completely reversed this effect ($n = 10$ cells).

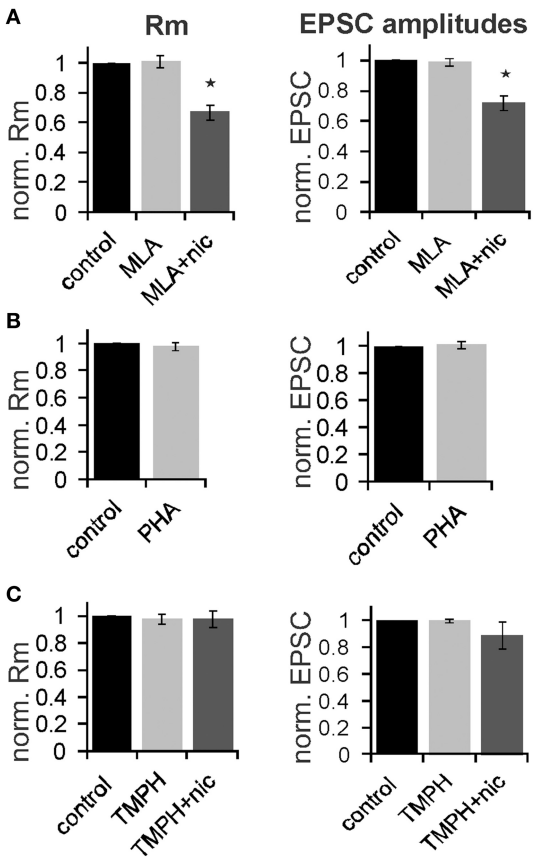

FIGURE 7 | The effect of nicotine on PnC giant neurons is mediated by non-α7-nicotine receptors. (A) Effect of the α7nAChR preferring antagonist MLA (100 nM). Membrane resistance (Rm) was normalized to the resistance of each cell under control conditions. MLA alone had no effect on Rm, however, the effect of nicotine persisted in presence of MLA. MLA alone did also not affect EPSC amplitudes, whereas nicotine reduced synaptic currents in presence of MLA ($n = 4$ cells, only trigeminal EPSC amplitudes were analyzed). **(B)** Effect of the α7 specific agonist PHA543-613 (30 μM) on Rm and EPSC amplitudes: PHA 543-613 had no effect on PnC giant neurons ($n = 6$ and $n = 4$, respectively). **(C)** Effect of the non-α7 preferring antagonist TMPH (100 nM): TMPH alone had no effect on Rm or EPSC amplitudes. In presence of TMPH, nicotine did also not affect Rm and synaptic currents, indicating that TMPH antagonizes the nicotine effect. Asterisk marks statistical significance with $p < 0.05$ ($n = 6$ cells).

as with MLA. We found no significant effect of drugs on the membrane resistance, indicating that pre-application of TMPH blocked the nicotine effect [$F_{(2,\ 16)} = 2.41$; $p = 0.15$; $n = 6$]. We also found no significant drug effect on EPSC amplitudes [$F_{(2,\ 16)} = 2.31$; $p = 0.19$; $n = 6$; **Figure 7C**].

In summary, our electrophysiological experiments support our behavioral results showing that the non-α7nAChR preferring antagonist TMPH blocks a portion of the cholinergic inhibition of startle neurons in the PnC, whereas the α7 antagonist MLA and the agonist PHA543-613 had no effect. Together with the behavioral results reported above our data indicate that non-α7nAChRs are expressed in the PnC and inhibit startle signaling, thereby contributing to PPI at 20–100 ms interstimulus intervals.

ARE NON-α7nAChRs IN THE PnC RESPONSIBLE FOR ENHANCEMENT OF PPI THROUGH SYSTEMIC NICOTINE?

Numerous studies have shown that acute systemic nicotine enhances PPI. We confirmed this PPI enhancing effect in our laboratory, using a low intensity prepulse of 75 dB. Subcutaneous (s.c.) injections of nicotine significantly affected PPI [$F_{(3,\ 29)} = 14.63$; $p < 0.0001$; $n = 8$ animals; **Figure 8A**]. A LSD *post-hoc* analysis revealed that doses of 0.01 and 0.1 mg/kg had little effect,

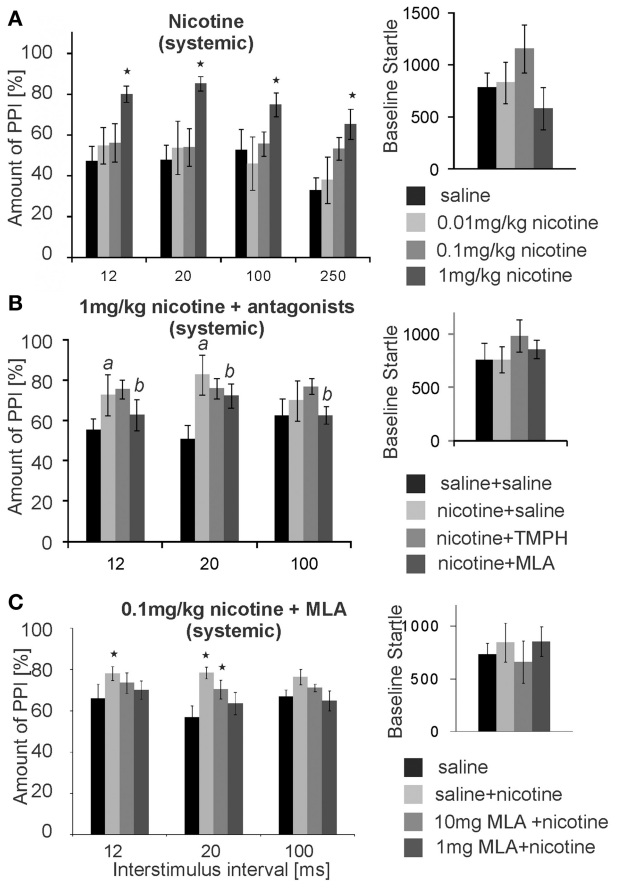

FIGURE 8 | Systemic nicotine enhances PPI, which can be reversed by systemic MLA, but not by systemic TMPH. (A) Subcutaneous injections of nicotine significantly enhanced PPI of startle at the highest dose of 1 mg/kg, without changing the baseline startle amplitude ($n = 8$ rats). A low prepulse of 75 dB was used here. Nicotine did not significantly change baseline startle responses. **(B)** Another batch of rats injected with 1 mg/kg nicotine also showed nicotine enhanced PPI (significant difference between saline and nicotine condition labeled by "*a*"). An additional intraperitoneal injection of 5 mg/kg MLA, but not of 5 mg/kg TMPH or saline, significantly reduced the nicotine enhanced PPI (significant difference between nicotine and nicotine + MLA condition labeled by "*b*"). There was no drug effect on the baseline startle response ($n = 8$ animals). **(C)** With a shorter protocol and only one nicotine injection per rat, subcutaneous nicotine at the dose of 0.1 mg/kg significantly enhanced startle at short ISIs of 12 and 20 ms. This was completely reversed by 1 mg/kg MLA administered i.p., and partly reversed by 10 mg/kg MLA i.p. Asterisk marks statistical significance with $p < 0.05$ ($n = 12$ animals).

showing a significant increase in PPI only at an ISI of 250 ms with the higher dose. Systemic injections of 1 mg/kg nicotine (free base) significantly increased PPI from ~50 to 80% for ISIs of 12, 20, 100, and 250 ms. Baseline startle amplitudes were not significantly affected by s.c. nicotine, when compared to saline controls ($n = 8$; $p = 0.25$; **Figure 8A**). Testing of the higher dose of 10 mg/kg was abandoned, since it occasionally led to seizures.

After establishing the nicotine enhancement of PPI, we combined systemic nicotine injections with systemic injections of subtype-specific antagonists in a different batch of rats in order to see whether or not the non-α7nAChRs that contribute to PPI are also responsible for the nicotine enhanced PPI. A low prepulse of 75 dB was also used here to allow for PPI enhancement by nicotine. There was a significant effect of drug [$F_{(2, 21)} = 50.27$; $p < 0.0001$; $n = 8$; **Figure 8B**]. LSD *post-hoc* again confirmed a PPI enhancing effect of nicotine. Further, systemic MLA, but not TMPH, significantly attenuated nicotine enhanced PPI at ISIs of 12 and 20 ms. There were no significant effects of either TMPH or MLA on baseline startle amplitudes, in comparison to saline controls ($p = 0.15$).

In a different batch of rats we repeated the MLA injections using different concentrations. In order to avoid the possibility that nicotine receptor up- or down-regulation affects our results, each rat received only one dose of nicotine and a saline control injection (pseudorandomized). We also measured PPI at ISIs of 12, 20, 100 ms only, cutting down the total time of startle testing in order to avoid the possibility that the short-lasting nicotine effect was wearing off during testing. With a shorter protocol and a single dose per rat the effect of the lower dose of 0.1 mg/kg nicotine was also significant. Co-administration of 1 and 10 mg/kg MLA reversed the PPI enhancing effect of nicotine in a dose dependent manner (**Figure 8C**). ANOVA revealed an effect of drug [$F_{(3, 56)} = 5.17$; $p = 0.003$; $n = 12$] and *post-hoc* tests showed a significant effect of nicotine for the nicotine only measurements at 12 ms ($p = 0.003$) and 20 ms ISI ($p = 0.04$), but no significant change to saline control when MLA was co-administered, except from the less efficient MLA dose at 20 ms ISI (**Figure 8C**). Systemic TMPH tested at these conditions had still no effect on nicotine enhanced PPI [$F_{(3,56)}$ =0.173; $p = 0.916$; $n = 12$; data not shown]. In summary, nicotine enhanced PPI seems to be dependent on α7nAChRs. Our previous *in vivo* and *in vitro* results indicated that nAChRs expressed in the PnC are of the non-α7nAChR type, since neither the α7nAChR agonist PHA 543-613 nor MLA applied to the PnC revealed any effect on PnC signaling or PPI (see above). In accordance with this, local microinfusions of MLA into the PnC did not affect nicotine enhanced PPI [$F_{(1, 23)} = 2.21$, $p = 0.34$; $n = 12$ animals; Supplementary Figure S3] or baseline startle ($p = 0.81$; $n = 12$; data not shown). In summary, systemic antagonism of α7nAChRs, but not local antagonism in the PnC, reversed the PPI enhancing effect of nicotine.

In conclusion, our present study indicates that nAChRs are functionally expressed in the PnC, which receives cholinergic input from the PPT. Both electrophysiological and behavioral data suggest that these are non-α7nAChRs and that they are activated during PPI, contributing to the inhibition of startle by prepulses. In contrast, the enhancement of PPI through systemic nicotine is mediated by α7nAChRs expressed elsewhere in the brain than in the PnC.

DISCUSSION

In order to identify the role of nAChRs in PPI, we employed systemic injections and stereotaxic microinfusions in combination with behavioral measurements and *in vitro* slice electrophysiology. Our studies suggest that non-α7nAChRs in the PnC contribute to startle inhibition by prepulses, whereas the PPI enhancing effect of systemic nicotine is mediated predominantly by α7nAChRs that are not located in the PnC.

nAChRs IN THE PnC

Stereotaxic microinfusions into the PnC as well as our electrophysiological experiments clearly showed the expression of functional nAChRs in the PnC. The fact that local microinfusion of both nicotine and the nicotine antagonist TMPH into the PnC inhibited PPI seems puzzling at first glance; however, the presence of exogenous nicotine presumably makes these receptors unavailable for additional activation by acetylcholine released from PPT projections, therefore nicotine basically acts as an antagonist. Alternatively, the persistent presence of a high concentration of nicotine following PnC microinfusion could also simply inactivate nAChRs (Revah et al., 1991). We saw no sign of a decay of nicotine effect on PnC neuron excitability during the 10 min. nicotine bath application, which favors the first possibility. However, we would expect to see a reduction of baseline startle after local nicotine microinfusion due to reduced PnC excitability, which we did not find. Baseline startle amplitudes, though, can be very variable and e.g., potentiated by the microinfusion procedure itself, since it is mildly stressful and might lead to startle sensitization. We therefore, might have missed changes in baseline startle.

Our findings are in line with results of Stevens et al. (1993) who showed an antagonistic effect of tubocurarine on nicotine induced effects on postsynaptic currents of medial pontine reticular formation neurons. A previous study showed that activation of muscarinic receptors in the PnC inhibits synaptic transmission presumably through a presynaptic mechanism (Bosch and Schmid, 2006). We found an additional non-muscarinic receptor mediated effect of carbachol that affected the membrane resistance of PnC neurons (Bosch and Schmid, 2006, 2008). Our present data using carbachol in combination with tubocurarine further indicates that there is a nicotinic component contributing to the effect of carbachol on PnC neuron signaling, and that this nicotinic component is not affecting presynaptic glutamate release. Nicotine receptors are known to be cation channels that activate and inactivate rapidly. Neither our nicotine microinfusions, nor the bath application of nicotine are suitable to capture any of these rapid and short-lasting effects of nicotine receptor activation. Instead, we observe a tonic effect of startle-mediating PnC neurons through nicotine that reduces the membrane resistance and EPSC amplitudes while shifting the resting membrane potential toward less negative values. It has to be noted that since we didn't correct our data for the liquid junction potential, the measured resting membrane potential of −48 mV during nicotine application may correspond to a real membrane potential of around −60 mV. The observed nicotine effects are independent from the activation of cadmium sensitive voltage-gated calcium channels commonly required for neurotransmitter release. Also, it doesn't affect paired-pulse ratio of the glutamatergic EPSCs. Together, this suggests that nicotine does not affect presynaptic glutamate release probability. Future studies will have to show whether calcium influx through nAChRs directly activates presynaptic glycine or GABA release onto PnC giant neurons, and/or whether postsynaptic nAChRs affect PnC excitability through e.g., the activation of potassium, cation and/or chloride channels (for review see Shen and Yakel, 2009).

TIMING

Our present and past studies show the functional expression of both muscarinic and nicotinic receptors in the PnC, the sensorimotor interface of the startle circuitry. Microinfusion of nicotine and TMPH into the PnC in our study, as well as of muscarinic antagonists by Fendt and Koch (1999), significantly attenuated PPI, indicating that the activation of both muscarinic and nAChRs are likely contributing to PPI of startle. The generally limited effects of cholinergic antagonists indicate that the cholinergic inhibition might not be restricted to the PnC, but also affect sensory neurons upstream (Gómez-Nieto et al., 2014), and that other neurotransmitters, such as GABA, glutamate, and glycine, also contribute to PPI, as proposed before (Yeomans et al., 2010; Geis and Schmid, 2011).

Both ionotropic and metabotropic cholinergic and GABAergic receptors mediate PPI in rats (Jones and Shannon, 2000b; Yeomans et al., 2010, present data). Ionotropic receptors within the primary startle and PPI pathway would be expected to mediate PPI at rather short ISIs, due to their rapid activation and inactivation. In particular, α7nAChRs and $GABA_A$ receptors have been shown to be most important for PPT at ISIs roughly from 20–100 ms (**Figures 2**, **3**; Yeomans et al., 2010). Muscarinic receptors are slower to activate and yield longer lasting effects and should therefore, affect PPI at intermediate and longer ISIs. Indeed, it has been shown that systemic injections of $GABA_B$ or muscarinic antagonists disrupt PPI at longer ISIs from 100–500 ms, but not at short ISIs (Jones and Shannon, 2000b; Fendt et al., 2001; Ukai et al., 2004; Yeomans et al., 2010), and PnC microinfusion of muscarinic antagonists as well as knock-out of muscarinic M4 receptors have been shown to disrupt PPI in rats at ISIs of 100 ms (Fendt and Koch, 1999; Koshimizu et al., 2012). This clearly corroborates the hypothesis that the serial activation of ionotropic and metabotropic receptors contribute to the fast, transient and the long lasting inhibition of startle by a prepulse, respectively.

SOURCE OF ACETYLCHOLINE

The mesopontine cholinergic projections from the pedunculopontine tegmentum (PPT) to the startle mediating giant neurons in the caudal pontine reticular nucleus (PnC) have been identified as a crucial structure for inhibiting startle during PPI (for review see Koch, 1999; Fendt et al., 2001). In general, the mesopontine cholinergic cell groups send mainly ascending projections to the collicullus, thalamus, and striatum promoting orienting and approach behavior, as well as eye saccades, while the descending projections seem to inhibit avoidance/escape responses (Fendt et al., 2001; Jones and Shannon, 2004; Mena-Segovia et al., 2008; Winn, 2008; Yeomans, 2012). Stimulation of the PPT has shown to increase acetylcholine release in the PnC (Lydic and Baghdoyan, 1993), however, based on our data we cannot assume that the only source of acetylcholine release in the PnC is the PPT projection. Lesions the adjacent laterodorsal tegmentum (LDT) has also been shown to disrupt PPI (Jones and Shannon, 2004), whereas in a recent study specific cholinergic lesions in the PPT almost completely abolished startle responses without necessarily impacting PPI (MacLaren et al., 2014). This indicates that either the LDT is directly involved in mediating PPI or that there is a

more complex cholinergic regulation of PnC inhibition on the level of LDT/PPT (see Kohlmeier et al., 2012).

Interestingly, our finding of fast nicotinic receptor activation followed by slower muscarinic activation parallels results shown for other PPT/LDT outputs, e.g., to the colliculus as shown in monkeys and rats (Isa and Hall, 2009), or to the thalamo-cortical arousal system for improving sensory processing in cats and rats (Steriade, 1993).

nAChR SUBTYPES

Nicotine receptor modulators, especially of the α7nAChR subtype, are currently evaluated in many studies as a potential target for enhancing cognitive function (Young and Geyer, 2013; Young et al., 2013). Both major nAChR subtypes found in the mammalian brain, the α4β2 and/or α7, were reported to be involved in PPI or in other forms of sensory gating (for review see Adler et al., 1998; Schreiber et al., 2002; Leiser et al., 2009). Our electrophysiological and behavioral data indicate that the nAChRs in the PnC are mainly of the non-α7 subtype since the non- α7 preferring antagonist TMPH reversed nicotine effects, whereas the α7 specific antagonist MLA and agonist PHA 546613 had no effect. TMPH antagonizes the majority of subtypes found in non-α7 containing neural nAChRs, which include the α3, α4, β2, and β4 subunits (Damaj et al., 2005). The α4β2 nAChR subtype is the most common neuronal non-α7nAChR and it has been shown to be involved in cognitive processing (Changeux, 2010). It is therefore, the most likely candidate for mediating ionotropic cholinergic effects in the PnC, but further research is required to confirm this.

The involvement of non-α7nAChR is surprising, since the α7nAChR has been implicated in the past in PPI and cognitive function: Specific α7nAChR agonists have been shown to ameliorate sensory gating deficits (Adler et al., 1998; Stevens et al., 1998; Suemaru et al., 2004; Kohnomi et al., 2010), and α7nAChR knock-out mice show a mild PPI deficit in a recent study (Azzopardi et al., 2013), although previous studies had not reported any PPI deficits in these mice (Paylor et al., 1998; Young et al., 2011). However, our data indicate that non-α7nAChRs in the PnC contribute a small portion to normal PPI, whereas α7nAChRs elsewhere in the brain can exert a powerful modulation of PPI when activated e.g., by exogenous nicotine (see below).

SYSTEMIC NICOTINE ENHANCED PPI

Previous studies have shown that systemic nicotine or nicotine agonists enhance PPI in rats (Acri et al., 1994; Curzon et al., 1994; Faraday et al., 1999; Schreiber et al., 2002). Our data extend this result by showing that a low dose of nicotine increased PPI at an ISI of 250 ms, whereas a higher dose of nicotine was more effective in enhancing PPI at shorter ISIs (between 12 and 50 ms), where maximum PPI is observed in rats. Both the timing of the systemic effect of nicotine, as well as the fact that systemic MLA, but not PnC microinfusions of any nicotine antagonists, reversed the systemic nicotine effect, indicate that systemic nicotine predominantly affects α7nAChR nAChRs in brain regions other than the PnC that extrinsically modulate PPI. Indeed, multiple brain areas that have been shown to modulate PPI (for review see Koch, 1999; Fendt et al., 2001), receive cholinergic input, and express α7nAChRs, which include the superior colliculus, thalamus, basal lateral amygdala, the substantia nigra (SN), the hippocampus, the striatum, and the medial prefrontal cortex (mPFC, Woolf, 1991). Most importantly, Azzopardi et al. (2013) has shown that α7nAChR knock-out mice do not show nicotine enhanced PPI, which is in accordance to our current pharmacological results in rats.

Almost all individuals with schizophrenia smoke cigarettes and show improvement in cognitive performance and normal PPI following nicotine consumption (Kumari et al., 1996; Forchuk et al., 1997; Adler et al., 1998; Postma et al., 2006). Our results indicate that this effect may be due to the activation of α7nAChRs. Interestingly, the α7 subunit of the nAChR has been identified as a susceptibility gene for schizophrenia (for review see Martin et al., 2004).

CONCLUSION

In conclusion, the present study shows that activation of non-α7nAChRs in the PnC contributes a small, but significant portion to PPI at short interstimulus intervals, whereas activation of α7nAChRs elsewhere in the brain predominantly mediate the PPI enhancing effect of an acute dose of nicotine. This is an important piece in understanding not only the role of cholinergic neurotransmission in arousal, orienting responses and startle inhibition, but also for the correct interpretation of behavioral, preclinical, and clinical data as well as for developing drugs for the amelioration of PPI deficits and the enhancement of cognitive function.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnbeh.2015.00030/abstract

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Impaired top-down modulation of saccadic latencies in patients with schizophrenia but not in first-degree relatives

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Impaired eye movements have a long history in schizophrenia research and meet the criteria of a reliable biomarker. However, the effects of cognitive load and task difficulty on saccadic latencies (SL) are less understood. Recent studies showed that SL are strongly task dependent: SL are decreased in tasks with higher cognitive demand, and increased in tasks with lower cognitive demand. The present study investigates SL modulation in patients with schizophrenia and their first-degree relatives. A group of 13 patients suffering from ICD-10 schizophrenia, 10 first-degree relatives, and 24 control subjects performed two different types of visual tasks: a color task and a Landolt ring orientation task. We used video-based oculography to measure SL. We found that patients exhibited a similar unspecific SL pattern in the two different tasks, whereas controls and relatives exhibited 20–26% shorter average latencies in the orientation task (higher cognitive demand) compared to the color task (lower cognitive demand). Also, classification performance using support vector machines suggests that relatives should be assigned to the healthy controls and not to the patient group. Therefore, visual processing of different content does not modulate SL in patients with schizophrenia, but modulates SL in the relatives and healthy controls. The results reflect a specific oculomotor attentional dysfunction in patients with schizophrenia that is a potential state marker, possibly caused by impaired top-down disinhibition of the superior colliculus by frontal/prefrontal areas such as the frontal eye fields.

Keywords: schizophrenia, relatives, eye movements, saccades, saccadic latencies, state marker

1. INTRODUCTION

Impaired eye movements are a well replicated finding in patients with schizophrenia, for a review see Levy et al. (1994) and Trillenberg et al. (2004). For example, scanpaths during exploratory eye movements are spatially limited and contain fewer fixations in patients with schizophrenia (Loughland et al., 2002; Minassian et al., 2005; Bestelmeyer et al., 2006; Benson et al., 2007; Nishiura et al., 2007; Takahashi et al., 2008; Suzuki et al., 2009; Elahipanah et al., 2011; Delerue and Boucart, 2013; Sprenger et al., 2013). When following a moving target, smooth-pursuit eye movements are impaired (Schwartz et al., 1995; Nkam et al., 2001, 2010; Nagel et al., 2012; Krishna et al., 2014). Further, more errors in antisaccade tasks were found (Fukushima et al., 1988; Petrovsky et al., 2009; Dyckman et al., 2011; Cutsuridis et al., 2014). Exploratory eye movements have been used as discriminator from controls, from other neurotic disorders, or from mood disorders in large-sample studies (Kojima et al., 2001; Suzuki et al., 2009; Benson et al., 2012). Impaired eye movements were also found in high-risk groups (Nieman et al., 2007; van Tricht et al., 2010), and fist-degree relatives (Holzman et al., 1974; Thaker et al., 2000; Radant et al., 2010; Kang et al., 2011; Aichert et al., 2013; Roberts et al., 2013), and people with schizotypal personality (O'Driscoll et al., 1998; Ettinger et al., 2005; Mitropoulou et al., 2011). Thus, impaired eye movements have been suggested to be an endophenotype (Allen et al., 2009) and to be related to genetics (Ross et al., 2002; Radant et al., 2010; Smyrnis et al., 2011), but not all of the various oculomotor parameters fully meet the criteria for a robust endophenotype (Calkins et al., 2008; Kallimani et al., 2009; Mazhari et al., 2011); this is not surprising due to the fact that they are associated with different cognitive processes and neural circuits (e.g., smooth pursuit, fixations, saccades, anti-saccades).

Already at the beginning of an eye movement, top-down processes can influence viewing behavior. Saccadic latency (SL) is the delay time after stimulus onset until a saccade is performed, due to the time required to program the motor command for a consecutive saccade with a specific direction and amplitude; this duration is usually around 200 ms. Studies of SL have shown that a task with higher cognitive demand (i.e., identification vs. simple observation) can reduce SL (Trottier and Pratt, 2005; Guyader et al., 2010). Shorter SL may be due to top-down disinhibition of the superior colliculus (SC), potentially mediated by the direct pathway connecting frontal/prefrontal cortex to the SC. However, previous studies that investigated the effect of tasks with various complexity on SL in patients with schizophrenia are sparse. In our previous study, we investigated top-down SL modulation by different visual tasks in schizophrenia and found impaired SL pattern compared to healthy controls (Schwab et al., 2013). However, familial association is an important criterion in the evaluation of endophenotypes and requires to expand studies with a group of first-degree relatives (Chen et al., 2006). Therefore, the purpose of this paper is to investigate the modulation of SL by higher- and lower-demand cognitive tasks in first-degree relatives and compare

these data with SL pattern in healthy controls and patients with schizophrenia to evaluate SL as potential trait or state marker. We used two different visual tasks: color recognition (low demand) and orientation recognition (higher demand) to study SL patterns.

2. MATERIALS AND METHODS

2.1. PARTICIPANTS

Ten first-degree relatives of patients with schizophrenia and 12 healthy controls underwent video-oculography during a visual task. Data of 13 patients and 12 healthy controls were used from a previous study (Schwab et al., 2013) and combined with the newly acquired data, resulting in a total dataset of 13 patients, 10 first-degree relatives, and 24 healthy controls. For demographic and clinical details, see **Table 1**. Patients either suffered from schizophrenia (10 paranoid, F20.0; 1 hebephrenic, F20.1) or acute polymorphic psychotic disorder (1 F23.0; 1 F23.1), according to ICD-10. All patients were recruited from the University Hospital of Psychiatry in Bern, Switzerland. First-degree relatives were unaffected parents of patients with schizophrenia who were recruited from a support group (Vereinigung der Angehörigen von Schizophreniekranken, VASK Bern). Healthy controls and first-degree relatives were not on any medications and had no history of schizophrenia or psychotic disorders. None of the subjects had a history of eye diseases, dichromacy, neurological diseases, diseases of the cervical spine, or shoulder/neck pain. From the newly acquired sample (controls and relatives), six subjects were excluded from analysis (four due to bad data quality, one due to cervical spine surgery, and one due to psychoactive medication). The study was approved by the ethics committee (Kantonale Ethikkommission Bern, No. 135/09). Written informed consent was obtained from all participants prior to the examination according to the tenets of the Declaration of Helsinki.

The groups significantly differed in age (see **Table 1**) because relatives (parents) were older compared to the other two groups ($F_{2,44} = 16.6$, $p < 0.001$), but patient ages were not significantly different from controls (Welch two-sample t-test: $t_{34,9} = 1.03$, $p = 0.31$). The groups had no significant gender differences ($\chi^2 = 2.17$, df $= 2$, $p = 0.34$). The groups were significantly different in amount of education; most prominently, the control group had four more years of education compared to patients ($F_{2,44} = 10.4$, $p < 0.001$). Visual acuity was not significantly different between groups (Wilcoxon rank sum test; patients vs. controls: $W = 125$, $p = 0.48$; patients vs. relatives: $W = 56.5$, $p = 0.83$; controls vs. relatives: $W = 131$, $p = 0.66$).

Eleven patients were being treated with atypical antipsychotics (usually risperidone or aripiprazole), one patient received both atypical and typical antipsychotics, and one patient was not taking any antipsychotic medication [chlorpromazine equivalent dosage (CED) in **Table 1**]. Five patients were taking additional medications: One patient was taking antidepressants (SSRI), two were taking both benzodiazepines and antidepressants (SSRI and tetracyclic), three were taking mood-stabilizers (sodium valproate), and one was taking an opioid. The positive and negative syndrome scale (PANSS) and the modified Rogers scale (MRS) were used to assess overall psychopathology (**Table 1**).

Table 1 | Demographic and clinical information.

	Patients with schizophrenia ($n = 13$)	Healthy controls ($n = 24$)	Relatives ($n = 10$)
Mean age (range)	33.8 (24–49)	37.9 (21–66)	62.3 (50–72)
Gender (male/female)	3/10	10/14	2/8
Mean years of education (range)	12.4 (9–18)	16.6 (10–23)	14.1 (12–20)
Mean duration of illness (SD)	11.9 (8.8)	n/a	n/a
Mean CED (SD)	435 (424)	n/a	n/a
Mean MRS (SD)	2.5 (4.0)	n/a	n/a
Mean PANSS positive (SD)	16.3 (5.4)	n/a	n/a
Mean PANSS negative (SD)	11.1 (4.5)	n/a	n/a
Mean PANSS total (SD)	53.8 (15.0)	n/a	n/a
Median visual acuity (IQR)	1.0 (0.5–1.0)	1.00 (0.7–1.0)	0.95 (0.7–1.0)

SD, standard deviation; IQR, interquartile range; CED, chlorpromazine equivalent dose; MRS, modified Roger's scale; PANSS, positive and negative syndrome scale; PANSS positive, positive symptoms subscale; PANSS negative, negative symptoms subscale; PANSS total, sum of all subscales.

2.2. APPARATUS

Eye movements of the dominant eye were recorded with a video-based infrared eye tracker (iView X HED-MHT, SMI, Germany) at a sampling rate of 200 Hz and a spatial resolution of 0.5°–1°. Stimuli were presented using our own software, which was based on PsychoPy (Peirce, 2008). Visual targets were presented in the center and periphery of the subject's visual field to induce a large saccade. The visual targets were colored squares (red and yellow, 6 cm × 6 cm, 4.3° visual angle), Landolt rings (upward- or downward-oriented, 6 cm × 6 cm, 4.3° visual angle). All targets (central and peripheral) were presented at a viewing distance of 80 cm at individual eye height; peripheral targets at 55° in the left and right periphery. Further details and methodological aspects are described in our methods paper (Schwab et al., 2012).

2.3. PROCEDURE

First, visual acuity (Snellen chart), color vision (Ishihara test), visual dominance (Porta test), and handedness (Edinburgh inventory) were determined. All subjects were screened for eye diseases, diseases of the cervical vertebrae, neck and shoulder pain, drug abuse, and medication consumption. We used a peripheral recognition task (Schwab et al., 2012) to invoke large saccades. In the paradigm, a black fixation dot was presented. Then, the first target appeared in the same position, followed by a second target on either the left or right side (50% probability of each; **Figure 1A**). In the color task, we used color squares (red and yellow), and in the Landolt orientation task, we used Landolt rings (up- or downward orientation) as visual targets. The task was to determine whether these two objects (first and second target) were identical in terms of color (color squares) or orientation (Landolt rings). The subjects were instructed to move their eyes and head naturally as required and to make quick and accurate responses. In this context, we could precisely record large gaze shifts with our eye-head tracker consisting of both head and saccadic components, but in this paper we only focused on the saccadic latency time. The

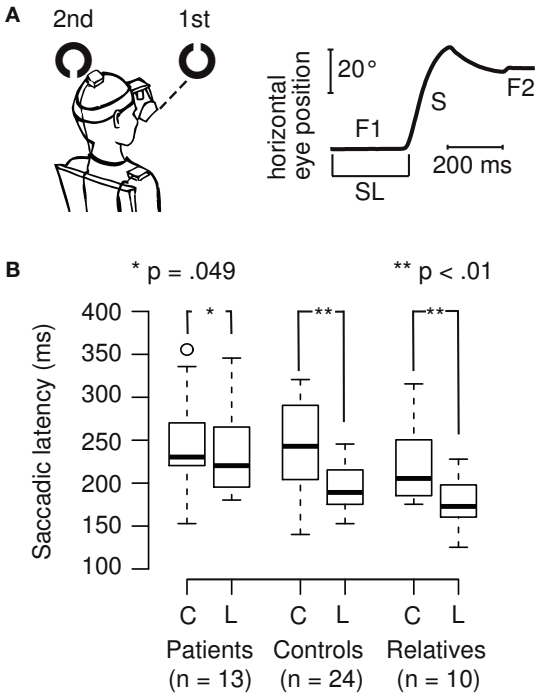

FIGURE 1 | (A) Example of a Landolt trial and eye position data. When the first target disappears and the second target appears, the eye changes in horizontal position after a short delay, the saccadic latency (SL), which usually is around 200 ms. The initial fixation (F1) is followed by a saccade (S) and a subsequent fixation (F2) on the second target. **(B)** SL is shorter during the Landolt task (L, higher cognitive demand) compared to the color task (C, lower cognitive demand) in control subjects (26% decrease) and first-degree relatives (20% decrease). In patients with schizophrenia, this task difference is smaller (7% decrease), and also reached statistical significance.

subjects pressed two buttons using their index ("Yes") and middle ("No") fingers of their dominant hands. In the experimental session, each subject performed 16 training trials, followed by 96 experimental trials spread across 3 blocks (32 trials per block). The trials involved color squares or Landolt rings (50% probability of each). For each subject, the conditions were randomly ordered and balanced within each block before the experiment started.

2.4. ANALYSIS

Based on our previous study (Schwab et al., 2013), we performed a power analysis. Comparing the color with the Landolt task (within-subject factor), we found a significant saccadic latency reduction of 22.3% in the 12 healthy controls (color: 269 ± 36 ms; Landolt 209 ± 23 ms; paired t-test: $t_{11} = 5.8$, $p = 0.0001$). The effect size is large (Cohen's $d = 2$), and a resulting power calculation based on this effect size, a significance level of 0.05, and a sample size of 10 subjects would result in a power of 0.99. Therefore, sample sizes of 10 and more subjects have sufficient statistical power to find significant effects (paired t-test power calculation; two-sided).

The data were preprocessed using our own custom MATLAB toolbox (Schwab et al., 2012). Eye recordings were transformed to visual angles (in degrees), low-pass filtered (750°/s), and smoothed (moving average over 20 ms). Saccades were detected using a velocity threshold algorithm with 60°/s, and 15°/s used as the onset and offset thresholds, respectively. SL values were then exported, and all statistics were performed in R (http://www.r-project.org/).

In order to classify the subjects based on the SL, we used Support Vector Machines (SVM, R package "e1071"). We trained to classify the subjects by using two values, the SL in the Landolt, and the SL in the color task. We compared the following classification accuracies: (1) classification of patients vs. controls, (2) classification of patients vs. relatives and controls, and (3) classification of patients and relatives vs. controls. In (1), we used $n = 13$ patients and a random sampling of $n = 13$ healthy controls (from a total of 24), in order to have equal group sizes. In (2), we used $n = 13$ patients and a random sampling of $n = 13$ of healthy controls and relatives (from a total of 34; 24 healthy controls and 10 relatives). In (3), we randomly sampled $n = 13$ patients and relatives (from a total 23, 13 patients and 10 relatives) and we randomly sampled $n = 13$ from the healthy controls (from a total of 24). Thus, our subsamples for the classification always contained a total 26 subjects ($n = 13$ per group) for all the three classification problems. We implemented leave-one-out-cross-validation, i.e., out training set always contained 25 subjects, and 1 subject was tested against the model prediction. This was implemented in a Monte Carlo run with 5×10^6 iterations for each of the three classification problems, and mean specificity and sensitivity were calculated.

3. RESULTS

Saccadic latencies (SL) in the different tasks (color and Landolt) were analyzed (**Figures 1A,B**). Patients had a small, significant reduction of only 6.5% between the two tasks (color: $M = 248$ ms, SD = 56 ms; Landolt: $M = 232$ ms, SD = 51 ms; two-sided paired t-test: $t_{12} = 2.19$, $p = 0.049$; $d = 0.3$). Controls had a large significant reduction of 26.2% in the Landolt task compared to the color task (color: $M = 261$ ms, SD = 100 ms; Landolt: $M = 193$ ms, SD = 27 ms; two-sided paired t-test: $t_{23} = 3.26$, $p = 0.003$; $d = 0.9$). First-degree relatives exhibited a large significant reduction of 19.5% in the Landolt task compared to the color task (color: $M = 221$ ms, SD = 49 ms; Landolt: $M = 178$ ms, SD = 30 ms; two-sided paired t-test: $t_9 = 3.58$, $p = 0.006$; $d = 1.1$).

We calculated the saccadic latency differences (SLD) between the tasks by subtracting the SL in the Landolt task from the SL in the color task (**Figure 2**). Positive SLD values denote the extent of SL reduction in the Landolt task. SLD was 16 ms for patients, which was significantly shorter than controls (68 ms; Welch two-sample t-test, $t_{28,3} = 2.35$, $p = 0.013$; $d = 0.6$) and relatives (43 ms; Welch two-sample t-test, $t_{15,4} = 1.90$, $p = 0.038$; $d = 0.8$). There was no significant difference between controls and relatives (Welch two-sample t-test, $t_{31,8} = 1.05$, $p = 0.15$). In patients, there was no correlation of SLD with their CED ($r = 0.17$, $t_{11} = 0.56$, $p = 0.59$).

We compared changes in SL variance in the Landolt task compared to the color task (**Figure 1B**). Patients showed no significant change in the variance in the Landolt task (10% increase; Levene's test, $F_{1,24} = 0.03$, $p = 0.86$). Controls had significantly smaller variance in the Landolt task (73% reduction; Levene's test, $F_{1,46} = 4.44$, $p = 0.041$). Relatives showed no significant change in

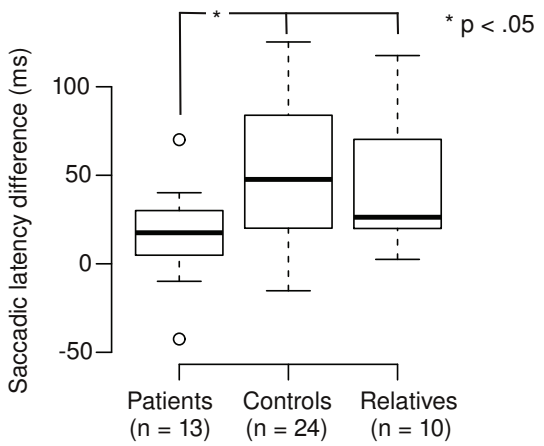

FIGURE 2 | Patients with schizophrenia exhibit smaller saccadic latency differences in the Landolt task (mean 16 ms) compared to control subjects (68 ms) and first-degree relatives (43 ms).

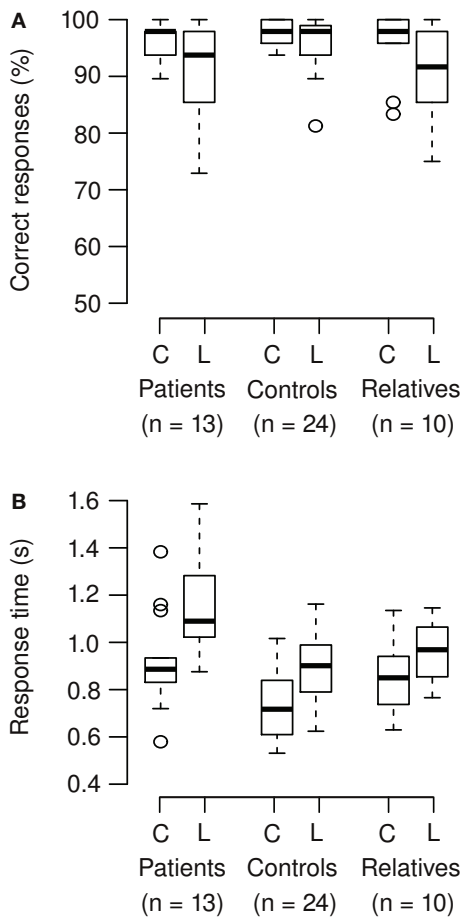

FIGURE 3 | (A) Participants correctly responded in 95% of the trials (mean). In patients and relatives, accuracy of responses was lower in the Landolt (L) compared to the color condition (C), but there was only a trend toward significance (patients: $p = 0.086$; relatives: $p = 0.050$). **(B)** The Landolt task produced longer response times across groups ($p < 0.001$). The patients exhibited longer response times across tasks ($p < 0.001$). Also, the relative increase of reaction time in the Landolt task was larger in patients compared to the other two groups (significant interaction of task × group; $p = 0.002$).

the variance during the Landolt task (38% reduction; Levene's test, $F_{1,18} = 1.46$, $p = 0.24$).

Correct responses and response times were analyzed (**Figures 3A,B**). On average, participants correctly responded in 95% of the trials. In patients and relatives, accuracy of responses was lower in the Landolt compared to the color condition, but this was not significant (Wilcoxon signed rank test; patients: $V = 70.5$, $p = 0.086$; relatives: $V = 39.5$, $p = 0.050$). The Landolt task produced longer response times across groups compared to the color task (mean increase 22%). The patients exhibited longer response times across tasks compared to the other groups (mean increase 24%). Also, the relative increase of reaction time in the Landolt task was larger in patients (28%) compared to the other two groups (12%). These results were confirmed by ANOVA (main effect group: $F_{2,44} = 8.65$, $p < 0.001$; main effect task: $F_{1,44} = 155$, $p < 0.001$; interaction task × group: $F_{2,44} = 7.01$, $p = 0.002$).

In the context that SL patterns may be useful for classification of the subjects, we used SVM with leave-one-out-cross-validation in a Monte Carlo experiment. We found that the SVM could classify the subjects (patients vs. relatives and healthy controls) with a mean sensitivity of 61%, and a specificity of 62% (**Figure 4**). The confusion matrix includes eight true positives, five false positives, eight true negatives, and five false negatives. Taken together, 16 subjects (62%) were correctly classified in average, 10 were incorrectly classified (38%). Performing other classifications resulted into lower accuracies: patients vs. healthy controls showed a mean sensitivity of 57%, and a specificity of 53%, and patients and relatives vs. healthy controls showed a mean sensitivity of 42%, and a specificity of 40%.

We tested five patients, two who were taking benzodiazepines, and three who were taking valproic acid for a potential medication effect of increased SL. During the color task, the five patients taking the medication mentioned above had a mean SL of 239 ms (SD = 56 ms), and the eight patients not taking this medication had a mean SL of 255 ms (SD = 59 ms), which was not a significant difference (Welch two-sample t-test: $t_9 = 0.49$, $p = 0.64$). In the Landolt task, the five patients taking the medication had a mean SL of 226 ms (SD = 68 ms), and the eight patients not taking this medication had a mean SL of 236 ms (SD = 42 ms), which was not a significant difference (Welch two-sample t-test: $t_{5,9} = 0.27$, $p = 0.79$).

In summary, the Landolt task caused (1) shorter SL and smaller within-group variability in controls and first-degree relatives compared to the patients, (2) longer response times consistently across groups, and (3) a relatively larger increase in response times of patients compared to the other two groups (interaction group × task). Further, classification based on SL works better merging the relatives and the controls and then classify into patients vs. healthy controls/relatives compared to merging the patients and relatives and then classy between patients and relatives vs. healthy controls.

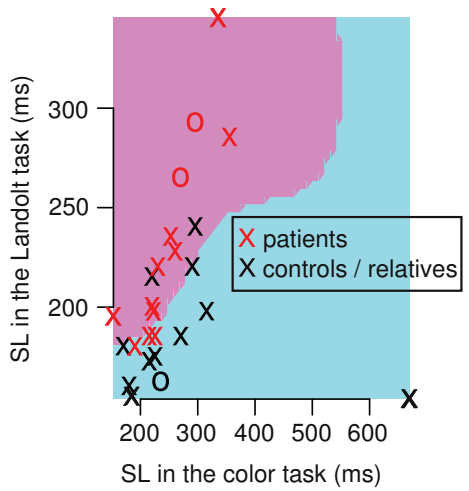

FIGURE 4 | Single example of a Monte Carlo iteration of the support vector machines (SVM) that classify between patients vs. relatives/healthy controls. The classification had a mean sensitivity of 61%, and a mean specificity of 62% over 5×10^6 iterations. In each iteration, we used 26 subjects (13 patients, 13 relatives/healthy controls) and implemented a leave-one-out-cross-validation (training-set: $n = 25$, test-set: $n = 1$). The single iteration above shows a radial kernel classifier (pink and blue area) of the training set. This model classifies subjects within the pink area as patients, and subjects within the blue area as controls/relatives. Note that the pink area covers longer SL in the Landolt task compared to the color task, and the blue area is associated with shorter SL in the Landolt task compared to the color task.

4. DISCUSSION

It has been shown that SL are strongly task dependent: higher cognitive demand causes shorter SL, while lower cognitive demand causes longer SL (Trottier and Pratt, 2005; Guyader et al., 2010). The present study demonstrates that SL modulation indeed fails in patients with schizophrenia but not in first-degree relatives during the two tasks of different cognitive demand. First-degree relatives and controls showed shorter SL in the Landolt task, and longer SL in the color task, consistent with previous findings (Trottier and Pratt, 2005; Guyader et al., 2010). The lack of SL modulation in patients may be caused by impaired top-down saccade generation in schizophrenia. Multiple brain areas are involved in the accurate execution of saccadic eye movements, most importantly the frontal eye field (FEF), which controls visual attention (Schall, 2004; Roux et al., 2014).

The fact that impaired SL modulation was not found in first-degree relatives does not support SL as possible trait marker. We found task-dependent modulation of SL in relatives and healthy controls, but not to the same extent in the patients. Also, our SVM classifier exhibited best accuracies during classification into a patient vs. a merged relatives/healthy control group. Thus, our data indicate impaired SL modulation may be a state marker, but a potential relation to psychopathology has yet to be studied. To take full advantage of this, however, further investigations, including longitudinal studies, will be required in order to relate objective measures such as SL to treatment outcomes or psychopathology scales (Walther et al., 2009). The results of this study are consistence with the literature showing that not all oculomotor parameters fully meet the criteria of an endophenotype, for example, antisaccade impairments (Levy et al., 2008). An explanation is that the various impaired oculomotor parameters in schizophrenia and the different experimental tasks (antisaccade, visual exploration, smooth pursuit, etc.) relate to different neurocognitive deficits arising from different brain circuits. Scanpath patterns likely relate to visual attention, fixations to visual memory, and antissaccades and SL to the control of executive and inhibitory frontal functions. Different parameters highlight a specific aspect of visual behavior and oculomotor control and contribute to the understanding of the underlying pathophysiology of schizophrenia.

Variance of SL decreased in the Landolt task compared to the color task, but this was only significant for the control group, indicating that this group became more homogeneous in their performance when faced with increased cognitive demand.

Performance data confirmed that the two tasks were of different cognitive demand. Landolt tasks produced fewer correct responses and longer response times. A possible explanation is that the Landolt orientation task required more foveal vision and mental rotation, while the color condition required peripheral vision during color detection. This is supported by larger saccade amplitudes found in the Landolt task than in the color task in our previous study (Schwab et al., 2013).

We now address some limitations of the study. First, most patients were taking psychoactive medication, which can affect oculomotor functions. For example, it was shown that atypical antipsychotic medication can impair performance in smooth pursuit (Lencer et al., 2008). On the other hand, it was found that antipsychotic medication can improve eye movement control (Burke and Reveley, 2002). Therefore, the overall effect of atypical antipsychotics on eye movement performance is ambiguous. Atypical antipsychotics have been suggested to even improve oculomotor markers, but evidence is lacking (Schmechtig et al., 2013). Three patients were taking valproic acid, and two were taking benzodiazepines, which was both associated with an increase of saccadic latency (Reilly et al., 2008; Larrison et al., 2011). However, the five patients who were taking valproic acid or benzodiazepines had no increase in SL compared to the other patients. Also, we observed that SL in patients were not generally increased compared to controls, which argues against a general slowing effect due to medication. Also, we could not find a correlation between SL and CED. Thus, even though our data suggest that the effects are not confounded by the type or the amount of medication, impaired saccadic latencies modulation observed in patients cannot be attributed to illness effects alone, thus the medication influence has to be further studied.

Second, it is unclear whether our findings are specific to schizophrenia, since we have not studied another pathological group (e.g., patients with mood disorders). Even though a recent review concluded that findings concerning anxiety and mood disorders have failed to support oculomotor impairments in these diseases (Toh et al., 2011), abnormal eye movements, but not specifically abnormal saccadic latencies, have been found in bipolar disorder (Bestelmeyer et al., 2006). In the context that symptoms and the brain systems affected in different psychiatric conditions can

overlap, it would be surprising to find abnormal eye movements only in schizophrenia. Nevertheless, eye movements seem to be a promising instrument for diagnosis and classification of patients with schizophrenia (Benson et al., 2012), and eye movement should in future studies be tested to classify different subtypes in schizophrenia, similar as eye movement can distinguish subtypes in Parkinson's disease and dementia (Willard and Lueck, 2014). The fact that the first-degree relatives are significantly older than the other two groups is not a real limitation of the study as we found significant saccadic latency modulation in the first-degree relatives, likewise as the younger healthy controls. The elderly group seems to perform as well as the younger healthy controls in view of saccadic latency modulation. Therefore, we conclude aging does not seem to affect saccadic latency adaption toward different tasks.

SL modulation during tasks with lower and higher cognitive demand is an important attentional feature to adapt to a changing environment by allowing the brain to save resources during low demand and to optimize performance during higher demand. The SL deficiencies found in patients with schizophrenia, but not in first-degree relatives, may be a state marker associated to their clinical condition. SL impairments may have their greatest impact during times of concentration. In this way, oculomotor deficits, may influence higher-order cognition (Butler and Javitt, 2005). For example, they could lead to difficulties determining which stimuli are relevant in the situations of daily life that require higher attentional resources.

AUTHOR CONTRIBUTIONS

SS and AA designed the experiments, MJ performed the experiments, and SS analyzed the data. SS wrote the first draft of the paper. All authors contributed to the final version and have approved the final manuscript.

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Disrupted thalamic resting-state functional networks in schizophrenia

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The thalamus plays a key role in filtering or gating information and has extensive interconnectivity with other brain regions. Recent studies provide evidence of thalamus abnormality in schizophrenia, but the resting functional networks of the thalamus in schizophrenia is still unclear. We characterize the thalamic resting-state networks (RSNs) in 72 patients with schizophrenia and 73 healthy controls, using a standard seed-based whole-brain correlation. In comparison with controls, patients exhibited enhance thalamic connectivity with bilateral precentral gyrus, dorsal medial frontal gyrus, middle occipital gyrus, and lingual gyrus. Reduced thalamic connectivity in schizophrenia was found in bilateral superior frontal gyrus, anterior cingualte cortex, inferior parietal lobe, and cerebellum. Our findings question the "disconnectivity model" of schizophrenia by showing the over-connected thalamic network during resting state in schizophrenia and highlight the thalamus as a key hub in the schizophrenic network abnormality.

Keywords: thalamus, resting-state, schizophrenia, functional connectivity, fMRI

INTRODUCTION

Schizophrenia, a significant mental illness of unclear etiology, seems to affect nearly every brain structure and many brain functions. An accumulating body of evidence so far has suggested that the thalamus, a nuclear complex with multiple connections to different parts of the brain regions may be abnormal in patients with schizophrenia (Jones, 1997). It is therefore believed that faulty connections between the thalamus and other cortical regions may be related to the wide diversity of behavioral and cognitive characteristics appeared in those patients (Mingoia et al., 2012). Although many *in-vivo* neuroimaging studies have implicated the thalamus in the pathophysiology of schizophrenia, limitations of constant methods of evaluating thalamic structure and function have prevented researchers from clearly understanding its role and findings have been largely inconsistent (Woodward et al., 2012).

While cortical organization considerations in prior research received much attention, an equally large literature has focused on thalamus and its role. Particularly, the thalamic dysfunction hypothesis has been a major recent model for schizophrenia and accounts for the onset and progression of this disease. Aberrant interconnectivity of the thalamus with other brain regions is presumed to play a central role in mental functions that are focal points of schizophrenia such as consciousness, perception, and the integration of thoughts (Bob and Mashour, 2011; Angelopoulos et al., 2014). In other words, a misconnectivity or disconnectivity syndrome would affect the organization of neurons in the brain creating a problem in neural connection (Schmitt et al., 2011). This assumption has been examined with functional magnetic resonance imaging (fMRI) and EEG (electroencephalography) data. Theoretically, brain regions, which have spontaneous synchronized neuronal activity are "functionally" connected (those regions exhibited synchronized activity because of the causal effect of one brain region had on another). EEG studies have revealed that patients with schizophrenia have decreased synchronization of neuronal oscillations, especially beta and gamma band activity (Uhlhaas and Singer, 2010). Studies using fMRI and the blood oxygenated level dependent (BOLD) contrast showed that patients with schizophrenia have decreased task and resting state functional connectivity (FC) of different brain regions (Whalley et al., 2005) and alterations of the default mode network (DMN; Mingoia et al., 2012). Although most researchers agree that cerebral connectivity may be altered in schizophrenia, still we cannot agree on a consistent set of alterations that can be used to characterize the mental problem.

To better understand whether connectivity differences between the thalamus and cortical regions may have contributed to schizophrenics' clinical symptoms, we investigated a conventional metric of resting state connectivity. Typically, brain functional resting-state networks (RSNs) characterize a corresponding set of significantly coherent brain areas with respect to low-frequency

BOLD signals during resting state (Zhang et al., 2008; Welsh et al., 2010). The correlations between sensorimotor cortical areas and DMNs during the resting state have been substantially mentioned in previous schizophrenia research (Tang et al., 2011; Klingner et al., 2014). However, the resting functional networks of deep gray matter structures, such as the thalamus, have been seldom studied with resting-state fMRI. Nevertheless, findings so far seem consistent to suggest a qualitative difference between patients' with schizophrenia and healthy controls in the thalamus related FC. Previous data showed a typical pattern of thalamic functional RSNs in healthy individuals that may involve several cortical regions (Tang et al., 2011). For example, motor and somatosensory areas were correlated strongly with ventral lateral and ventral posterior-lateral portions of the thalamus. Using ROI (region of interest) based approach, the prefrontal cortex was found functionally connected to anterior and dorsomedial regions of the thalamus, whereas the temporal lobe and occipital cortex were found to be correlated with posterior medial and lateral areas of the thalamus that appeared consistent with the medial geniculate nucleus and the lateral geniculate nucleus, respectively (Zhang et al., 2010). Studies also indicated that patients with schizophrenia suffered from disruption in their thalamic RSNs, manifesting both hyper- and hypo-regulation of thalamocortical FC. A recent study by Woodward et al. (2012) suggested a significant group difference in the prefrontal-thalamic connectivity and motor/somatosensory-thalamic connectivity (Woodward et al., 2012). Group comparisons revealed significantly reduced prefrontal-thalamic connectivity and increased motor/somatosensory-thalamic connectivity in schizophrenia, after controlling the local gray matter content within the thalamus and their antipsychotic medication dosage.

Built upon the literature, our purpose in this study was to understand the neural correlates of the thalamus by using resting-state fMRI and to investigate whether thalamic RSNs are different in patients with schizophrenia. Advanced from the prior research, here we surveyed the FC/coupling between a seed region (thalamus) and other cortical and subcortical areas in the whole brain during the resting condition. The analysis of intrinsic functional architectural changes by using resting-state fMRI in schizophrenia may help us to identify the pathophysiological mechanisms of disease, to improve the clinical observation and diagnosis and suggest better interventions.

MATERIALS AND METHODS

PARTICIPANTS

Seventy two patients with schizophrenia (58 males, 14 females, see **Table 1**), according to DSM-IV diagnostic criteria, were studies from the dataset released from the Center for Biomedical Research Excellence (COBRE), University of New Mexico.[1] Patients with the presence of DSM-IV Axis I diagnoses of other disorders such as depression, a history of any substance dependence, or a history of clinically significant head trauma were excluded from the analysis. The treatment details of schizophrenia patients were listed in **Table 2**. Meanwhile, 74 age-compatible healthy controls were recruited (two were dis-enrolled). All of the control participants were free of the DSM-IV diagnoses of schizophrenia and other DSM-IV Axis I diagnoses of mental disorders. None of them had neurological diseases, a history of any substance dependence, or a history of clinically significant head trauma.

[1]http://coins.mrn.org/dx and http://fcon_1000.projects.nitrc.org/indi/retro/cobre.html

Table 1 | Sample Demographics.

Measure	Schizo (*n* = 72)		HC (*n* = 74)		Statistics
	Mean	SD	Mean	SD	*P*
Age (year)	38.17	13.89	35.82	11.58	0.27
Gender					0.15
Male	58		51		
Female	14		23		
Handness					< 0.01[b]
Right	60		71		
Left	10		1		
Both	2		2		
IQ	(*n* = 68)		(*n* = 67)		
Verbal	97.88	16.73	106.79	11.16	< 0.01[b]
Performance	102.68	16.64	114.03	12.32	< 0.01[b]
Sum	99.59	16.86	108.33	11.83	< 0.01[b]
Education (year)	12.99	1.84	13.52	1.75	< 0.01[b]
Illness duration (year) (*n* = 71)	16.03	12.41			
PANSS					
Positive scale (*n* = 72)	14.96	4.83			
Negative scale (*n* = 72)	14.53	4.83			
General (*n* = 72)	29.22	8.34			

Note: Demographic information for the patient sample and control sample. Mean and standard deviation are provided for the continuous variable (e.g., age). Schizo = schizophrenic patients. HC = healthy controls. [a]Fisher's exact test, p < 0.01, [b]Student's t test, p < 0.01.

MRI DATA ACQUISITION

All subjects underwent structural and functional MRI scans in a single session using a 3 T Siemens Trio scanner with foam padding/paper tape to restrict head motion. All images were acquired parallel to anterior-commissure-posterior-commissure line with an auto-align technique.

For the reference image of anatomy and ROI analyses, a five-echo multiecho magnetization-prepared rapid gradient echo (MEMPR) sequence was used to acquire a whole brain high-resolution T1-weighted MR image in a coronal view [TE (echo times) = 1.64, 3.5, 5.36, 7.22, 9.08 ms, TR (repetition time) = 2.53 s, inversion time = 1.2 s, flip angle = 7°, number of excitations = 1, slice thickness = 1 mm, FOV (field of view) = 256 mm, resolution = 256 × 256]. The total scan time was 6.325 min and 150 time points were collected. Echo-planar images (336 images across three runs) were collected using a conventional single-shot, gradient-echo echoplanar pulse sequence [TR = 2,000 ms; TE = 29 ms; flip angle = 75°; FOV = 240 mm; matrix size = 64 × 64; 33 slices; voxel size = 3.75 × 3.75 × 4.55 mm^3]. The first image of each run was eliminated to account for T1 equilibrium effects (Mayer et al., 2013).

Table 2 | Treatment details of schizophrenia patients.

Treatment	Case number
Risperdal/Risperidone	15
Seroquel/Quetiapine	12
Aripiprazole/Abilify	11
Clozapine/Clozaril	11
Zyprexa/Olanzapine	11
Risperdal Consta/Risperidone Microspheres	11
Ativan/Razepam	8
Sertraline/Zoloft	8
Geodon/Ziprasidone	7
Benzotropine/Cogentin	6
Propranolol/Inderal	6
Holdol Dec/Haloperidol decanoate	6
Trazaone/Desyrel	4
Clonazepam/Klonopin	3
Buproprion/Wellbutrin	3
Citalopram/Celexa	3
Fluoxetine/Prozac	3
Zolpidem/Ambien	2
Divalproex/Depakote	2
Diazepam/Valium/Diastat/Diastat	1
Zaleplon/Sonata	1
Lexapro/Escitalopram	1
Fluvoxamine/Luvox	1
Mirtazapine/Remeron	1
Effexor/Venlafaxine	1
Tegretol/Carbamazepine	1
Permatil/Fluphenazine	1
Haloperidol/Haldol	1
Trilafon/Perphenazine	1
Thiothixene/Navane	1

The resting state fMRI was performed with single-shot full k-space echo-planar imaging (EPI) with ramp sampling correction using the intercomissural line (AC-PC) as a reference. The fMRI acquisition parameters were as follows: TR/TE = 2000 ms/29 ms, FOV = 256 mm × 256 mm, matrix = 64 × 64, 32 slices, voxel size: $3 \times 3 \times 4$ mm^3 (see http://coins.mrn.org/dx for more details).

IMAGE PROCESSING

The first 5 volumes were not analyzed to allow for signal equilibration effects. The fMRI data were then preprocessed using SPM8 software (available at: http://www.fil.ion.ucl.ac.uk/spm) implemented in a MATLAB suite (Mathworks, Inc, Natick, Massachusetts). Images were realigned to correct for motion, corrected for errors in slice timing, spatially transformed to standard stereotaxic space (based on the Montreal Neurologic Institute coordinate system), and smoothed with a 6 mm full-width half-maximum gaussian kernel. Data were then bandpass filtered from 0.01 to 0.08 Hz, to remove low frequency noise (including slow scanner drifts) and influences of higher frequencies reflecting cardiac and respiratory signals (Cordes et al., 2001).

Functional connectivity analysis was carried out by applying a seed-region approach using the left and right thalamus as defined in the automated anatomical labeling atlas (AAL; Tzourio-Mazoyer et al., 2002). For each ROI, individual participant analyses were carried out using the General Linear Model (GLM) with the time series for the ROI, as well as for the nuisance covariates (white matter, cerebrospinal fluid, and six motion parameters) as predictors. These nuisance signals are typically adjusted for in resting-state FC studies (Yu et al., 2014a,b), in order to remove the influence of global signal fluctuations of nonneuronal origin (e.g., physiological artifacts associated with variables such as cardiac and respiratory cycles, CSF motion, and scanner drift) (Fox and Raichle, 2007).

To address head motion concerns in resting-state fMRI analyses, we calculated the voxel-specific mean framewise displacement (FD) for accounting head motion at group-level analysis (Power et al., 2012, 2013, 2014; Van Dijk et al., 2012). FD measure indexes the movement of the head from one volume to the next and is calculated as the sum of the absolute values of the differentiated realignment estimates (by backward differences) at every time point (Power et al., 2012). Then, we repeated the above analyses after removing frames with FD > 0.5 mm ("scrubbing"). One time point before "bad" time points and one time points after "bad" time points were deleted. Eleven participants in the schizophrenia group and two in the control group were excluded because that more than 70% of the volumes have FD > 0.5 mm in these subjects. In the remaining 60 schizophrenia, 22 ± 19 (mean ± SD) percent of time points were removed, leaving 113 ± 27 time points. There was no significant difference between the excluded schizophrenia subjects and the included schizophrenia subjects in symptoms (i.e., PNASS and illness duration), p values >0.2, indicating that greater movement in those subjects were not due to worse symptoms. The mean FD in the schizophrenia group was significant larger than that in the control group, $t = 3.12$, $p < 0.005$. Thus, the FD value for each subject was added as covariate of no interest in all fMRI analysis.

STATISTICAL ANALYSIS

Contrast images were generated for each subject by estimating the regression coefficient between all brain voxels and each seed's time series, respectively. The resultant z-transformed β-value maps were then included in group (second-level) random effects analyses, adopting a 2 × 2 mixed design, factorial model (group [control, patient] by hemisphere [right seed, left seed]). Moreover, adding age, sex, and education level as additional covariate of no interest did not change the results. We also used regression analyses to examine whether clinical scores (e.g., PANSS and IQ scores) were related to thalamic FC, when considering the schizophrenia participants alone. The threshold was $P < 0.05$, family wise error (FWE) correction for multiple comparisons at the cluster level. The threshold of $P < 0.001$ uncorrected was used as cluster forming threshold. The cluster level correction method has better sensitivity than voxel-level correction at the cost of poor localization power. Maps are displayed at the same threshold (corrected at cluster level). Images are in radiologic format with subject left on image right. All coordinates are reported in MNI coordinates by SPM.

RESULTS

Table 1 represents the sample demographics. For the schizophrenia group, one subject was removed because only

67 time pointed were collected for this subject. As shown in the **Table 1**, the average age of our 72 schizophrenia patients was 38.17 ± 13.89 years. The PANSS scales were reported in the patients' group. In contrast, the average age of control subjects was 35.82 ± 11.58 years. There was a significant group difference in their personal educational attainment, $p = 0.008$. This was expected, as patients' psychiatric disorders often hold back learning achievement in other schizophrenia samples (Guo et al., 2013; Yu et al., 2014a). Within these participants, ten in the schizophrenia group and only one in the control group were left-handed. Demographic data also suggests our sample groups did not significantly differ in gender distribution ($\chi^2 = 2.033$, $p = 0.154$) or average age (t test, $p = 0.270$).

Across groups, FC analyses for the thalamus seeds revealed significant positive correlations with ACC/dorsal medial prefrontal cortex (PFC), bilateral insula, bilateral striatum, midbrain, and cerebellum, as well as negative correlations bilaterally with ventral medial frontal gyrus, precentral gyrus, cuneus, precuneus, and lingual gyrus (see **Figure 1**).

Group analyses revealed that schizophrenia patients exhibited reduced thalamus-based FC with bilateral superior frontal gyrus, anterior cingualte cortex, inferior parietal lobe, and cerebellum. Patients also showed increased thalamus-based FC with bilateral precentral gyrus, dorsal medial frontal gyrus, middle occipital gyrus, and lingual gyrus (see **Table 3**; **Figure 2**). There was no significant interaction between group and hemisphere.

Regression analysis found no significant thalamic FC with clinical variables.

Table 3 | Brain regions showing differences in the thalamus-based functional connectivity between controls and patients with schizohrenia.

Brain Regions	Voxels	Z-scores	MNI Coordinates		
			X	*Y*	*Z*
Control vs.Schizophrenia					
Thalamus	176	5.32	12	−9	3
Thalamus			−12	−5	0
Midbrain			6	−15	−15
Superior Frontal Gyrus	400	6.53	−33	54	30
Inferior Parietal Lobule	122	5.32	−60	−48	42
Anterior Cingualte Cortex	65	3.80	−12	33	27
Cerebellum	1737	6.46	33	−63	−54
Cerebellum	442	5.24	−30	−60	−39
Schizophrenia vs. Control					
L Superior Temporal Gyrus	3394	6.54	−60	−6	−9
Precentral Gyrus		6.25	−42	−21	66
Precentral Gyrus		5.83	35	−27	69
Posterior insula		5.27	−55	−18	0
Dorsal medial PFG		5.05	3	−30	63
R Superior Temporal Gyrus	728	6.39	−21	−50	−3
Posterior insula		6.03	45	−36	6
Lingual Gyrus	1738	4.97	54	−9	−12
Middle Occipital Gyrus	231	4.62	36	−72	9
Middle Occipital Gyrus	283	5.79	−54	−63	3

All values $P < 0.05$ FWE-corr at cluster-level after t maps thresholded at $P < 0.001$ uncorrected.

DISCUSSION

In this study, we reexamined the fMRI data of a large group of schizophrenia patients obtained from the COBRE database, University of New Mexico regarding thalamic FC during resting state. Thalamus-based FC was established across all brain regions in all subjects. However, we found significant regional differences in the connectivity patterns of patients. The results partly supported the findings by Woodward et al. (2012) and displayed both over and under connectivity in the thalamic network during resting state (Woodward et al., 2012), supporting the view that schizophrenia is characterized by a mixture of hypo- and hyper-connectivity (Pettersson-Yeo et al., 2011).

In our study, schizophrenia patients exhibited increased thalamic FC in several brain areas, including bilateral precentral gyrus, dorsal medial frontal gyrus, middle occipital gyrus, and lingual gyrus. Our findings were not altogether consistent with those of previous studies. Similar to many prior studies, thalamic hyper-connectivity was detected in motor and visual cortices. These hyper-connectivity findings challenge the disconnectivity/hypoconnectivity hypothesis of schizophrenia which states that too many synapses are eliminated in development, specifically during adolescence, in schizophrenia (Beaumont and Dimond, 1973; Friston and Frith, 1995; Friston, 1999). Our results are in favour of the misconnectivity hypothesis that the regulation of synaptic strength in the connections between cortical neurons is altered in schizophrenia and may be manifested as both hyper- and hypo-connectivity (Crow, 1998; Innocenti et al., 2003). Using voxel-based morphometry and Granger causality analysis, a recent study demonstrated that the causal connectivity of the integrated prefrontalthalamic (limbic)-cerebellar (sensorimotor) circuit was partly affected by structural deficits in first-episode, drug naive schizophrenia (Guo et al., 2015), although no age thalamic connectivity was found in that study. A recent study further examined whether and how FC patterns during rest change with task engagement in schizophrenia (Cetin et al., 2014). Using independent component analysis (ICA) combined with experimental tasks with different levels of sensori-motor processing, this study found higher functional network connectivity between thalamus and multiple sensorimotor regions across tasks, highlighting the importance of the thalamus as a gateway to sensory input (Cetin et al., 2014). This study, using a different data analysis approach (i.e., ICA), provides convergent evidence that thalamic FC with other sensorimotor areas is exaggerated in schizophrenia and further shows that this pattern persists even during sensory load tasks. Taken together, these findings highlighted a key role of thalamus in the neurodevelopment of FC network in schizophrenia.

Hyper-connectivity was observed in dorsal medial frontal gyrus, while hypo-connectivity has been identified in PFC (Woodward et al., 2012). We suspect that the hyper-connectivity between thalamus and dorsal medial prefrontal gyrus (dmPFG) might be related to the impaired decision making found in schizophrenics. Patients with schizophrenia are characterized by making decisions based on less information, which results in fast and incorrect decisions. This behavior is referred to as jumping to conclusions bias (Averbeck et al., 2011; Krug et al., 2014).

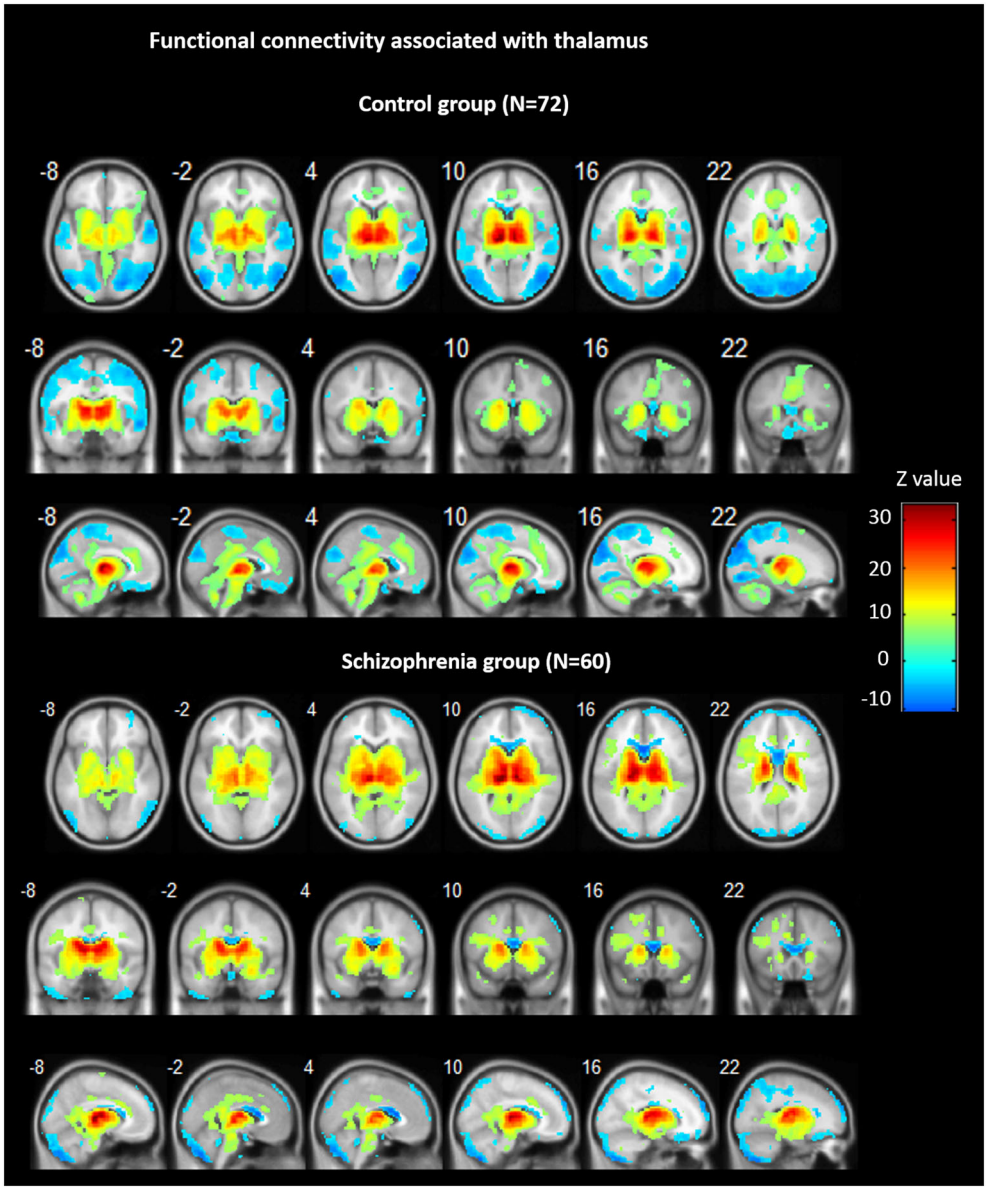

FIGURE 1 | Functional connectivity (FC) associated with the thalamus in schizophrenia and in controls. Hot color represents positive FC with thalamus, whereas blue cold color represents negative FC. Images are in radiologic format with subject left on image right.

The underlying mechanism might be linked with the crucial thalamic function of filtering and gating information (Andreasen, 1997) and the function of medial frontal gyrus in high-level executive functions and decision-related processes (Mansouri et al., 2009). Talati and Hirsch (2005) noted that medial frontal gyrus was associated with information collection of perceptual go/no-go decision making. Specifically, lower-level processing biases were determined and left hemisphere of the medial frontal gyrus processes perceptual decisions depending on temporal and object information. The right hemisphere of the medial frontal gyrus conducted analogous decisions based on spatial information. As a result, thalamus-dorsal medial prefrontal gyrus hyper-connectivity might readily activate this pathway and exert influences on the decision making of schizophrenia patients.

In our results, thalamic hyper-connectivity was also found in lingual gyrus in schizophrenics. Lingual gyrus and middle occipital gyrus are parts of the occipital lobe, where visual cortex locates. Starting in the 1950s, the symptom of visual perception disturbances has been widely reported in schizophrenia patients (Silverstein and Keane, 2011). This might provide an illustration that schizophrenics typically performed worse in visual contrast (Dakin et al., 2005; Yang et al., 2013) and visual motion and orientation tasks (Yang et al., 2013). In addition, the lingual gyrus contributes to visuomotor processing and plays a key role in generating or recalling dreams during sleep (Bischof and

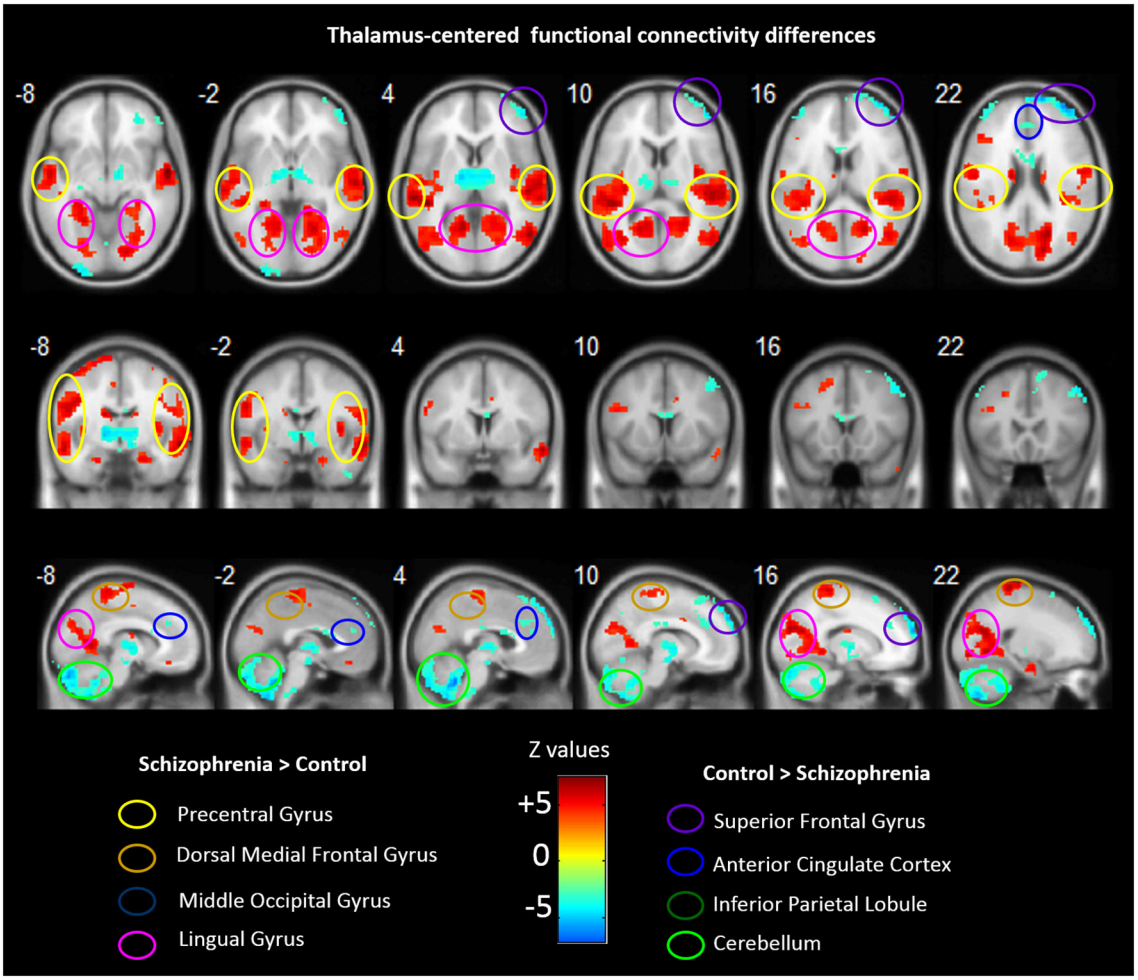

FIGURE 2 | Abnormal FC associated with the thalamus in schizophrenia. Hot color represents higher FC with thalamus in schizophrenia, whereas blue cold color represents lower FC in schizophrenia. Images are in radiologic format with subject left on image right.

Bassetti, 2004). Thus, this alternation of connectivity between the lingual gyrus and the thalamus might be related to an underlying sleep disturbance in schizophrenic patients. Perhaps the increased connectivity between the lingual gyrus and the thalamus caused severe sleep latency and sleep disorders in schizophrenia patients.

Consistent with several studies reported decreased thalamic connectivity in PFC (Woodward et al., 2012), our data indicated thalamic hypo-connectivity in subregions of the PFC, including bilateral superior frontal gyrus. In schizophrenia patients, decreased connectivity between PFC and working memory related regions such as thalamus was found to be linked to deficits in the central executive component of working memory (Barch and Csernansky, 2007; Barch and Ceaser, 2012). Our finding provided more detailed locations of brain areas affected by schizophrenia. In an fMRI study, Tully et al. (2014) reported that thinner cortex was found in a region in the superior frontal gyrus (Brodmann area 10) of schizophrenia patients and was associated with declined role and real-world functioning. Thinner cortex and poor function in the superior frontal cortex might result from hypo-connectivity between the area and thalamus. Subsequently, thalamic hypo-connectivity caused declined role and real-world functioning in schizophrenics.

In addition, our results showed thalamic hypo-connectivity in anterior cingulate cortex, inferior parietal lobs, and cerebellum. In an electrophysiological study, Mulert et al. (2001) suggested that anterior cingulate cortex activity of schizophrenics was reduced as indicated by the attenuated N1 potential (60–150 ms). Yan et al. (2012) presumed that decreased resting-state connectivity in anterior cingulate cortex was crucial in schizophrenia symptom expression and was correlated with Stroop colour-word test performance and disease severity. Stroop colour-word test was used to test executive control function. The study therefore concluded that deficits in executive control function were related to schizophrenics' abnormal functional and structural connectivity in anterior cingulate cortex. Our results provided further evidence for the implication of anterior cingulated cortex in schizophrenia.

On the other hand, studies regarding a DMN may shed light on the thalamic hypo-connectivity in inferior parietal lobes. The DMN comprised medial prefrontal cortex, posterior cingulate cortex/precuneus, inferior parietal lobule, and lateral temporal cortex. In passive setting and when direct attention was not required, the DMN was activated (Raichle et al., 2001; Buckner et al., 2008). Previous studies demonstrated diverse DMN connectivity patterns (Zhou et al., 2007; Whitfield-Gabrieli et al., 2009; Ongür et al., 2010; Rotarska-Jagiela et al., 2010; Jang et al., 2011; Calhoun et al., 2012; Mingoia et al., 2012), which might explain the heterogeneous symptoms of this disease. In our current data, schizophrenia patients had a different pattern of DMN, where thalamus and inferior parietal lobule displayed lower FC in schizophrenics than that did in normal controls. Our findings also indicated a reduced FC in medial prefrontal cortex/ACC and supported the assumption of decreased connectivity in DMN found in schizophrenics (Rotarska-Jagiela et al., 2010; Camchong et al., 2011; Jang et al., 2011). This atypical intrinsic connectivity is thought to associate with the difficulties in self-referential, introspective processing as well as theory of mind which often seen in schizophrenia patients (Zhou et al., 2007; Camchong et al., 2011; Liu et al., 2012). Interestingly, in some other references, first-degree relatives of schizophrenia patients, including unaffected siblings, have been reported to reveal similar atypical intrinsic connectivity that is observed in patients, although patients tend to exhibit more severe and widespread aberrances (van Buuren et al., 2012; Chang et al., 2014; Guo et al., 2014). It seems that the degree of this intrinsic connectivity dysfunction might be related to the risk of developing schizophrenia the illness manifestation. It also worth noting that the DMN may not be a single unit but is composed of substructures with substantially different connectivity patterns. Schizophrenia might be associated with abnormalities in certain subcomponents of the DMN but not others (Calhoun et al., 2012).

Consistent with a prior study that employed the same dataset provided by the COBRE, our results also showed decreased cerebellar-thalamic FC (Wang et al., 2014). Several resting-state fMRI studies as well have observed the same results of thalamic hypo-connectivity in cerebellum (Collin et al., 2011; Chen et al., 2013). In a diffusion tensor tractography study, schizophrenia patients had a declined fractional anisotropy in fiber tracks between cerebellum and thalamus (Magnotta et al., 2008). Previous literature has reported that the cortico-cerebellar-thalamic-cortical circuit (CCTCC) dysfunction might cause some symptoms of schizophrenia (Andreasen et al., 1998, 1999). Cerebellum impairment might result in the CCTCC dysfunction and subsequent schizophrenia symptoms. Other than movement synchrony, cerebellum was also responsible for cognitive and affective functions (Leiner et al., 1993; Stoodley et al., 2012). The hypo-connectivity between the cerebellum and thalamus might elucidate the neurological soft signs of schizophrenia patients because those patients also exhibited cerebellar dysfunction (Ho et al., 2004; Varambally et al., 2006).

Our study has several limitations. First, schizophrenia patients may have diverse antipsychotic medication statuses, which might affect their brain activities (Lui et al., 2010; Sambataro et al., 2010) and cognitive functions (Meltzer and McGurk, 1999). According to the literature, after receiving 6-week treatment of second-generation antipsychotic drugs, synchronized brain activity and declined integration function across brain networks were identified among schizophrenics (Meltzer and McGurk, 1999). Sambataro et al. (2010) also acknowledged that olanzapine treatment increased DMN connectivity in schizophrenics. Second, we need empirical and longitudinal data to establish the causal relationship between FC and schizophrenia symptoms. Our analysis simply reanalysed an existing dataset; therefore, lacking hypothesis-driven experiment design to determine the causal relationship between brain structures and schizophrenia symptoms. Future studies can develop longitudinal studies to ascertain the causal relationship. Finally, the current study used the entire left and right thalamus as seeds. However, it is probable that there is some gradient in connectivity within the thalamus. Future studies may use sub-regions in the thalamus as seeds to further explore the FC within different parts of thalamus.

This study re-examined a dataset with a great number of schizophrenia patients. The analytical method adopted by this study yielded results of both thalamic hyper- and hypo-connectivity in more detailed and specific brain regions. For example, we identified thalamic hyper- and hypo-connectivity in two subregions of the PFC, dorsal medial frontal gyrus and bilateral superior frontal gyrus, respectively. In conclusion, our results demonstrated a coherent finding that schizophrenics had a loosely integrated and more diverse FC across the brain. Precise locations of brain regions involved in thalamic FC not only showed a clearer brain mapping of schizophrenia patients but also provided more clues for future researchers.

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Anatomical constitution of sense organs as a marker of mental disorders

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It is challenging to set the spatial limits of the mind. This was suggested in classical philosophy by Aristotle, when he stated that the mind is "in a way all existing things" (Aristotle, 1986) but it is also a trendy topic studied by the extended mind theorists, for example (Clark and Chalmers, 1998). The latter propose that the mind goes beyond the body in the act of cognition, since the environment plays a crucial role in mental activity. In this opinion article we do not go that far, but we pose an important question: where in the body shall we look for markers of mental disorders? Our proposal is that the bodily constitution of sense organs may be an indicator of mental characteristics, and therefore it can be affected in cases of disorders such as schizophrenia. We support this opinion with empirical papers and we suggest new lines of research to improve the understanding of this devastating disease.

Ever since Occidental thought began to flourish amongst the Greeks, sensibility and intelligence have been considered different faculties. They are intimately related, but have different objects. In general terms, lower faculties nourish higher capacities, with intelligence being the highest of them. This intuitive description is very much common sense: the sense organs are stimulated and transfer signals to the central nervous system through peripheral nerves. The faculty of sensibility lies in sense organs, and higher faculties are related to the brain, which is in charge of processing and elaborating a response. This way of explaining animal and human behavior is computational in the sense that we have an input, a process and an output. Taking the visual system as an example, the input is the stimulation of cones and rods by photons, which elicits an action potential; the process takes place in the lateral geniculate nucleus of the thalamus and the visual cortex, and the output is the perceived image. Nowadays we know that top-down signals are as important as bottom-up influences in perception, and it is widely accepted that anatomical constitution is an important factor to guarantee the accuracy of these signals. In the case of the bottom-up flow of information, the constitution of sense organs involves not only neural tissue (i.e., the retina), but also non-neural related structures (i.e., crystalline lens, eye muscles). Moreover, anatomical constitution plays an important role in perception and mental disorders (Korn, 2000; Eskelinen et al., 2013).

From a computational point of view, mental disorders such as schizophrenia are mainly considered an impairment at the level of the "process," and therefore their physical correlates are searched for in the brain. Furthermore, errors in the process are expressed as a disturbance of the output, that is, behavior. Hence, the diagnosis of mental disorders is mostly carried out after observations, interviews, and the comparison of the results with diagnostic manuals. In any case, research on psychiatric conditions has focused on the search for brain correlates at the level of the process. For example, an aberrant migration of cortical interneurons during development has been found to be associated with schizophrenia.The migration of interneurons in the embryo is fundamental for correct mental function, as it determines the final positioning of the neurons, thereby establishing the basis for the correct wiring of neural circuitry (Marín, 2013). Multiple investigations suggest that schizophrenia and other neuropsychiatric disorders are associated with interneuron dysfunction (Marín, 2012),

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and that schizophrenia patients carry mutations in certain genes that affect the migration of the cortical interneurons during embryonic development (Valiente and Marín, 2010). Another clear example of a physical correlate is the fact that a progressive loss of brain volume occurs in schizophrenia, affecting both gray and white matter. Brain volume loss in schizophrenia seems to be related to a combination of early developmental processes (reflected in intracranial volume reduction) as well as the progression of the illness (Haijma et al., 2013).

There are a plethora of studies that support a physical modification of the brain in schizophrenia, which correlates with a disruption in the "computational process." In addition, we have known for decades that perception also plays an important role in the understanding of schizophrenia and the detection of its symptoms (McGhie and Chapman, 1961). There are several studies that show a correlation between schizophrenia and the alteration of visual perception (such as visual masking, Green et al., 2012, luminance, size, contrast, orientation and motion perception, Yang et al., 2013; Serrano-Pedraza et al., 2014), as well as eye movements (Silverstein and Keane, 2011). However, vision is not the only sense affected in schizophrenia: aberrant processing of auditory (Micoulaud-Franchi et al., 2014), olfactory (Moberg et al., 2014), gustative (Compton et al., 2013) and tactile (Ferri et al., 2014) stimuli also occurs. These are important discoveries for improving our understanding of schizophrenia.

The main message of these introductory paragraphs is that in schizophrenia there are perceptual anomalies whose physical substrate has been searched for at the level of the process, that is, in the brain. We will suggest a possible new approach to diagnosing mental disorders such as schizophrenia, i.e., looking for physical differences directly in the constitution of sense organs rather than impairments in the processing of the signals coded by them. In order to simplify our arguments and make them as clear as possible, we will focus on the study of vision in schizophrenia.

Previous reports have shown an association between visual perception anomalies, as well as aberrant eye-related movements, and schizophrenia. An extensive review of the topic is beyond the scope of this article, so we will only mention some examples to show how this association has been studied in this disorder. A special section of the journal *Schizophrenia Bulletin*, published in 2011 and edited by Silverstein and Keane (2011), analyzes the contribution of vision science to this mental illness. According to the editors, this special section refers to "psychophysical, electrophysiological and imaging data," focusing on intermediate vision—mainly object processing. The extensive research that begins the section describes several behavioral, neuroimaging and neurophysiological studies published between 2005 and 2010, all of which demonstrate perceptual organization impairment in schizophrenia. They point to an association between aberrant object processing and a compromised neural signal in visual extrastriate areas and the temporal, parietal and frontal regions related to the integration of visual information. This mainstream view is supported by a recent review on visual illusions and schizophrenia, interpreted via a Bayesian perspective (Notredame et al., 2014). In this case, perception is interpreted as the interplay between top-down expectations and bottom-up sensory inputs; therefore, the cortical influence on sensory signals is crucial in object processing. Visual illusions will emerge when expectations and bottom-up signals are abnormally integrated, as Dima et al. (2010) empirically demonstrate: in their experiment, healthy control subjects seem to experience visual illusions due to the strong influence of top-down expectations; on the other hand, schizophrenia patients overcome the illusions through a strengthened bottom-up input. With respect to eye-related movements, which are an indicator of the constitution of sense organs, a recent report shows that simple tests for the detection of abnormal eye movements can accurately discriminate between schizophrenia patients and controls (Benson et al., 2012). These researchers evaluated different oculomotor tasks and found differences in nearly all of them. Moreover, fixation dispersal during free viewing was the task that best discriminated between patients and controls. With respect to the etiology of this finding, the authors hypothesize that the eye movement disturbance should result from aberrant physiological processes directly connected to those which are responsible for the pathophysiology of schizophrenia.

The new perspective we propose here is based on the actual unity of sensibility and cognition or, in other words, the integrity of the input-process-output framework. In our opinion, mental diseases can be accompanied by physical anomalies in the sense organs; this should not be overly surprising if we consider that some disorders, such as schizophrenia, are even associated with alterations in non-neural peripheral tissues (Fernandez-Egea et al., 2009; Dieset et al., 2014). Our statement is based on two well-proven facts: (1) schizophrenia has been proven to have a genetic component (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014); (2) the physical substrate of sense organs and neural tissue derive from the same embryonic layer, the ectoderm, which is in a continuous interplay with the mesoderm, where the non-neural structures of the sense organs originate from. In fact, going back to the case of vision, our theoretical hypothesis is already supported by several recent studies that suggest anatomical and functional anomalies of the retina in schizophrenia (Fountoulakis, 2010; Hébert et al., 2010; Chu et al., 2012; Lee et al., 2013; Lavoie et al., 2014). At a genetic level, there is also evidence that supports this novel approach. As we have noted in a recent article (Güell, 2014), four of the five known genes involved in the disrupted tangential migration of cortical interneurons (NRG1, ERBB4, GRIN1, and DTNBP1) are involved in the expression of molecules related to visual structures. We believe that it could be of great interest to assess how the expression of these genes (and their mutations) may impact the anatomical constitution of the visual system, since this feature could be used as a new diagnostic tool in schizophrenia. Indirectly, it may also be interesting to analyze the differences in eye morphology in controls and schizophrenia patients who carry mutations in any of these genes.

This hypothesis is supported by other experiments. The work by Benson and collaborators cited above (Benson et al., 2012) focuses on the physiological (functional) level of the visual system and we wonder whether the anomalies they found could be associated with a modification of eye morphology. In this sense, it could be interesting to enrich these functional studies with an analysis of the anatomical features of the eye and eye-related

structures, such as retinal nerve fiber layer, macular volume, proprioceptive information of eye muscles (Korn, 2000; Chu et al., 2012; Lee et al., 2013), or using techniques such as adaptive optics (Saleh et al., 2014). Furthermore, Yang et al. (2013) suggest than anomalous perceptual experiences might lie at the base of the chronic difficulties confronted by individuals with schizophrenia, as they attempt to navigate the complex, fast-moving and constantly changing world. Going back to the causal chain, even though part of these perceptual anomalies might be due to top-down expectations from the cerebral cortex, it should be taken into account that subtle changes in the morphological features of the receptor organs could also contribute to these aberrant experiences. Since perception is a continuous interplay between top-down and bottom-up signals, we believe the study of the constitution of the sense organs could be as important as analyzing the anatomy and function of the brain.

We are not trying to identify perception and cognition in our proposal. Rather, we suggest that the diagnosis of a complex mental disorder could be done by looking for these morphological traits in the sense organs, as a complement of neuroimaging techniques. Therefore, we propose that research on schizophrenia and other mental disorders could profitably focus on the anatomical constitution of the input structures, as well as on brain correlates. Thus, the notion of "endophenotype" introduced by Gottesman and Shields (1973) as "internal phenotypes discoverable by a biochemical test or physiological marker" could be expanded to the morphological features of the peripheral nervous system and its associated structures. This could provide new tools to improve diagnosis, adequately understand the etiology of the disease, and eventually open new paths for the treatment of these disorders.

As a final note, we would like to clarify that this potential association between the morphology of input structures and mental disorders does not mean a radical determinism. Even though certain morphological configurations may be related with a disease, they would not necessarily imply that a person is "schizophrenic," or even as being on the "schizophrenic spectrum." Rather, it would point to a constitutional predisposition (Güell, 2014) to suffer the disease, open to various environmental factors.

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frontiers in Behavioral Neuroscience

GENERAL COMMENTARY
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Commentary: Anatomical constitution of sense organs as a marker of mental disorders

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Keywords: schizophrenia, retina, pattern electroretinogram, pathophysiology, dopamine

A Commentary on

Anatomical constitution of sense organs as a marker of mental disorders
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Research in neuroscience and psychiatry is limited by the difficulty to accurately access brain functioning. There is currently a need to develop new methods assessing the neurobiological underpinning of brain dysfunctions (London et al., 2013; Lavoie et al., 2014; Güell and Bernácer, 2015; Laprevote et al., 2015; Schwitzer et al., 2015a,b). On this basis, Güell and Bernácer in an exciting article recently discussed the relevance of studying visual perception in mental disorders and especially in schizophrenia (Güell and Bernácer, 2015). Among other deficits, they outlined retinal functional and anatomical deficits detected in schizophrenia using respectively flash electroretinogram (fERG) and optical coherence tomography (OCT). However, we would like to suggest herein that the pattern electroretinogram (PERG), a retinal functional recording, might provide a relevant complementary measurement to enhance understanding of biological mechanisms underlying brain disorders in schizophrenia.

Flash and pattern ERG allow for the assessment of specific cell types of the neural retina and give different information on the pathophysiology. Using a light stimulation, the fERG mainly assesses the electric biopotential evoked by the first stages of visual processing namely photoreceptors and bipolar-Müller cell complex (Holder et al., 2010). As previously found, these first stages are altered in schizophrenia (Warner et al., 1999; Balogh et al., 2008; Hébert et al., 2015). However, the fERG does not significantly provide information concerning the ganglion cells, the axons of which form the optic nerve. The ganglion cells constitute the ultimate retinal relay before the transmission of the visual information from the retina to the visual cortex. The functional properties of these cells can be assessed by the PERG using the central presentation of reversing black and white checkerboards (Bach et al., 2013). The On-Off organization of the ganglion cells receptive fields makes these cells particularly sensitive to the alternate changes in contrast levels of the checkerboards, and leads to large responses in the PERG (Holder et al., 2010). The electrical signal transmitted to ganglion cells originates from photoreceptor and bipolar cells and is under the influence of interneurons cells (amacrine and horizontal cells). The signal elicited at the ganglion cell level thus results from the integration of several retinal stages. Since it is more integrated there than at the photoreceptor and bipolar cell level we suggest that its measurement might be a useful complementary test to approach the neural function in schizophrenia. Furthermore, the layer of the ganglion cells is the first retinal stage providing information in the form of action potentials.

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Trustworthy evidence underlines that PERG protocols are good candidates to investigate dopamine transmission, a neurotransmitter known to be involved in schizophrenia. Indeed, the manipulation of the contrast level of the reversing black and white checkerboards during PERG informs on retinal contrast processing, which is largely influenced by retinal dopaminergic amacrine cells (Djamgoz et al., 1997). Moreover, alterations of retinal contrast processing detected with reversal PERG stimulation were found in Parkinson's disease, a dopaminergic pathology (Garcia-Martin et al., 2014). Importantly, these changes appear to be good biological markers, inasmuch as they predict quality of life and disease severity in Parkinson's disease.

PERG shares several advantages with fERG. Measurements of PERG are non-invasive, relatively fast, easy-to-use, and inexpensive. Importantly, PERG also has some additional advantages relative to fERG. Unlike most protocols of fERG, there is no need to dilate the pupil, making the PERG less invasive than fERG, with no alteration of visual perception. Second, whereas the fERG is evaluated after adaptation periods in darkness and light, the PERG is entirely measured in light conditions without any adaptation period (Bach et al., 2013; McCulloch et al., 2015). These methodological advantages may facilitate the use of PERG in patients with schizophrenia. As the PERG contrast processing appears sensitive to dopaminergic dysfunction, it might represent a suitable approach for monitoring anti-psychotic response, an important part of patient care. Finally, the PERG can be coupled with other retinal measurements such as the fERG to provide a more thorough picture of the pathophysiology of the disease.

As previously described, measures of PERG assess the functional properties of the retinal ganglion cells, the axons of which form the optic nerve. Anatomical constitution and organization of these cells can be evaluated by retinal imaging techniques, namely OCT. Several studies found retinal nerve fiber layer (RNFL) thinning in schizophrenia, which was observed with spectral domain OCT (Chu et al., 2012; Lee et al., 2013; Silverstein et al., 2015). RNFL is constituted by the fibers of the optic nerve and its thinning is a direct reflection of a loss of ganglion cell axons. We suppose that anatomical alterations of retinal ganglion cells observed in schizophrenia could lead to functional deficits of these cells. Such impairments could be detected by PERG. Importantly, adding functional measurements like PERG to imaging techniques such as OCT may provide a potential structure-function correlation.

Although PERG might appear as a relevant measure to indirectly approach the pathophysiology of schizophrenia, there are several limitations and a large number of steps required to demonstrate the usefulness of this exam in the better understanding of the disease. To this date, PERG measurements have not been evaluated in schizophrenia patients yet and consequently there is no certainty that differences between patients and healthy controls would be observed. Accordingly, case-control studies with a large number of subjects and standardized protocols are needed to eventually demonstrate differences between patients and controls. This constitutes the starting point to investigate the relevance of this method. Additionally, the pathophysiology of schizophrenia remains to this date elusive and involves more complex mechanisms than only dopaminergic dysfunctions (Khandaker et al., 2015). As a consequence, only one exam such as PERG does not alone give sufficient information on central dysfunctions but should be coupled with other assessments to provide a clearer picture of the disease. Then, in the case where differences between groups would be observed, control groups of patients with other dopaminergic pathologies are crucial to conclude on the specificity of PERG. Also, as we can consider that it is impossible to have medication-free schizophrenia patients, the effects of different types of psychotropic drugs such as antipsychotics must be tested on PERG recordings to differentiate the potential effects of medications or disease. Finally, although there is currently an increasing interest for PERG assessments in psychiatric research (Schwitzer et al., 2015a, 2016), the precise mechanisms underlying PERG anomalies should be investigated to understand the biological underpinning of brain dysfunctions.

Approximately 0.5% of the population suffers from schizophrenia, which has psychiatric and cognitive consequences. A current challenge in neuroscience research is to develop reproducible measures providing an indirect access to the brain functioning. In the case of schizophrenia, there is an urgent need for biological markers enabling early detection of the disease. Eventually, the PERG might be used as a useful complementary measurement to allow a better understanding of the pathophysiology of schizophrenia.

AUTHOR CONTRIBUTIONS

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

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frontiers in Behavioral Neuroscience

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Neural circuits containing olfactory neurons are involved in the prepulse inhibition of the startle reflex in rats

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Many neuropsychiatric disorders, such as schizophrenia, have been associated with olfactory dysfunction and abnormalities in the prepulse inhibition (PPI) response to a startle reflex. However, whether these two abnormalities could be related is unclear. The present investigations were designed to determine whether theblockage of olfactory sensory input by zinc sulfate infusion in the olfactory naris (0.5 ml, 0.17 M, ZnE) can disturb the PPI response. Furthermore, a bilateral microinjection of lidocaine/MK801 in the olfactory bulb (OB) was administered to examine whether the blockage of olfactory sensory input could impair the PPI response. To identify the neural projection between olfaction and PPI-related areas, trans-synaptic retrograde tracing with the recombinant pseudorabies virus (PRV) was used. Our results demonstrated that blockage of olfactory sensory input could disturb olfactory behavior. In the function studies, we demonstrated that blockage of olfactory sensory input could impair the pre-pulse inhibition of the startle response following decreased c-Fos expression in relevant brain regions during the PPI responses. Furthermore, similar and more robust findings indicated that blockage of olfactory sensory input by microinjection of lidocaine/MK801 in the OB could impair the PPI response. In the circuit-level studies, we demonstrated that trans-synaptic retrograde tracing with PRV exhibited a large portion of labeled neurons in several regions of the olfactory cortices from the pedunculopontine tegmental nucleus (PPTg). Thus, these data suggest that the olfactory system participates in the PPI regulating fields and plays a role in the pre-pulse inhibition of the startle response in rats.

Keywords: olfactory dysfunction, sensory stimulus, prepulse inhibition (PPI), NMDA receptor, neural trace

Introduction

An intact nervous system engages a variety of mechanisms to suppress or "filter out" irrelevant information in sensory, cognitive and motor domains to enable an individual to focus on the most salient stimuli in the environment (Braff and Geyer, 1990). The prepulse inhibition reflex (PPI) is a measure of the suppression of irrelevant information, which measures an individual's decreased response to unexpected auditory stimuli, referred to as an acoustic startle response (ASR), when a weak auditory pulse precedes the unexpected stimuli (Braff and Geyer, 1990). Clinical

studies have demonstrated that many neuropsychiatric disorders exhibit an impaired ability to suppress irrelevant information. For example, patients with schizophrenia exhibited impairments in automatically filtering irrelevant thoughts and sensory stimuli (Geyer et al., 2002). Clinical observations have also demonstrated that olfactory dysfunction occurred in neuropsychiatric disorders (Martzke et al., 1997). Furthermore, in rodents, some animal models of neuropsychiatric disease have been demonstrated to exhibit olfactory dysfunction (Huckins et al., 2013). Currently, whether there is a relationship between olfactory dysfunction and an abnormal PPI is unclear.

To explore the contribution of blockage of olfactory sensory input in the PPI response in rats, an animal model was established by naris infusion of zinc sulfate (ZnE) to create an olfactory blockage model (Alberts and Galef, 1971). The activities of the PPI-related cortex were evaluated by c-Fos expression, which was used to map the distribution of neurons activated by physiological and pharmacological stimuli (Sagar et al., 1988). Previous studies have indicated that the amygdala and hippocampus were part of the PPI-related neural circuit (Swerdlow et al., 2001). Furthermore, the pedunculopontine tegmental nucleus (PPTg) played an important role in the PPI response (Takahashi et al., 2007).

The olfactory bulb (OB) transmits smell information from the nose to the brain and is necessary for the proper sensation of smell (Wacker and Ludwig, 2012). To further determine whether the blockade of olfactory sensory input affects the PPI response, OB transmission was inhibited by a local microinjection of lidocaine or MK-801 in the OB to measure the effect on the PPI response. To better understand the neural substances that underlie the contribution of olfactory areas to the PPI response, a recombinant pseudorabies virus (PRV614) that expressed monomeric red fluorescent protein (mRFP) was simultaneously microinjected into the PPTg, which was included in a classic PPI circuit (Fendt et al., 2001); various regions were retrogradely labeled that innervated the PPTg (Banfield et al., 2003) to explore the olfactory related cortices that participated in PPI response regulation.

Materials and Methods

Animals

Male Sprague Dawley rats (11–12 weeks) that weighed 220–250 g were obtained from the Experimental Animal Center, Kunming Medical College, Kunming, China. The rats were housed in standard cages (4 rats per cage) at a constant temperature of 25 ± 0.5°C and 60 ± 5% relative humidity with an alternating 12 h light/12 h dark cycle (lights on at 7:00 AM). Food and water were available ad libitum. All animals were treated in accordance with the Wuhan Institute of Physics and Mathematics (WIPM), Chinese Academy of Sciences (CAS) guidelines regarding the practice of animal care. The animal facilities and experimental protocols adhered to the Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Drugs

MK-801 (dizocilpine, (5R, 10S)-(+)-5-Methyl-10, 11-dihydro-5 H-dibenzo [a, d] cyclohepten-5, 10-imine hydrogen maleate) and lidocaine hydrochloride were purchased from Sigma (St. Louis, MO, USA) and dissolved in 0.9% saline as the vehicle. Ketamine hydrochloride, chloral hydrate, and zinc sulfate were purchased from Shanghai Pharmaceutical Group Co., Ltd (Shanghai, China). Chloral hydrate was dissolved in deionized water as the vehicle. Lemon (Sigma–Aldrich, #262600) or mint (Sigma–Aldrich, #49599) essences were dissolved in mineral oil (Sigma–Aldrich, #91975).

Behavioral Experiments

Intranasal Effusion of Zinc Sulfate

The intranasal administration of zinc sulfate (0.17 M, ZnE) and the naïve control were performed under general anesthesia (ketamine hydrochloride, 40 mg/kg, i.p.) prior to behavioral testing. Secretion and swelling in two nostrils were simultaneously induced by zinc sulfate treatment, which led to dyspnea. Two preventive measures were performed in our experiment. First, atropine was injected prior to ZnE treatment to inhibit the secretion from reducing the respiratory obstruction. Second, one of the nostrils was treated by ZnE, and 2 h later, the second nostril was treated by ZnE. The animals were placed on their backs, and 50 μl of either zinc sulfate solution or saline were stretched into each naris with a blunted syringe.

Odor Habituation/Discrimination Test

The odor habituation/discrimination measurement was designed to measure the breadth times of cross-habituation among different odorants and specify the degree of spontaneous discrimination between different odorants. The test was performed in a compartment (L × W × H: 0.75 × 0.5 × 0.5 m) with two glass plates placed 15 cm apart in the diagonal position. An odor (10–6v/v, lemon or mint essence dissolved in mineral oil) was introduced on one side of the compartment, and a control odor (mineral oil) was introduced on the other side. The animals were acclimatized to the compartment once per day for 5 min on three consecutive days (between 09:00 and 15:00 each day) prior to behavioral testing. On the fourth day, the habituation-discrimination behaviors were recorded. The odor and control solutions (25 μl each) were applied to filter paper and placed on the glass plate. During the trials, the animal was first presented with the lemon odor on one side of the compartment and the control odor on the other side in five consecutive 5-min trials; each trial was separated by a 15 min break interval after acclimation. In the sixth trial, lemon was replaced by a novel odor (mint), and the animals were subsequently exposed to the mint odor for 5 min. The amount of time that the animal spent investigating (sniffing) the mint or mineral oil was recorded. Sniffing was defined as when the animal placed its nose within 1 cm of the glass plate surface. During each experimental break interval, the compartment was cleaned with 75% ethanol. Odor discrimination was considered to be impaired if the animal spent less time investigating the new (mint) odor in the sixth trial.

Locomotion in an Open Field

The open-field apparatus was a square plywood arena with a smooth and waterproof surface (high: 35 cm, width: 50 cm). The floor of the arena was divided into 20 equal units with black lines; there were 9 central units and 11 edge units. The apparatus was lighted from above with a 40-w light bulb. The animals were habituated to the apparatus for 30 min on three consecutive days. The animals were subsequently placed in the center of the open field and were allowed to move freely for 5 min. During each experimental interval (10 min), the compartment was cleaned with 75% ethanol to clear the scent mark. The behavior and movement of each animal were recorded with a video camera positioned above the open field, and the total locomotion and time spent in the central area were measured on a computer using infrared tracing software. All open-field tests were conducted between 9:00 and 15:00 h.

Prepulse Inhibition Measurement Procedure and Apparatus

The acoustic startle response and prepulse inhibition were measured according to a previously described procedure (Chen et al., 2010; Meng et al., 2010). All animals were housed in a sound-attenuated room with a 60-dB ambient noise level. During the experiments, the rats were placed in a plexiglass cylinder (10 cm in diameter and 28 cm in length) in a four-unit automated ASR testing instrument (Yilike Co. LTD., Kunming, China) and were exposed to 65 dB of SPL white background noise. The small plexiglass cylinder in each of the four units was fixed to a platform; a sensitive sensor was attached underneath the platform and was connected to a PC in an adjacent room to collect the startle-response data.

The rats were provided an acclimation period prior to the test session. During the test session, the rats were placed in the test cylinder for a 5-min period, and ten trials of randomly delivered 115-dB SPL pulses or prepulsed startle stimuli were presented. After the acclimation period, the rats were exposed to 50 trials of randomly delivered acoustic stimuli delivered through a speaker above the cylinder. The acoustic stimuli consisted of 10 trials of a 115-dB SPL pulse stimulus, 10 trials without a delivered stimulus (NOSTIM) and 30 trials of a prepulsed startle stimulus. The 115 dB SPL pulse consisted of a single 20 ms sound presentation. The prepulsed startle stimuli consisted of 100 ms of 20-ms white-noise pulses that contained non-startling stimuli of 5, 10 or 15 dB SPL above the 65 dB SPL background noise (PPI 5, PPI 10 and PPI 15, respectively) followed by a single 20 ms 115-dB SPL pulse. Inter-trial intervals (ITI) of 27–32 s were used between the stimuli presentations.

Experimental Design

All rats underwent behavioral tests 2 weeks after arrival to acclimate them to the testing environment. For the rats that underwent stereotaxic surgery (Experiments 2), the tests occurred 1 week after surgical recovery. All independent experiments were completed in independent animals, and the number of each group was 7–10.

ZnE Treatment Effects on the Odor Habituation/Discrimination and Locomotion in the Open Field

In Experiment 1, the rats were randomly assigned to naïve control and ZnE groups (n = 7–10). All treatments and tests were conducted similar to the description in ***Behavioral experiments***. To determine whether ZnE treatment impaired the rats' olfactory sensory, an odor habituation/discrimination test was used 24 h after the ZnE or naïve control treatment. The locomotor activity was subsequently measured after the odor habituation/discrimination measurement to reduce the possibility that ZnE treatment may impair locomotion to disturb the PPI response. Following the completion of the olfactory and locomotion tests, the ZnE/naïve rats were measured for the PPI response.

Effects of a Lidocaine/MK-801 Microinjection into the Olfactory Bulb on the PPI Response

To confirm the hypothesis that the blockage of the olfactory sensory impaired the PPI response, the activity of the OB in the rats was selectively inactivated/blocked by lidocaine/MK-801. The rats were anesthetized with chloral hydrate (10%, 0.4 ml/100 g) 1 week prior to the experiments. A pair of stainless steel guide cannula (26G, 0.4 mm i.d., 0.5 mm o.d.) were stereotaxically implanted bilaterally into the OB (±1.5 mm lateral to the midline, −7.08 mm anterior to Bregma, and 4 mm dorsoventral to the dura) and mounted onto the skull with dental cement. A guide cannula was maintained in place throughout the experiment. On the eighth day, an infusion cannula was inserted through the guide cannula until it protruded 1 mm beyond the respective inner end.

Microinjections into the OB were performed using two needles (33-gauge, RWD Life Science, Shenzhen, China), which were connected to a 5 L syringe (RWD Life Science, Shenzhen, China) by PE-10 tubing (RWD Life Science, Shenzhen, China). A multi-channel syringe pump (RWD Life Science, Shenzhen, China) was used to bilaterally inject the agents into the OB at the same time at a rate of 0.2 μl/min for 10 min (final volume: 2 μl per side). In all animals, the infusion cannula was maintained in place for 10 min to enable adequate diffusion of the solution. This lidocaine volume has been effectively used to reversibly block cortical activity for approximately 40 min (Tehovnik and Sommer, 1997). Immediately following the lidocaine/MK-801 infusion, the animals were subjected to the PPI test. After the PPI test, the location of the infusion cannula was assessed in the post-mortem brains. The methods have been previously described (Contreras et al., 2007). The animals were sacrificed by decapitation, and the brains were removed, frozen and cut into coronal sections. The path of the infusion cannula was examined to verify that the cannula location was within the selected area of the OB.

Viral Transsynaptic Tracing from the PPTg in Rats

To further explore the relation between the olfactory-related cortex and PPI circuits, independent animals were administered a stereotactic microinjection of a recombinant PRV (PRV614) that expressed the mRFP into the PPTg to retroactively trace the organizational circuitry of the olfactory system that

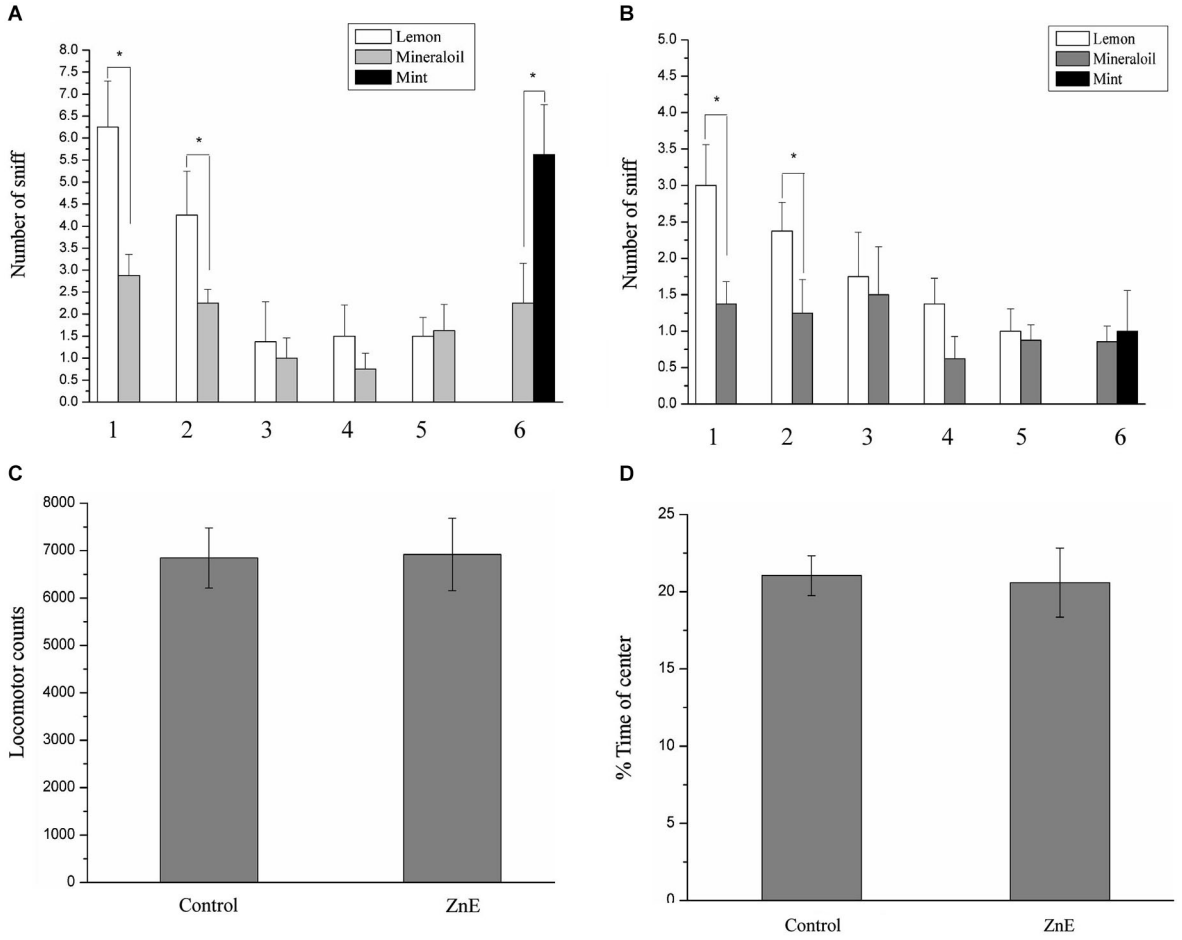

FIGURE 1 | The performance of ZnE-treated rats in a habituation/discrimination task and locomotion in an open field. (A,B) Odor habituation/ discrimination task. Values represent the mean ± SD (n = 8–10). * indicates $p < 0.05$. **(C)** Effect of ZnE treatment on the locomotor activity of rats in the open field. The rats were allowed to freely explore the open field for 5 min (mean ± SEM). There was no significant difference between the groups ($F_{(2,21)} = 0.005$, $p > 0.05$). **(D)** The effect of ZnE treatment on the time spent in the central area of the open field. No differences were identified between the groups with respect to the time spent in the central area of the open field ($F_{(2,21)} = 0.897$, $p > 0.05$) compared with the naïve control group.

may modulate the PPI response. A detailed procedure of the microinjection has been previously described. Briefly, the animals were anesthetized with chloral hydrate (0.75 mg/kg) and then mounted in a stereotaxic apparatus. A hole was drilled on the skull above the PPTg (1.32 mm anteroposterior, ±1.90 mm mediolateral from the interaural, and 9 mm dorsoventral to the dura). Injections were performed with a glass micropipette (tip diameter—10–15 um) connected to a 10 μl Hamilton syringe. A recombinant PRV that expressed mRFP was controlled by the cytomegalovirus promoter. The PRV614 (1×10^8 pfu/ml) was mixed with 0.17 μm green fluorescent beads (1:100 diluted, P7220). Then, 200 nl of the mixture were injected into the PPTg at a rate of 20 nl/min. After the injection, the glass micropipette was maintained in the brain for an additional 15 min to prevent the viral solution from diffusing away from the inject site. Four days after the injection, the rats were deeply anesthetized with 10% urethane and transcardially perfused with 500 ml of physiological saline followed by 500 ml of 4% paraformaldehyde (PFA) solution. The brain was dissected and subsequently post-fixed in 4% PFA at 4°C overnight. Coronal brain sections (40–50 μm) were sequentially prepared on a Leica VT1000S vibrating microtome and transferred into 24-well plates. The sections were subsequently wet mounted on slides with the Vecta-Shield mounting medium and were visualized with wide-field upright fluorescence microscopy (Olympus BX51). The images were further manipulated for brightness and contrast, and a montage was created using Adobe Photoshop version CS2. Scale bars were added with image pro plus (IPP 6.0).

C-Fos Expression in PPI-Sensitive Brain Fields by Viral Transsynaptic Tracing

To identify whether PPI-sensitive fields by viral transsynaptic tracing were a part of the neuroanatomical substrates in the PPI response, c-Fos expression in the PRV tracing-signal positive fields was confirmed by immunohistochemistry (IHC). For this

experiment, independent animals were needed for treatment by different stimuli, including the control (no stimulus), PPI and Pre stimulus. After the stimulus, c-Fos immunostaining in the PRV tracing-signal positive fields was performed as previously described (Arime et al., 2012). c-Fos expression was measured to occur from 1–4 h after an acute stimulation. To confirm the fields activated by PPI stimulation, the animals were deeply anesthetized with chloral hydrate (450 mg/kg, i.p., Shanghai Chemical Reagent Co., LTD, China) 2 h after the PPI stimulus. The rats were then transcardially perfused with 0.9% saline followed by a fixative that comprised 4% PFA in 0.01 M phosphate-buffered saline (PBS, pH = 7.4). The brains were removed followed by locate in 10, 20 and 30% sucrose-0.01 M PBS to completely dehydrate the tissues. The brains were then frozen-sectioned coronally at 20 μm thickness. After permeabilization and blocking, the frozen coronal sections in the sensitive brain fields were incubated with rabbit anti-c-Fos antibody (1:200; sc-52, Santa Cruz Biotechnology) overnight at 4°C. They were washed with 0.01 M PBS buffer and incubated with biotinylated goat anti-rabbit antibody (1:200; vector Lab, CA, USA) for 20 min in 37°C. The streptovidin-conjugated horse radish peroxidase was then added, with 3, 39-diaminobenzidine (DAB) used as the chromagen (Vectastain Elite ABC Kit, Vector Laboratories, CA, USA). The sections were dehydrated through a graded ethanol series and coverslipped with resinene. The sections were examined under a 40× objective lens magnification, and c-Fos immunoreactivity (CMR) was measured with Image-Pro Plus software (Media Cybernetics, Silver Spring, USA). Statistical analyses were conducted with the SPSS statistical package.

Statistical Analyses

The data are presented as the mean ± standard error of the mean. The percentage of the PPI was calculated using the following equation: [(startle response to a 115-dB SPL startle stimulus—response to pulses with a prepulse)/startle response to a 115-dB SPL startle stimulus × 100]. The data were analyzed for variables of interest using the statistical package for the social sciences (SPSS v. 13.0). A paired-samples Student's *t*-test was used for odor and locomotor evaluations. The c-Fos data were analyzed using one-way ANOVA to identify group differences. The alpha level for significance was set at 0.05.

Results

ZnE Treatment on the Odor Habituation/Discrimination

Our primary goal was to test whether there is a causal relationship between PPI response impairment and olfactory deficiency. We first examined odor-guided behavior in an odor habituation/discrimination task, which enabled measurements of novel odor investigation, odor learning and memory (habituation), and odor discrimination within a single behavioral test (Yang and Crawley, 2009). In this test, the rats were presented with lemon and a control odor during five consecutive days and subsequently exposed to a novel odor (mint odor, see Section Materials and Methods). Analysis of variance, using the five habituation trial numbers as the within-subject factor and the experimental group as the between-subjects factor, identified significant main effects ($F_{(4,160)} = 50.869$, $p < 0.05$) and interaction effects ($F_{(20,160)} = 2.015$, $p < 0.05$). This finding indicated that habituation to the lemon odor progressed across successive trials and was affected by the different treatment methods. *Post hoc* analysis indicated that in the odor habituation experiments (**Figures 1A,B**), the animals in the naïve control and ZnE groups exhibited a significant decrease in the sniffing time from the first odor (lemon) presentation through the subsequent presentations of the same odor. This decrease in sniffing behavior suggested that the animals successfully habituated to the repeated lemon odor presentations. When the lemon odor was replaced by a novel odor (mint) in the discrimination test, pairwise comparisons indicated that the control animals spent more time investigating the mint odor than the mineral oil ($p < 0.05$), whereas the animals that

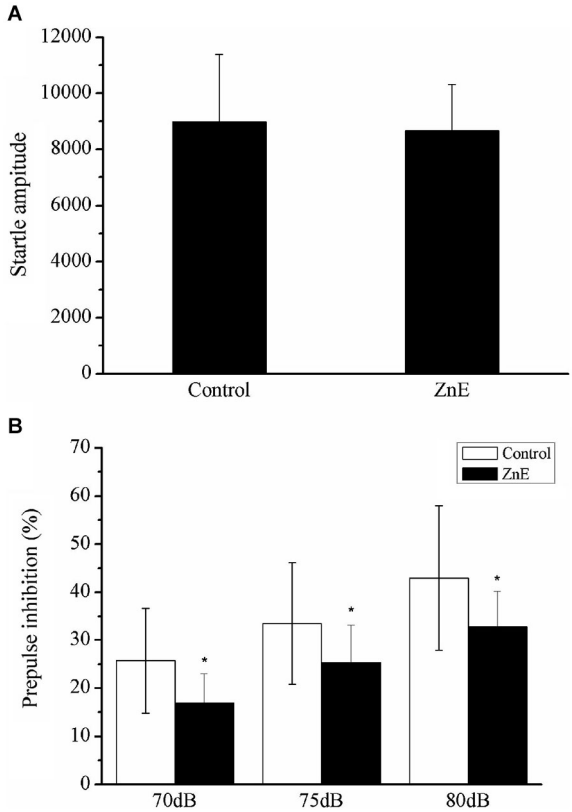

FIGURE 2 | The effects of ZnE on the startle magnitude and PPI response. (A) The effect of ZnE treatment on the startle response magnitude. An independent sample t test identified a significant difference between the groups ($F_{(1,21)} = 10.044$, $p > 0.05$). **(B)** The effect of ZnE on the percentage change induced by a prepulse prior to an ASR PPI. A two-way ANOVA identified a main treatment effect ($F_{(2,42)} = 4.836$, $p < 0.05$), but there was no significant interaction (intensity × treatment) ($F_{(4,42)} = 1.689$, $p > 0.05$). A *post hoc* (LSD) test indicated that the ZnE treatment significantly altered the PPI response (naïve control vs. ZnE, $p < 0.05$). The data are presented as the mean ± SD. * indicates a significant difference compared with the naïve control group ($p < 0.05$).

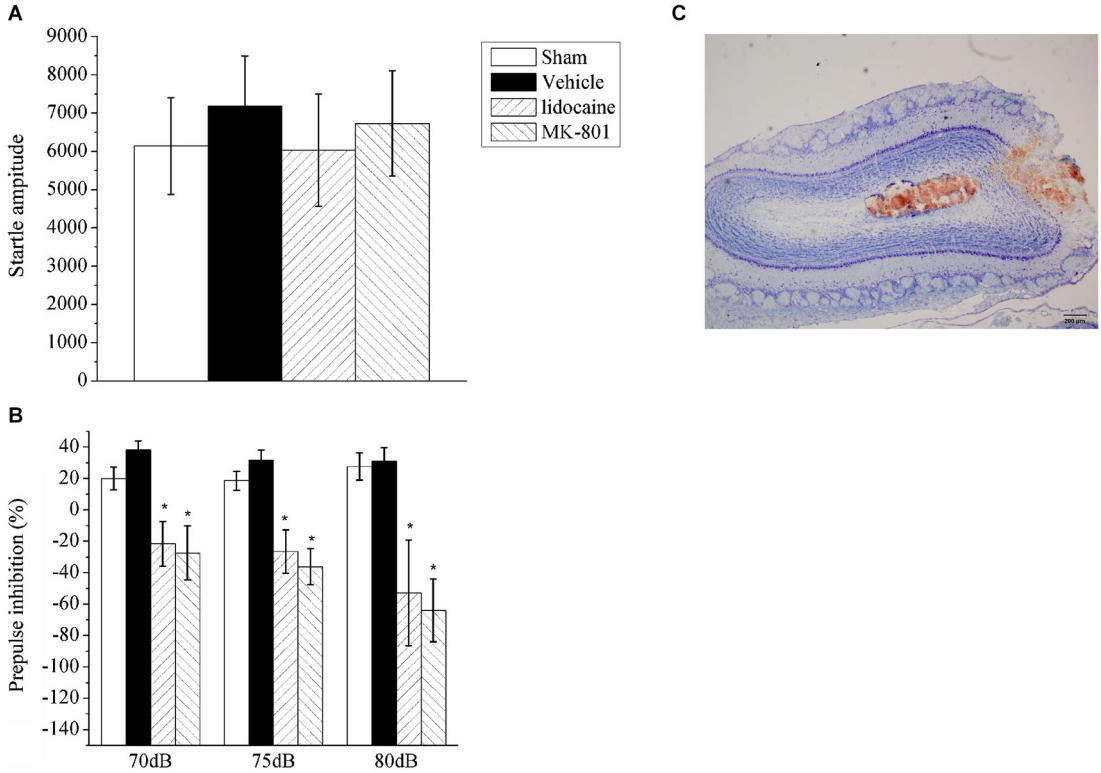

FIGURE 3 | The effect of lidocaine or MK801 bilateral microinjections into the OB on the ASR and PPI. (A) Acoustic startle amplitude of the rats administered bilateral microinjections of lidocaine or MK801 in the OB. A one-way ANOVA indicated there was no significant difference between the groups. **(B)** The PPI (%) of the rats with the OB inhibited with lidocaine or MK801 at three different auditory prepulse intensities (70, 75 and 80 dB). A repeated measures ANOVA of the prepulse data identified significant effects of drug treatment ($F_{(3,20)}$ = 5.74, p < 0.05). The values indicate the mean ± SD (n = 6). * indicates a significant difference compared with the vehicle group (p < 0.05). **(C)** A representative photograph of the OB that indicates the microinjection site.

received the ZnE application to the naris did not exhibit a significant difference in their sniffing times between the mint and control odors (**Figures 1A,B**). These results suggested that the animals that received the ZnE application exhibited impaired discrimination between familiar and novel odorants ($p > 0.05$).

Effects of ZnE on Locomotion in an Open Field

There is a possibility that locomotor activity was disturbed by ZnE to affect the PPI response. To test this possibility, we examined the locomotor activity of the rats treated by ZnE treatment. **Figures 1C,D** shows that there were no significant differences in total locomotion between the naïve control and ZnE groups ($p > 0.05$). In addition, no difference was identified in the time spent in the central area of the open field ($p > 0.05$) for either group. These findings indicated that no gross motor dysfunction was caused by ZnE.

Effects of ZnE Treatment on the ASR and PPI

Figure 2 shows the effect of a prepulse on the acoustic startle reflex in the ZnE and naïve control rats. The animals treated with ZnE were evaluated for their ASR and their prepulse inhibition to determine if olfaction has an impact on PPI and ASR. ZnE treatment caused significant changes in the animal's PPI, but the ASR was unclear. The effects of ZnE treatments on the PPI and ASR are illustrated in **Figure 2**. An independent samples t-test of the ASR indicated that there was no significant difference between the groups (**Figure 2A**; $p > 0.05$). A two-way ANOVA of the PPI test identified a main treatment effect ($F_{(2,42)}$ = 4.836, $p < 0.05$), but there was no significant interaction between the groups and stimulus intensities (**Figure 2B**; $F_{(4,42)}$ = 1.689, $p > 0.05$). A *post hoc* analysis (LSD) indicated that ZnE treatment decreased the PPI ($p < 0.05$: naïve control vs. ZnE). These results indicated that ZnE treatment significantly reduced the PPI at all auditory intensities tested. Further analysis of the relationship between ZnE treatment and prepulse intensity was used to examine and differentiate the main effects caused by increasing the auditory intensity of the prepulse. Significant differences between the ZnE treatment and naïve control groups were identified at 70, 75 and 80 dB (**Figure 2B**). ZnE treatment disrupted the PPI expression in the 75 and 80 dB tests and may have led to a decrease in the startle response after an acoustic prepulse compared with the naïve control group (one way ANOVA, $F_{(2,21)}$ = 5.379, $p < 0.05$; $F_{(2,21)}$ = 4.820, $p < 0.05$).

Effects of Lidocaine/MK801 Microinjections into the Olfactory Bulb (OB) on the PPI

In the previous section, the nasal epithelium was directly stimulated; thus, the effects identified could be mediated by these effects rather than olfaction. To further test the role of olfactory neurons in PPI, we examined the PPI response after the inactivation of OB neurons with a stereotaxic injection of either lidocaine or MK-801. Bilateral microinjections were administered in the OB of four groups of rats, including the lidocaine ($n = 6$), MK801 ($n = 6$), vehicle ($n = 6$) or sham surgery ($n = 6$) groups; the animals were then subjected to the PPI test. A repeated measures ANOVA of the data identified significant effects for drug treatment ($F_{(3,20)} = 5.74$, $p < 0.05$) and the intensity of the auditory prepulse ($F_{(2,40)} = 1.007$, $p > 0.05$), but not for the interaction between drug treatment and prepulse intensity ($F_{(6,40)} = 0.886$, $p > 0.05$). Inactivation of the OB neurons by lidocaine or MK801 injection had no effect on the startle amplitude (**Figure 3A**), but it significantly enhanced the startle response following the prepulse (**Figure 3B**). The injection sites in the OB were identified using Nissl staining (**Figure 3C**).

Regions in the Olfactory Cortices Give Rise To Afferent Circuitry to the PPTg

Although the PPTg has been shown to play a critical role in mediating the PPI of the ASR (Takahashi et al., 2007), the exact role of olfactory contribution to the PPI response is unclear. Retrograde viral tracing was used to investigate whether neurons in the olfactory cortices directly participated in the regulation of the PPI of the ASR. The virus PRV614, which expressed mRFP, was injected into the PPTg and allowed to spread retrogradely for 4 days (**Figure 4A**). On the fourth day, the rats exhibited moderate signs of viral infection. At this point, the infected rats were anesthetized with 20% urethane, perfused transcardially with PFA, and the location of viral expression was examined with fluorescence microscopy. Labeled neurons were identified near the targeted injection site and were specifically restricted to the PPTg (**Figure 4B**). Several regions that have been shown to regulate the PPI of the ASR were retrogradely labeled in the ipsilateral hemisphere. The most prominently labeled regions were the basolateral amygdala (BLA), basomedial amygdala (BMA) and CA1 of the ventral hippocampus (**Figures 4C,D**). These results indicated that the PRV614-mRFP trans-synaptic tracing successfully identified areas found to regulate the PPI of the ASR, which is consistent with the areas identified using lesion, electrophysiological and pharmacological approaches (Contreras et al., 2007).

In addition to the regions known to regulate the PPI of the ASR, a large number of labeled neurons were distributed in several areas of the primary olfactory cortex, including the anterior olfactory nucleus (AON), the piriform cortex (Pcx) and the lateral entorhinal cortex (LEnt; **Figure 5**). The majority of the labeled neurons in the AON were distributed in the dorsal aspect (**Figure 6A**). Most labeled neurons in the Pcx were identified in layer II of the Pcx and were distributed from the anterior (APc) to posterior (PPc) aspects. The neurons in layer III of the Pcx

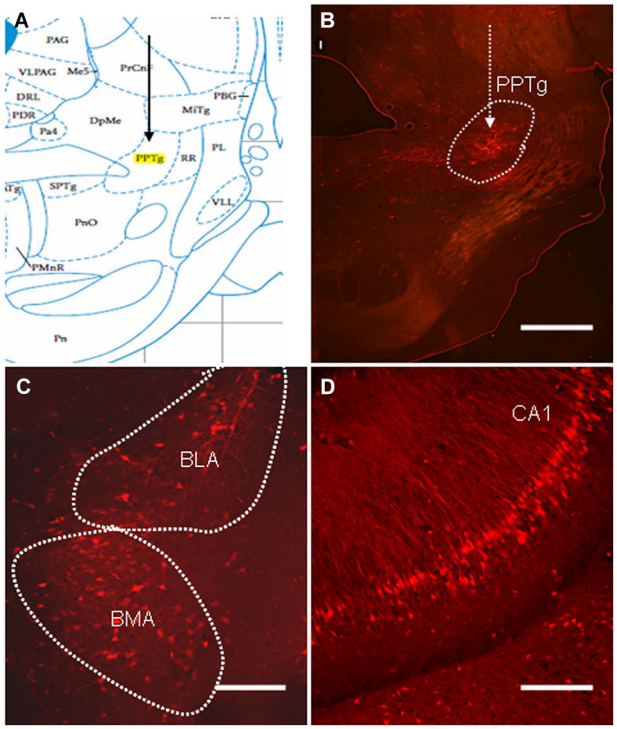

FIGURE 4 | The distribution of PRV614-mRFP trans-synaptically labeled neurons in regions known to mediate PPI after 4 days of infection. (A) A schematic map of the midbrain (PPTg is highlighted in yellow). **(B)** PRV614-mRFP labeled neuron distribution is restricted to the PPTg near the injected site. **(C)** Trans-synaptically labeled neurons in the BLA and the basomedia amygdala (BMA). **(D)** Trans-synaptically labeled neurons specifically distributed in the CA1 of the ventral hippocampus. Scale bar in **B** = 1 mm; scale bars in **C** and **D** = 200 μm.

were also labeled to some extent (**Figures 5B,C**). The neurons in the LEnt were prominently labeled in both layers II and III (**Figure 5D**).

C-Fos Expression

To verify that the regions identified with viral transsynaptic tracing are a part of the neuroanatomical structure that participates in the PPI response, we examined c-Fos protein expression in the mPFC, amygdala and hippocampus fields in rat brains from the naïve control ($n = 4$), PPI ($n = 4$) and Pre ($n = 4$) groups using an IHC method. After the PPI and Pre stimulus, a significant difference in c-Fos expression was identified in the mPFC ($F_{(2,12)} = 58.17$, $p < 0.05$), BLA ($F_{(2,12)} = 34.95$, $p < 0.05$), BMA ($F_{(2,12)} = 38.84$, $p < 0.05$) and CA1 ($F_{(2,12)} = 87.92$, $p < 0.05$). Representative photomicrographs of c-Fos staining in the mPFC are shown in **Figure 6**.

Discussion

In the present study, to determine whether blockage of olfactory sensory input induced by zinc sulfate infusion in the olfactory naris (0.5 ml, 0.17 M, ZnE) can disturb the PPI response, we performed behavioral and pharmacological tests in rats,

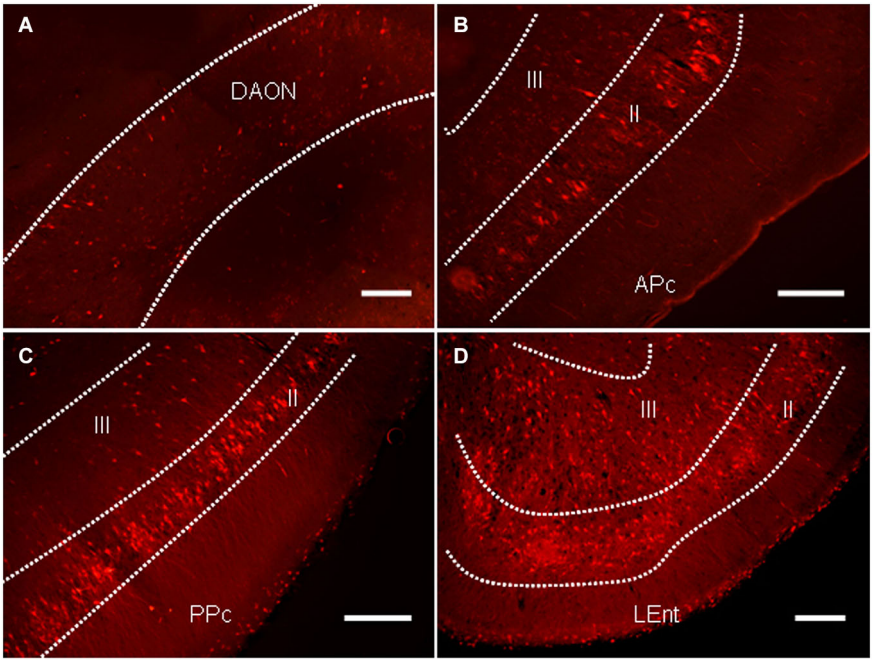

FIGURE 5 | The distribution of PRV614-mRFP trans-synaptically labeled neurons in several regions of the olfactory cortices after 4 days of infection. (A) The distribution of trans-synaptically labeled neurons in the dorsal aspect of the anterior olfactory nucleus (DAON). **(B)** The distribution of trans-synaptically labeled neurons in the anterior piriform cortex (APc). **(C)** The distribution of trans-synaptically labeled neurons in the posterior piriform cortex (PPc). **(D)** The distribution of trans-synaptically labeled neurons in the lateral entorhinal cortex (LEnt). Scale bar = 200 μm.

including an odor habituation/discrimination test, locomotion test, IHC, and virus tracing. Our results demonstrated that blockage of olfactory sensory input by ZnE treatment impaired the PPI response with no effect on locomotion and inhibited the expression of c-Fos in PPI-related sensitive fields. Importantly, the reversible blockade of activity in the OB by lidocaine or MK-801 also impaired the PPI response. In addition, PRV614 tracing studies demonstrated that many fields in the brain were identified between the olfaction-related cortex and PPI-related brain areas (PTTg area) (Fendt et al., 2001). Thus, it is highly likely that olfactory impairment can disturb the function of the PPI system. More importantly, as part of forebrain structures, this study reveals that the olfactory sensory input contribute to regulate the PPI response by mean of top-down.

Previous studies have demonstrated that ZnE impaired the sensory neurons in the olfactory epithelium and widely disturbed olfactory sensory and perception functions (Niu et al., 2013). For example, ZnE treated rats, in this study and previous studies (Meng et al., 2010), exhibited disabilities in investigation, discrimination and habituation in response to various odors. In addition to the assessment of olfactory function in ZnE-treated rats, the locomotor activity in the animals was also assessed in this study to exclude possible anxiety related effects and depressive behaviors associated with excessive ZnE treatments, which may have resulted in confounding issues that affected the PPI response (Ahmari et al., 2012). Previous studies have indicated that bulbectomy may have caused anxiety or depression, as well as PPI impairment (Bilkei-Gorzo et al., 2002). However, our results indicated that the impairment of olfactory perception could disturb the PPI response with no effect on locomotor. Namely, the results are based on the olfactory lesion but not locomotor impairment.

Furthermore, OB played a vital role in olfactory relaying from environmental stimulus to sensory cortex (Kay and Sherman, 2007). From the anatomy, olfactory cortex was parts of forebrain structure, which projected to subcortical structures including the PPC and amygdala.

Olfactory cortex stimulation altered the forebrain reward and motivational behavior. The olfaction-driven behaviors required an intact olfactory system to detect and discriminate the environmental stimulus from background stimuli (Fitzgerald et al., 2014). At the same time, the abilities of relaying the olfactory information into forebrain structures including emotional and reward-related brain structures were required in top-down pattern modulating (Wilson and Mainen, 2006; Wilson and Sullivan, 2011; Fitzgerald et al., 2014).

The olfactory system would interact with these forebrain structures that were involved in modulating the primary PPI pathway and then regulated these brain fields' activities by the mean of top-down (Li et al., 2009; Du et al., 2010, 2011; Ma and Luo, 2012). Although the primary neural pathways mediating PPI response were located in the brainstem (Davis and Gendelman, 1977; Li and Frost, 2000; Fendt et al., 2001), the forebrains were included in primary neural pathways including PPTg and were top-down modulated. For example the primary

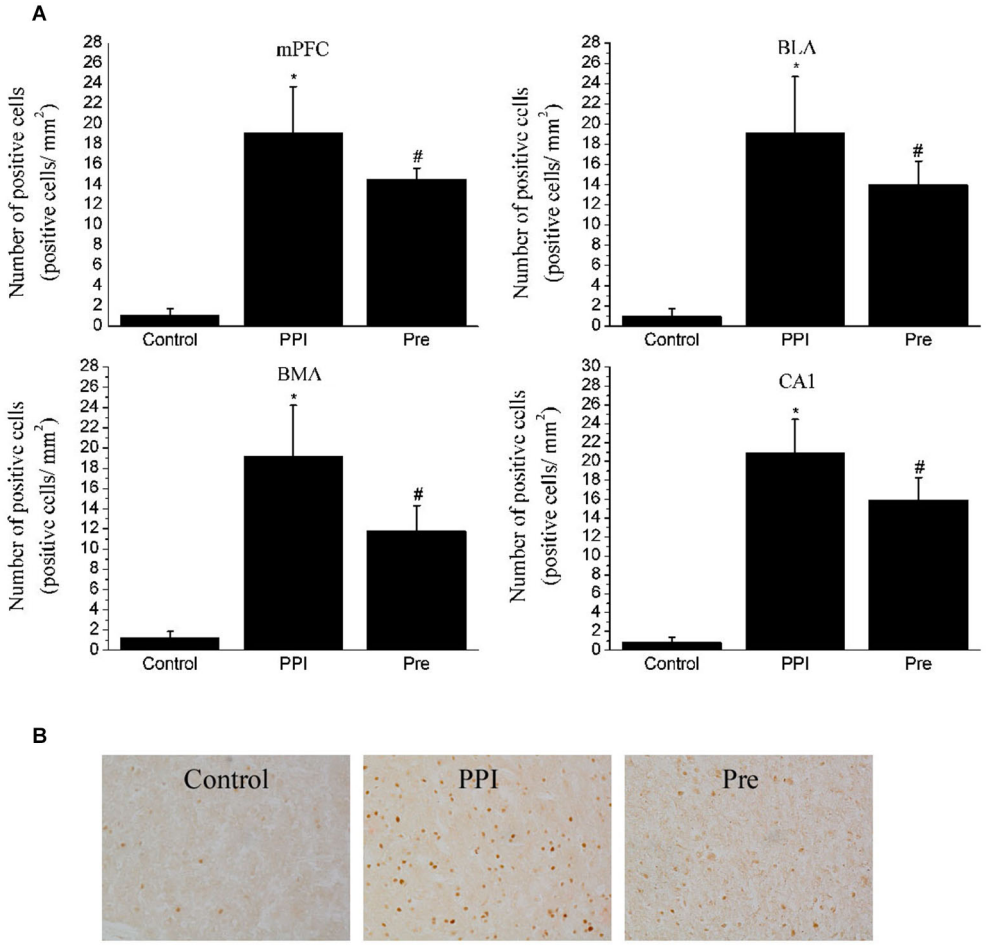

FIGURE 6 | Expressions of c-Fos in selected areas of the rats in the naïve control, PPI and prepulse groups. (A) One-way ANOVA analysis: mPFC, $F_{(2,12)} = 58.17$, $p < 0.05$; BLA, $F_{(2,12)} = 34.95$, $p < 0.05$; BMA, $F_{(2,12)} = 38.84$, $p < 0.05$; CA1, $F_{(2,12)} = 87.92$, $p < 0.05$. * indicates $p < 0.05$ vs. the naïve control group and # indicates $p < 0.05$ vs. the PPI group. Values indicate the mean ± SD ($n = 4$). **(B)** Representative photomicrographs of c-Fos staining in the mPFC are shown (20X).

auditory cortex, lateral nucleus of the amygdala and posterior parietal cortex also played a role in the top-down modulation of PPI in rats (Bakshi and Geyer, 1998; Miller et al., 2010; Du et al., 2011). At the same time; some higher-order cognitive processes could modulate the PPI response (Neumann, 2007; Du et al., 2010). Some evidences confirmed that directing attention to the prepulse stimulus could enhance PPI response (Heekeren et al., 2004; Annic et al., 2014). In patients, attentive deficit was found in anosmia cases (Roberts et al., 2010; Garcia-Sanchez et al., 2011). Olfactory sensory stimulus affected on the attentional response by top-down regulations via descending axonal projections from forebrain structures (Georgoussi et al., 1990; Yochman et al., 2013). So we concluded that olfactory sensory stimulus modulated the PPI response by affecting the attention.

To further examine the role of olfactory sensory input and its disruption of PPI, the rats were transiently microinjected with lidocaine in the OB. The OB is the first relay station that transmits olfactory sensory information to the higher olfactory cortex (Lledo et al., 2005). The PPI was similarly reduced in the lidocaine treated and ZnE treated rats. Furthermore, MK801, an N-methyl-D-aspartate (NMDA) receptor antagonist (Lledo et al., 2005), injection into the OB also reduced the PPI response. These results suggested that glutaminergic neurons in the OB may have played a regulatory role in the startle reflex and possibly the PPI phenomenon. The reduced PPI effect was dependent on olfactory specific locations: limiting olfactory stimulus by both ZnE in the nasal epithelium and lidocaine blockage of the OB resulted in an impaired PPI phenomenon (**Figures 2**, **3**). These results emphasized the fact that an olfactory stimulus plays an important role in the regulation of the PPI response.

Although these experiments demonstrated that blockage of olfactory sensory input could impair the PPI response, the circuit basis that the olfactory system interacted with the PPI system was not identified. Previously, a retrograde transsynaptic tracer (PRV) was used to identify the CNS regions that innervated the target areas (Rodriguez-Sierra and Terasawa, 1979). Thus,

the PRV614 retrograde tracing from the PPTg was used to demonstrate that circuits existed between the olfactory systems in the PPTg, which may provide a potential neural circuit for olfaction to regulate the PPI response. Our data demonstrated PRV614-mRFP trans-synaptically labeled neurons were present in several regions of the olfactory cortices, including the dorsal aspect of the anterior olfactory, APc, PPc, and lateral entorhinal cortex (LEnt), after 4 days of PRV injection. Simultaneously, the labeled neurons of classic PPI networks, such as the mPFC, BLA, BMA and CA1 of the hippocampus, were also identified after 4 days of PRV injection (Swerdlow et al., 2001). The results demonstrated that the olfactory system was indirectly connected with the PPI fields. Furthermore, olfactory information was first transmitted through the OB to the limbic circuitry and then relayed to PPI-mediating regulatory circuitry in the brainstem.

Previous studies have demonstrated that the neural circuits that included the PPI pathways can be labeled by c-Fos mapping. This method is suitable and applicable to detect neurons activated by various stimuli (Knapska et al., 2012). Our data indicated these fields traced by PRV were also confirmed by c-Fos expression. Studies have demonstrated the mPFC, BLA, BMA and CA1 of the hippocampus were included in the regulation of PPI (Schwabe et al., 2004; Stevenson and Gratton, 2004).

In conclusion, ZnE-treated rats exhibited normal locomotor activity but an impaired PPI to a startle reflex, which suggested that olfactory dysfunction played a role in sensorimotor gating, but not in overall physiological functions. PRV retrograde tracing from the PPTg identified important circuitry interactions between the olfactory system and other neural circuits that regulate PPI. Although olfactory dysfunction has not been commonly used by clinicians, the results presented here highlight a connection between olfactory function and psychiatric illness.

Author Contributions

HN, QZ and XH have bred and maintained the animal colonies and litter details including litter size and litter survival within this study. HN and TZ performed the behavior test and IHC experiments. TZ and XS performed blind data scoring for c-Fos test. ZZ and XH performed the PRV tracing experiment. YQ, FX and MH conceived the study and participated in its design and coordination and drafted the manuscript. All authors read, edited and approved the final manuscript.

Acknowledgments

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Altered neuronal excitability underlies impaired hippocampal function in an animal model of psychosis

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Psychosis is accompanied by severe attentional deficits, and impairments in associational-memory processing and sensory information processing that are ascribed to dysfunctions in prefrontal and hippocampal function. Disruptions of glutamatergic signaling may underlie these alterations: Antagonism of the N-methyl-D-aspartate receptor (NMDAR) results in similar molecular, cellular, cognitive and behavioral changes in rodents and/or humans as those that occur in psychosis, raising the question as to whether changes in glutamatergic transmission may be intrinsic to the pathophysiology of the disease. In an animal model of psychosis that comprises treatment with the irreversible NMDAR-antagonist, MK801, we explored the cellular mechanisms that may underlie hippocampal dysfunction in psychosis. MK801-treatment resulted in a profound loss of hippocampal LTP that was evident 4 weeks after treatment. Whereas neuronal expression of the immediate early gene, Arc, was enhanced in the hippocampus by spatial learning in controls, MK801-treated animals failed to show activity-dependent increases in Arc expression. By contrast, a significant increase in basal Arc expression in the absence of learning was evident compared to controls. Paired-pulse (PP) facilitation was increased at the 40 ms interval indicating that NMDAR and/or fast GABAergic-mediated neurotransmission was disrupted. In line with this, MK801-treatment resulted in a significant decrease in GABA(A), and increase in GABA(B)-receptor-expression in PFC, along with a significant increase of GABA(B)- and NMDAR-GluN2B expression in the dentate gyrus. NMDAR-GluN1 or GluN2A subunit expression was unchanged. These data suggest that in psychosis, deficits in hippocampus-dependent memory may be caused by a loss of hippocampal LTP that arises through enhanced hippocampal neuronal excitability, altered GluN2B and GABA receptor expression and an uncoupling of the hippocampus-prefrontal cortex circuitry.

Keywords: hippocampus, schizophrenia, NMDA receptor hypofunction, MK801, GABA, *in vivo*

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Introduction

Changes in neurotransmission, mediated by the glutamatergic, dopaminergic and GABAergic systems may underlie pathophysiological changes in the brain that lead to psychosis (Laruelle et al., 1996; Kristiansen et al., 2006; Abi-Dargham et al., 2012; Stan and Lewis, 2012). However, dopamine receptor ligands treat only a subset of the symptoms (Chue and Lalonde, 2014),

and pharmacological treatment to regulate neurotransmission through GABA(A) receptors, fails to ameliorate symptoms (Dold et al., 2013). In psychosis, hypofunction of glutamatergic N-methyl-D-aspartate receptors (NMDAR) is believed to occur (Gunduz-Bruce, 2009). Furthermore, NMDAR-antagonists trigger psychotic episodes in healthy adults and exacerbate symptoms in psychosis patients (Lahti et al., 2001). An interplay with the dopaminergic and GABAergic systems is likely (Healy and Meador-Woodruff, 1996; Cochran et al., 2003; Hashimoto et al., 2003; Kinney et al., 2006). Moreover, a reciprocal regulation of NMDAR and GABA-receptor function may lie at the core of psychosis (Gordon, 2010; Wiescholleck and Manahan-Vaughan, 2013a) and mediate dopaminergic dysfunction in the disease (Schwartz et al., 2012).

Early clinical intervention in psychosis greatly increases the chances of improved clinical outcome (Álvarez-Jiménez et al., 2012). Thus, an understanding of the mechanisms that accompany the early stages of the disease is key to the development of effective therapeutic strategies. Notwithstanding their limitations, animal models of NMDAR-hypofunction emulate many of the symptoms of psychosis seen in humans (Neill et al., 2010; Wiescholleck and Manahan-Vaughan, 2013a). Prolonged treatment of rats with the irreversible NMDAR-antagonist, MK801, emulates the chronic and advanced phase of psychosis (Wiescholleck and Manahan-Vaughan, 2013a). Furthermore, following acute, one-off systemic treatment, animals exhibit symptoms of first-episode psychosis (Wöhrl et al., 2007; Manahan-Vaughan et al., 2008a,b), but also show long-lasting cognitive deficits (Manahan-Vaughan et al., 2008a,b; Wiescholleck and Manahan-Vaughan, 2012, 2013b; Galizio et al., 2013; Lobellova et al., 2013). These changes emulate deficits in hippocampus-dependent memory, behavioral flexibility, attention, and working memory that are known to occur in psychosis patients (Rajji et al., 2009).

Hippocampal synaptic plasticity may underlie information storage (Kemp and Manahan-Vaughan, 2007), and cognitive processes such as the learning of spatial scenes, or contextual fear memory are tightly associated with LTP (Straube et al., 2003; Whitlock et al., 2006; Kemp and Manahan-Vaughan, 2007). LTP-like plasticity and learning are deficient in schizophrenic patients after transcranial magnetic, or direct current stimulation (Frantseva et al., 2008; Hasan et al., 2011), whereby both induce synaptic plasticity in the cortex of healthy adults (Stefan et al., 2000; Liebetanz et al., 2002). Strikingly, in close parallel to these observations in humans, a profound loss of hippocampal LTP that is persistent (>months), and is accompanied by a significant loss of hippocampus-dependent memory occurs in the MK801-animal model of psychosis (Manahan-Vaughan et al., 2008a,b; Wiescholleck and Manahan-Vaughan, 2012, 2013b). This suggests that the hippocampus may be at the center of cognitive changes that occur in psychosis and raises the question as to the mechanisms that underlie these alterations.

NMDAR-hypofunction reduces inhibitory GABAergic neurotransmission (Schwartz et al., 2012). We postulated that the loss of LTP and associated changes in memory function seen in humans and rodents could arise from changes in glutamatergic neurotransmission and increased neuronal excitability in the hippocampus. To clarify this, we examined the mechanisms that underlie LTP impairments and loss of hippocampus-dependent memory (Manahan-Vaughan et al., 2008a,b; Wiescholleck and Manahan-Vaughan, 2012, 2013b) that occur following an acute systemic treatment with the NMDAR-antagonist, MK801 (Wiescholleck and Manahan-Vaughan, 2013a). We observed that a chronic elevation in neuronal excitability occurred in the hippocampus that was accompanied by a disruption of inhibitory control in the prefrontal cortex. These changes were mediated by alterations in expression of the NMDAR subunit GluN2B in the hippocampus, along with changes in GABA(A) and GABA(B) expression in both the hippocampus and prefrontal cortex. We propose that in psychosis, deficits in hippocampus-dependent memory are caused by a loss of hippocampal LTP that arises through enhanced hippocampal neuronal excitability, altered glutamate and GABA receptor expression, and an uncoupling of the hippocampus-prefrontal cortex circuitry.

Materials and Methods

Animals

The present study was carried out in accordance with the European Communities Council Directive of September 22nd 2010 (2010/63/EEC) for care of laboratory animals and after approval of the local government ethics committee (Bezirksamt, Arnsberg). Male Wistar rats (7–8 weeks, Charles River, Germany) were housed individually in a temperature- and humidity-controlled vivarium with a constant 12-h light-dark cycle (lights on from 6 a.m. to 6 p.m.) with *ad libitum* food and water access. All surgical procedures and experiments were conducted during the day.

Compounds and Drug Treatment

The NMDAR antagonist [+]-5-methyl-10, 11-dihydro-5H-dibenzo-[a, d]-cyclohepten-5, 10-imine maleate (MK801, Tocris, Germany) was dissolved in 0.9% physiological saline. MK801 (5 mg/kg) or vehicle (10 ml/kg) were injected intraperitoneally (i.p.) 7 days before commencement of experiments. The concentration of MK801 was chosen in accordance with previous studies conducted by our group (Wöhrl et al., 2007; Manahan-Vaughan et al., 2008a,b), in which the same dose proved to be effective in inducing long-lasting effects. A single high-dose treatment, as opposed to chronic low-dose treatment, was chosen in order to specifically emulate the very first acute psychosis-related experience (Wiescholleck and Manahan-Vaughan, 2013a). Directly after injection, psychosis-like behaviors (locomotion, ataxia and stereotypy) were observed. Twenty-four hours after treatment the animals' behavior was not different from vehicle injected controls, as described previously (Wöhrl et al., 2007). No differences in locomotion ability, grooming or rearing behavior was observed over multiple days after treatment, in line with our previous findings (Manahan-Vaughan et al., 2008a).

Electrophysiology Procedures

Surgical Implantations

Animals were anesthetized (52 mg/kg pentobarbital via intraperitoneal injection, i.p.) and underwent chronic implantation of electrodes as described previously (Wiescholleck and Manahan-Vaughan, 2013b). Specifically, a monopolar recording electrode (1 mm diameter, 3.1 mm posterior to bregma, 1.9 mm lateral to the midline) was placed in the dentate gyrus granule cell layer and a bipolar stimulating electrode was placed in the medial perforant pathway (1 mm diameter, 6.9 mm posterior to bregma, 4.1 mm lateral to the midline). The animals were allowed between 7 and 10 days to recover from surgery before experiments were conducted. Pre- and post-operative analgesia was implemented using meloxicam (0.2 mg/kg, i.p.) and local administration of xylocaine.

Responses were evoked in freely behaving animals by stimulating at low frequency (0.025 Hz, 0.2 ms stimulus duration, 10,000 Hz sample rate). For each time-point, five evoked responses were averaged. Dentate gyrus population spike (PS) amplitude, as well as field excitatory postsynaptic potential (fEPSP) slope were monitored. Each experiment started with an input-output (i/o) curve (maximal stimulation 900 μA) to determine the stimulus intensity required to elicit a PS that was of 40% of the maximum obtained in the i/o curve. The i/o-curves between vehicle- and MK801-treated animals did not differ at any time-point after treatment. To ensure stability of recordings and to assess basal synaptic transmission, all animals were tested in a baseline experiment first, where only test-pulse stimulation was applied.

LTP was induced by high-frequency stimulation (HFS) (10 bursts of 15 pulses at 200 Hz with 10 s interburst interval) and was recorded 1 week before (pretreatment LTP control) and 4 weeks after MK801- or vehicle-treatment. PS and fEPSP values for pretreatment LTP did not differ significantly in between the two experimental groups. Short-term potentiation (STP) was induced by weak HFS (wHFS, 3 bursts of 15 pulses at 200 Hz with 10 s interburst interval).

Paired-pulse (PP) measurements, as a tool for assessment of general excitability and neurotransmission, were performed by PP stimulation every 40 s with interpulse intervals (IPI) of 20, 25, 40, 50, 100, 300, 500 ms and 1 s. The whole protocol was applied 3 times with an interval of at least 20 min.

At the beginning of each experiment, baseline evoked potentials were assessed by averaging the response to stimulation (five sweeps at 40 s intervals), every 5 min over a period of 60 min. At this point, either HFS or wHFS was given, and three additional measurements at 5 min intervals were taken, followed by recordings at 15 min intervals for 24 h.

Histology

At the end of the electrophysiological study, brains were removed and histological verification of electrodes and cannula localization was carried out, as described previously (Goh and Manahan-Vaughan, 2013). Brain sections (16 μm) were stained according to the Nissl method using 1% toluidine blue and then examined using a light microscope. Data from animals, in which an incorrect electrode or cannula localization was found, were excluded from analysis.

Compartment Analysis of Temporal Activity by Fluorescence *in situ* Hybridization

We examined Arc mRNA expression in rats that received a systemic MK801 injection 4 weeks previously, compared to the Arc mRNA expression in rats that received a systemic vehicle injection. In a separate study, we analyzed Arc mRNA expression that is triggered by novel holeboard learning in rats that were treated with vehicle or MK801. Testing was conducted in a gray perspex box (40 × 40 × 50 cm) that contained a hole (5.5 cm diameter and 4.5 cm deep) in the floor of each corner of the box (Kemp and Manahan-Vaughan, 2008a). Typically, novel exploration of a novel empty holeboard facilitates LTP that lasts for at least 24 h in rats (Kemp and Manahan-Vaughan, 2008a), and is prevented by pharmacological antagonists that interfere with LTP (Kemp and Manahan-Vaughan, 2008b; Lemon and Manahan-Vaughan, 2012). Holeboard learning in the absence of afferent stimulation, is also impaired by the same antagonists (Hagena and Manahan-Vaughan, 2012). All animals were handled for 2 days before experiments. The rats participated in the following behavioral protocol: One day before the experiment, they were habituated for 1 h to a recording chamber. On the experimental day, they were acclimatized to the same chamber again for at least 60 min. Following this, the empty holeboard was inserted. After 6 min of exploration time, starting after the first direct hole exploration, brains were removed. The exploration time of each hole was counted separately.

On each experiment day, the holeboard experiments were performed for the same number of vehicle and MK801 injected rats. However, the animals that did not perform holeboard exploration were sacrificed on a different day than the rats that performed holeboard exploration. Thus, no direct statistical comparison between these two groups (Arc expression in the absence of learning vs. Arc expression following learning) was conducted.

After extraction, the brains were quick-frozen and stored at −80°C. The unfixed brains were cut coronally 20 μm thick on a Cryostat (Leica CM 3050S) at approximately −20°C, mounted directly on superfrost plus slides (Gerhard Menzel GmbH, Braunschweig, Germany) and stored at −80°C until further processing.

Compartment analysis of temporal activity by fluorescence *in situ* hybridization (catFISH) was conducted by adapting the procedure used by Guzowski and Worley (2001). The Arc cDNA was produced by Entelechon (Bad Abbach, Germany) according to the sequence of Lyford et al. (1995). Using the linearized DNA, the antisense RNA probe labeled with fluorescein at the uridine-5'-triphosphate site was created using the *in vitro* transcription kit Ambion MaxiScript Kit (Invitrogen, Carlsberg, USA). The length of the RNA probe was monitored by gel electrophoresis.

Following fixation with 4% paraformaldehyde (PFA), brain slices were deposited in a solution of acetic anhydride (Acros Organics, New Jersey, USA) with diethylpyrocarbonate water (C. Roth, Karlsruhe, Germany) and triethanolamine followed

by an acetone/methanol (1:1) solution for 5 min. Afterwards, the slides were set in a humid chamber that was prepared with a 50% deionized formamide solution (Sigma-Aldrich, St.Louis, USA). After covering with 1×-prehybridization buffer (Sigma-Aldrich, St.Louis, USA), they were incubated for 30 min.

For the hybridization step, for each glass slide 1 ng mRNA probe was dissolved in 1 μg 1×-hybridization buffer (Sigma-Aldrich, St.Louis, USA), heated at 90°C for 5 min and placed directly on ice. The slices were incubated with the Arc mRNA in a humidity chamber at 56°C overnight. Then, 10 μg/ml ribonuclease A (from bovine pancreas, Sigma-Aldrich) was added for 15 min at 37°C. To block endogenous peroxidase, the slices were pretreated with 3% H_2O_2 for 15 min, and in order to inhibit unspecific binding of proteins, a solution of 10% normal goat serum (n-Goat) was added for 60 min in the humid chamber. Anti-fluorescein-peroxidase (polyclonal sheep Fab-fragment, 11426346910, Roche Diagnostics, Mannheim, Germany) was applied in a dilution of 1:400 in 1% n-Goat for 2 h. After washing 3 times 5 min with tris-buffered saline (TBS)-Tween 20 (Polysorbate 20), a solution of biotinylated tyramine (bT), H_2O_2 (0.02%), and TBS was applied (1:1:100) for 30 min to enhance the fluorescence signal. Then, the slices were incubated in a dilution of StrepAvidin Cy5 (Dianova, Hamburg, Germany) 1:3000 with 1% bovine serum albumin (BSA, Sigma Aldrich, St. Louis, USA) in TBS-Tween for 30 min. In order to label the nuclei of the cells, 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsberg, USA) was added in a concentration of 1:10000.

For glutamic acid decarboxylase 2 (GAD67) staining, the slices were incubated in 10% n-Goat and 0.2% Triton X (Tx, Sigma Aldrich, St. Louis, USA) for 90 min on the next day. Afterwards, they were treated with the GAD67 antibody (AB) (1:100, monoclonal mouse AB, MAB-5406, Merck Millipore, Billerica, USA) and 1% n-Goat and 0.2% overnight. On the next day, the slices were incubated with Dylight-488 (1:250, Cy2-labeled goat-anti-mouse AB, 115-485-166, Dianova) and 1% n-Goat and 0.2% Tx and coverslipped with Vectorshield HardSet mounting medium for fluorescence (Vector Laboratories, Burlingame, USA).

As a dilution medium, saline-sodium citrate buffer were used until the anti-fluorescein step, followed by TBS. DAPI and the following procedures were conducted with phosphate buffered saline (PBS) as the dilution medium.

Immunohistochemistry

All animals were handled for 2 days before experiments. To arrest further brain degradation processes, the rats were perfused with 4% PFA and the extracted brains were stored and cryoprotected in PFA followed by sucrose (30%) for 5 days at 4°C. Serial coronal sections (30 μm thick) were prepared using a freezing microtome. To minimize errors, or variations in the processing of data sets, slices from vehicle and MK801-treated animals were processed together.

In order to reduce unspecific background staining, endogenous peroxidase was blocked by 0.3% H_2O_2 and then, endogenous biotin and electrostatic loadings of proteins were reduced by 20% avidin (Avidin-biotin blocking kit, Vector Laboratories, Burlingame, USA). Afterwards, the primary antibodies (AB) were applied in 20% biotin (Avidin-biotin blocking kit) overnight in their corresponding concentration: **GABA(A)** (1:400, monoclonal mouse AB, MAB341, Merck Millipore, Billerica, USA), **GABA(B)** (1:500, monoclonal mouse AB, ab55051, Abcam, Cambridge, UK), **GluN1** (1:400, monoclonal mouse AB, 556308, PharMingen, Becton, Dickinson and Company, Franklin Lakes, USA). **GluN2A** (1:750, polyclonal rabbit AB, sc-9056, Santa Cruz Biotechnology, Santa Cruz, USA), and **GluN2B** (1:200, polyclonal goat AB, sc-1469, Santa Cruz Biotechnology, Santa Cruz, USA), The secondary AB, rat-absorbed biotinylated horse-anti-mouse (BA-2001), goat-anti-rat (BA-1000), and horse-anti-goat antibodies (BA-9500) (all from Vector Laboratories, Burlingame, USA), were added 1:500 for 90 min. After applying the ABC-Elite detection system 1:1000 for 90 min, the reaction was visualized by incubation in 0.05% 3, 3'-Diaminobenzidine-solution (DAB, Sigma-Aldrich, St.Louis, USA) and 0.01% H_2O_2 for 10 min.

For staining of GABA(A), GABA(B) and GluN2B the dilution medium contained 10% normal serum (Vector Laboratories, Burlingame, USA) diluted in PBS containing Tx. For staining of GluN1 and GluN2A, 1% BSA in TBS containing Tx was used as the dilution medium.

To enhance the staining of GluN1 and GluN2A, the sections were left in the primary AB solutions for 5 days at 4°C. Following the secondary AB, the sections were enhanced with bT as described by Adams (1992). After mounting on gelatinized slides and dehydration, they were cover-slipped with DePeX (Serva, Heidelberg, Germany).

To verify antibody specificity, we conducted western blotting of GABA(A), GABA(B) and GluN1 receptors, and used an immunoprecipitation approach to assess GluN2A and GluN2B (Burry, 2000; **Figure 1**). Hippocampal tissue from 6–8 week old male Wistar rats was used. Hippocampi were homogenized at 4°C in Tris-HCI buffer (20 mM, pH 7.4) containing 10% sucrose. Homogenates were centrifuged at 4°C for 30 min with 14,000 g. Pellets were resuspended in ice-cold Tris-HCl buffer, pH 7.4 that contained cocktail of protease inhibitors (Sample buffer). The total protein concentration of each sample was determined using a Bradford protein assay.

For western blot experiments, 20 μg protein was applied to each gel. Gel electrophoresis was conducted using 8% sodium dodecyl sulfate (SDS) polyacrylamide gels run on a mini-gel apparatus (Pequlab), for 1.5 h at room temperature. The western blot transfer was conducted in cold transfer buffer (wet conditions) using a western blot transfer system (Consort) at 400 V, 250 mA for 1.5 h. Membranes were incubated for 1 h in TBS buffer (100 mM Tris-HCl; 0.9% NaCl, 1% Tween 20, pH 7.4) containing 5% non-fat dry milk to block nonspecific binding sites. Blots were subsequently incubated overnight at 4°C with antibodies that labeled GluN1 (1:750 dilution), Glun2A (1:250 dilution), GluN2B (1:500

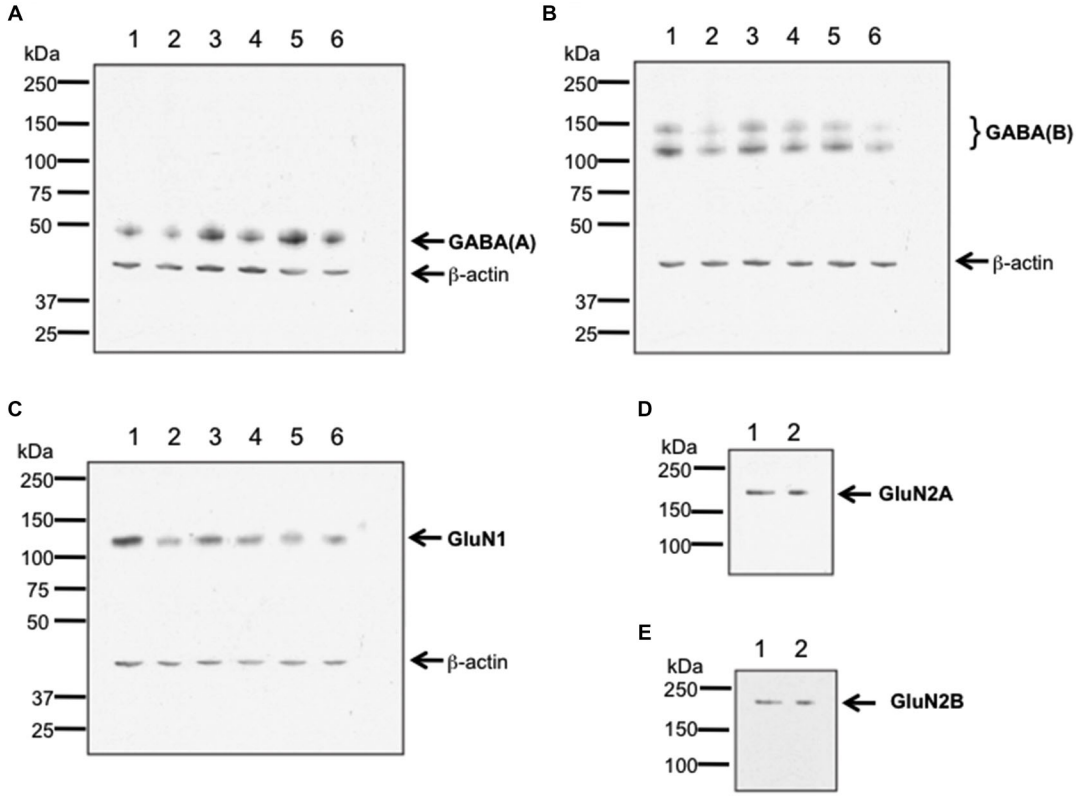

FIGURE 1 | Verification of antibody specificity. **(A–C)** Examples of western blots showing binding specificity of the antibodies used in the immunohistochemistry experiments: the GABA(A) antibody labeled a band of ca. 50 kDa **(A)** corresponding to the target receptor (Olsen and Tobin, 1990). Two bands were labeled by the GABA(B) receptor antibody **(B)**, that correspond to the GABA(B) receptor subtypes 1 and 2 (Kaupmann et al., 1998). The GluN1 antibody **(C)** labeled a ca. 120 kDa band, corresponding to the reported kDa weight of GluN1 (Riou et al., 2012). β-actin (42 kDa) was used as a protein-loading control. Hippocampal tissue was used for assessment. Each lane corresponds to a separate sample. **(D,E)** Examples of immunoblots showing binding specificity of GluN2A **(D)** and GluN2B **(E)**. The GluN2A and GluN2B antibodies labeled ca. 177 and ca. 178 kDa bands, respectively. These bands correspond to the reported kDa weights of the target receptors (Riou et al., 2012). Hippocampal tissue was used for assessment. Each lane corresponds to a separate sample.

dilution), GABA(A) (1:500 dilution), or GABA(B) (1:500 dilution). As a loading control, we used β-actin that was labeled using a monoclonal antibody (1:20000 Sigma, St. Louis, USA).

For immunoprecipitation experiments, a total of 200 μg of protein of tissue lysate was filled up to 400 μl of total volume with sample buffer and incubated with either anti-GluN2A or anti-GluN2B antibody. Immunocomplexes were captured by incubating overnight with protein A Sepharose beads at 4°C. Beads were washed three times in Sample buffer. Immunoprecipitated proteins were eluted with Laemmli buffer 2x and immunoblotted using the indicated primary antibodies (1:250 dilution).

For both western blots and immunoblots, immunoreactivity was revealed using HRP-coupled anti-rabbit or anti-goat secondary antibodies (1:10,000 dilution, GE Healthcare, Freiburg, Germany) and the enhanced ECL detection system (GE Healthcare, Freiburg, Germany). Quantification was done by densitometric analysis using the programs ScanJet (Hewlett Packard, Palo Alto, USA) and ImageJ 1.45S (Wayne Rasband, National Institutes of Health, USA, Java 1.6.0_20 (64 bit)). The lanes in **Figure 1** correspond to separate hippocampal samples.

Brain Slice Assessment

For both the *in situ* hybridization and immunohistochemistry, hippocampal brain areas were assessed at approximately −3.3 mm and −4.3 mm relative to bregma (**Figure 2**). For the *in situ* hybridization, we analyzed representative and randomly chosen small areas of the DG, the CA3 and the CA1 region. For the immunohistochemistry, we analyzed the whole area of the DG, the CA4, the CA2/3 region and the CA1 region. Additionally, for the immunohistochemistry, prefrontal areas at approximately 3.2 mm and 2.2 mm relative to bregma were used, corresponding to the medial prefrontal cortex. For both techniques, Nissl staining using 1% toluidine blue was made for surveillance of quality and spatial orientation. In addition, negative controls were prepared for supervision of specificity. For this purpose each primary antibody and each secondary antibody was used separately following the

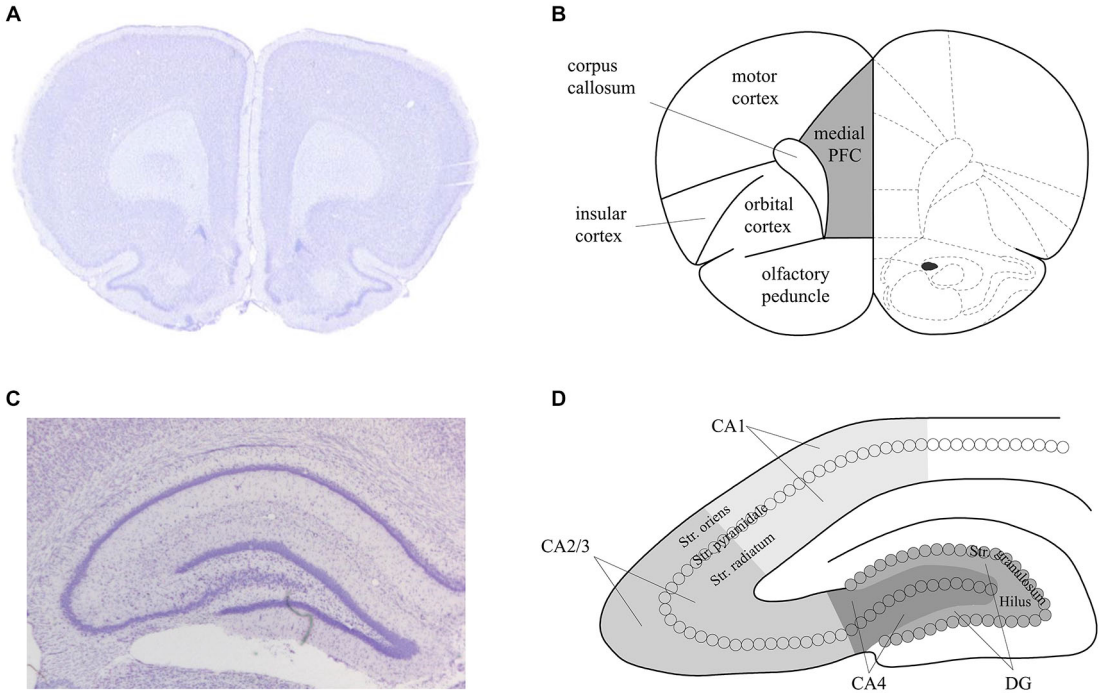

FIGURE 2 | Schema of the areas analyzed in the prefrontal cortex and hippocampus of the rat. Nissl stained sections of the prefrontal cortex (PFC) **(A)** and hippocampus **(C)** that correspond to the analysed areas of the medial PFC **(B)** and dorsal hippocampus **(D)** that were assessed in the present study. **Figure 1B** is based on Paxinos and Watson (1986).

staining protocols. No staining could be observed in these negative controls, indicating that the staining observed was specific.

To analyze the amount of receptor expression, we either counted the cells per mm^2 or we measured the optical density within the subregions of the hippocampus using the software ImageJ (densitometry of the DAB-labeled immunohistochemistry). To correct for differences in the optical density, we subtracted the optical density measured for background staining (within the corpus callosum) from the optical density measured within the subregions of the hippocampus, for each individual slice. This was done to address differences in staining intensity between sections.

Data Analysis and Statistics

In all electrophysiological experiments, data were expressed as mean % pre-injection values ± standard error of the mean. A mixed ANOVA with the repeated measures factor (TIME) and between-groups factor (GROUP) was used to evaluate differences in plasticity between control experiments and experiments after MK801, or vehicle application.

In the catFISH experiments, MK801 and vehicle-injected groups were compared. For each animal, we labeled three consecutive slices, and z-stacks were obtained from the CA1, CA3 and DG region of one hemisphere of each slice using a Zeiss ApoTome (63× magnification). Only cells were included where the entire nucleus was visible in the z-stack. The percentage of intranuclear Arc-positive cells within the hippocampal regions was analyzed. All GAD67-positive interneurons and glial cells were excluded from the analysis. To control for bias, measurements were carried out blind and additionally spot-checked by two additional individuals. A multifactorial one-way ANOVA using a between-group factor TREATMENT (vehicle vs. MK801) and a between-group factor REGION (PFC, DG, CA4, CA2/3 and CA1 for immunohistochemistry; DG, CA3 and CA1 for *in situ* hybridization) was used to analyze the immunohistochemistry or catFISH data. The catFISH data was furthermore separated according to the behavioral protocol (without holeboard exploration or after holeboard exploration). All data were shown as mean ± standard error of mean. Statistical analysis was performed using the SPSS software (version 19). The level of significance was set at $p < 0.05$.

Results

Hippocampal LTP in Behaving Rats is Impaired after MK801-Treatment

In line with previous reports (Manahan-Vaughan et al., 2008a,b; Wiescholleck and Manahan-Vaughan, 2012, 2013b), LTP that was induced by HFS (200 Hz) was significantly impaired in MK801-treated animals 1 and 4 weeks after treatment, compared to animals that received vehicle $F_{(1,6)} = 36.351$, $p < 0.005$, $n = 4$, **Figure 3**). Control animals expressed robust LTP that lasted for over 24 h, whereas MK801-animals showed an immediate

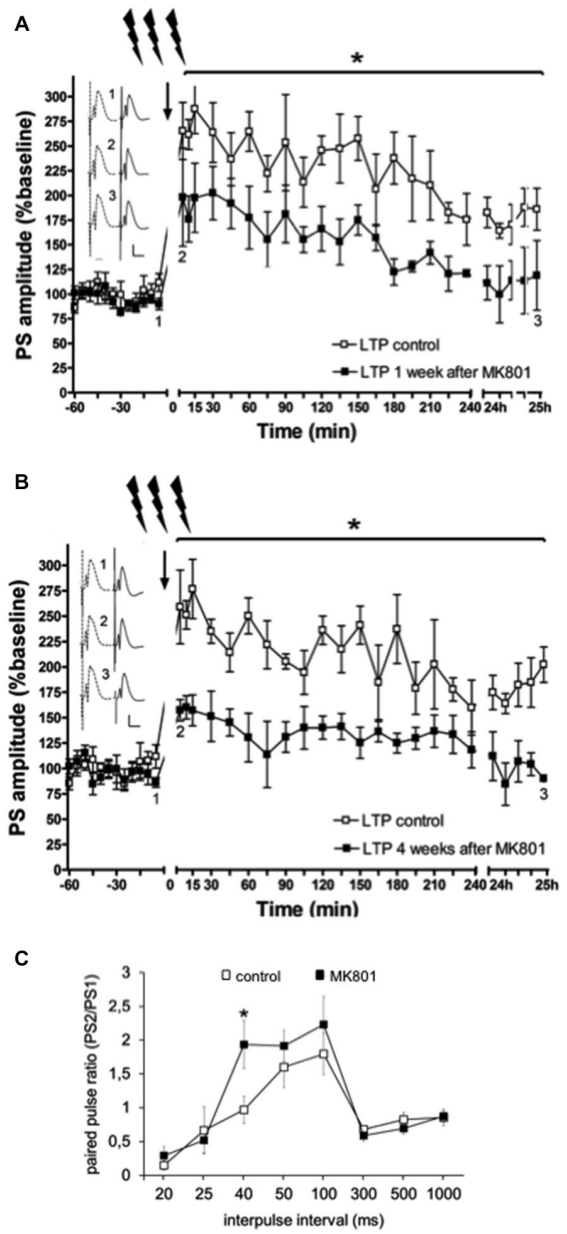

FIGURE 3 | Electrophysiological responses following MK801-treatment. (A) MK801-treatment impairs LTP. High frequency stimulation (HFS) elicits robust LTP that persists for over 24 h in animals that were treated 1 week **(A)**, or 4 weeks **(B)** previously with vehicle. In contrast, LTP is profoundly impaired 1 **(A)**, or 4 weeks **(B)** after MK801-treatment. Insets: Analogs (control—dashed line, MK801—black line) were obtained **1**, pre-HFS, **2**, 5 min post-HFS and **3**, 24 h post-HFS. Vertical scale-bar corresponds to 5 mV, horizontal scale-bar to 10 ms. *$p < 0.05$. **(C)** Paired-pulse (PP) responses are facilitated at the 40 ms interval after MK801-treatment. The graph shows PP responses at the interpulse intervals (IPI) of 20, 25, 40, 50, 100, 300, 500 ms and 1 s. The PP ratio was determined by dividing the amplitude of the second population spike (PS) in mV divided by the amplitude of the first PS (PS2/PS1). PP facilitation was evident at the 40 ms IPI after a single MK801-injection. Responses were unaffected at all other IPIs. *$p < 0.05$.

impairment of LTP after HFS. This suggests that a single treatment with MK801 is sufficient to cause prolonged effects on hippocampal function. In line with this, we have observed that hippocampus-dependent learning is also chronically impaired (Manahan-Vaughan et al., 2008a,b; Wiescholleck and Manahan-Vaughan, 2012, 2013b).

Neuronal Arc Expression is Enhanced in the Hippocampus Following Spatial Learning in Vehicle-Treated Rats. MK801-Treatment Increases Basal Arc Gene Expression in the Hippocampus, but does not Increase Arc Expression after Spatial Learning

The immediate early gene, Arc, is a very useful marker for activity-dependent neuronal activity. Arc gene transcription is rapidly induced by synaptic activity and precipitates in neuronal dendrites of non-GABAergic recently activated neurons (Link et al., 1995; Lyford et al., 1995). Furthermore, Arc is linked to learning and behavior, NMDAR-LTP, homeostatic scaling and structural plasticity (Korb and Finkbeiner, 2011). We therefore used compartment analysis of temporal activity by fluorescence *in situ* hybridization (catFISH) of Arc (Guzowski et al., 1999) to examine whether experience-dependent neuronal activity is altered following learning in the MK801-model.

We first assessed basal Arc gene expression in MK801-treated animals compared to vehicle-injected controls (**Figures 4A–C**, **5**). A multifactorial ANOVA showed, for the group without holeboard exploration, an effect for the factor TREATMENT ($F_{(1,24)} = 20.61$, $p < 0.001$) and the factor REGION ($F_{(2,24)} = 4.69$, $p < 0.05$). No interaction effect TREATMENT * REGION was observed ($F_{(2,24)} = 0.15$, $p = 0.86$). The *post hoc* test showed that 4 weeks after injection, MK801-treated animals ($n = 5$) exhibited significantly higher basal Arc expression in the CA1 region ($p < 0.01$, **Figure 4A**), the CA3 region ($p < 0.03$, **Figure 4B**) and the dentate gyrus ($p < 0.05$, **Figure 4C**) in the MK801-treated animals compared to vehicle-injected controls ($n = 5$). These findings suggest that hippocampal neurons of MK801-treated animals have lower excitability thresholds and may engage in redundant information encoding. Psychosis is characterized by an excessive processing of irrelevant stimuli as well as memory deficits (Anticevic and Corlett, 2012). We therefore asked the question if a learning event, that is known to promote the expression of LTP and result in long-term memory (Kemp and Manahan-Vaughan, 2004) results in increased Arc expression in MK801-animals.

We exposed the animals to a novel holeboard and then examined Arc-related expression in hippocampal neurons. After spatial learning the MK801 treatment did not result in increased Arc expression compared to vehicle injected animals (**Figures 4A–C**, **5**): A multifactorial ANOVA did not reveal an effect for the factor TREATMENT ($F_{(1,24)} = 2.30$, $p = 0.14$), the factor REGION ($F_{(2,24)} = 0.16$, $p = 0.85$) or the interaction TREATMENT * REGION ($F_{(2,24)} = 0.11$, $p = 0.90$).

To rule out that the differences in basal Arc expression observed can be explained by the behavior of the rats, we compared the rats' activity during exploration of the empty holeboard. We measured the time that each rat spent exploring the different holes, i.e., dipping their heads into one of the holes,

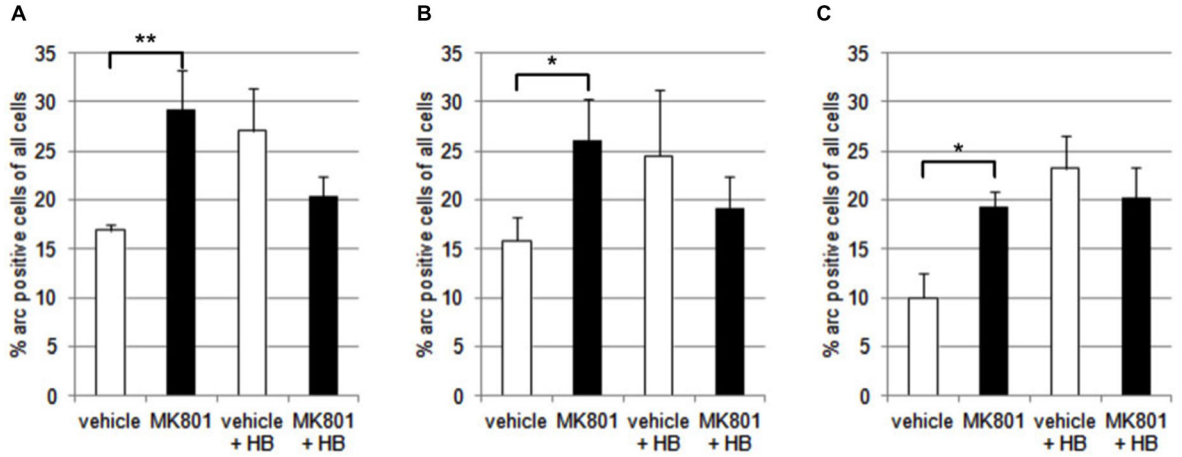

FIGURE 4 | Overview of Arc expression in naïve animals or following spatial learning. Bar charts summarize Arc expression in naïve animals that were treated with vehicle or with MK801 4 weeks previously. In the CA1 region **(A)**, the CA3 region **(B)** and dentate gyrus **(C)**, basal Arc expression is significantly increased in MK801-treated animals compared to vehicle-injected controls. In a separate animal cohort, novel spatial exploration resulted in significant elevations of neuronal Arc expression in the hippocampus of vehicle-treated animals compared to their naïve state. This was not the case for MK801-treated animals. Here, no difference was detected in Arc gene expression after novel spatial exploration. $^{*}p < 0.05$, $^{**}p < 0.01$.

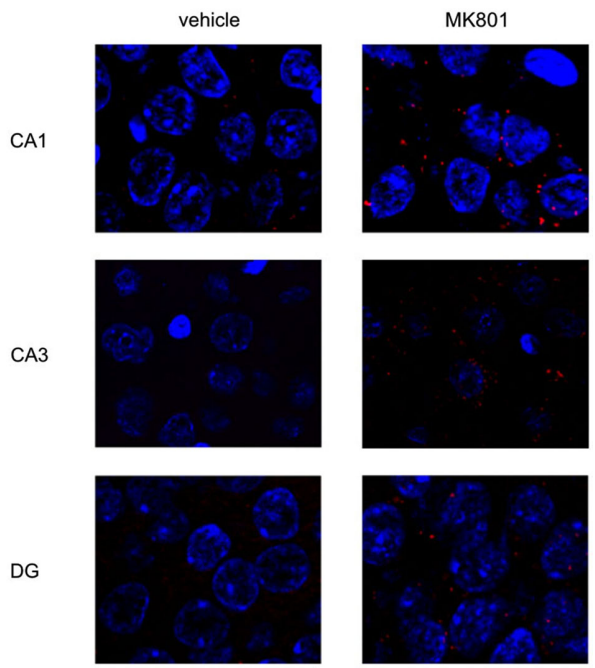

FIGURE 5 | MK801-treatment alters basal Arc gene expression in the hippocampus. Spatial learning elicits increases in Arc expression in control, but not MK801-treated animals. The images show Arc expression (red dots) in the CA1 and CA3 regions and dentate gyrus (DG), in naïve animals, after vehicle (left images) or MK801 injection (right images). MK801-treated animals exhibit a significantly higher basal Arc expression in the CA1 and CA3 regions compared to vehicle-treated animals. The nuclei were stained in blue using DAPI. The pictures were taken using a 63× objective.

within a 6 min period (not shown). The average exploration time of vehicle-treated animals ($n = 5$) was 10.57 ± 1.9 s, and of MK801-treated animals ($n = 5$) was 13.21 ± 1.9 s. No significant difference was evident between the groups (t-test: $p > 0.05$).

These findings suggest that the aberrantly enhanced expression of Arc in MK801-treated animals reflects intrinsic elevations in neuronal excitability thresholds that impair signal-to-noise ratios to such an extent, that a novel learning event cannot be effectively encoded. To clarify this, we examined PP responses.

Paired-Pulse Facilitation is Increased at the 40 ms Interval after MK801-Treatment

Neurotransmission was examined in control animals ($n = 12$) and in animals treated with MK801 ($n = 14$), by stimulating the perforant path with pulse pairs, of constant stimulus intensity, at inter-pulse intervals of 20, 25, 40, 50, 100, 300, 500 and 1000 ms. We found that MK801-treated animals ($n = 5$) showed a PP facilitation at the 40 ms interval compared to control rats ($F_{(1,24)} = 5.156$, $p < 0.05$, $n = 5$) **Figure 3C**). At all other inter-pulse intervals, there were no differences in PP response between control animals and those that were treated with MK801 prior to testing (20 ms: $F_{(1,24)} = 0.940$, n.s.; 25 ms: $F_{(1,24)} = 0.156$, n.s.; **40 ms**: $F_{(1,24)} = 5.156$, $\boldsymbol{p < 0.05}$; 50 ms. $F_{(1,24)} = 0.734$, n.s.; 100 ms: $F_{(1,24)} = 0.654$, n.s.; 300 ms: $F_{(1,24)} = 0.816$, n.s.; 500 ms: $F_{(1,24)} = 0.951$, n.s.; 1000 ms: $F_{(1,24)} = 0.334$, n.s).

This specific interval reflects neurotransmission mediated by NMDAR and GABA(B) receptors, and suggests that neurotransmission is enhanced at perforant path-dentate gyrus synapses of MK801-treated animals. Inhibitory tonus is regulated by GABA(A) and GABA(B) receptors (Mann et al., 2009). Thus, we explored whether expression of these receptors is altered after MK801-treatment. Given its role in memory and its proposed dysfunction in psychosis, we also examined expression in the prefrontal cortex.

GABA(A) Expression is Decreased in the Prefrontal Cortex, whereas GABA(B) Expression is Increased in Prefrontal Cortex and Dentate Gyrus after MK801-Treatment

GABA(A) expression was significantly decreased 4 weeks after MK801 treatment ($n = 5$) compared to vehicle-treated controls ($n = 5$, **Figures 6**, **7**) (multifactorial ANOVA: TREATMENT $F_{(1,40)} = 14.92$, $p < 0.001$, REGION $F_{(4,40)} = 80.48$, $p < 0.001$). Furthermore, a Duncan's *post hoc* test revealed that the prefrontal cortex was specifically affected (vehicle: 72.50 ± 1.47 vs. MK801: 61.07 ± 3.32; $p < 0.01$), whereas no significant changes were evident in the hippocampal subfields (**Figures 5**, **6**).

GABA(B) expression was significantly increased in MK801-treated animals ($n = 5$) compared to controls ($n = 5$, **Figures 6**, **7**) (multifactorial ANOVA: TREATMENT $F_{(1,40)} = 42.586$, $p < 0.001$, REGION $F_{(4,40)} = 555.14$, $p < 0.001$, interaction TREATMENT × REGION $F_{(4,40)} = 20.33$, $p < 0.001$). In this case, the increases in expression occur in both the prefrontal cortex (Duncan's *post hoc* test, vehicle: 375.25 ± 14.6 vs. MK801: 542.5 ± 28.79, $p < 0.001$) and the dentate gyrus (Duncan's *post hoc* test, vehicle: 73.11 ± 3.49 vs. MK801: 118.37 ± 9.99, $p < 0.01$) (**Figures 6**, **7**).

These findings suggest that the alterations in neuronal excitability, and dysfunctional information processing that we detected in MK801-treated animals results from alterations in GABA receptor expression in both the hippocampus and prefrontal cortex.

GluN2B, but not GluN2A or GluN1 Expression is Increased in the Dentate Gyrus 4 Weeks after MK801 Treatment

With respect to the expression of the NMDAR subunits, GluN1 and GluN2A, no statistically significant differences between vehicle and MK801-treatment could be observed in the prefrontal cortex or the different hippocampal subregions 4 weeks after treatment with either MK801 or vehicle (multifactorial ANOVA, n.s., **Figures 6**, **7**). However, GluN2B expression was significantly increased (multifactorial ANOVA, factor TREATMENT $F_{(1,40)} = 14.259$, $p < 0.001$, factor REGION $F_{(4,40)} = 13.55$, $p < 0.001$, **Figures 6**, **7**). These increases were specific to the dentate gyrus (Duncan's *post hoc* test, vehicle: 28.60 ± 2.11 vs. MK801: 38.13 ± 2.61, $p < 0.01$).

Discussion

The results of this study show that deficits in hippocampal LTP in the MK801-animal-model of psychosis are associated with elevations in neuronal excitability that are accompanied by changes in GABA-receptor expression in the hippocampus and prefrontal cortex, along with increased GluN2B-expression in the dentate gyrus. We propose that neuronal hyperexcitability in the hippocampus, coupled with an imbalance of GABAergic inhibitory control in the prefrontal cortex, results in an uncoupling of hippocampus-prefrontal cortex communication, and comprises a key aspect of hippocampal and memory dysfunction in psychosis.

Acute MK801-treatment results in transient behavioral changes that emulate a psychotic episode (Wöhrl et al., 2007; Manahan-Vaughan et al., 2008a). A severe LTP impairment is triggered in the dentate gyrus, that doesn't recover with time (Wiescholleck and Manahan-Vaughan, 2013b). In the present study, we observed that 4 weeks after MK801-treatment, LTP was still impaired. There are two possible explanations for this deficit: irreversible NMDAR-antagonism chronically disables NMDAR-dependent LTP, or neuronal excitability becomes persistently elevated, such that a further potentiation of synaptic efficacy by LTP is no longer possible. In the dentate gyrus, LTP is not exclusively NMDAR-dependent (Manahan-Vaughan et al., 1998), thus perpetual NMDAR-knock-down may not necessarily result in a complete failure of LTP. In contrast, it has been demonstrated that pushing synaptic potentiation to its upper limits by repeated afferent tetanisation, not only disables the ability of the affected hippocampal synapses to express LTP, but it also prevents new spatial learning (Moser et al., 1998; Moser and Moser, 1999). Given the fact that psychosis patients exhibit a loss of sensory (Javitt, 2009) and sensorimotor gating (Braff et al., 1978) and higher resting BOLD responses in multiple cortical areas (Medoff et al., 2001), we postulated that excitability levels may *increase* in the hippocampus following MK801-treatment, and explored if activity-dependent neuronal activity becomes altered.

Arc is expressed in an activity-dependent manner in response to hippocampus-dependent learning (Guzowski et al., 1999). It is transcribed at a low level under basal (naïve) conditions (Guzowski et al., 2001; Korb and Finkbeiner, 2011). Strikingly, we observed that basal Arc expression was significantly higher in MK801-treated animals compared to controls. In other words, neuronal activation is elevated in MK801-treated animals *even in the absence* of hippocampus-dependent learning. This suggests that the hippocampi of MK801-treated rats may be encoding stimuli and experiences that would normally be dismissed as irrelevant by healthy rats. This is an interesting possibility, as it is known that psychotic patients exhibit excessive processing of irrelevant stimuli that result on the one hand, in cognitive misrepresentations, and on the other hand, in memory deficits (Anticevic and Corlett, 2012). We therefore asked the question if novel spatial learning, that is known to cause a transient and activity-dependent elevation of Arc expression hippocampal neurons, and strengthens LTP (Kemp and Manahan-Vaughan, 2007), can trigger additional Arc expression in MK801-treated animals, that occurs in addition to the already enhanced Arc expression. Strikingly MK801-animals failed to show an increase compared to control animals after the same learning event. This suggests that the elevated levels of basal Arc expression interfere with the encoding of novel spatial experience. We postulate that this aberrantly enhanced expression of Arc in MK801-treated animals reflects intrinsic elevations in neuronal excitability thresholds that impair signal-to-noise ratios to such an extent, that a novel learning event cannot be effectively encoded.

In line with this, paired-pulse (PP) responses were enhanced at the 40 ms interval. In the PP paradigm, two stimuli are delivered with different intervals in the range of 20–1000 ms.

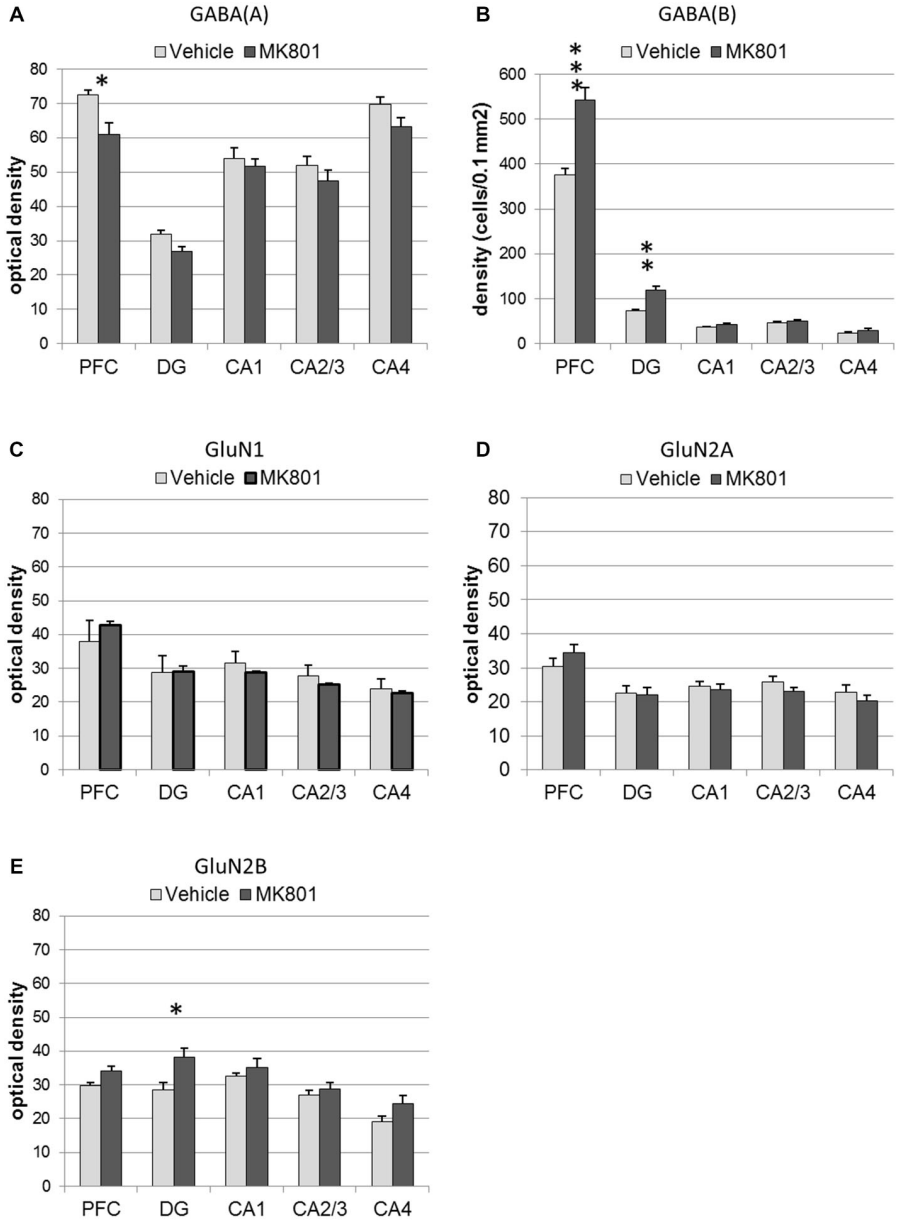

FIGURE 6 | MK801-treatment alters GABA receptor and N-methyl-D-aspartate receptor (NMDAR) subunit expression in the hippocampus and prefrontal cortex. Bar charts represent receptor expression in the hippocampal subregions (CA1, CA2/3, CA4, dentate gyrus:DG), and in the prefrontal cortex (PFC) 4 weeks after MK801-treatment (derived from densitometry of the DAB-labeled immunohistochemistry). In MK801-treated animals, **(A)** GABA(A) expression was reduced in the prefrontal cortex but unchanged in the hippocampus, whereas GABA(B) expression **(B)** was significantly increased in both the dentate gyrus and prefrontal cortex compared to vehicle-treated controls. Whereas GluN1 **(C)** and GluN2A **(D)** expression was unaffected by MK801-treatment, GluN2B expression **(E)** was significantly increased in the dentate gyrus compared to vehicle-treated controls $^*p < 0.05$.

The population spike (PS), which reflects the number and synchrony of granule cell discharges (Andersen et al., 1971), exhibits a depression of the 2nd PS at PP-intervals of 20–40 ms, followed by facilitation at PP-intervals of 40–200 ms, and depression at longer intervals. At PP-intervals of 20–40 ms, the PS-depression most likely occurs due to GABA(A)-activation, which in turn leads to an increase in chloride conductance (Albertson and Joy, 1987). At 40–200 ms stimulus intervals, GABA(B)-autoreceptors are active, thereby inhibiting GABA release. At the same time NMDAR are activated (McNaughton, 1982; Albertson and Joy, 1987). The enhanced facilitation we saw at the 40 ms PP-interval may thus relate to a disinhibition of principle cells. This could be triggered by changes in expression of GABA(B) -receptors, or of NMDAR.

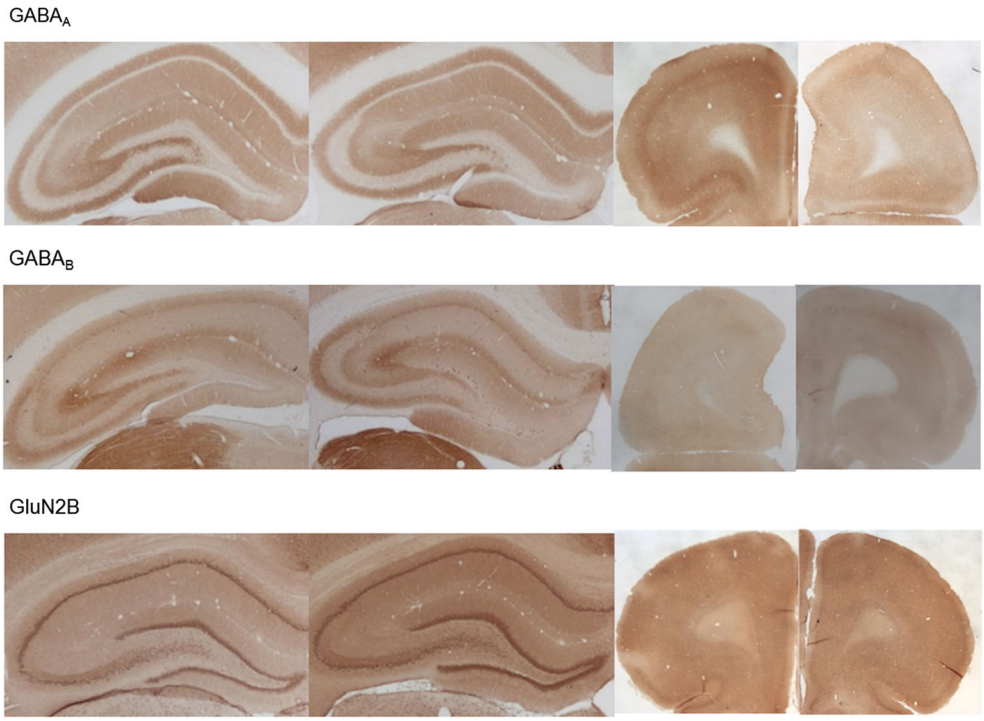

FIGURE 7 | Photomicrographs of expression of GABA(A), GABA(B) and GluN2B in the hippocampus and prefrontal cortex. Photomicrographs show examples of receptor expression in the hippocampus (left panels) and prefrontal cortex (PFC) (right panels) 4 weeks after MK801-treatment. In each case, the left panel for the respective subregion shows the control examples. Examples for GABA(A), GABA(B), and GluN2B are shown. Scale bar, hippocampus 1 mm, PFC 5 mm. The examples highlight the decrease of GABA(A)-receptor expression in the prefrontal cortex, the increase of GABA(B)-receptor expression in the dentate gyrus and prefrontal cortex and the increase of GluN2B expression in the dentate gyrus 4 weeks after MK801-treatment.

To explore this we examined the expression and distribution of GABA(A) and GABA(B)-receptors, as well as the GluN1, GluN2A and GluN2B subunits of the NMDAR in the hippocampus.

After MK801-treatment, GABA(B)-receptor and GluN2B expression was enhanced in the dentate gyrus. The CA-subfields of the hippocampus were unaffected. This suggests that increases in neuronal excitability in the dentate gyrus underlie the deficits in LTP we observed. It also explains why PP-responses were increased at the 40 ms interval. GABA(B)-receptors regulate basal inhibitory tonus (Mann et al., 2009), but in the dentate gyrus, they play a very important role in *stimulating* excitability of granule cells, by reducing synaptic inhibition that is mediated by hilar interneurons (Burgard and Sarvey, 1991; Mott and Lewis, 1991; Mott et al., 1993). This serves to promote transfer of incoming information from the entorhinal cortex, via the perforant path, to the hippocampus. Thus, an enhancement of GABA(B)-receptor expression in the dentate gyrus may not only result in neuronal hyperexcitability, it could also result in the processing of irrelevant information by the hippocampus, due to a weakening of gate control of entorhinal inputs. This alteration will be compounded by the increase in GluN2B expression that was specific to the dentate gyrus. GluN2B-containing NMDAR require intense postsynaptic depolarization to remove the voltage-dependent Mg^{2+}-block of the NMDAR and result in channel opening (Köhr et al., 2003; Erreger et al., 2005; Berberich et al., 2007), but when activated, they support twice as much charge-transfer (as GluN2A-containing NMDAR), deactivate slower and support a greater Ca^{2+}-influx per unit of current (Vicini et al., 1998; Sobczyk et al., 2005). An increase in GluN2B-expression will increase the ease with which incoming stimuli can result in LTP, but if this occurs on a background of enhanced neuronal excitability, it will not necessarily result in persistent or appropriate long-term encoding (Moser and Moser, 1999). Taken together, the enhanced expression of GABA(B)-receptors and GluN2B comprise a highly potent partnership, that can not only be expected to raise elevate neuronal excitability in the dentate gyrus, but mediate redundant synaptic information encoding, impaired LTP, and excessive encoding of experiences that would normally be ignored by the hippocampus. The alterations in dentate gyrus information processing can also be expected to impair information processing in the CA-subfields of the hippocampus (Bikbaev et al., 2008). In line with this, activity-dependent Arc expression was elevated throughout the hippocampus in MK801-treated animals, although increased expression of GABA(B) and GluN2B was localized to the dentate gyrus.

Both hypoactivation and hyperactivation of the prefrontal cortex may contribute to psychosis, and both, when mediated by changes in GABAergic inhibition, lead to attentional deficits (Pezze et al., 2014). Although it is unclear if NMDAR are affected in the prefrontal cortex of psychotic patients (Akbarian et al., 1996; Toro and Deakin, 2005), ketamine-induced psychosis alters metabolic response in this structure (Breier et al., 1997). Thus, we explored if GABA-receptors or GluN-subunits are altered in the prefrontal cortex. Here, we saw no change in GluN-subunits, whereas GABA(A) expression was decreased and GABA(B) expression was increased. These changes can be expected to cause a potent suppression of GABAergic transmission in the prefrontal cortex. Whereas a reduction in GABA(A)-expression under conditions of normal GABA tonus would be expected to result in increased neuronal excitability, the increase in GABA(B)-expression may counteract this to some extent, because basal GABAergic tonus is likely to be reduced (Mann et al., 2009). Nonetheless, it is possible that localized neuronal excitability is increased due to deficits in fast GABAergic transmission, mediated by GABA(A) (Nicoll et al., 1990), leading to aberrant information processing. The increased GABA(B)-expression can be expected to have far-reaching effects. This receptor is important for the modulation of network activity in the prefrontal cortex (Wang et al., 2010), and for the regulation of cortical upstates (Mann et al., 2009) that are essential for the synchronization of long-range cortical activity (Volgushev et al., 2006). Impairments in these processes can be expected to result in attentional and working memory deficits (Major and Tank, 2004), as occur in psychosis (Millan et al., 2012). We propose that the changed expression of GABA-receptors in the prefrontal cortex, could reflect an uncoupling of hippocampus-prefrontal cortex communication: the neuronal hyperexcitability within the hippocampus and associated enhanced output to the prefrontal cortex, will be met by decreased information processing and cortical informational relay that compounds the deficits in hippocampus-dependent long-term memory. These changes may be initiated in the hippocampus: intraperitoneal infusion of MK801 results in an acute enhancement of medial prefrontal cortex potentials that are evoked by hippocampal stimulation (Blot et al., 2015). Thus, alterations in neuronal excitability in the hippocampus may result in adaptive processes in the prefrontal cortex that comprise changed GABA-receptor expression and are aimed to return hippocampus-mediated elevations in excitability back to normal levels.

Conclusions

Increases in neuronal excitability in the hippocampus of schizophrenics, as indicated by elevations in regional cerebral blood flow, have been reported (Medoff et al., 2001). Furthermore, hypomagnesaemia causes neuronal excitability and leads to psychosis (Morris, 1992). Our data indicate that neuronal hyperexcitabillty in the hippocampus, together with a disruption of inhibitory control in the prefrontal cortex, contribute to the loss of LTP and may comprise a mechanism underlying impairments of learning that occur in this model of first-episode psychosis.

Author Contributions

Valentyna Dubovyk conducted the antibody specificity experiments (**Figure 1**), contributed to data analysis and presentation, and the writing of the paper and approved the final version. Valentina Wiescolleck conducted the elctrophysiology experiments described in **Figure 3**, contributed to data analysis and presentation, and the writing of the paper and approved the final version. Verena Aliane conducted the behavioral and *in situ* hybridization experiments described in **Figures 4**, **5**, contributed to data analysis and presentation, and the writing of the paper and approved the final version. Thomas Grüter conducted the immunohistochemical experiments described in **Figures 6**, **7**, and assisted in the behavioral and *in situ* hybridization experiments described in **Figures 4**, **5**. He also contributed to data analysis and presentation, and the writing of the paper and approved the final version. Denise Manahan-Vaughan designed the experimental approach, created the concept and the hypothesis for the study, supervised all experimental work, analysed the data, and was the main person to write the paper. All authors are accountable for the work described in this paper and worked together to ensure the integrity and reproducibility of the data described.

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GENERAL COMMENTARY
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A commentary on "Antipsychotic-induced Parkinsonism is associated with working memory deficits in schizophrenia-spectrum disorders"

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A commentary on

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In a recent study, Potvin et al. (2014) used a multivariate statistical approach to identify socio-demographic, neurological, drug induced extrapyramidal symptoms (EPS), and psychiatric predictors of cognitive impairments in patients with a DSM-IV diagnosis of schizophrenia (aged 18–60). Specifically, the study focused on antipsychotic-induced EPS, such as Parkinsonism which is characterized by hypokinesia, tremors, muscle rigidity, and/or bradykinesia (Tandon and Jibson, 2002). EPS were assessed with the Extrapyramidal Symptoms Rating Scale (ESRS; Chouinard and Margolese, 2005) and neurocognition was measured using the Cambridge Neuropsychological Test Automated Battery (CANTAB, Eclipse version 2.0; Elliott et al., 1998). The depressive and psychiatric symptoms of schizophrenia were evaluated using the Calgary Depression Scale for Schizophrenia (CDSS; Addington et al., 1992) and the Positive and Negative Syndrome Scale (PANSS; Kay et al., 1987).

Potvin et al. (2014) found an association between antipsychotic-induced Parkinsonism and working memory deficits in schizophrenia. Further, it was found that negative symptoms were associated with increased deficits in cognitive flexibility, working memory, planning, and visual paired association learning in schizophrenic patients (Elliott et al., 1998). Socio-demographic variables were also found to be significantly related to cognitive impairment in schizophrenia patients. These variables included the number of hospitalizations, age, gender, and education level (Potvin et al., 2014). Prior studies support these findings as it is known that lower SES are correlated with lower education levels and riskier health behaviors.

It should be noted, however, that while SES was shown by Shah et al. (2012) to be correlated with cognitive impairments in dementia patients and by Potvin et al. (2014) in schizophrenia patients, education or income variation alone may not be the reason why low SES is correlated with cognitive impairments in clinical patients. It could be the differing life choices, occupations and environments resulting in different levels of propensity across SES groups to experience life stresses and suffer from chronic or acute illnesses (Schurer et al., 2014). However, it cannot be discounted that SES factors could simply be comorbid factors with no causal or contributing

links to cognitive impairments in schizophrenia patients. Further research is needed to find more definitive causal links or at least diminish SES factors as confounding variables in understanding drug induced EPS in schizophrenia patients. These results were found using multiple hierarchical linear regression analyses which explored the associations between drug induced EPS and cognition, while taking into consideration the effect of socio-demographic and psychopathology variables.

The Potvin et al. (2014) results are in agreement with previous studies, suggesting a small-to-moderate association between negative symptoms (but not positive symptoms) and cognitive impairments in schizophrenia patient. These cognitive impairments included deficits in problem solving, attention, reasoning, speed of processing, working memory, and verbal/visual memory (Ventura et al., 2009).

Potvin et al. (2014) also posited that the number of hospitalization/duration of illness was found to be associated with cognitive impairments in schizophrenia patients. These results are supported by prior studies (Rajji and Mulsant, 2008; Shah et al., 2012). In the Potvin et al. study, males outperformed females across the three groups: schizophrenia patients ($N = 218$), unaffected first degree subjects ($N = 438$), and healthy non-relatives volunteers ($N = 123$), which in agreement to a cross-sectional study conducted by Torniainen et al. (2011).

Importantly, Potvin et al. (2014) found an association between antipsychotic-induced Parkinsonism and working memory deficits in schizophrenia patients. These are consistent with a study conducted by Jellinger (2010) on Parkinson's disease patients with no psychotic disorders, showing moderate working memory impairments. Further, Potvin et al.'s suggestion of an association between Parkinsonism and working memory impairments is supported by the literature. This is likely related to frontal lobe deficits in first-episode psychosis patients (Cuesta et al., 2014) and supported by imaging studies showing abnormal prefrontal dorsolateral cortex activations in schizophrenia patients while performing memory tasks (Van Snellenberg et al., 2006). Other studies have also reported a relationship between motor symptoms (e.g., akinesia or difficulty moving) and working memory in Parkinson's disease (Moustafa et al., 2014).

Potvin et al. (2014) study was naturalistic, that is, participants were not intentionally recruit based on increased levels of EPS or for specific symptoms (positive or negative). Rather, patients were recruited based on their clinical diagnosis, which makes the association of drug induced Parkinsonism and memory impairment in schizophrenia patients notable considering memory deficits are generally considered as an endophenotype of schizophrenia. While generally speaking schizophrenia endophenotypes can lead to occupational and social impairments, cognitive, specifically working memory, deficits, are one of the leading known causes of impairment in occupational and social functioning in schizophrenia patients (Bowie and Harvey, 2005; Barch et al., 2012).

Regardless of SES, prescribing antipsychotics is a common practice when treating for schizophrenia, even with the well-documented range of side effects, which include but is not limited to weight gain, hypotension, sedation, sexual dysfunctions, emotional instability, and EPS. Further, first-generation antipsychotics (FGAs), such as chlorpromazine and haloperidol, are more likely than second-generation antipsychotics (SGAs), such as Clozapine and Olanzapine, to produce EPS (Leucht et al., 2013). Additionally, a meta-analysis conducted by Leucht et al. (2013) on 15 different antipsychotics (including, Olanzapine, Clozapine, Risperidone, Chlorpromazine, Zotepine, Asenapine, and Haloperidol) had shown that different antipsychotics had varying effect sizes and ranges on patients' experience of EPS. Importantly, some studies found that first-generation (typical) antipsychotics, such as haloperidol, have higher affinity to D2 receptors and more associated with Parkinsonism than second-generation (atypical) antipsychotics, such as clozapine (Abi-Dargham and Laruelle, 2005; Joy et al., 2006; Juckel et al., 2006). It is also important to note that some studies found that some typical antipsychotics may similar effects to atypical antipsychotics on the brain and cognition and that some atypical antipsychotics are difference from each other, suggesting that the differences among these drugs can be related to their pharmokinetics, rather than being typical vs. atypical drug (Kapur et al., 1999).

As such, the results of Potvin et al. (2014) may be affected by lacking some experimental control in relation to the dosage and comparing prescribed antipsychotics. This could be rectified by calculating and substituting a chlorpromazine equivalent for participants in this study to rule out any confounding dosage and drug variations. Further, using unaffected relatives and healthy control group for comparative data of performance could have provided further insight on drug induced Parkinsonism in schizophrenia patients.

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Fronto-limbic novelty processing in acute psychosis: disrupted relationship with memory performance and potential implications for delusions

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Recent concepts have highlighted the role of the hippocampus and adjacent medial temporal lobe (MTL) in positive symptoms like delusions in schizophrenia. In healthy individuals, the MTL is critically involved in the detection and encoding of novel information. Here, we aimed to investigate whether dysfunctional novelty processing by the MTL might constitute a potential neural mechanism contributing to the pathophysiology of delusions, using functional magnetic resonance imaging (fMRI) in 16 unmedicated patients with paranoid schizophrenia and 20 age-matched healthy controls. All patients experienced positive symptoms at time of participation. Participants performed a visual target detection task with complex scene stimuli in which novel and familiar rare stimuli were presented randomly intermixed with a standard and a target picture. Presentation of novel relative to familiar images was associated with hippocampal activation in both patients and healthy controls, but only healthy controls showed a positive relationship between novelty-related hippocampal activation and recognition memory performance after 24 h. Patients, but not controls, showed a robust neural response in the orbitofrontal cortex (OFC) during presentation of novel stimuli. Functional connectivity analysis in the patients further revealed a novelty-related increase of functional connectivity of both the hippocampus and the OFC with the rostral anterior cingulate cortex (rACC) and the ventral striatum (VS). Notably, delusions correlated positively with the difference of the functional connectivity of the hippocampus vs. the OFC with the rACC. Taken together, our results suggest that alterations of fronto-limbic novelty processing may contribute to the pathophysiology of delusions in patients with acute psychosis.

Keywords: novelty, hippocampus, orbitofrontal cortex, rostral anterior cingulate cortex, ventral striatum, schizophrenia, psychosis, fMRI

Introduction

Schizophrenia is characterized by a combination of negative symptoms such as attention deficits, blunted affect, or anhedonia, and positive symptoms that include (auditory) hallucinations and bizarre delusions. While negative symptoms typically persist chronically, positive symptoms are pronounced during psychotic episodes that last for several weeks or months. Delusions, uncorrectable beliefs not shared by others, are a hallmark positive symptom of schizophrenia (Frith, 2005). A common form of delusions that has been classified as a first rank symptom of schizophrenia by Schneider are delusional perceptions, i.e., the delusional, often self-referential, interpretation of *a priori* unimportant stimuli. Patients who experience delusional perceptions typically attribute a direct personal relevance to such stimuli. More generally, altered salience attribution is such a characteristic feature of schizophrenia spectrum disorders that re-classification as a "salience syndrome" has been suggested during recent revision processes of DSM and ICD (van Os, 2009). From a cognitive neuroscience perspective, pathological salience attribution in schizophrenia has been suggested to reflect abnormal mismatches between expectancy and percept and could be considered as pathological prediction errors (Corlett et al., 2010).

An influential model of positive symptoms suggests that developmentally dysfunction of temporal lobe limbic structures leads to impaired interactions between the prefrontal cortex (PFC) and the mesolimbic dopaminergic system, resulting in a cortical dopamine deficit, but also in inadequate subcortical dopamine release. The hyperdopaminergic state in subcortical structures gives rise to a blunted signal-to-noise ratio in prediction error coding, leading to aberrant salience attribution to irrelevant events. Such aberrant salience attributions clinically present as psychotic symptoms such as hallucinations or delusions (Heinz, 2002; Kapur, 2003; Heinz and Schlagenhauf, 2010).

Multiple brain structures have been implicated in processing of salience, i.e., the propensity of a stimulus to attract attention. A "salience network" consisting primarily of the anterior insula and the dorsal anterior cingulate cortex (dACC; Seeley et al., 2007) enables switching between different large-scale neural networks involved in task-related (i.e., externally directed processes) or self-referential (i.e., internally directed) processes, respectively (Menon and Uddin, 2010; Ham et al., 2013). Dysfunction of this network in schizophrenia has been suggested to lead to aberrant salience attribution resulting in delusions and hallucinations (Kapur, 2003; White et al., 2010). Albeit not typically considered part of the salience network, a set of fronto-limbic brain structures has been shown to respond to behaviorally salient stimuli, most notably the ventral striatum (VS), the rostral anterior cingulate cortex (rACC), the adjacent medial prefrontal cortex (mPFC), and the orbitofrontal cortex (OFC). The VS has been implicated to the processing of reward by coding reward prediction and prediction errors in a dopamine-dependent manner (Knutson et al., 2001; Wittmann et al., 2005; Pessiglione et al., 2006; Schott et al., 2008). The OFC, in addition to responding to behavioral salience of reward stimuli, has been shown to code reward value (Sescousse et al., 2010), particularly in the lateral region (Rothkirch et al., 2012). In patients with schizophrenia, structural alterations of the OFC have been reported and linked to duration of the first psychotic episode (Malla et al., 2011). At a functional level, a region within the medial prefrontal cortex (mPFC) that extends into the OFC has been shown to exhibit an abnormal salience response during reward feedback processing, and the magnitude of this atypical mPFC/OFC response correlated with severity of positive symptoms (Schlagenhauf et al., 2009). It must be kept in mind that the fronto-limbic cortices (rACC, dACC, OFC, and mPFC) show considerable degree of functional specialization that is subject to ongoing investigation. While the OFC has been implicated in salience processing and representation of value (Kahnt et al., 2010; Sescousse et al., 2010; Rothkirch et al., 2012), more medial portions of the fronto-limbic complex have been specifically associated with personal preference (Ludwig et al., 2014). This may reflect the well-replicated observation that the adjacent rACC and ventral mPFC are involved in self-referential processing (Kelley et al., 2002; Qin and Northoff, 2011).

While most neurobiological models of schizophrenia have focused on dysfunctional interactions between the PFC and the mesolimbic dopaminergic system, structural anatomical investigations have repeatedly shown hippocampal alterations that are detectable already in newly diagnosed and unmedicated patients and progress with disease duration (Honea et al., 2005; Pujol et al., 2014). The hippocampus, along with adjacent medial temporal lobe (MTL) structures, plays a critical role in explicit memory and, compatibly, patients with schizophrenia commonly show memory deficits (Boyer et al., 2007; Ranganath et al., 2008). In the healthy brain, a prominent function of the hippocampus within its multifaceted contribution to explicit memory is the detection and rapid encoding of novel stimuli in their spatial and temporal context. Patients with schizophrenia have been shown to exhibit increased distractibility by novel stimuli (Cortiñas et al., 2008), and this phenomenon has been linked to increased attention shifting towards unexpected outcomes (Núñez Castellar et al., 2012). Converging evidence from human and animal studies highlights the behavioral salience of novel stimuli, and hippocampal novelty processing has been shown to trigger mesolimbic dopamine release from ventral tegmental area (VTA) neurons via a polysynaptic pathway that encompasses GABAergic neuronal populations in the VS/nucleus accumbens (NAcc) and the ventral pallidum (Lisman and Grace, 2005). Based on the well-studied dysfunction of the mesolimbic dopamine system in schizophrenia and the ability of the hippocampus to promote subcortical dopamine release, Lisman and colleagues proposed that chronic, dysfunctional hyperactivity of the hippocampus might contribute to the pathophysiology of positive symptoms in patients with schizophrenia (Lisman et al., 2008, 2010).

A fundamental limitation in the investigation of complex cognitive function in patients with schizophrenia is that most patients in clinical situations regularly take antipsychotic

medication that exerts profound influence on behavioral and neural measures of cognition. On the other hand, unmedicated acutely psychotic patients often have difficulties performing more complex experimental tasks. In the present functional magnetic resonance imaging (fMRI) study, we sought to circumvent this problem by using a simple target detection task (Bunzeck and Düzel, 2006) in which participants have to respond to a previously specified target image and view all other stimuli passively (**Figure 1**). We hypothesized that in patients the positive relationship between the hippocampal novelty response and successful memory encoding would be attenuated (Zierhut et al., 2010). Because all patients had positive symptoms at the time of participation, we further hypothesized that they would show increased novelty-related activation of brain regions involved in salience processing and motivation, namely the striatum and fronto-limbic structures like the rACC/mPFC or the OFC.

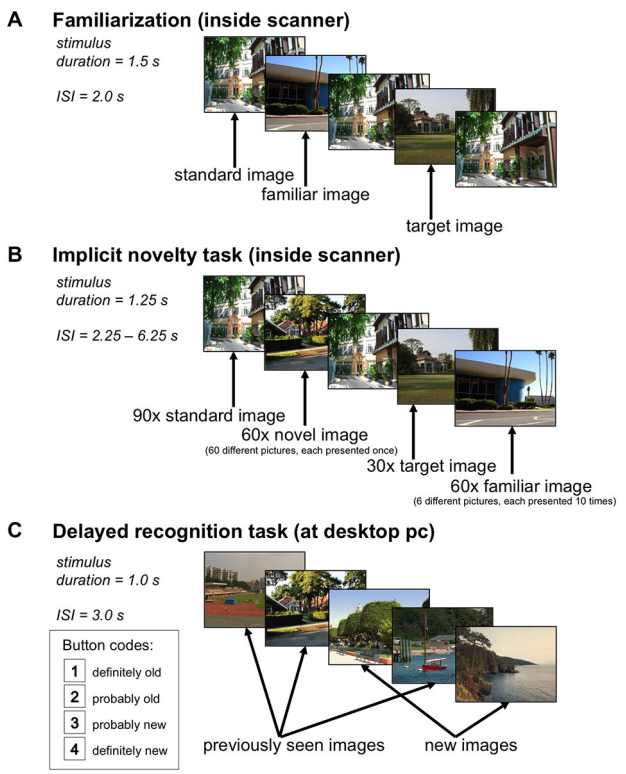

FIGURE 1 | Experimental Paradigm. **(A)** In the familiarization phase, a standard image and a target image were presented 66 times each, and six additional pictures were presented ten times each, the latter ones serving as rare familiar stimuli in the main experiment. **(B)** During the implicit novelty task, 60 novel images were presented randomly intermixed with 60 familiar images (each familiar stimulus was repeated 10 times), 90 repetitions of the standard image, and 30 repetitions of the target image. Participants were instructed to respond to the target image only. **(C)** 24 h after the functional magnetic resonance imaging (fMRI) experiment, participants underwent a delayed recognition memory task in which the 60 novel images from the main experiment were presented randomly intermixed with 60 previously unseen images, and participants were instructed to respond whether or not they had seen the image on the previous day, using a four-step confidence judgment.

Methods

Participants

Seventeen patients (13 male, 4 female) with acute psychosis were recruited from the Department of Psychiatry and Psychotherapy (Charité Campus Mitte and St. Hedwig Hospital), Charité Universitätsmedizin Berlin. All patients met ICD-10 and DSM IV diagnostic criteria of paranoid schizophrenia (ICD-10: F20.0) as assessed by at least one consultant psychiatrist. Exclusion criteria were history of neurological disorders, brain abnormalities in T1-weighted MRIs and co-morbid Axis I psychiatric disorders [Note: past depressive episodes were no exclusion criterion]. All patients were free of antipsychotic medication at the time of participation for at least four half-lives of their most recently used antipsychotic and had not used any centrally acting drugs (including benzodiazepines) for at least 12 h before participation. Psychopathological symptoms were assessed using the Scale for the Assessment of Positive Symptoms (SAPS; Andreasen, 1984a) and Scale for the Assessment of Negative Symptoms (SANS; Andreasen, 1984b). All patients exhibited positive symptoms such as delusions (mean score for "global delusions" 3.1 ± 1.0, see **Table 1**).

Twenty-four healthy control subjects (20 male, 4 female) matched for age, gender, handedness, educational level (depicted by educational years) and smoking habits were recruited by public postings at the university and via Internet advertisements. Exclusion criteria in healthy controls were lifetime history of any psychiatric or neurological disorder, systemic medical illness, use of any centrally acting or illicit drugs at the time of participation, or a family history of psychosis or bipolar disorder in first-degree relatives. Patients and healthy controls performed the Brief Assessment of Cognition in Schizophrenia (BACS; Keefe et al., 2004) to evaluate their cognitive performance in different domains like verbal memory, working memory, or processing speed.

One female patient and four male healthy controls were excluded from data analysis due to excessive head movement or technical problems during data acquisition, resulting in a final study cohort of 16 patients and 20 matched healthy controls.

Paradigm

We employed a modified version of a previously reported visual novelty paradigm (Schott et al., 2011), using the same stimulus material. **Figure 1** displays the experimental setup of the task. During acquisition of the anatomical MR image used for normalization (see below), participants underwent a familiarization phase in which they viewed a total of eight photographs of outdoor scenes on a back projection screen. A standard picture and a target picture were repeated 66 times. Six additional images were repeated 10 times in a pseudo-random Latin square order (**Figure 1A**) and served as familiar rare items in the main task. After the familiarization phase, participants were explicitly reminded which picture was the target.

The fMRI experiment consisted of a single scanning session. Novel and familiar stimuli (photographs of outdoor scenes) were

TABLE 1 | Demographic information.

	Patients	Controls	*p*
N	16	20	
Gender (m/f)	13/3	16/4	0.740 (X^2 test)
Age (± SD)	30.4 ± 6.9	31.5 ± 8.8	0.711 (*t*-test)
Educational level			
Below middle school	1	0	
Middle school	5	7	
High school (*Abitur*)	10	13	0.522 (X^2 test)
First language (German/other)	11/5	14/6	0.777 (X^2 test)
Handedness (r/l)	15/1	19/1	0.571 (X^2 test)
Smoking status (Fagerström scale 0–6)	1.38 ± 2.1	0.9 ± 1.9	0.204 (Mann-Whitney's U-test)
SAPS/SANS—total score	93.5 ± 23.6	-	
SAPS—total score	48.2 ± 16.2	-	
SANS—total score	45.2 ± 15.3	-	
Delusions Subscore	18.5 ± 5.3	-	
Global delusions item	3.1 ± 1.0	-	

SD, standard deviation; SAPS/SANS, Scale for Assessment of Positive/Negative Symptoms.

presented, randomly intermixed with a standard image and a target image (stimulus duration = 1.25 s), with an interstimulus interval (ISI) jittered between 2.25 s and 6.25 s using a near-exponential distribution, to optimize estimation of the blood oxygen level-dependent (BOLD) response (Hinrichs et al., 2000). A total of 240 picture stimuli were presented, including 90 repetitions of the standard image, 30 repetitions of the target image, 60 rare familiar scenes (the six additional pictures from the familiarization phase, each repeated 10 times), and 60 rare novel scenes (see **Figure 1B**). Participants were instructed to respond via button press whenever the target picture was presented, but just passively viewed all other images. The order of images was newly randomized across participants, as was the subset of novel targets, which consisted of 120 images, with the other half being used as distracters in the delayed recognition phase (see below).

Twenty-four hours after the fMRI experiment, participants performed a delayed recognition test (**Figure 1C**). The 60 novel targets from the fMRI experiment were presented again, randomly intermixed with 60 previously unseen photographs. Participants responded via mouse button whether or not they recognized the pictures from the previous day. False positive responses were explicitly discouraged.

Behavioral Data Analysis

The primary behavioral variable of interest was performance in the delayed recognition memory test. To obtain measures of memory performance that account for both hits and false alarms, we computed d' values for each subject (Stanislaw and Todorov, 1999). In three subjects (two controls, one patient), false alarm rates were 0 and were therefore adjusted by adding an error of 0.5/N (MacMillan and Kaplan, 1985). The resulting adjusted d' values were used for brain-behavior correlations (see below).

MRI Acquisition

MR images were acquired on a Siemens Sonata 1.5T MRI system (Siemens, Munich, Germany) using a standard head coil. 450 T2*-weighted echo-planar images [EPIs; TR = 2.0 s; TE = 35 ms; 35 axial slices (64 × 64 in-plane resolution); voxel size = 3.5 × 3.5 × 3.5 mm] were acquired in an ascending order (from bottom to top). Six volumes were acquired at the beginning of each run to allow for magnetic field stabilization and discarded from analysis. A co-planar T1-weighted MPRAGE image was acquired before the functional session and used for optimized normalization (see below).

Functional MRI Data Processing and Analysis

Data were analyzed using Statistical Parametric Mapping (SPM8, Wellcome Trust Center for Neuroimaging, London, UK). EPIs were corrected for acquisition delay and head motion. The subjects' individual T1-weighted MPRAGE image was then coregistered to the mean EPI and segmented using the segmentation algorithm implemented in SPM8. EPIs were then normalized into a common stereotactic reference frame (ICBM)[1] using the normalization parameters obtained from segmentation of the MPRAGE image [voxel size: 3 × 3 × 3 mm]. Normalized EPIs were smoothed using a Gaussian kernel [FWHM = 8 × 8 × 8 mm]. A high pass filter with a cut off frequency of 128 s was applied to the data.

Statistical analysis was performed in a two-stage Mixed Effects model. In the first stage, neural activity was modeled by a delta function at stimulus onset. Event-related BOLD responses were modeled by convolving these delta functions with a canonical hemodynamic response function (HRF). The resulting time courses formed the regressors of interest (novel and familiar target stimuli, standard picture) in a General Linear Model (GLM). The six rigid-body movement parameters determined from motion correction were included as covariates of no interest, plus a single constant representing the mean over scans. GLM parameters were estimated using a restricted maximum likelihood (ReML) fit. To assess the interaction

[1]http://loni.usc.edu/ICBM/

between novelty and diagnosis, single subjects' contrast images (novel vs. familiar) were submitted to a second level random effects analysis with diagnosis as fixed factor and age as covariate. Regions of interest (ROIs) were defined anatomically for the hippocampus (based on a probabilistic localization of the CA regions and the subiculum; SPM Anatomy Toolbox; Eickhoff et al., 2005) and the striatum (anatomical automated labeling, AAL; WFU Pickatlas, Wake Forest University) and by a combined anatomical and literature-based probabilistic approach (Schubert et al., 2008; Zweynert et al., 2011) for the OFC, the rACC and the VS (the Matlab script for ROI generation and the full coordinate lists are available from the authors upon request). The significance threshold was set to $p < 0.05$, small-volume-corrected for family-wise error (FWE) within the respective ROIs. For illustrative purposes only, figures display activations at $p < 0.005$, uncorrected. Peak activations (contrasts of parameter estimates) of significant between-group differences in the hippocampus were extracted and submitted to *post hoc* correlation analyses with memory performance (adjusted d' values) using robust Shepherd's pi correlations (Schwarzkopf et al., 2012; see Section Results).

Functional Connectivity Analysis

In order to assess alterations in functional connectivity of the hippocampus and OFC during novelty processing in psychotic patients relative to healthy controls, we employed the psychophysiological interaction approach (PPI; Friston et al., 1997). PPI is defined as the change in contribution of one brain area to another with experimental or psychological context (Friston et al., 1997; Gitelman et al., 2003). Based on the critical role of the hippocampus in novelty processing and on the pronounced novelty response of the OFC in the patient group (see Section Results) we used the hippocampus and OFC as seed regions. At the single subject level, separate PPI models were computed. For each participant, the first eigenvariate time series from a sphere seeded around the voxel with the highest variance explanation within the hippocampus and OFC ROIs, respectively, were extracted and deconvolved with the canonical HRF. This combined anatomical and functional definition of the seed regions was chosen to achieve a reasonable tradeoff between anatomical specificity and signal-to-noise ratio. The resulting time series were convolved with the psychological function of novelty (novel vs. familiar rare images) and subsequently reconvolved with the HRF, yielding the new variables X, which were entered as primary covariates of interest into new GLMs. The original BOLD eigenvariates and the psychological variable P (novel vs. familiar) convolved with the HRF formed further covariates in the GLM design matrices. We also included the regressors of the standard and target pictures and the six movement parameters determined as covariates of no interest, plus a constant representing the mean over scans. At second level, a two-way between-subjects ANOVA (patients vs. controls × hippocampal vs. orbitofrontal seed region) with age and smoking status (Fagerström score) as covariates was computed. A small-volume FWE-corrected significance level of 0.05 was applied, correcting for combined anatomical and probabilistic ROIs of the rACC and of the VS/NAcc. In the patients, peak contrasts of parameter estimates of significant between-group differences in the rACC/mPFC were extracted and submitted to *post hoc* correlation analyses with SAPS global delusion scores using robust Shepherd's pi correlations (Schwarzkopf et al., 2012; see Section Results).

Results

Behavioral Results

Table 2 displays the descriptive statistics of the target detection task and of the delayed memory task in patients and controls. During the fMRI experiment, all participants performed the target detection task with high accuracy, with no significant difference in hit rates between patients and controls ($Z = 0.36$, $p = 0.359$; two-sample Mann-Whitney U-test). Patients, however, showed a slightly higher false alarm rate ($Z = -1.77$, $p = 0.038$; two-sample Mann-Whitney U-test) and a trend for longer reaction times ($T_{30} = -1.74$, $p = 0.046$, one-tailed) [*Note*: Due to technical difficulties, behavioral data from the fMRI sessions were not available in two controls and two patients, and those subjects were excluded from these behavioral data analysis].

In the delayed memory task, both groups exhibited above-chance recognition performance, with hit rates being significantly higher than false alarm rates in both groups (controls: $T_{18} = 4.91$, $p < 0.001$; patients: $T_{15} = 3.74$, $p = 0.001$). There were no significant between-group differences in adjusted d' values ($T_{34} = 0.19$, $p = 0.850$) [*Note*: Corrected hit rates (hits—false alarms) were significantly higher in the control group; $T_{34} = 2.12$, $p = 0.041$, two-tailed].

Hippocampal Novelty Processing in Patients and Controls

Both, healthy controls and patients exhibited a robust response of the right hippocampus to novel as compared to familiar rare pictures ($p_{FWE} = 0.006$), and there was no significant difference in novelty-related hippocampal activation between patients and controls (**Figure 2A**). We also observed a trendwise activation of the left hippocampus to novel vs. familiar rare pictures in both patients and controls ([xyz] = [−30 −28 −17]; $p_{FWE} = 0.051$).

TABLE 2 | Descriptive statistics of the behavioral results.

	Patients	Controls
Target detection task (fMRI)		
Hits	0.983 ± 0.031	0.976 ± 0.061
RT hits	607 ± 92	565 ± 43
False alarms	0.008 ± 0.011	0.006 ± 0.021
Delayed memory task		
Hits	0.23 ± 0.19	0.38 ± 0.19
False alarms	0.17 ± 0.19	0.24 ± 0.14
Adjusted d'	0.407 ± 0.423	0.438 ± 0.512

RT, reaction time. RTs for false alarms are not reported due to low numbers. The d' values were adjusted for false alarm rates of 0 in two controls and one patient.

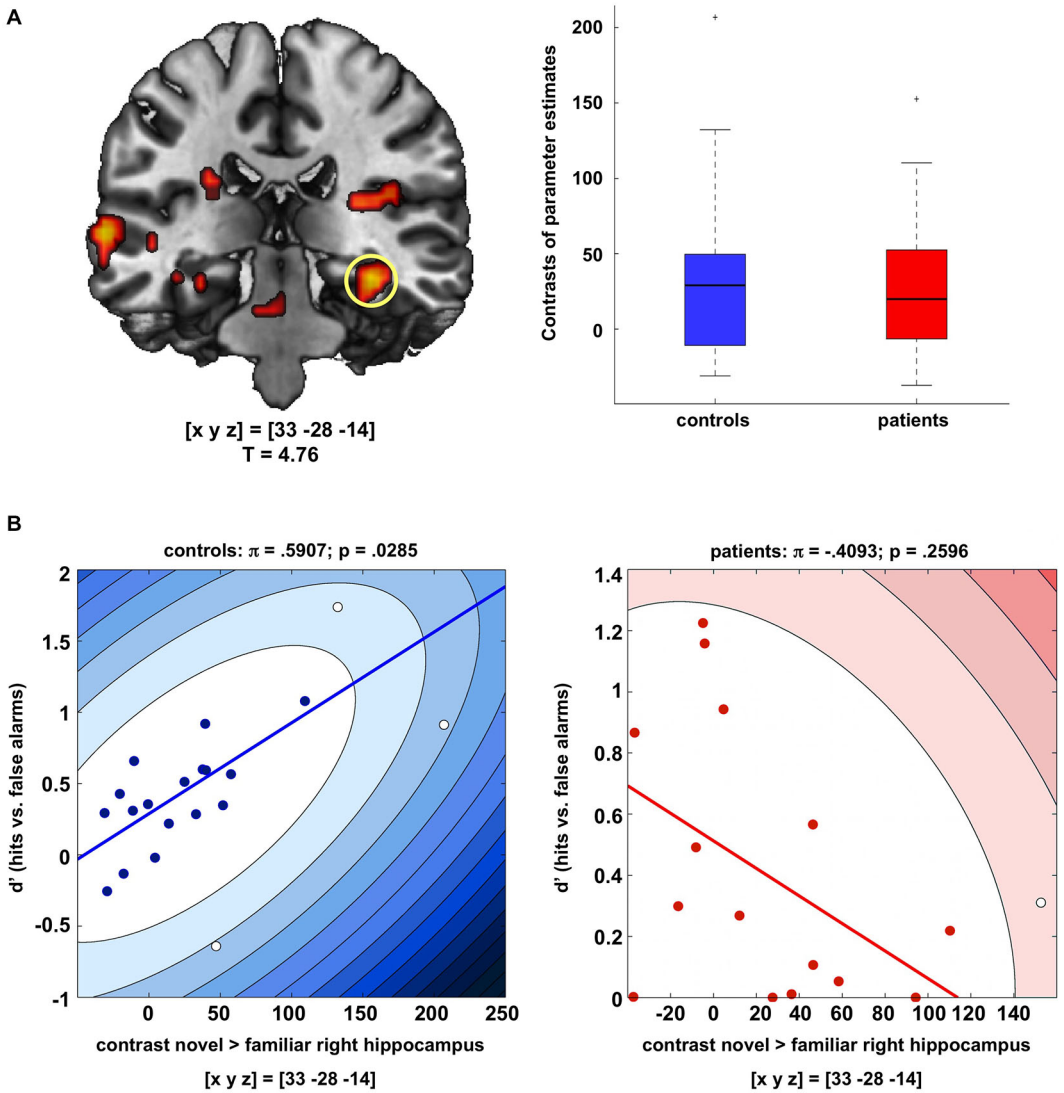

FIGURE 2 | Hippocampal novelty processing in patients and controls. **(A)** Both patients and healthy controls exhibited reliable activation of the right and, to a lesser extent, also of the left hippocampus during presentation of novel as compared to familiar rare images ($p < 0.05$; FWE-corrected for anatomical hippocampus ROI). Bar plots depict contrasts of parameter estimates at peak voxels +/− standard errors. Activations are shown at $p < 0.005$, uncorrected, for illustrative purpose only. **(B)** In the healthy controls, hippocampal activation to novel images was positively correlated with successful recognition of the images after 24 h (d' values, adjusted in participants with false alarm rates of 0), whereas no significant correlation was observed in the patients. Scatter plots depict Shepherd's *pi* correlations, separately for controls (left) and patients (right).

To assess the relationship between hippocampal activity during novelty detection and delayed memory performance, correlations were computed between the peak activation in the right hippocampus to novel vs. familiar pictures and recognition performance in the delayed memory test (d' values, adjusted for false alarm rates of 0 in two controls and one patient), separately for patients and controls. Because brain-behavior correlations have been criticized for their sensitivity to outliers (Rousselet and Pernet, 2012; Schwarzkopf et al., 2012), we employed robust Shepherd's *pi* correlations, in which outliers are first excluded based on the bootstrapped Mahalnobis distance, followed by a non-parametric Spearman correlation (Schwarzkopf et al., 2012). Controls exhibited a moderately strong positive correlation between novelty-related hippocampal activation and delayed recognition performance ($\pi = 0.591$, $p = 0.0285$, two-tailed; **Figure 2B**, left panel). In the patients, the correlation between hippocampal novelty responses and d' values was not statistically significant and nominally negative ($\pi = -0.409$, $p = 0.2596$, two-tailed; **Figure 2B**, right panel). A direct comparison of the correlation coefficients of controls vs. patients using Fisher's Z test yielded a highly significant difference ($Z = 2.83$; $p = 0.0047$, two-tailed) [*Note*: When using Spearman's correlations without outlier detection, this pattern remained qualitatively unchanged (controls: $\rho = 0.559$, $p = 0.0103$; patients: $\rho = -0.339$, $p = 0.1990$)].

Orbitofrontal Novelty Response in Patients with Schizophrenia

In a direct comparison of patients' and controls' novelty contrasts, patients showed a robust fMRI response in the right OFC to novel vs. familiar rare pictures that was absent in healthy controls (**Figure 3**). Similar to the novelty response in the novelty-related hippocampus, novelty-related OFC activation in patients did not significantly correlate with delayed recognition memory performance (adjusted d' values; $\pi = 0.1254$, $p = 1.0$).

Novelty-Related Hippocampal and Orbitofrontal Functional Connectivity

To further investigate potential neural networks underlying the OFC novelty response in the patients, we computed a functional connectivity analysis using the PPI approach with the hippocampus and OFC as seed regions and novelty vs. familiarity as psychological variable (see Section Methods for details). While there was no direct novelty-related functional connectivity increase between the hippocampus and OFC in the patients, both seed regions exhibited increased novelty-related functional connectivity with the rACC in patients when compared to healthy controls ($p = 0.015$, FWE-corrected for ROI volume; 2×2 random effects ANOVA model, T-test-based comparison of hippocampal and OFC PPI contrasts; see **Figure 4A**). This finding suggests that the rACC might function as a hub linking hippocampal and OFC novelty responses in the patients. To test whether this increased functional connectivity of the hippocampus and OFC with the rACC might be related to delusions, we computed Shepherd's *pi* correlations of the sum and the difference of their contrasts of parameter estimates and the global delusions subscale of the SAPS. While there was no significant correlation of the sum, the difference (hippocampal—orbitofrontal connectivity to the rACC), was positively correlated with the global delusions score in the patients ($\pi = 0.661$; $p = 0.0290$, two-tailed, Bonferroni-corrected; see **Figure 4B**).

A significant connectivity increase of both the hippocampus and the OFC in the patients was observed in the VS/nucleus accumbens (small-volume FWE-corrected $p = 0.033$; see **Figure 4A**, bottom). However, this functional connectivity increase did not correlate with global delusion scores (all $p > 0.290$).

Discussion

Our results provide further evidence for hippocampal dysfunction in schizophrenia, as evident from the absent relationship between the hippocampal novelty response and long-term memory performance. Moreover, patients exhibited a novelty-related OFC activation that was absent in the controls. Functional connectivity results further suggest that acute psychotic states might be accompanied by an processing of novel stimuli in fronto-limbic structures.

Disrupted Relationship Between Hippocampal Novelty Processing and Memory Performance in Schizophrenia

Patients, like healthy controls, exhibited a robust hippocampal response to novel vs. familiar stimuli, suggesting a preserved hippocampal novelty response in acute psychosis. The relationship between novelty-related hippocampal activation and successful memory encoding was, however, disrupted in the patients. While the hippocampal novelty response was positively correlated with later recognition memory performance in the control cohort, no such relationship was observed in patients. Several previous studies in healthy participants (Hariri et al., 2003; Bertolino et al., 2006; Schott et al., 2011, 2014; Barman et al., 2014) and neurological patient

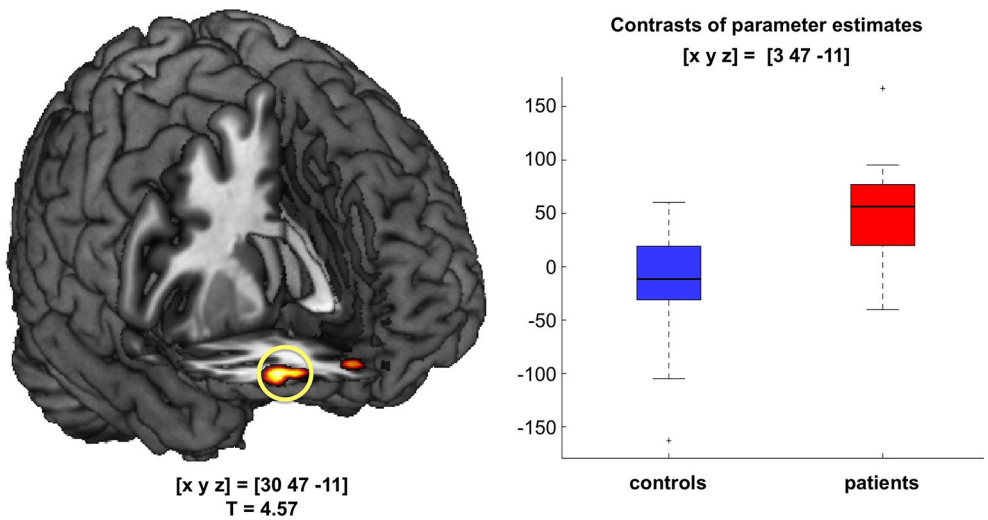

FIGURE 3 | Orbitofrontal novelty processing in patients with acute psychosis. During presentation of novel images, patients, but not controls, exhibited robust activation of the right orbitofrontal cortex (OFC; $p < 0.05$; FWE-corrected for a combined anatomical and literature-based ROI of the OFC). Bar plots depict contrasts of parameter estimates at peak voxels +/− standard errors.

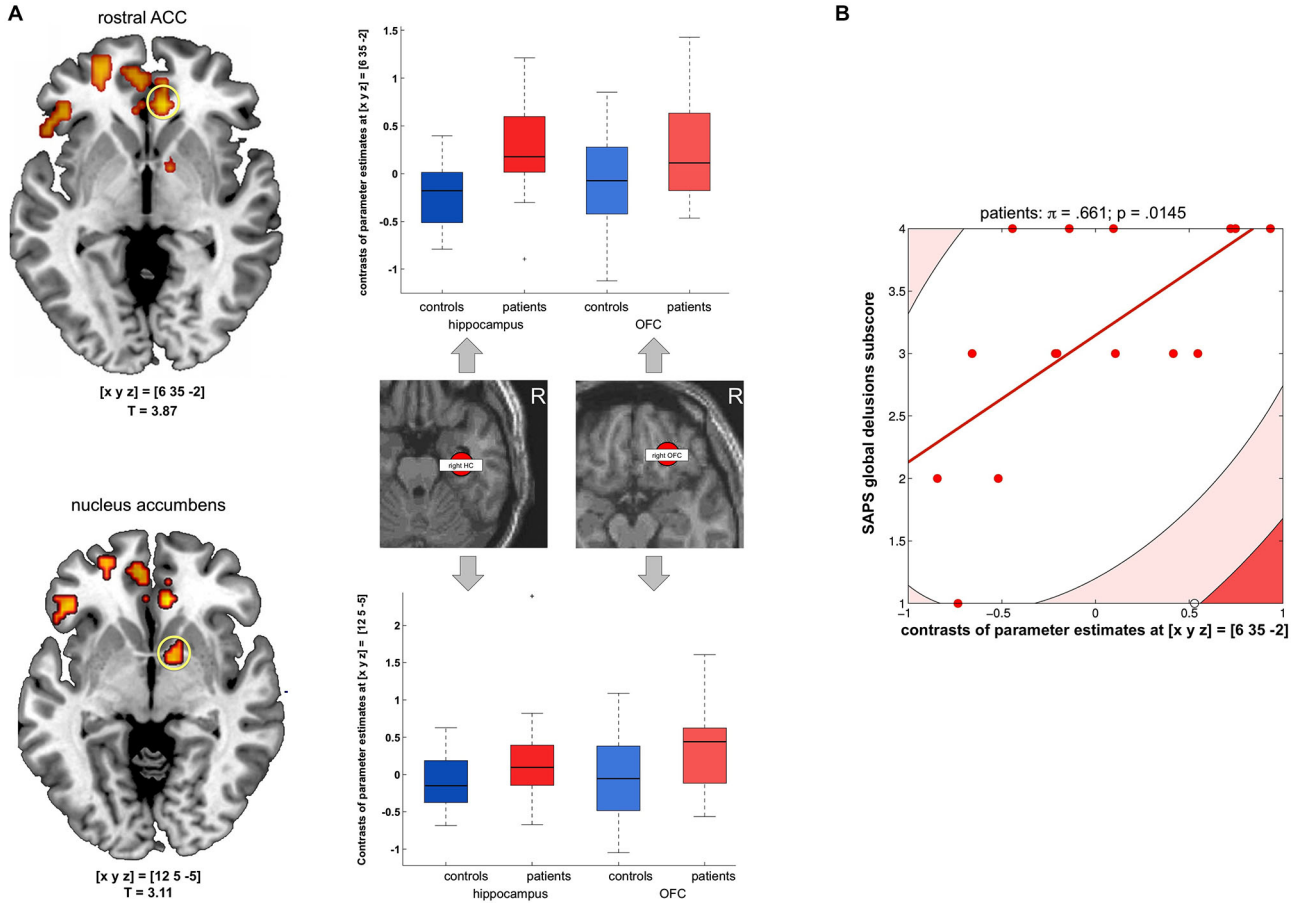

FIGURE 4 | Functional connectivity of the hippocampus and OFC to the rACC. **(A)** Left panel: Patients exhibited stronger novelty-related functional connectivity from both the hippocampus and OFC to the rostral anterior cingulate cortex (rACC, top) and to the nucleus accumbens (NAcc, bottom); activations are displayed at $p < 0.005$, uncorrected for illustrative purposes only. Right panel: Box plots display contrasts of parameter estimates of novelty-related functional connectivity (median +/− 25 percent quantiles, separated by group and anatomical seed region) to the rACC (top) and NAcc (bottom). All $p < 0.05$, small-volume FWE-corrected for a combined anatomical and probabilistic rACC ROI (Holtmann et al., 2013). **(B)** Scatter plot depicting Shepherd's *pi* correlation of the difference between hippocampal and orbitofrontal connectivity (contrasts of parameter estimates) and global delusion subscores of the scale for the assessment of positive symptoms (SAPS) (applicable to patients only). The correlation survived Bonferroni correction for the two correlations computed (sum and difference of the contrasts of parameter estimates reflecting hippocampal and orbitofrontal connectivity to the rACC).

populations (Oedekoven et al., 2014; Hulst et al., 2015) have suggested a positive relationship between fMRI activation of the hippocampus during novelty processing or encoding and memory performance. In patients with schizophrenia, however, that relationship appears to be disrupted. For example, patients with schizophrenia exhibit a modulation of hippocampal activity by level of processing (LoP), but that hippocampal activation in the patients does not predict memory performance (Ragland et al., 2006a,b; Zierhut et al., 2010). Our results expand those observations by showing, similar to LoP, stimulus novelty does elicit a hippocampal neural response, but this response is not associated with successful encoding, and the present data therefore suggest a disruption of the relationship between hippocampal novelty responses and memory formation in patients with acute psychosis. While the lack of such a correlation in the patients may not be considered a deficit *per se*, it might nevertheless provide further—at least indirect—evidence for the previously suggested role of the hippocampus in the pathophysiology of schizophrenia. As discussed by Lisman et al. (2008, 2010), hippocampal pyramidal cells are most likely overactive in patients with schizophrenia. While tonically increased activity of the hippocampus might not be readily detectable by means of activation-based fMRI, it is conceivable that a decreased signal-to-noise ratio resulting from such increased tonic activity may attenuate the relationship between hippocampal BOLD signal and successful memory formation. In a recent high-field fMRI study, it could be demonstrated that, in healthy humans, hippocampal input structures (dentate gyrus, CA3, apical CA1) primarily respond to novelty while successful encoding has been associated with activation of output structures (pyramidal CA1, subiculum; Maass et al., 2014). Notably, computational

models of MTL pathology suggest that deficits in recall-based and particularly in context-specific memory processes in patients with schizophrenia might result from the disruption of intra-MTL connectivity (Talamini et al., 2005; Talamini and Meeter, 2009). In the present study, a 1.5T MR tomograph was employed, and the spatial resolution of our EPIs did not allow for a differential investigation of hippocampal subfields. We can therefore only tentatively suggest that in unmedicated patients with schizophrenia there might exhibit a disruption of intra-hippocampal functional connectivity at the level of either hippocampal output regions or intra-hippocampal neural circuitry.

A recent influential model suggests that chronic disinhibition of hippocampal pyramidal cells and resulting overstimulation of mesolimbic dopaminergic nuclei by a polysynaptic pathway originating in the hippocampus might trigger positive symptoms (Lisman et al., 2008, 2010). Hippocampal novelty detection has been suggested to elicit increased stimulation of the NAcc by the hippocampus, which in turn reduces the tonic inhibition of the VTA by the ventral pallidum, ultimately promoting dopamine release in the NAcc and hippocampus (Lisman and Grace, 2005). In healthy humans, novelty detection has been linked to co-activation of the hippocampus and dopaminergic midbrain (Schott et al., 2004; Bunzeck and Düzel, 2006). In a series of studies, it could be further demonstrated that moderate enhancement by either reward-related enhancement of endogenous dopaminergic activity (Bunzeck et al., 2009) or pharmacological stimulation of dopamine release via the dopamine precursor L-dopa (Eckart and Bunzeck, 2013) elicits accelerated novelty processing in healthy humans, while the presumably further increased dopamine release by combination of reward and L-dopa has been associated with delayed novelty signals and impaired recognition (Apitz and Bunzeck, 2013). One potential explanation for this oberservation might be an inverse U-shaped function of dopaminergic action in the hippocampus, similar to the well-characterized modulation of prefrontal function by dopamine levels (Meyer-Lindenberg and Weinberger, 2006). Moreover, an imbalance of tonic vs. phasic dopaminergic activity also differentially affects memory performance with pharmacologically enhanced phasic dopaminergic activity being associated with improved performance while increased tonic dopaminergic stimulation has the opposite effect (Knecht et al., 2004; Breitenstein et al., 2006). Given the well-documented increased presynaptic dopaminergic activity (Bonoldi and Howes, 2013) and the dysregulation of tonic vs. dopamine action (Goto et al., 2007) in schizophrenia, dysfunction of the dopaminergic system might constitute an additional pathomechanism underlying the disrupted translation of hippocampal novelty signals into successfully encoded engrams.

One limitation of the present study is that, while we did observe a robust hippocampal signal to novel stimuli in both, controls and patients, no midbrain activation was found in either group. The most likely reason for this is that the study was conducted on a 1.5T MR tomograph, and most studies in which midbrain activity could be reliably detected had employed field strengths of at least 3T, providing an inherently higher signal-to-noise ratio and higher spatial resolution (Bunzeck and Düzel, 2006; D'Ardenne et al., 2008; Schott et al., 2008; Krebs et al., 2009a,b). An additional—or alternative—explanation might be that, given the absence of a direct co-activation of the hippocampus and the striatum, including the NAcc, the stimuli might not have been sufficiently salient engage the hippocampal-VTA loop, at least in the control group. In the patients, on the other hand, an indirect activation might have occurred, as indexed by the functional connectivity increase between the hippocampus and OFC on the one hand and the striatum on the other.

Fronto-Limbic Novelty Processing and its Potential Role in Delusions

While novelty-related hippocampal activation in patients did not correlate with later memory performance, the patients showed more pronounced activation and functional connectivity increases to stimulus novelty in fronto-limbic structures, most prominently in the OFC (**Figure 3**). The human OFC is functionally strongly connected with the mesolimbic dopamine system (Gurevich et al., 1997; Cole et al., 2012). OFC activation in response to novelty has previously also been observed in healthy humans (Bunzeck et al., 2012), and it has been linked to explicit, voluntary encoding of information into long-term memory. In a previous study using the same stimulus material, we had also observed an orbitofrontal novelty response in healthy humans (Schott et al., 2011), but, importantly, in that study, participants had performed an explicit novelty/familiarity decision, novelty was thus a task-relevant feature. Here, on the other hand, novel stimuli were processed implicitly while participants focused on the detection of a target stimulus. The OFC has been implicated in the processing of salient information, for example by coding reward value (Kahnt et al., 2010; Rothkirch et al., 2012), although other studies have suggested that the OFC and adjacent mPFC are primarily involved in conveying a more general, probability-related salience signal, while the actual value is coded by the VS (Knutson et al., 2005).

One apparently straight-forward and plausible explanation of the novelty-related OFC activation in the patient group might therefore be that patients might attribute atypical salience to the novel, but task-irrelevant stimuli, which would be in line with the previously demonstrated attention orienting towards novel stimuli in patients with schizophrenia (Cortiñas et al., 2008; Núñez Castellar et al., 2012). In a study of feedback processing in patients with schizophrenia, abnormal activation of the ventral mPFC to negative feedback in a monetary incentive delay (MID) task, and the mPFC activation during feedback processing was correlated with severity of delusions in the patient group (Schlagenhauf et al., 2009). No such correlation, however, was found in the present study, but instead, the SAPS global delusions score correlated positively with the difference of the hippocampal vs. orbitofrontal functional connectivity to the rACC/mPFC (**Figure 4B**). We therefore suggest that, in our study, the OFC activation to novelty observed in patients is unlikely to directly reflect dysfunctional salience processing

leading to delusions. Recent studies point to considerable functional specialization within the OFC (Kahnt et al., 2012) and suggest that OFC subregions might be differentially involved in coding implicit overall salience and value of a stimulus (Rothkirch et al., 2012). Specifically, activation of the medial OFC—close to the region where a correlation between atypical feedback responses and delusions was observed by Schlagenhauf et al. (2009)—was associated with implicit salience, irrespective of value, whereas a region in the right lateral OFC—close to the OFC region in which patients exhibited a novelty response in the present study—showed a parametric modulation of activity by stimulus value. In the present study, novel and familiar distracter stimuli did not differ in terms of motivational value or semantic content, making a higher (extrinsic) motivational value of the novel stimuli unlikely. We therefore suggest that the OFC novelty response and the novelty-related functional increased connectivity between the OFC and rACC might reflect a more intrinsic stimulus evaluation process in the patients.

When stimulus evaluation in the OFC is impaired, for example as a result of psychosis-related structural alterations (Malla et al., 2011), there might be an imbalance of hippocampal vs. orbitofrontal functional connectivity with the rACC. The observation that the relative novelty-related functional connectivity of the hippocampus vs. the OFC with the rACC correlated positive with delusions raises the possibility that hippocampal-rACC interactions during processing of novel stimuli might contribute to the pathophysiology of delusions, while novelty processing within the (lateral) OFC might actually confer a protective effect. The rACC/mPFC is part of the so-called Default Mode Network (DMN) that has been implicated in social and self-referential processing (Gusnard et al., 2001) and has been specifically been linked to self-reference (Kelley et al., 2002). Erroneous self-attribution is a hallmark feature of delusions in schizophrenia and particularly of delusional perceptions (Bovet and Parnas, 1993). Patients with schizophrenia and people at high risk for psychosis exhibit impaired deactivation of the DMN (Landin-Romero et al., 2015) and fail to suppress the rACC/mPFC during task that involve no self-reference (Pauly et al., 2014; Falkenberg et al., 2015). Moreover, MTL activation during self-referential processing in patients with schizophrenia has been shown to correlate with positive symptoms (Pauly et al., 2014). Together with our present results, these data raise the possibility that dysfunctional interactions of the MTL and rACC/mPFC might give rise to aberrant self-attribution of stimuli, which may manifest clinically as delusions.

Future studies are warranted to further characterize the functional parcellation of fronto-limbic structures within this network. With respect to the OFC region that was found activated to novel stimuli in the present study, we can thus far only speculate that it could reflect an evaluation process of the stimuli that might to some extent moderate the hippocampal-rACC interactions, possibly by computing stimulus value (Rothkirch et al., 2012).

Functional Connectivity of the Ventral Striatum

Hippocampal novelty processing has previously been linked to stimulation of dopamine release by VTA neurons (Lisman and Grace, 2005), and a hyperactive hippocampal-VTA loop has been suggested to be involved in the generation of psychotic symptoms (Lisman et al., 2008, 2010). In addition to the rACC, the VS also exhibited increased functional connectivity with the hippocampus and OFC in the patients (**Figure 4A**, bottom). Ventral striatal activation during reward processing has been shown to correlate with dopamine release (Schott et al., 2008), and it appears plausible that novelty-related stimulation of dopamine release in the patients might be involved in the generation of an abnormal response salience attribution. This notion is well in line with our predictions as we had hypothesized that patients in an acute state of psychosis might attribute abnormal salience to novel, but otherwise irrelevant stimuli, which might be a putative neural basis for delusional phenomena. However, unlike MTL vs. OFC to rACC functional connectivity, the connectivity between these structures and the NAcc did not correlate with psychopathology in the present study, and the behavioral relevance of the increased ventral striatal functional connectivity in the patients cannot be conclusively resolved by the present study.

Conclusion

The present study suggests that patients in an acute psychotic state exhibit atypical processing of novel information in two ways. First, the hippocampal novelty response is decoupled from successful hippocampus-dependent encoding of the novel information into episodic memory. Second, patients exhibited a specific novelty response in the OFC and increased novelty-related fronto-limbic functional connectivity. With the hippocampal-rACC functional connectivity showing a positive correlation with delusions, our results highlight the possibility that delusions might arise from abnormal processing of novel stimuli in fronto-limbic cortices.

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Comprehensive behavioral analysis of voltage-gated calcium channel beta-anchoring and -regulatory protein knockout mice

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Calcium (Ca^{2+}) influx through voltage-gated Ca^{2+} channels (VGCCs) induces numerous intracellular events such as neuronal excitability, neurotransmitter release, synaptic plasticity, and gene regulation. It has been shown that genes related to Ca^{2+} signaling, such as the *CACNA1C*, *CACNB2,* and *CACNA1I* genes that encode VGCC subunits, are associated with schizophrenia and other psychiatric disorders. Recently, VGCC beta-anchoring and -regulatory protein (BARP) was identified as a novel regulator of VGCC activity via the interaction of VGCC β subunits. To examine the role of the BARP in higher brain functions, we generated BARP knockout (KO) mice and conducted a comprehensive battery of behavioral tests. BARP KO mice exhibited greatly reduced locomotor activity, as evidenced by decreased vertical activity, stereotypic counts in the open field test, and activity level in the home cage, and longer latency to complete a session in spontaneous T-maze alteration test, which reached "study-wide significance." Acoustic startle response was also reduced in the mutants. Interestingly, they showed multiple behavioral phenotypes that are seemingly opposite to those seen in the mouse models of schizophrenia and its related disorders, including increased working memory, flexibility, prepulse inhibition, and social interaction, and decreased locomotor activity, though many of these phenotypes are statistically weak and require further replications. These results demonstrate that BARP is involved in the regulation of locomotor activity and, possibly, emotionality. The possibility was also suggested that BARP KO mice may serve as a unique tool for investigating the pathogenesis/pathophysiology of schizophrenia and related disorders. Further evaluation of the molecular and physiological phenotypes of the mutant mice would provide new insights into the role of BARP in higher brain functions.

Keywords: voltage-gated calcium channels, voltage-gated calcium channel beta-anchoring and -regulatory protein, knockout mouse, behavior, psychiatric disorders

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Introduction

In calcium (Ca^{2+}) signaling pathways, voltage-gated calcium channels (VGCCs) initiate numerous intracellular events including neuronal excitability, neurotransmitter release, synaptic plasticity, and Ca^{2+}-induced gene regulation (Catterall, 2011). VGCCs are heteromultimeric protein complexes composed of the pore-forming α1 subunit, designated as CaV, and auxiliary subunits α2/δ, β, and γ (Ertel et al., 2000). The α1 and β subunits interact to enhance functional channel trafficking to the plasma membrane (Mori et al., 1991; Bichet et al., 2000), to modify multiple kinetic properties (Varadi et al., 1991), and to regulate the stability of VGCC complexes (Campiglio et al., 2013). β subunits are thought to be the structural and functional platform of VGCC complexes, because they contain an SH3-HOOK-GK module that places them within a family of proteins called the membrane-associated guanylate kinases (Buraei and Yang, 2010; Nakao et al., 2013). The β subunits directly interact with various proteins, including small G-proteins such as Kir/Gem, Rem, and Rad, to regulate VGCC activity (Béguin et al., 2001; Finlin et al., 2003). At the presynaptic active zones, β subunits interact with Rab3-interacting molecules (RIMs), Bassoon, and CAST (Kiyonaka et al., 2007, 2012; Uriu et al., 2010; Chen et al., 2011), and these interactions are critical for VGCCs and active zone proteins to couple functionally to regulate neurotransmitter release. Interestingly, several studies have demonstrated that the β4 subunit directly regulates activity-dependent gene regulation (Subramanyam et al., 2009; Tadmouri et al., 2012). In this context, the β4 subunit also binds to a transcription factor for DNA binding, a phosphatase for histone dephosphorylation, and heterochromatin protein 1γ for nucleosome association (Hibino et al., 2003; Xu et al., 2011; Tadmouri et al., 2012). Recently, VGCC beta-anchoring and -regulatory protein (BARP) was identified (Béguin et al., 2014). BARP modulates the localization of β subunits and their association with the α1 subunit to negatively regulate VGCC activity (Béguin et al., 2014).

Among the genes involved in psychiatric disorders, genes encoding VGCC subunits have attracted attention (Ripke et al., 2014). In 2002, a family-based association study revealed for the first time that CACNA1C, which encodes the VGCC pore-forming α1 subunit, is associated with bipolar disorder (Sklar et al., 2002). Genome-wide association studies have identified that CACNA1C and CACNB3, which encodes the VGCC β subunit, are involved in the development of bipolar disorder (Ferreira et al., 2008; Ripke et al., 2011; Sklar et al., 2011). Notably, the latest large-scale genome-wide association study shows that CACNA1C is ranked fourth in terms of the degree of association (*p*-value) with schizophrenia out of 108 schizophrenia-associated genetic loci (Ripke et al., 2014). In addition, CACNB2 and CACNA1I are nineteenth and thirty-ninth, respectively, of the 108 loci (Ripke et al., 2014).

CaV1.2, encoded by CACNA1C, has been shown to play critical roles in higher brain function in mouse models. Conditional deletion of CACNA1C in the mouse hippocampus and cortex resulted in a severe impairment of hippocampus-dependent spatial memory in the Morris water maze test (Moosmang et al., 2005; White et al., 2008). Acute pharmacological blockade of CaV1.2, but not chronic genetic inactivation, impaired acquisition of fear learning (Langwieser et al., 2010). Furthermore, an anterior cingulate cortex-limited deletion of CACNA1C in mice impaired observational fear learning (Jeon et al., 2010). Constitutive CACNA1C heterozygous mice, forebrain-specific conditional CACNA1C knockout (KO) mice, and prefrontal cortex-specific CACNA1C knockdown mice show increased anxiety-like behavior in the elevated plus maze (Lee et al., 2012).

It has been reported that genes encoding VGCC subunits, especially CACNA1C, are associated with psychiatric disorders (Sklar et al., 2002, 2011; Ferreira et al., 2008; Ripke et al., 2011, 2014). Considering the fact that BARP regulates VGCC activity including CaV1.2 encoded by CACNA1C (Béguin et al., 2014), we hypothesized that BARP deficiency may result in some behavioral abnormalities related to psychiatric disorders. We generated BARP KO mice using gene targeting by homologous recombination, and then conducted a comprehensive behavioral test battery, which covers many distinct behavioral domains from simple sensorimotor functions to higher brain functions including learning and memory, on the BARP KO mice (Powell and Miyakawa, 2006; Takao and Miyakawa, 2006a).

Materials and Methods

Generation of Mutant Mice

The targeting vector was constructed using the genomic PCR products from 129/SvJ mouse genome DNA, synthetic linkers carrying the loxP sequence (Shibata et al., 1997), the neomycin-resistance gene from pMC1 Neo (Stratagene), the diphtheria toxin gene from pMC1-DT-A, and pBluscriptII SK(−) (Stratagene). J1 ES cells were transfected with the linearized targeting vector and selected using G418. Of ∼550 clones isolated, Southern blotting analysis identified the clone carrying the homologous mutation. Chimeric mice produced with the ES clones were crossed with C57BL/6J mice and could transmit the mutant gene to the pups. To determine the mouse genotype, PCR with the primer sets of mDos_genoR_Not(+), mDos_genoL_Not2(−), and LacZ_Aat(−) was carried out using genomic DNA from mice; sequences are mDos_genoRNot(+) (5′-GAGCTGAACCTG AGCTGGCTCTATG-3′), mDos_genoL_Not2(−) (5′-GTG GGTGCCGTTGTCCAGATAAGTAG-3′), and LacZ_Aat(−) (5′-CGCTGATTTGTGTAGTCGGTTTATG-3′). For β-galactosidase staining, mice were perfused with a 2% paraformaldehyde solution buffered with 0.12 M sodium phosphate (pH 7.4) under pentobarbital anesthesia, and the brains were immersed immediately in ice-cold 25% sucrose. Frozen coronal sections (40 μm thickness) were stained with the X-Gal reagent (Nishi et al., 1997).

Abbreviations: BARP, beta-anchoring and -regulatory protein; lx, lux; PPI, prepulse inhibition; s, second; VGCC, voltage-gated calcium channel.

Animals and Experimental Design

We used male BARP KO mice ($n = 22$), which were backcrossed to the C57BL/6J background for at least seven generations, and their male wild type (WT) littermates ($n = 22$). The ages of the mice at the time of the experiment are shown in Supplementary Table 1. Wild-type and mutant mice were group housed (total three to five per cage; one to three for each genotype) in a room with a 12 h light/dark cycle (lights on at 7:00 a.m.), with access to food and water *ad libitum*. Room temperature was kept at 23 ± 2°C. Behavioral testing was performed between 9:00 a.m. and 6:00 p.m. After the tests, all apparatus was cleaned with diluted sodium hypochlorite solution to prevent a bias due to olfactory cues. To minimize the effects of previous test experiment on subsequent behavior, we performed the behavioral test battery in a specific order, in which the less stressful tests preceded the more stressful tests (Supplementary Table 1). Each behavioral test was separated from the others at least by 1 day. All behavioral testing procedures were approved by the Institutional Animal Care and Use Committee of Fujita Health University.

Behavioral Tests

Most of the behavioral tests were performed as previously described (Yamasaki et al., 2008), unless otherwise noted.

Neurological Screen and Neuromuscular Strength Test

The righting, whisker twitch, and ear twitch reflexes were evaluated. Physical features, including the presence of whiskers or bald hair patches, were also recorded. A grip strength meter (O'HARA & Co., Tokyo, Japan) was used to assess forelimb grip strength. Mice were lifted by holding the tail so that their forepaws could grasp a wire grid. The mice were then gently pulled backward by the tail with their posture parallel to the surface of the table until they released the grid. The peak force applied by the forelimbs of the mouse was recorded in Newtons (N). Each mouse was tested three times, and the greatest value measured was used for data analysis. In the wire hang test, the mouse was placed on a wire mesh that was then slowly inverted, so that the mouse gripped the wire in order not to fall off. Latency to fall was recorded, with a 60 s cut-off time.

Light/Dark Transition Test

A light/dark transition test was conducted as previously described (Takao and Miyakawa, 2006b). The apparatus consisted of a cage (21 × 42 × 25 cm) divided into two sections of equal size by a partition with a door (O'HARA & Co., Tokyo, Japan). One chamber was brightly illuminated (390 lx), whereas the other chamber was dark (2 lx). Mice were placed into the dark chamber and allowed to move freely between the two chambers with the door open for 10 min. The total number of transitions, latency to first enter the lit chamber, distance traveled, and time spent in each chamber were recorded by ImageLD software (see Section, "Data Analysis").

Open Field Test

Each mouse was placed in the corner of the open field apparatus (40 × 40 × 30 cm; Accuscan Instruments, Columbus, OH, USA). The apparatus was illuminated at 100 lx. Total distance traveled (cm), vertical activity (rearing measured by counting the number of photobeam interruptions), time spent in the center area (20 × 20 cm), and beam-break counts for stereotyped behaviors (defined by the number of breaks of the same beam) were recorded. Data were collected for 120 min.

Elevated Plus Maze Test

An elevated plus maze test was conducted as previously described (Komada et al., 2008). The elevated plus maze consisted of two open arms (25 × 5 cm) and two enclosed arms of the same size with 15 cm high transparent walls, and the arms were connected by a central square (5 × 5 cm) (O'HARA & Co., Tokyo, Japan). The open arms were surrounded by a raised ledge (3 mm thick and 3 mm high) to avoid mice falling off the arms. The arms and central square were made of white plastic plates and were elevated 55 cm above the floor. Arms of the same type were located opposite from each other. Each mouse was placed in the central square of the maze, facing one of the enclosed arms. The number of entries into the open and enclosed arms and the time spent in the open or enclosed arms were recorded during a 10-min test period. Percentage of entries into open arms, time spent in open arms (s), number of total entries, and total distance traveled (cm) were calculated. Data acquisition and analysis were performed automatically, using ImageEP software (see Section, "Data analysis").

Hot Plate Test

The hot plate test was used to evaluate sensitivity to a painful stimulus. Mice were placed on a 55.0°C (± 0.1°C) hot plate (Columbus Instruments, OH, USA), and latency to the first fore- or hind-paw response was recorded. The paw response was defined as either a paw lick or a foot shake.

Social Interaction Test in a Novel Environment

In the social interaction test, two mice of identical genotypes that were previously housed in different cages were placed in a box together (40 × 40 × 30 cm) (O'HARA & Co., Tokyo, Japan) and allowed to explore freely for 10 min. Behavior was recorded and analyzed automatically using ImageSI program (see Section, "Data analysis"). The total number of contacts, total duration of active contacts, total contact duration, mean duration per contact, and total distance traveled were measured. If the two mice contacted each other and the distance traveled by either mouse was longer than 10 cm, the behavior was classified as an "active contact." Images were captured at 3 frame per s, and distance traveled between two successive frames was calculated for each mouse.

Rotarod Test

Motor coordination and balance were tested with the rotarod test. This test, which uses an accelerating rotarod (UGO Basile, Comerio, VA, Italy), was performed by placing mice on rotating drums (3 cm diameter) and measuring the time each animal was able to maintain its balance on the rod. The speed of the rotarod accelerated from 4 to 40 rpm over a 5-min period.

Crawley's Sociability and Social Novelty Preference Test

This test is a well-designed method to investigate the effect of complex genetics on sociability and preference for social novelty (Crawley, 2004; Moy et al., 2004). The testing apparatus consisted of a rectangular, three-chambered box and a lid with an infrared video camera (O'HARA & Co., Tokyo, Japan). Each chamber was 20 × 40 × 47 cm and the dividing walls were made from clear Plexiglas, with small square openings (5 × 3 cm) allowing access into each chamber. We modified the method described by Moy et al. (2004) as follows: a habituation session was performed in the apparatus for 10 min the day before the sociability test, and the wire cages in the lateral compartments were located in the corners of each compartment. In the sociability test, an unfamiliar C57BL/6J male mouse (stranger), that had no prior contact with the subject mice, was placed in one of the side chambers. The location of the stranger mouse (stranger side) in the left vs. right side chamber was systematically alternated between trials. The stranger mouse was enclosed in a small round wire cage, which allowed nose contact between the bars, but prevented fighting. The cage was 11 cm in height, with a bottom diameter of 9 cm, and vertical bars 0.5 cm apart. The subject mouse was first placed in the middle chamber and allowed to explore the entire test box for a 10-min session. The amount of time spent in each chamber was measured with a camera fitted on top of the box. In the social novelty preference test, each mouse was tested in a 10-min session to quantify social preference for a new stranger. After the first 10-min session, a second unfamiliar mouse was placed in the chamber that had been empty during the first 10-min session. This second stranger was also enclosed in an identical small wire cage. The test mouse thus had a choice between the first, already-investigated unfamiliar mouse (familiar side), and the novel unfamiliar mouse (unfamiliar side). The amount of time spent in each chamber during the second 10-min session was measured as described above. Data acquisition and analysis were performed automatically using ImageCSI (see Section, "Data analysis").

Startle Response/Prepulse Inhibition (PPI) Test

A startle reflex measurement system (O'HARA & Co., Tokyo, Japan) was used to measure acoustic startle response and PPI. A test session began by placing a mouse in a plastic cylinder where it was left undisturbed for 10 min. White noise (40 ms) was used as the startle stimulus for all trial types. The startle response was recorded for 400 ms (measuring the response every 1 ms) starting with the onset of the startle stimulus. The background noise level in each chamber was 70 dB. The peak startle amplitude recorded during the 140 ms sampling window was used as the dependent variable. A test session consisted of six trial types (i.e., 2 types for startle stimulus-only trials, and 4 types for PPI trials). The intensity of the startle stimulus was 110 or 120 dB. The prepulse sound was presented 100 ms before the startle stimulus, and its intensity was 74 or 78 dB. Four combinations of prepulse and startle stimuli were used (74–110, 78–110, 74–120, and 78–120 dB). Six blocks of the six trial types were presented in a pseudo-random order, such that each trial type was presented once within a block. The average inter-trial interval was 15 s (range 10–20 s).

Porsolt Forced Swim Test

A transparent plastic cylinder (20 cm height × 10 cm diameter) filled with water (21–23°C) up to a height of 7.5 cm was put in a white plastic chamber (31 × 41 × 41 cm) (O'HARA & Co., Tokyo, Japan). Mice were placed into the cylinder, and both immobility and the distance traveled were recorded over a 10-min test period. Images were captured at 2 frame per s. For each pair of successive frames, the amount of area (pixels) within which the mouse moved was measured. When the amount of area was below a certain threshold, mouse behavior was classified as "immobile." When the amount of area equaled or exceeded the threshold, the mouse was classified as "moving." The optimal threshold to judge moving was determined by adjusting it to the amount of immobility measured by human observation. Immobility lasting for less than 2 s was not included in the analysis. Data acquisition and analysis were performed automatically, using ImageTS/PS software (see Section, "Data analysis").

Barnes Maze Test

The Barnes maze task was conducted on "dry land," a white circular surface 1.0 m in diameter, with 12 holes equally spaced around the perimeter (O'HARA & Co., Tokyo, Japan). A black Plexiglas escape box (17 × 13 × 7 cm), which had paper cage bedding on its bottom, was located under one of the holes. The hole above the escape box represented the target, analogous to the hidden platform in the Morris water maze task. The location of the escape box (target) was consistent for a given mouse but randomized across mice. The maze was rotated daily, with the spatial location of the target unchanged with respect to the distal visual room cues, to prevent a bias based on olfactory or proximal cues within the maze. The mice that could not find the box were guided to it and allowed to enter it to remain there for 30 s. Two trials per day were conducted for 6 successive days. Each trial ended when the mouse entered the escape box or after 5 min had elapsed. The amount of time that the mice took to enter the box (escape latency), the number of errors (defined by the animal placing its nose in a hole that did not lead to the escape box), and the number of omission errors (defined by the visit to the target hole without subsequent entry into the target hole) were recorded by ImageBM software. On day 7, a probe test was conducted without the escape box, to assess memory based on distal environmental room cues. Another probe trial was conducted 30 days after the last training session to evaluate memory retention. The time spent around the target hole was recorded in these probe tests by the software.

T-Maze Test

Spontaneous Alternation Test

The spontaneous alternation test was conducted using an automated T-maze apparatus (O'HARA & Co., Tokyo, Japan). It was constructed of white plastic runways with walls 25 cm high. The maze was partitioned off into 6 areas by sliding doors that could be opened downward. The stem of the T was composed of area S2 (13 × 24 cm), and the arms of the T were composed

of areas A1 and A2 (11.5 × 20.5 cm). Areas P1 and P2 were the connecting passageways from the respective arm (area A1 or A2) to the start compartment (area S1). Mice were subjected to a forced alternation protocol (see below) for 5 days (one session consisting of 10 trials per day; cut-off time, 50 min). Each trial had first and second runs. On the sample run, the mouse was forced to choose one of the arms of the T (area A1 or A2). After the mouse stayed more than 10 s, the door that separated the arm (area A1 or A2) and the connecting passageway (area P1 or P2) would be opened, and the mouse could return to the starting compartment (area S1) via the connecting passageway. The mouse was then given a 3 s delay in area S1, followed by a free choice between both T arms. The correct response was choosing the other arm that had not been chosen on the first trial of the pair. The location of the sample arm (left or right) was varied pseudo-randomly across trials using the Gellermann schedule so that mice received equal numbers of left and right presentations. A variety of fixed extra-maze clues surrounded the apparatus. On days 6–8, a delay (3, 10, 30, or 60 s) was applied after the sample trial. Data acquisition, control of sliding doors, and data analysis were performed by ImageTM software (see Section, "Data analysis").

Forced Alternation Test Using Food Reward

This test was conducted as previously described (Shoji et al., 2012). The forced alternation test used an automated T-maze apparatus (O'HARA & Co., Tokyo, Japan). The end of each arm was equipped with a pellet dispenser that could provide a food reward (a sucrose pellet). The pellet sensors were able to record pellet intake by the mice automatically. One week before the pre-training, mice were deprived of food until their body weight was reduced to 80–85% of their initial weight. Mice were kept on a maintenance diet throughout the forced alternation and left-right discrimination tests in the T-maze. Before the first session, mice were subjected to 30-min habituation session, during which they were allowed to freely explore the T-maze with all doors open and both arms baited with food. On the day after the habituation session, mice were subjected to a forced alternation protocol for 5 days (one session consisting of 10 trials per day; cut-off time, 50 min). Each trial had first and second runs. On the forced run, the mouse was forced to choose one of the arms of the T (area A1 or A2), and received the reward at the end of the arm. After the mouse either consumed the pellet or stayed more than 30 s without consuming the pellet (defined as an "Omission Error"), the door that separated the arm (area A1 or A2) and the connecting passageway (area P1 or P2) was opened and the mouse could return to the starting compartment (area S1) via the connecting passageway. In this way, the potential stress was reduced compared to the traditional forced alternation paradigm in which the human experimenter brings the mouse back to the start box by hand. The mouse was then given a 3 s delay in area S1, followed by a free choice between both T arms, and was rewarded for choosing the other arm that had not been chosen on the first trial of the pair. If the mouse failed to eat the pellet within 30 s, the response was recorded as an "Omission Error". Choosing the incorrect arm resulted in no reward and confinement to the arm for 10 s. The location of the sample arm (left or right) was varied pseudo-randomly across trials using the Gellermann schedule so that mice received equal numbers of left and right presentations. A variety of fixed extra-maze clues surrounded the apparatus. On days 6–12, a delay (3, 10, 30, 60, or 120 s) was applied after the sample trial. Data acquisition, control of sliding doors, and data analysis were performed by ImageTM software (see Section, "Data analysis").

Left-right Discrimination Test

This test was conducted as previously described (Shoji et al., 2012). The mice were placed in the stem of the T-maze. The door leading to the straight alley was opened and the mice were able to freely choose either the right or left arm of the T-maze. A sucrose pellet was always delivered to the food tray within one of the arms, which was the goal arm. Mice had to learn to enter the goal arm. If a mouse chose the goal arm, it was allowed to consume the food reward. The mouse was then allowed to return to the starting compartment. The correct arm was assigned to each mouse randomly. On day 8, the correct arm was changed to test reversal learning. Data acquisition, control of sliding doors, and data analysis were performed by ImageTM software (see Section, "Data analysis").

Tail Suspension Test

The tail suspension test was performed for a 10-min test session. Mice were suspended 30 cm above the floor of a white plastic chamber (31 × 41 × 41 cm) (O'HARA & Co., Tokyo, Japan) in a visually isolated area by adhesive tape placed ∼1 cm from the base of the tail, and behavior was recorded over a 10-min test period. Images were captured at 2 frame per s. Similar to the Porsolt forced swim test, immobility was judged by the application program according to a certain threshold. Immobility lasting for less than 2 s was not included in the analysis. Data acquisition and analysis were performed automatically, using ImageTS/PS software (see Section, "Data analysis").

Contextual and Cued Fear Conditioning Test

A contextual and cued fear conditioning test was conducted as previously described (Shoji et al., 2014). Each mouse was placed in a transparent acrylic chamber (33 × 25 × 28 cm) with a stainless-steel grid floor (0.2 cm diameter, spaced 0.5 cm apart) (O'HARA & Co., Tokyo, Japan) illuminated at 100 lx, and allowed to explore freely for 2 min. A 55 dB white noise, which served as the conditioned stimulus (CS), was presented for 30 s, followed by a mild (2 s, 0.3 mA) foot shock, which served as the unconditioned stimulus (US). Two more CS-US pairings were presented with a 2-min inter-stimulus interval. Context test was conducted 24 h after conditioning in the same chamber. Cued test with altered context was conducted after conditioning using a triangular box (33 × 29 × 32 cm) made of white opaque Plexiglas, which was located in a different room. Thirty-five d after fear conditioning, mice were presented with the tone alone for 15 min in the altered context (extinction training phase). Twenty-four h after extinction training, mice were presented with the tone alone in the altered context (extinction test phase). The test chamber was illuminated at 30 lx. Data acquisition, control of stimuli (i.e., white noises and shocks), and data analysis were performed automatically, using ImageFZ software (see Section,

"Data analysis"). Images were captured at 1 frame per s. For each pair of successive frames, the amount of area (pixels) by which the mouse moved was measured. When this area was below a certain threshold (i.e., 30 pixels), the behavior was classified as "freezing." When the amount of area equaled or exceeded the threshold, the behavior was classified as "non-freezing." The optimal threshold (amount of pixels) used to classify freezing was determined by adjusting it to the amount of freezing measured by human observation. "Freezing" that lasted less than the defined time threshold (i.e., 2 s) was not included in the analysis. The parameters were constant for all mice assessed.

Social Interaction Test in Home Cage

The social interaction monitoring system comprised the home cage and a filtered cage top with an infrared video camera (31 × 19 × 30 cm; 25 × 15 × 23.5 cm, inside dimensions). Two mice of the same genotype that had been housed separately were placed together in a home cage. To evaluate social interaction, their location was then monitored for 1 week. Output from the video camera was fed into a Windows computer. Images from each cage were captured at a rate of 1 frame per s. Social interaction was measured by counting the number of objects detected in each frame: two objects indicated that the mice were not in contact with each other, and one object indicated contact between the two mice. We also measured locomotor activity during these experiments by quantifying the number of pixels that changed between each pair of successive frames. Analysis was performed automatically using ImageHA software (see Section, "Data analysis").

Data Analysis

Behavioral data were obtained automatically through applications based on the ImageJ program, and they were modified for each test by Tsuyoshi Miyakawa (available through O'HARA & Co., Tokyo, Japan). Statistical analysis was conducted using StatView (SAS Institute, Cary, NC, USA). Data were analyzed using two-tailed *t*-tests, Two-Way ANOVAs, Two-Way repeated measures ANOVAs, Mann–Whitney *U*-tests, or correlation *Z*-tests. Values in graphs are expressed as mean ± SEM or box plots (median, interquartile, and 90 and 10th percentiles). We defined "study-wide significance" as the statistical significance that survived Bonferroni correction for 89 indices employed in the behavioral test battery. "Nominal significance" was defined as the one that achieved a statistical significance in an index ($p < 0.05$) but did not survive this correction.

Results

General Characterization of BARP KO Mice

To study the physiological importance of BARP and its *in vivo* effects on psychiatric disorders, we generated transgenic mice in which BARP expression was knocked out (Supplementary Figure 1). We confirmed the deficiency of BARP in BARP KO mice using Southern, Northern, and Western blot analyses and genomic PCR analysis (Supplementary Figures 1B–E). As expected from the targeting construct for generation of BARP deficient mice (Supplementary Figure 1A), neurons expressing the BARP protein were detected by β-galactosidase activity in the BARP KO mice (data not shown). The regions that showed positive X-gal staining in the brains of BARP KO mice were the cortex, hippocampus, and cerebellum. This expression pattern is consistent with a previous report (Béguin et al., 2014).

We analyzed behaviors of BARP KO mice using a behavioral test battery (Powell and Miyakawa, 2006; Takao and Miyakawa, 2006a). The BARP KO mice weighed significantly less than their WT littermates ($t = 5.308$, $df = 42$, $p < 0.0001$) (**Figure 1A**). The difference reached study-wide significance. The BARP KO mice showed no obvious abnormalities on body temperature (**Figure 1B**), or on a gross inspection of their fur, whiskers, posture, righting reflex, whisker touch reflex, ear twitch reflex, and the response to key jangling (**Table 1**). The grip strength of BARP KO mice was nominally weaker than WT mice, suggesting that the BARP KO mice had muscle weakness and decreased motivation ($t = 3.392$, $df = 42$, $p = 0.0015$) (**Figure 1C**). In the wire hang test, there was no significant difference in the latency to fall off the wire grid between BARP KO and WT mice (**Figure 1D**). There were no significant differences in motor coordination (rotarod test, **Figure 1E**) and pain sensitivity (hot plate test, **Figure 1F**) compared with WT mice.

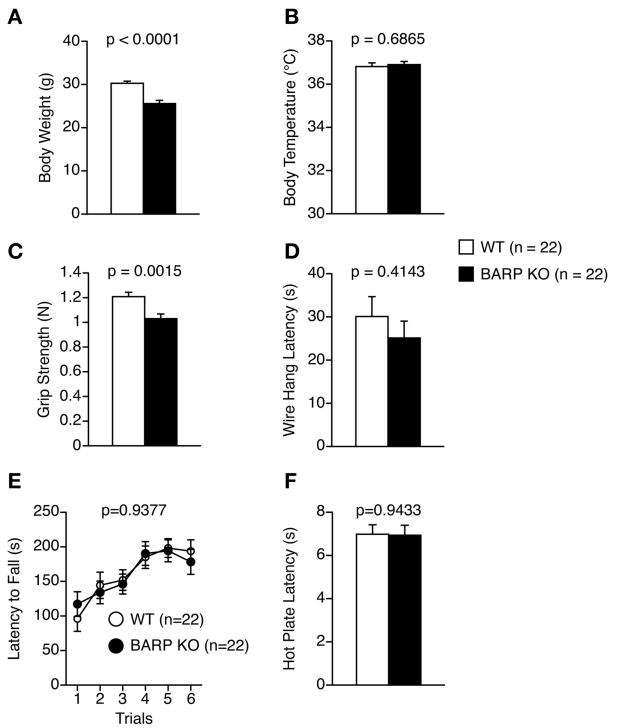

FIGURE 1 | General health and neurological screening, motor coordination/motor learning, and pain sensitivity in beta-anchoring and -regulatory protein (BARP), knockout (KO), mice, and wild type (WT) mice. (A) Body weight. **(B)** Body temperature. **(C)** Grip strength score. **(D)** Wire hang latency. **(E)** Latency to fall off the rotating rod in the rotarod test. **(F)** Latency to the first fore- or hind-paw response in the hot plate test. Data represent the mean ± SEM. The *p*-values indicate a genotype effect in a *t*-test **(A–D,F)** or a Two-Way repeated measures ANOVA **(E)**.

TABLE 1 | Summary of a comprehensive behavioral test battery for beta-anchoring and–regulatory protein (BARP) knockout (KO) mice in comparison with wild type (WT) mice.

Test	Measure	Alteration from WT
General health, neurological, and wire hang	Gross inspection of their fur	→
	Whiskers	→
	Posture	→
	Righting reflex	→
	Whisker touch reflex	→
	Ear twitch reflex	→
	Response to key jangling	→
	Body weight	↓↓
	Body temperature	→
	Grip Strength	↓
	Wire hang time	→
Rotarod	Latency to fall	→
Hot plate	Latency	→
Light/dark transition	Transitions	↓
Elevated plus maze	Entries into open arms	→
Open field	Total distance	↓ (0–60 min)
	Vertical activity	↓↓
	Center time	↓ (30 and 55 min time points)
	Stereotypic Counts	↓↓
Porsolt forced swimming	Immobility	→
Tail suspension	Immobility	→
Social interaction (novel environment)	Total duration of contacts	→
	Number of contacts	→
	Total duration of active contact	→
	Mean duration / Contact	→
	Distance traveled	→
Social interaction (Crawley ver.)	Time spent in chamber	See Results
	Time spent around cage	See Results
Social interaction (home cage)	Mean number of objects	↓
	Activity level during overall period	↓↓
	Activity level during dark period	↓↓
	Activity level during light period	↓
Prepulse inhibition	Acoustic startle response	↓↓
	Prepulse inhibition	↑
Fear conditioning test	Freezing in during conditioning	See Results
	Freezing during the context test	See Results
	Freezing during cued test with altered context	See Results
Barnes maze	Latency to enter into the target hole	→
	Probe test (1 day after training)	→
	Probe test (30 days after training)	→
T-maze (spontaneous test)	Correct responses during training	↑ (4 and 5 sessions)
	Correct responses in the delay session	↑
	Latency to complete a session	↑↑
T-maze (forced alteration test)	Correct responses during training	→
	Correct responses in the delay session	↑ (120 s)
	Latency to complete a session	→
T-maze (left-right discrimination test)	Correct responses (reversal)	↑
	Latency to complete a session	→

Symbols: →, no significant difference ($p > 0.05$); ↑↑, increase that reached study-wide significance; ↓↓, decrease that reached study-wide significance; ↑, nominally significant increase; ↓, nominally significant decrease; in comparison with WT littermates.

Decreased Locomotor Activity in BARP KO Mice

In the light/dark transition test, BARP KO mice showed a nominally significant decrease in total distance traveled in the light and dark chambers, and number of transitions compared with WT mice [genotype effect, $F_{(1, 42)} = 4.945$, $p = 0.0316$; genotype × time effect, $F_{(1, 42)} = 0.100$, $p = 0.7536$ and $t = 2.939$, $df = 42$, $p = 0.0053$, respectively] (**Figures 2A,C**). However, BARP KO and WT mice showed similar stay time in the light chamber ($t = 1.883$, $df = 42$, $p = 0.0666$), and similar latency to the first transition ($t = 0.963$, $df = 42$, $p = 0.3409$) (**Figures 2B,D**).

In the elevated plus maze test, no significant differences between BARP KO and WT mice were found in any of the parameters examined (**Figures 2E–H**).

In the open-field test, BARP KO mice showed a nominally significant decrease in distance traveled compared with WT mice in the initial 60 min [**Figure 2I**; total, genotype effect, $F_{(1, 42)} = 1.184$, $p = 0.2827$; genotype × time effect, $F_{(23, 966)} = 3.087$, $p < 0.0001$; 0–60 min, genotype effect, $F_{(1, 42)} = 4.747$, $p = 0.0350$]. Vertical activity and stereotypic behavior counts were significantly reduced in BARP KO mice compared with WT mice [$F_{(1, 42)} = 25.380$, $p < 0.0001$, and $F_{(1, 42)} = 26.134$, $p < 0.0001$, respectively] (**Figures 2J,L**). These differences reached study-wide significance. BARP KO mice showed a nominally significant decrease in time spent in the center of the open field apparatus at the 30 and 55 min time points [total, genotype effect, $F_{(1, 42)} = 0.534$, $p = 0.4692$; genotype × time effect, $F_{(23, 966)} = 1.877$, $p = 0.0075$; 30 min, simple main effect, $F_{(1, 42)} = 4.542$, $p = 0.0390$; 55 min, simple main effect, $F_{(1, 42)} = 5.738$, $p = 0.0211$] (**Figure 2K**).

Normal Depression-Like Behavior in BARP KO Mice

For the Porsolt forced swim test, there were no significant effects of genotype on the percentage of immobility time on day 1 [genotype effect, $F_{(1, 42)} = 2.679$, $p = 0.1092$; genotype × time effect, $F_{(9, 378)} = 0.663$, $p = 0.7425$] and on day 2 [genotype effect, $F_{(1, 42)} = 1.835$, $p = 0.1828$; genotype × time effect, $F_{(9, 378)} = 1.054$, $p = 0.3962$] (**Figure 3A**). In the tail suspension test (**Figure 3B**), no significant genotype effect was found for the percentage of immobility time [genotype effect, $F_{(1, 42)} = 1.501$, $p = 0.2273$; genotype × time effect, $F_{(9, 378)} = 1.000$, $p = 0.4392$].

Nominally Significant Increase in Sociability in BARP KO Mice

In the social interaction test, there were no significant differences between BARP KO and WT mice in their total duration of contacts ($t = 0.710$, $df = 20$, $p = 0.4858$) (**Figure 4A**), number of contacts ($t = 0.910$, $df = 20$, $p = 0.3736$) (**Figure 4B**), total duration of active contacts ($t = 0.932$, $df = 20$, $p = 0.3625$) (**Figure 4C**), mean duration per contact ($t = 0.053$, $df = 20$, $p = 0.9582$) (**Figure 4D**), or distance traveled ($t = 0.317$, $df = 20$, $p = 0.7546$) (**Figure 4E**).

The three-chamber social approach test consists of a sociability test and a social novelty preference test, defined by the time spent around a wire cage containing a stranger mouse vs. an empty cage in the sociability test, and an unfamiliar mouse vs. a familiar mouse in the social novelty preference test (Moy et al., 2004). In the sociability test, the time spent in chamber was statistically indistinguishable between BARP KO and WT [genotype effect, $F_{(1, 42)} = 1.829$, $p = 0.1835$; genotype × side, $F_{(1, 42)} = 1.788$, $p = 0.1883$] (**Figure 4F**). A Two-Way repeated measures ANOVA showed no significant genotype effect, while there was marginally significant interaction in time around cage [genotype effect, $F_{(1, 42)} = 1.425$, $p = 0.2393$; genotype × side interaction, $F_{(1, 42)} = 3.790$, $p = 0.0583$]. Paired and unpaired t-tests showed that there were nominally significant differences in time spend around cage in BARP KO ($t = 3.724$, $df = 21$, $p = 0.0013$, stranger side vs. empty cage side) (**Figure 4G**), and in time spent around cage with stranger between genotypes ($t = 2.284$, $df = 42$, $p = 0.0275$, BARP KO vs. WT) (**Figure 4G**). In the social novelty preference test, a Two-Way repeated measures ANOVA showed no significant genotype effect in time spent in chamber [$F_{(1, 42)} = 3.036$, $p = 0.0887$]. Since there was marginally significant interaction in time spent in chamber [$F_{(1, 42)} = 3.200$, $p = 0.0808$], we conducted unpaired and paired t-tests on time spent in chambers. There was a nominally significant difference between genotypes in time spent in chamber of unfamiliar side ($t = 2.250$, $df = 42$, $p = 0.0298$, BARP KO vs. WT) (**Figure 4H**). The time spent in each chamber was statistically indistinguishable between the familiar and unfamiliar sides in BARP KO mice ($t = 0.851$, $df = 21$, $p = 0.4042$) (**Figure 4H**), while WT mice tended to spend more time in the chamber of unfamiliar side than that of familiar side ($t = 1.831$, $df = 21$, $p = 0.0813$) (**Figure 4H**). There was no statistically significant genotype effect nor genotype × side interaction in time spent around cage [genotype effect, $F_{(1, 42)} = 0.098$, $p = 0.7559$; genotype × side interaction, $F_{(1, 42)} = 0.728$, $p = 0.3985$] (**Figure 4I**). To avoid false-positives caused by the multiple statistical tests used, Bonferroni correction was applied to the results in the three-chamber social approach test (the adjusted p-value at the 0.05 significance level for 16 indices was 0.003125). After the correction, results remained significant for time spent around the cage by BARP KOs in the sociability test ($t = 3.724$, $df = 21$, $p = 0.0013$, stranger side vs. empty cage side) (**Figure 4G**).

It should be noted that the time spent by WT mice around a stranger mouse was similar to that spent around an empty cage (**Figure 4G**). In this study, a habituation session was performed in the apparatus for 10 min the day before the sociability test, while it is conventionally conducted immediately before the sociability test (Moy et al., 2004). The time spent around a stranger cage could be determined by approach-avoidance conflict and the protocol we used could have shifted the conflict toward avoidance behavior by increasing the relative novelty of the environment.

The social interaction of BARP KO mice in the home cage under familiar conditions was observed over a 7 days period (**Figures 4J,K**). In the social interaction test in the home cage, the mean number of objects usually increases when mice are active and decreases when mice are sleeping in one place. BARP KO mice spent less time separated from each other than WT

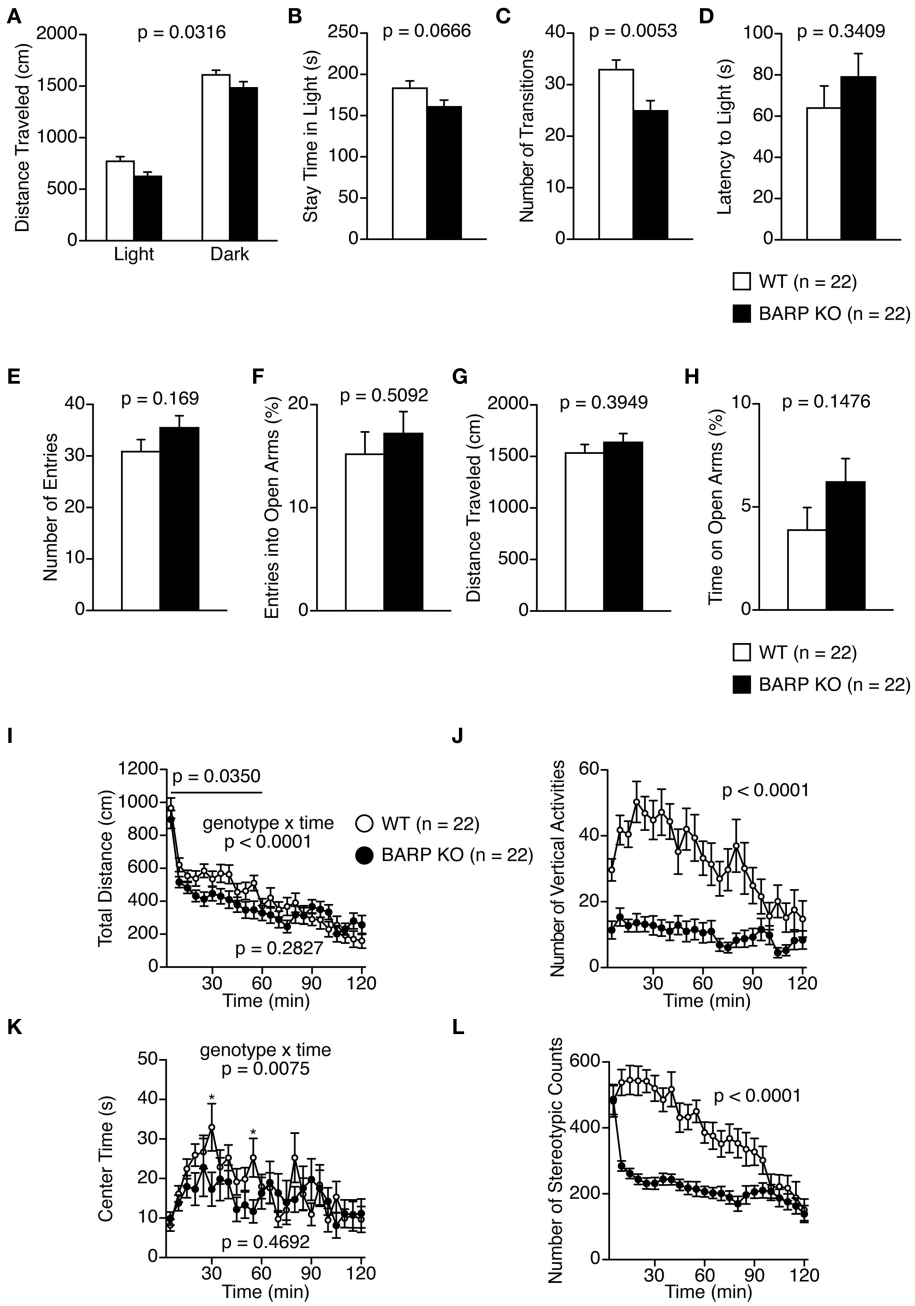

FIGURE 2 | Decreased locomotor activity in beta-anchoring and -regulatory protein (BARP) knockout (KO) mice. (A–D) Light/dark transition test: the distance traveled in the light/dark compartments **(A)**, time spent in the light compartment **(B)**, number of light/dark transitions **(C)**, and latency to enter the light compartment **(D)** are shown. **(E–H)** Elevated plus maze test: the number of arm entries **(E)**, percentage of entries into open arms **(F)**, distance traveled **(G)**, and percentage of time spent in open arms **(H)** are shown. **(I–L)** Open field test: the total distance traveled **(I)**, vertical activity **(J)**, time spent in the center area **(K)**, and stereotypic behavior counts **(L)**. Data represent the mean ± SEM. The *p*-values indicate the genotype effect in a *t*-test **(B–H)** and a Two-Way repeated measures ANOVA **(A,I–L)**.

mice in the light ($p = 0.0165$) and dark periods ($p = 0.0165$) (**Figures 4J,K**). This result suggests that the duration of social interaction was increased in BARP KO mice compared with WT mice. In addition, locomotor activity was lower in BARP KO mice in the overall period [genotype effect, $F_{(1, 20)} = 25.223$, $p < 0.0001$; genotype × time interaction, $F_{(167, 3340)} = 6.521$, $p < 0.0001$], light period [$F_{(1, 20)} = 12.578$, $p = 0.0020$], and dark period [$F_{(1, 20)} = 21.489$, $p = 0.0002$] (**Figure 4L**). The difference in the overall period and dark period reached study-wide significance.

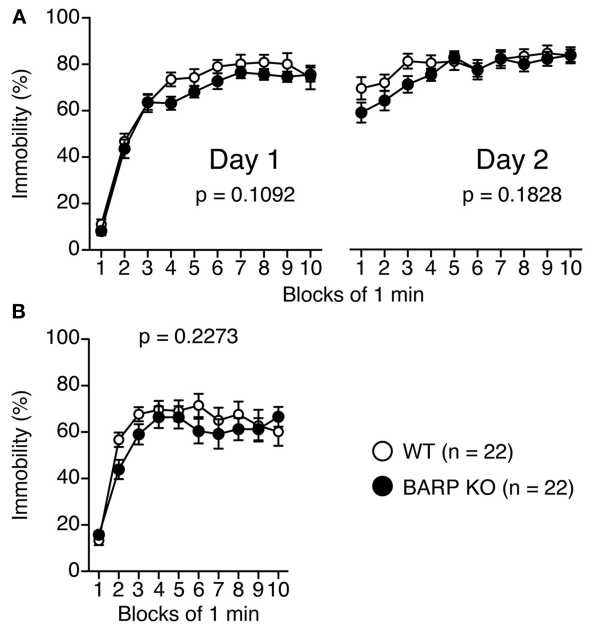

FIGURE 3 | Normal depression-like behavior in beta-anchoring and -regulatory protein (BARP) knockout (KO) mice. Immobility on day 1 and day 2 for BARP KO and wild type (WT) mice in the Porsolt forced swim test **(A)**. Immobility time for BARP KO and WT mice in the tail suspension test **(B)**. Data represent the mean ± SEM. The *p*-values indicate a genotype effect in a Two-Way repeated measures ANOVA.

Decreased Acoustic Startle Response and Nominally Significant Increase in PPI in BARP KO Mice

The acoustic startle response of the BARP KO mice at 110 and 120 dB was significantly decreased [$F_{(1, 42)} = 43.015$, $p < 0.0001$], which reached study-wide significance (**Figure 5A**). PPI, whose decrease often reflects psychiatric disturbance (Swerdlow et al., 2000), was significantly increased at 120 dB in the BARP KO mice [genotype effect, $F_{(1, 42)} = 1.671$, $p = 0.2032$; genotype × prepulse sound level effect, $F_{(1, 42)} = 0.062$, $p = 0.8042$ in startle response at 110 dB; genotype effect, $F_{(1, 42)} = 6.296$, $p = 0.0160$ in startle response at 120 dB] (**Figure 5B**). Since it is possible that the apparent increase in PPI was due to a secondary effect of a decreased acoustic startle response in BARP KO mice, we examined the correlation between startle amplitude and PPI. There was no significant correlation between startle amplitude and PPI [WT: startle amplitude (110 dB) and PPI (74–110 dB), $r = 0.176$, $p = 0.4377$, startle amplitude (110 dB) and PPI (78–110 dB), $r = 0.051$, $p = 0.8277$, startle amplitude (120 dB) and PPI (74–120 dB), $r = 0.202$, $p = 0.3716$, startle amplitude (120 dB) and PPI (78–120 dB), $r = 0.175$, $p = 0.4417$; BARP KO: startle amplitude (110 dB) and PPI (74–110 dB), $r = -0.147$, $p = 0.5184$, startle amplitude (110 dB) and PPI (78–110 dB), $r = 0.149$, $p = 0.5134$, startle amplitude (120 dB) and PPI (74–120 dB), $r = 0.110$, $p = 0.6293$, startle amplitude (120 dB) and PPI (78–120 dB), $r = 0.127$, $p = 0.5789$) (Supplementary Figures 3A–D). Thus, it is unlikely that the apparent increase in PPI was due to a secondary effect of a decreased acoustic startle response in BARP KO mice.

Nominally Significant Increase in Suppression Ratio during Pre-Tone Period at 1 d after Conditioning in BARP KO Mice

Pavlovian fear conditioning forms a strong association between a CS and an aversive US that can trigger stereotypic fear responses such as freezing (Maren, 2001; Pape and Pare, 2010). On the first day, BARP KO and WT mice were placed into a chamber and conditioned with a tone (the CS) paired with a foot shock (the US) (conditioning). On the second day, the mice were returned to the chamber and the incidences of freezing were examined in the absence of the tone and foot shock (context test). Next, conditioned mice were placed in a novel chamber, where freezing behavior was measured after the presentation of the tone without the foot shock (cued test). During the conditioning period, BARP KO mice showed lower levels of freezing before, during, and after foot shocks [$F_{(1, 42)} = 5.026$, $p = 0.0303$] (**Figure 6A**). There was no significant difference in the distance traveled during and after each foot shock in the conditioning period [foot shock 1, genotype effect, $F_{(1, 42)} = 0.056$, $p = 0.8139$; genotype × time effect, $F_{(22, 924)} = 0.504$, $p = 0.9726$; foot shock 2, genotype effect, $F_{(1, 42)} = 0.01$, $p = 0.9216$; genotype × time effect, $F_{(22, 924)} = 0.47$, $p = 0.9824$; foot shock 3, genotype effect, $F_{(1, 42)} = 0.179$, $p = 0.6748$; genotype × time effect, $F_{(22, 924)} = 0.422$, $p = 0.9914$] (**Figure 6B**). To control for baseline activity, we classified the distance traveled during the first 2 min of training as "baseline activity" and used a suppression ratio [suppression ratio = (activity during testing)/(activity during baseline + activity during testing)] as a secondary index of fear (Anagnostaras et al., 2000; Frankland et al., 2004). In the context test 1 day after conditioning, BARP KO mice showed reduced levels of freezing, although the suppression ratio was indistinguishable between BARP KO and WT mice [freezing, genotype effect, $F_{(1, 42)} = 10.69$, $p = 0.0022$; genotype × time effect, $F_{(4, 168)} = 0.506$, $p = 0.7310$; suppression ratio, genotype effect, $F_{(1, 42)} = 2.799$, $p = 0.1017$; genotype × time effect, $F_{(1, 42)} = 1.194$, $p = 0.2807$] (**Figures 6C,D**). In the cued test with altered context 1 day after conditioning, BARP KO mice showed a nominally significant reduction in levels of freezing during pre-tone period [genotype effect, $F_{(1, 42)} = 13.741$, $p = 0.0006$] and during the CS [genotype effect, $F_{(1, 42)} = 5.589$, $p = 0.0228$] (**Figure 6C**). Suppression ratio during pre-tone period showed a nominally significant decrease in BARP KO [genotype effect, $F_{(1, 42)} = 12.025$, $p = 0.0012$], while the one during the CS was statistically indistinguishable between BARP KO and WT mice [$F_{(1, 42)} = 3.408$, $p = 0.0719$; genotype × time effect, $F_{(1, 42)} = 0.386$, $p = 0.5379$] (**Figure 6D**). In the context test 35 days after conditioning, there were no significant genotype effects nor genotype × time interactions in freezing [genotype effect, $F_{(1, 42)} = 0.719$, $p = 0.4011$; genotype × time effect, $F_{(4, 168)} = 1.078$, p = 0.3689] and in suppression ratio [genotype effect, $F_{(1, 42)} = 0.269$, $p = 0.6065$; genotype × time effect, $F_{(1, 42)} = 1.975$, $p = 0.1672$] (**Figures 6E,F**). In the cued test 35 days after conditioning, levels of freezing during pre-tone period [genotype effect, $F_{(1, 42)} =$

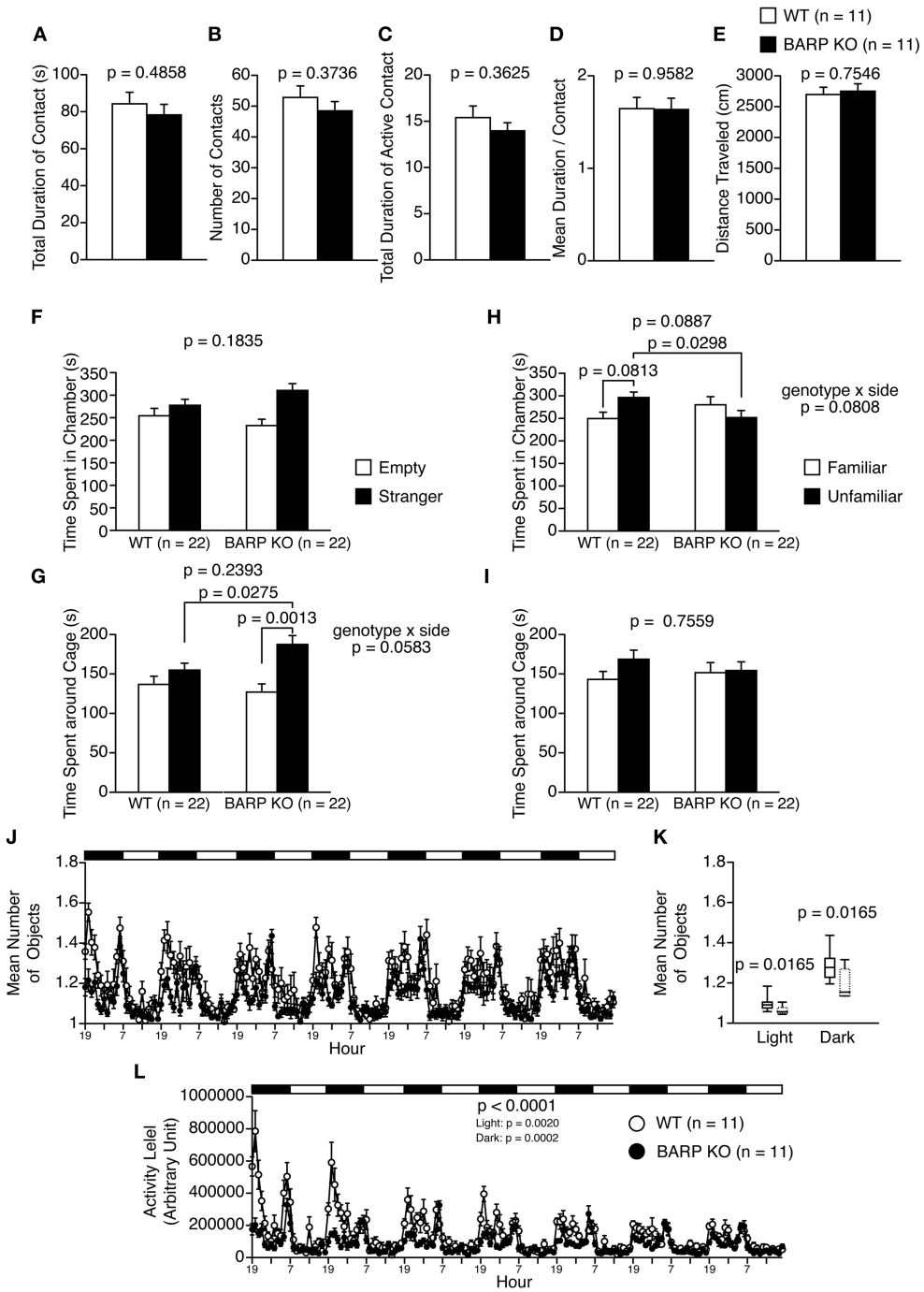

FIGURE 4 | Nominally significant increase in sociability in beta-anchoring and -regulatory protein (BARP) knockout (KO) mice. (A–E) Social interaction test: the total duration of contacts **(A)**, number of contacts **(B)**, total duration of active contacts **(C)**, mean duration of each contact **(D)**, and total distance traveled **(E)** are shown. **(F–I)** Sociability test **(F,G)** and social novelty preference test **(H,I)**: the time spent in chamber in the sociability test **(F)**, time spent around cage in the sociability test **(G)**, time spent in chamber in the social novelty preference test **(H)**, and time spent around cage in the sociability test **(I)**. In the sociability test, the time spent in the chamber with the empty cage (empty side) and with the cage containing a stranger (stranger side) are shown, and the time spent in the vicinity of the empty cage (empty side) vs. the cage containing a stranger (stranger side) are shown. In the preference for social novelty test, the time spent in the chamber with the cage containing a stranger (unfamiliar side) and with the cage containing a familiar (familiar side) are shown, and the time spent in the vicinity of the cage containing a stranger (unfamiliar side) and the cage containing a familiar (familiar side) are shown. **(J–L)** 24-h home cage social interaction test: mean number of objects detected **(J)**, box plot distributions of mean number of objects detected (WT, solid line; BARP KO, dashed line) **(K)**, and activity level **(L)** over 7 days. Data are shown as mean ± SEM or in box plots (median, interquartile, and 90 and 10th percentiles). The *p*-values indicate the genotype effect in a *t*-test **(A–I)**, a Mann–Whitney *U*-test **(K)** and a Two-Way repeated measures ANOVA **(L)**.

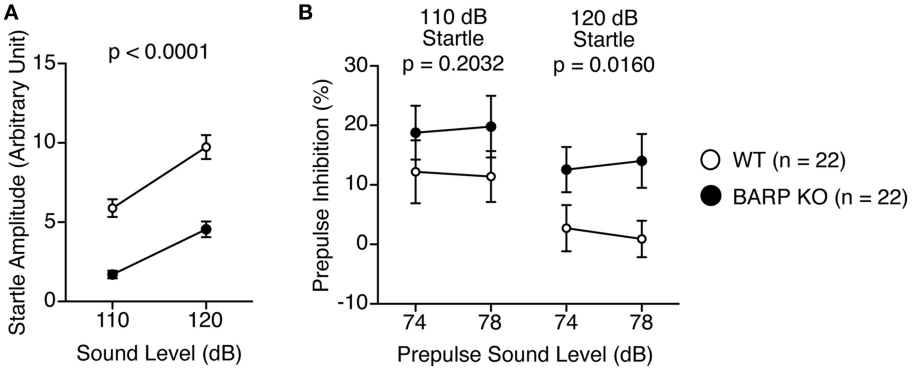

FIGURE 5 | Decreased acoustic startle response and nominally significant increase in prepulse inhibition (PPI) in beta-anchoring and -regulatory protein (BARP) knockout (KO) mice. (A) Amplitude of the startle response. **(B)** Percentage of PPI. Data represent the mean ± SEM. The *p*-values indicate a genotype effect in a Two-Way repeated measures ANOVA.

3.181, $p = 0.0817$; genotype × time effect, $F_{(2, 84)} = 0.659$, $p = 0.5199$] and during the CS [genotype effect, $F_{(1, 42)} = 0.816$, $p = 0.3715$; genotype × time effect, $F_{(14, 588)} = 0.518$, $p = 0.9236$] were statistically indistinguishable between BARP KO and WT (**Figure 6E**). There were no significant genotype effects nor genotype × time interactions in suppression ratio during pre-tone period [genotype effect, $F_{(1, 42)} = 2.011$, $p = 0.1635$; genotype × time effect, $F_{(1, 42)} = 0.086$, $p = 0.7710$] and during the CS [genotype effect, $F_{(1, 42)} = 0.440$, $p = 0.5109$; genotype × time effect, $F_{(1, 42)} = 1.600$, $p = 0.2129$] (**Figure 6F**).

To examine the role of BARP on cued fear extinction, we exposed WT and BARP KO mice to repeated non-reinforced CS presentations for 15 min at 35 days after fear conditioning (**Figure 6E**, right). A cued test with altered context was conducted 1 day after extinction training (**Figures 6G,H**). There were no significant genotype effects nor genotype × time interactions in freezing during pre-tone period [baseline, genotype effect, $F_{(1, 42)} = 3.300$, $p = 0.0764$; genotype × time effect, $F_{(2, 84)} = 0.372$, $p = 0.6905$] or during the CS [genotype effect, $F_{(1, 42)} = 0.302$, $p = 0.5855$; genotype × time effect, $F_{(2, 84)} = 0.364$, $p = 0.6958$]. We failed to detect the statistically significant differences in suppression ratio during pre-tone period [genotype effect, $F_{(1, 42)} = 0.339$, $p = 0.5637$; genotype × time effect, $F_{(1, 42)} = 0.397$, $p = 0.5318$] or during the CS [genotype effect, $F_{(1, 42)} = 0.301$, $p = 0.5864$; genotype × time effect, $F_{(1, 42)} = 0.220$, $p = 0.6415$].

Normal Acquisition and Retention of Spatial Reference Memory in BARP KO Mice

In the Barnes maze test, there was no significant effect of genotype on the latency to find the target hole [$F_{(1, 42)} = 0.083$, $p = 0.7751$] (**Figure 7A**), the number of search errors made [$F_{(1, 42)} = 0.030$, $p = 0.8634$] (**Figure 7B**), the distance to reach the target hole during acquisition [$F_{(1, 42)} = 0.086$, $p = 0.7713$] (**Figure 7C**), or the number of omission errors [$F_{(1, 42)} = 0.184$, $p = 0.6698$] (**Figure 7D**), indicating normal acquisition of spatial reference memory in BARP KO mice. Probe trials where the escape box was removed were performed 24 h and 30 days after the last day of training. There were no significant differences between genotypes in the time spent around the target during these probe tests ($t = 0.916$, $df = 42$, $p = 0.3649$, and $t = 0.225$, $df = 42$, $p = 0.8231$, respectively) (**Figure 7E**).

Nominally Significant Increase in Working memory and Flexibility in BARP KO Mice

Mice were subjected to the spontaneous alteration, forced alternation using food reward, and left-right discrimination tests in the T-maze. In the T-maze spontaneous alternation test, BARP KO and WT mice were subjected to five consecutive sessions (**Figures 8A–C**). One session consisted of 10 trials. Each trial had first and second runs. On the first run of each trial, mice were forced to choose one of the goal arms (the other being blocked by a removable door). During the second run in which the mice had to remember the location of the visited arm in the first run, both goal arms were open and the mouse was able to choose one arm freely. BARP KO mice showed increased correct responses compared with WT mice in sessions four and five [total, genotype effect, $F_{(1, 42)} = 1.734$, $p = 0.195$; genotype × time effect, $F_{(4, 168)} = 2.751$, $p = 0.0299$; sessions four and five, genotype effect, $F_{(1, 42)} = 10.206$, $p = 0.0027$] (**Figure 8A**). BARP KO mice exhibited longer latency to complete a session in spontaneous T-maze alteration test ($F_{(1, 42)} = 28.733$, $p < 0.0001$) (**Figure 8B**). This difference reached study-wide significance. With 10, 30, and 60 s delays between the forced and free choices, there was a significant difference in the percentage of correct choices between BARP KO and WT mice [genotype effect, $F_{(1, 41)} = 5.202$, $p = 0.0278$] (**Figure 8C**).

Next, mice were subjected to the T-maze forced alternation tests using food reward after food restriction. BARP KO and WT mice were subjected to daily training sessions consisting of five consecutive sessions where the mice had to remember the location of the previously visited arm, similar to the T-maze spontaneous alternation test, to get a food reward. During training sessions, there was no significant difference between BARP KO and WT mice (**Figures 8D–F**, Supplementary Figure 4B). With 10, 30, and 60 s delays between the forced and free choices, the number of correct choices was indistinguishable between BARP KO and WT mice [genotype effect, $F_{(1, 42)} =$

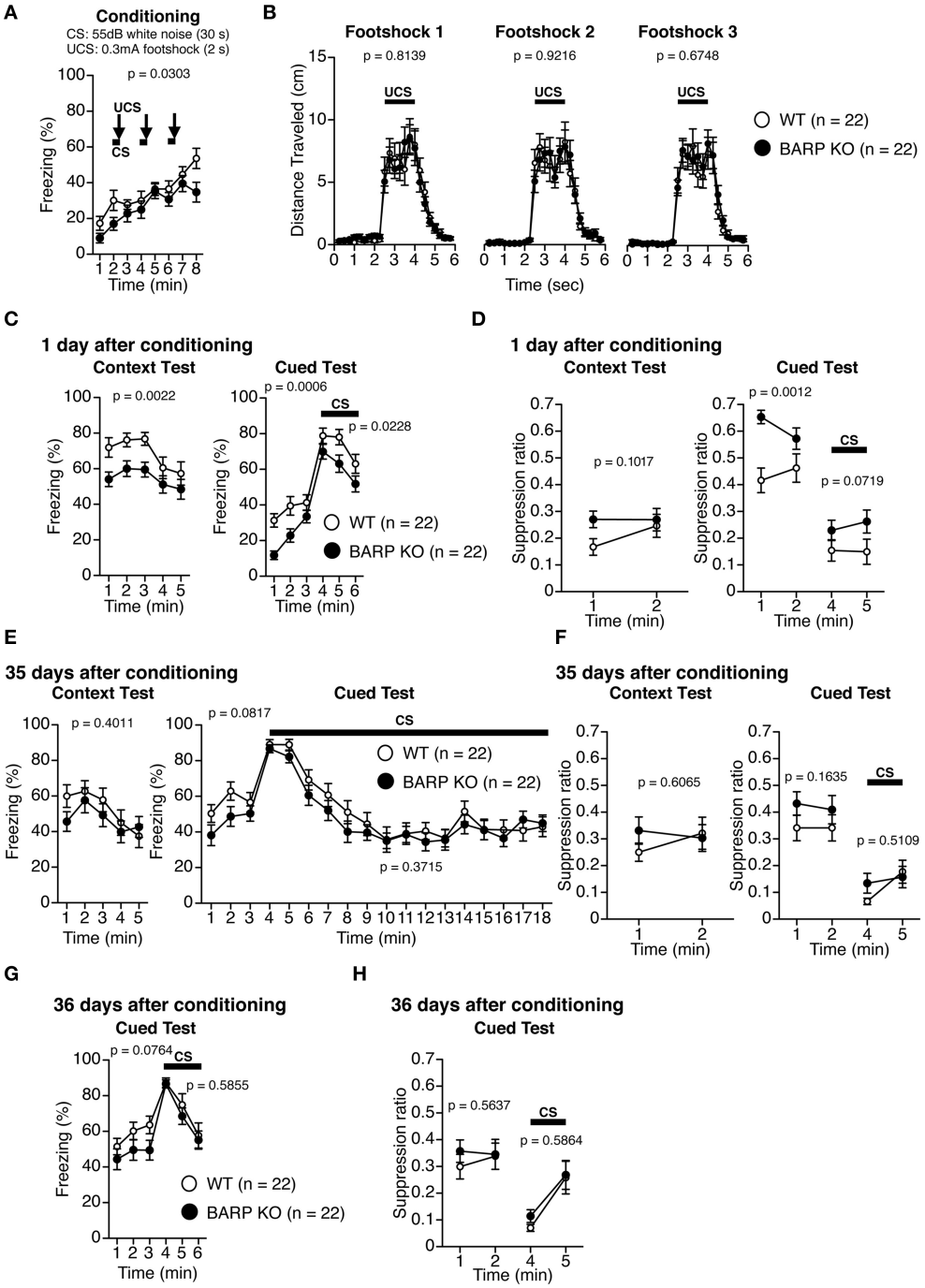

FIGURE 6 | Nominally significant increase in suppression ratio during pre-tone period at 1 day after conditioning in beta-anchoring and -regulatory protein (BARP) knockout (KO) mice. (A) Freezing during conditioning. **(B)** Distance traveled during and after each foot shock in the training phase. **(C)** Freezing during the context test (left) and the cued test (right) 1 day after conditioning. **(D)** The suppression ratio in the context (left) and cued tests (right) 1 day after conditioning. **(E)** Freezing during the context test (left) and the cued test (right) 35 days after conditioning. **(F)** The suppression ratio in the context (left) and cued tests (right) 35 days after conditioning. **(G)** The cued test 36 days after conditioning. **(H)** The suppression ratio in the cued test 36 days after conditioning. Data represent the mean ± SEM. Data were analyzed using a Two-Way repeated measures ANOVA, and the *p*-values indicate a genotype effect.

2.139, $p = 0.151$; genotype × delay effect, $F_{(3,\ 126)} = 0.657$, $p = 0.5798$] (**Figure 8G**). To increase the difficulty of the task, a delay period (3, 30, 60, or 120 s) was applied. Under these conditions, BARP KO mice showed a nominally significant increase in spatial working memory with the 120 s delay [genotype effect, $F_{(1,\ 42)} = 1.293$, $p = 0.2619$; genotype × delay effect, $F_{(3,\ 126)} = 3.420$,

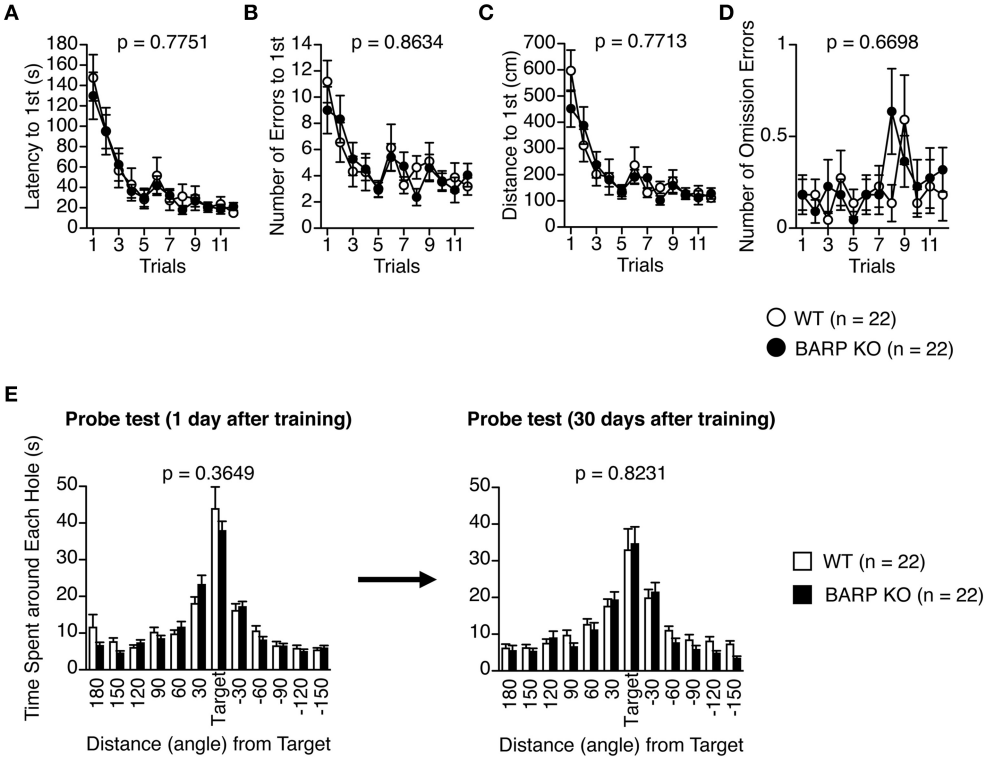

FIGURE 7 | Normal acquisition and retention of spatial reference memory in beta-anchoring and -regulatory protein (BARP) knockout (KO) mice. (A) Latency to reach the target hole. **(B)** Number of errors before reaching the target hole. **(C)** Distance to reach the target hole. **(D)** Number of omission errors before reaching the target hole. Data were analyzed by Two-Way repeated measures ANOVA. Data are presented as means of three trials. The *p*-values indicate a genotype effect in a Two-Way repeated measures ANOVA. **(E)** Time spent around each hole in the probe trial conducted 24 h (left) and 1 month (right) after the last training session. The *p*-values indicate a genotype effect in a *t*-test.

$p = 0.0194$; 120 s delay, genotype effect, $t = 2.609$, $df = 42$, $p = 0.0125$] (**Figure 8H**).

We then performed the left-right discrimination test in the T-maze to investigate perseveration tendency (**Figures 9A–C**). In the first seven sessions, where the baited arm was fixed to one side, correct responses were indistinguishable between BARP KO and WT mice [genotype effect, $F_{(1, 42)} = 0.370$, $p = 0.5465$; genotype × blocks of two sessions effect, $F_{(6, 252)} = 0.251$, $p = 0.9585$] (**Figure 9A**). However, in the following 8 days, when the baited arm was changed to the opposite side, the correct choice percentage was significantly higher in the BARP KO than the WT mice [$F_{(1, 42)} = 4.528$, $p = 0.0393$] (**Figure 9A**), suggesting a nominally significant increase in flexibility in the mutant mice.

Discussion

In this study, we found that BARP KO mice have hypo-locomotor activity as evidenced by decreased vertical activity, stereotypic counts in the open field test, and activity level in the home cage, and longer latency to complete a session in spontaneous T-maze alteration test, which reached "study-wide significance." In addition, some suggestive evidence showed that BARP KO mice may have increased anxiety-like behavior and multiple behavioral phenotypes that are seemingly opposite to those seen in the mouse models of schizophrenia and its related disorders.

The most pronounced behavioral phenotype of BARP KO mice is a decreased locomotor activity. It has been reported that CaV2.2 KO mice display hyperactivity (Beuckmann et al., 2003). Considering that BARP down-regulates activities of VGCCs including CaV2.2 (Béguin et al., 2014), it is possible that this behavioral phenotype of BARP KO mice could be mediated through decreased negative regulation of CaV2.2 by the lack of BARP.

BARP KO mice also exhibited contrasting behavioral phenotypes to those typically seen in mouse models of schizophrenia and its related disorders. BARP KO mice showed increased working memory ability, PPI, and social interaction, and decreased locomotor activity and perseveration, which are opposite to those generally considered as core features of schizophrenia (Braff and Geyer, 1990; Crider, 1997; Elvevag and Goldberg, 2000; Powell and Miyakawa, 2006), though many of the behavioral results suggesting these phenotypes achieved only nominal statistical significance and require further replication. In addition, greatly reduced vertical activity and stereotypic behavior counts in the open-field test in the mice are also in contrast to the behavioral phenotypes of some mouse models of schizophrenia and its related disorders,

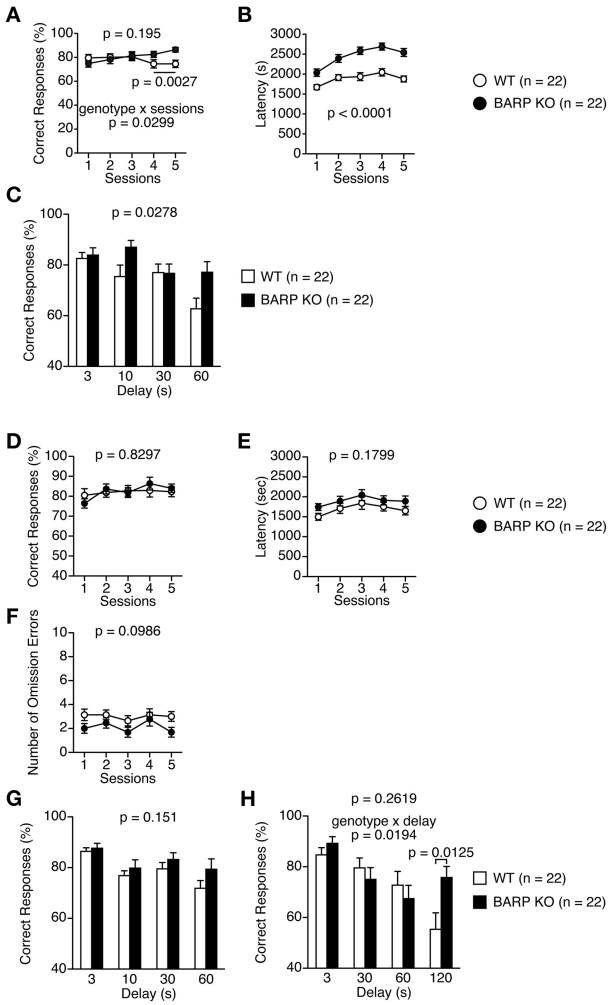

FIGURE 8 | Nominally significant increase in working memory in beta-anchoring and- regulatory protein (BARP) knockout (KO) mice. (A–C) T-maze spontaneous alternation test: the percent correct responses **(A)** and latency **(B)** in the training session, and correct responses in the delay session **(C)** in BARP KO and wild type (WT) mice. **(D–H)** T-maze forced alternation test: the percent correct responses **(D)**, latency **(E)**, and omission errors **(F)** in the training session, and correct responses in the delay session **(G,H)** in BARP KO and WT mice. Data represent the mean ± SEM. Data were analyzed using a Two-Way repeated measures ANOVA and a *t*-test. The *p*-values indicate a genotype effect.

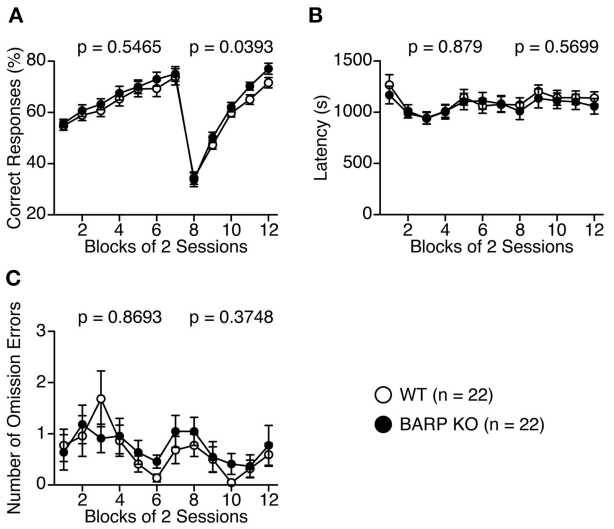

FIGURE 9 | Nominally significant decrease in a perseveration tendency of beta-anchoring and -regulatory protein (BARP) knockout (KO) mice in the T-maze left-right discrimination test. Percent correct responses **(A)**, latency **(B)**, and omission errors **(C)** in BARP KO and wild type (WT) mice. The baited arm was changed to the opposite side from session eight onwards. Data represent the mean ± SEM. Data were analyzed using a Two-Way repeated measures ANOVA and the *p*-values indicate a genotype effect.

such as dopamine transporter KO mice, dominant-negative Disrupted-in-Schizophrenia-1 transgenic mice, Schnurri-2 KO mice, forebrain-specific calcineurin KO mice, and α-CaMKII heterozygous KO mice (Giros et al., 1996; Miyakawa et al., 2003; Hikida et al., 2007; Yamasaki et al., 2008; Takao et al., 2013). Molecules related to Ca^{2+} signaling, such as calcineurin and α-isoform of Ca^{2+}/calmodulin dependent protein kinase II (α-CaMKII), act downstream of VGCC activation (Catterall, 2011), which is negatively regulated by BARP. Schizophrenia model mice such as forebrain-specific calcineurin KO mice and α-CaMKII heterozygous KO mice show hyperactivity, decreased working memory, and decreased PPI (Miyakawa et al., 2003; Yamasaki et al., 2008). It is possible that the deficit in BARP induces abnormal Ca^{2+} signaling via these molecules, contributing to these behavioral phenotypes.

We obtained some results that suggest increased anxiety-like behavior in BARP KO mice. The mutants exhibited nominally significant reductions in the number of transitions in the light/dark transition test. The number of transitions is considered as a core anxiety measure (Crawley et al., 1997). The BARP KO mice also showed a nominally significant reduction in time spent in the center of the open field apparatus, which is considered as an index of anxiety-like behavior, at a few time points. Since the *p*-values did not reach study-wide significance, there is the possibility that these results could be just false-positive and replication of behavioral study is needed. The possible increase of anxiety in the mice should be also examined by measuring other indices of anxiety, such as stress hormone level in the blood and expression level of immediately early genes in the amygdala.

It has been reported that genes encoding VGCC subunits, especially CACNA1C, are associated with psychiatric disorders, such as schizophrenia and bipolar disorder (Sklar et al., 2002, 2011; Ferreira et al., 2008; Ripke et al., 2011, 2014). We compared the behavioral phenotypes of BARP KO mice with those of mice with genetic or pharmacological inactivation of CaV1.2 encoded by CACNA1C (**Table 2**). We expected that BARP KO mice may show behavioral phenotypes opposite to those of CACNA1C transgenic mice, since BARP down-regulates VGCC activity (Béguin et al., 2014). However, behavioral phenotypes of BARP KO mice were not in consentient with such initial expectation (**Table 2**). One possibility to explain this inconsistency is that the lack of VGCC activity regulation by BARP could be compensated

TABLE 2 | Behavioral phenotypes in mice with genetic or pharmacological inactivation of $Ca_V1.2$ and beta-anchoring and -regulatory protein (BARP) knockout (KO).

	BARP KO	Hippocampus and cortex CACNA1C KO	Acute pharmacological blockade of CaV1.2	ACC-specific conditional CACNA1C KO	Constitutive CACNA1C heterozygous	Forebrain-specific conditional CACNA1C KO	PFC-specific CACNA1C knockdown
Anxiety-like behavior	Decreased number of transitions in the LD	Normal in the OF	N/D	Normal in the EP, LD, and OF	Decreased time spent in open arms in the EP Normal in the OF and LD	Decreased time spent in the open arms in the EP, in the center in the OF, and in the light in the LD	Decreased time spent in open arms in the EP
Memory	Better performance in the T-maze	Decreased performance during the training phase and in the remote memory probe test in the Morris water maze	Decreased performance at 1 day after conditioning in in cued fear conditioning paradigms	Impaired observational fear learning Normal in NOR and predator exposure Normal at 1 day after conditioning in cued and contextual fear conditioning paradigms	N/D	N/D	N/D
Other behavioral phenotypes	See **Table 1**	Normal in the rotarod test	N/D	N/D	N/D	N/D	Normal in locomotor activity
References	Present study	Moosmang et al., 2005; White et al., 2008	Langwieser et al., 2010	Jeon et al., 2010	Lee et al., 2012	Lee et al., 2012	Lee et al., 2012

Abbreviations: ACC, anterior cingulate cortex; EP, elevated plus-maze test; LD, light-dark transition test; N/D, not determined; NOR, novel object recognition; PFC, prefrontal cortex; PS, Porsolt forced swim test.

for by other VGCC β subunit-interacting proteins, such as Kir/Gem, Rem, Rad, RIMs, Bassoon, and CAST (Béguin et al., 2001; Finlin et al., 2003; Kiyonaka et al., 2007, 2012; Uriu et al., 2010; Chen et al., 2011). Another possibility is that a battery of multiple behavioral tests was applied to the same mice in the present study, while only a few behavioral tests were conducted for a mouse in the previous studies on CACNA1C. As mice were subjected to various kinds of behavioral tests, the stress/experience of multiple different tests might have affected the results in this study.

BARP KO mice showed lower levels of basal freezing before experiencing foot shocks in the fear conditioning test, although they were hypoactive in the open field test and home cage. One possibility to explain this inconsistency is that the increased locomotor activity displayed by BARP KO mice could be due to this particular environment (e.g., the grid floor). In the context and cued tests 1 day after conditioning, BARP KO mice also showed decreased freezing, though differences in the suppression ratio failed to reach significance between BARP KO and WT mice during the context test and during cue presentation in the cued test, possibly due to the lower levels of basal freezing in BARP KO mice. However, suppression ratio during pre-tone period in the new context in the cued test was significantly greater in the BARP KO mice, suggesting that they may have a better ability of pattern separation and/or decreased generalized fear. It should be noted that impaired pattern separation is proposed as a cognitive deficit of the patients with schizophrenia (Tamminga et al., 2010).

Taken together, our study shows that BARP KO mice are hypoactive and may possibly have increased anxiety. Moreover, the mice showed multiple behavioral phenotypes that are opposite to those considered as the behavioral abnormalities related to schizophrenia, suggesting that they may serve as a unique tool for studying the mechanisms of psychiatric disorders and their endophenotypes. Since many of the results obtained in this study are statistically weak, replications of the results are needed. Hence, further evaluation of the molecular and physiological phenotypes of BARP KO mice could provide new insights into the role of BARP in higher brain functions.

Acknowledgments

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnbeh.2015.00141/abstract

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Prepulse inhibition predicts spatial working memory performance in the inbred Roman high- and low-avoidance rats and in genetically heterogeneous NIH-HS rats: relevance for studying pre-attentive and cognitive anomalies in schizophrenia

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Animal models of schizophrenia-relevant symptoms are increasingly important for progress in our understanding of the neurobiological basis of the disorder and for discovering novel and more specific treatments. Prepulse inhibition (PPI) and working memory, which are impaired in schizophrenic patients, are among the symptoms/processes modeled in those animal analogs. We have evaluated whether a genetically-selected rat model, the Roman high-avoidance inbred strain (RHA-I), displays PPI deficits as compared with its Roman low-avoidance (RLA-I) counterpart and the genetically heterogeneous NIH-HS rat stock. We have investigated whether PPI deficits predict spatial working memory impairments (in the Morris water maze; MWM) in these three rat types (Experiment 1), as well as in a separate sample of NIH-HS rats stratified according to their extreme (High, Medium, Low) PPI scores (Experiment 2). The results from Experiment 1 show that RHA-I rats display PPI and spatial working memory deficits compared to both RLA-I and NIH-HS rats. Likewise, in Experiment 2, "Low-PPI" NIH-HS rats present significantly impaired working memory with respect to "Medium-PPI" and "High-PPI" NIH-HS subgroups. Further support to these results comes from correlational, factorial, and multiple regression analyses, which reveal that PPI is positively associated with spatial working memory performance. Conversely, cued learning in the MWM was not associated with PPI. Thus, using genetically-selected and genetically heterogeneous rats, the present study shows, for the first time, that PPI is a positive predictor of performance in a spatial working memory task. These results may have translational value for schizophrenia symptom research in humans, as they suggest that either

by psychogenetic selection or by focusing on extreme PPI scores from a genetically heterogeneous rat stock, it is possible to detect a useful (perhaps "at risk") phenotype to study cognitive anomalies linked to schizophrenia.

Keywords: prepulse inhibition, spatial working memory, cognitive deficits, schizophrenia-relevant symptoms, schizophreniform rat model, Roman high-avoidance rats, Roman low-avoidance rats, genetically heterogeneous rats

Introduction

Schizophrenia symptoms are usually grouped in three categories: positive (hallucinations, delusions, and other thought disorders); negative (anhedonia, avolition, poverty of thought, and content of speech) and cognitive impairment (working memory and attention deterioration). Their complexity, diversity, and bizarreness preclude a full modeling of the entire constellation in animals, just as schizophrenic patients do not manifest every possible symptom. Some of the most commonly used animal models rely on the similarity between the effects that psychotomimetic and psychostimulant drugs trigger in both animals and humans, i.e., those animal analogs mostly reproduce positive (psychotic) symptoms of schizophrenia, while other rodent analogs focus on modeling negative (e.g., impaired social behavior) or cognitive (e.g., impairments of spatial learning, working memory) symptoms of the disorder. In addition, some rat and mouse models may be used to assess sensorimotor gating (pre-attentive) or attention-related processes which are impaired in schizophrenic patients (e.g., reviews by Sawa and Snyder, 2002; Powell and Miyakawa, 2006; Jones et al., 2011; Del Río et al., 2014).

One of these pre-attentive processes is prepulse inhibition (PPI), which refers to the ability of an acoustic stimulus of relatively weak intensity (i.e., prepulse) to diminish the acoustic startle response (ASR) caused by a subsequent acoustic pulse of higher intensity. PPI is an operational measure of the pre-attentive filtering process known as sensorimotor gating, which reflects the neural filtering of redundant or unnecessary stimuli that takes place in complex systems (Freedman et al., 1987; Koch and Schnitzler, 1997; Cromwell et al., 2008; García-Sánchez et al., 2011; Kohl et al., 2013). Since PPI is a cross-species phenomenon, it can be measured in both mammals and humans with the same experimental procedure, thereby providing a very useful paradigm for translational research. PPI is impaired in schizophrenic patients, among other mental disorders, and thus it is widely considered an endophenotype of the disorder (e.g., reviews by Freedman et al., 1987; Koch and Schnitzler, 1997; Cromwell et al., 2008; García-Sánchez et al., 2011; Kohl et al., 2013).

Genetically-based rat models of schizophrenia-related symptoms, derived from selective breeding programs, may have the advantage of symptom stability (within and across generations), and may lead to the identification of clusters of associated/related symptoms. Hence, genetic models represent a useful approach to study the neurobiological and, importantly, genetic mechanisms underlying the symptoms of schizophrenia. Examples of these genetically-based rat models are, for instance, the APO-SUS and APO-UNSUS rat lines (Ellenbroek et al., 1995; van der Elst et al., 2006, the "three hit" Low-PPI rat line (Petrovszki et al., 2013; Kekesi et al., 2015) and the Low-PPI/High-PPI rat lines (Freudenberg et al., 2007; Schwabe et al., 2007), which are rat lines presenting impairments in PPI (APO-SUS, "three hit low-PPI" and Low-PPI) and other schizophrenia-related symptoms, like latent inhibition or some cognitive functions (Ellenbroek et al., 1995; van der Elst et al., 2006; Freudenberg et al., 2007; Schwabe et al., 2007; Petrovszki et al., 2013; Kekesi et al., 2015; see review by Del Río et al., 2014).

The Roman High- (RHA) and Low-avoidance (RLA) rat lines/strains (depending on whether they are outbred –i.e., lines-, or inbred –i.e., strains-), may constitute another of such genetic models. They have been selectively and bidirectionally bred for their rapid (RHA) vs. extremely poor (RLA) ability to acquire the two-way active avoidance task (Bignami, 1965; Broadhurst and Bignami, 1965). The extensive research conducted over the last 40 years (e.g., Zeier et al., 1978; Driscoll and Bättig, 1982; Escorihuela et al., 1995a) has led to the conclusion that anxiety/fear and stress sensitivity are among the most prominent behavioral traits that distinguish the two Roman lines/strains. In fact, compared to their RLA counterparts, RHA rats (both from the outbred line –RHA/Verh- or from the inbred strain –RHA-I-) show a phenotype characterized by: (1) low unconditioned and conditioned anxiety/fear (López-Aumatell et al., 2009a,b,c; Díaz-Morán et al., 2012, 2013c; Martínez-Membrives et al., 2015), (2) a proactive coping style (Steimer and Driscoll, 2003, 2005; Driscoll et al., 2009; Díaz-Morán et al., 2012), (3) decreased sensitivity to reward-loss-induced frustration (e.g., Torres et al., 2005; Rosas et al., 2007; Gomez et al., 2009), (4) lowered activation of the hypothalamus–pituitary–adrenal (HPA) axis in response to stress (Steimer and Driscoll, 2003; Carrasco et al., 2008), (5) enhanced central GABA-A/benzodiazepine complex function (which is known to be critically involved in the regulation of anxiety/frustration; Corda et al., 1997; Bentareha et al., 1998) and (6) increased novelty- and drug-seeking behavior (e.g., Fernández-Teruel et al., 1992, 1997; Escorihuela et al., 1999; Giorgi et al., 2007; Manzo et al., 2014). Most important to assess whether RHA/RLA rats display differential schizophrenia-relevant features, the RHA strain/line displays a poorer performance in several learning/memory tasks (Nil and Bättig, 1981; Driscoll et al., 1995; Escorihuela et al., 1995b; Aguilar et al., 2002) and enhanced impulsive behavior in the 5-CSRTT and DRL-20 operant tasks (Zeier et al., 1978; Moreno et al., 2010; Klein et al., 2014). These profiles suggest that RHA rats may have some value for modeling certain deficits of executive function present in schizophrenia. Compared with the RLA line/strain and/or with standard rat

strains, RHA rats display relative deficits in latent inhibition threshold (Fernández-Teruel et al., 2006; and unpublished results from our laboratory), augmented mesocortical dopaminergic response to stress (Giorgi et al., 2003), enhanced locomotor as well as mesolimbic dopaminergic sensitization to repeated (DAergic) psychostimulant administration (Corda et al., 2005; Giorgi et al., 2007; Guitart-Masip et al., 2008), and neurochemical and neuromorphological evidence of decreased hippocampal function (Sallés et al., 2001; Meyza et al., 2009; Garcia-Falgueras et al., 2012). Remarkably, we have recently found that RHA-I rats show a dramatically reduced expression of mGluR2 in prefrontal cortex and hippocampus and increased cortical 5HT2AR expression (Klein et al., 2014). Thus, RHAs display a series of neurobehavioral traits that resemble some schizophrenia—relevant symptoms or associated neural processes.

As said above, schizophrenias usually present (or are associated with) complex clusters of symptoms. Knowing which of them are inter-related or which are orthogonal is important for both, progress in neurobiological research and for the development of novel treatments addressed to particular symptoms or clusters of symptoms. In this context, clinical researchers are studying the relationships among pre-attentive processes, attention, memory, cognition, and executive functions in schizophrenics and healthy human volunteers (Bitsios and Giakoumaki, 2005; Bitsios et al., 2006; Giakoumaki et al., 2008; Csomor et al., 2009). Using this approach it has been shown that PPI may be positively correlated with some cognitive functions, including working memory, in healthy human volunteers (Bitsios and Giakoumaki, 2005; Bitsios et al., 2006; Giakoumaki et al., 2008; Csomor et al., 2009; Singer et al., 2013). In a recent study in mice, Singer et al. have also shown associations between PPI and working memory (Singer et al., 2013).

In the present study we aimed at evaluating possible links between PPI and working memory in the genetically-selected inbred RHA-I and RLA-I rats (supposedly "altered" because of the psychogenetic selection, and therefore representing a parallel of a "clinical" or "at risk" sample), and in the genetically heterogeneous (i.e., outbred) NIH-HS rat stock as a plausible parallel of a normative and healthy human sample. The genetically heterogeneous NIH-HS rat stock (i.e., "National Institutes of Health Genetically Heterogeneous Rat Stock") was developed by Hansen and Spuhler (1984) through an eight-way cross from eight inbred rat strains and they were bred for more than 50 generations. The NIH-HS rats are a unique tool to study the genetic basis of complex traits due to their broad phenotypic variation and high degree of genetic recombination compared to the usual laboratory rat strains (e.g., Spuhler and Deitrich, 1984; López-Aumatell et al., 2008, 2009a,b, 2011; Johannesson et al., 2009; Vicens-Costa et al., 2011; Díaz-Morán et al., 2012, 2013a,b,c, 2014; Baud et al., 2013, 2014a,b; Estanislau et al., 2013; Palència et al., 2013; Alam et al., 2014; Tsaih et al., 2014). Moreover, NIH-HS rats have been shown to closely resemble RLA-I rats in their coping style and stress sensitivity profiles (e.g., López-Aumatell et al., 2009a; Díaz-Morán et al., 2012, 2013c; Estanislau et al., 2013). Taking into account these characteristics, we used the NIH-HS rats because of their potentially high translational value.

Therefore, the aim of this research was to investigate possible sensory gating-working memory relationships by: (1) characterizing the performance of the three rat strains/stocks (RHA-I, RLA-I, and NIH-HS) for PPI and spatial working memory in the delayed-matching-to-place in the Morris Water Maze (MWM); and (2) evaluating PPI-working memory associations in a sample of heterogeneous NIH-HS rats stratified by their extreme (low or high) PPI levels.

On the basis of the results reviewed above, we hypothesized that (1) RHA-I rats would show PPI and working memory deficits as compared to RLA-I and NIH-HS rats (note that, as said above, RLA-Is and NIH-HS have a very similar behavioral/neuroendocrine profiles in several tests/tasks), and (2) PPI levels would be positively associated with spatial working memory.

Materials and Methods

Animals

The animals used were males of the inbred Roman High- (RHA-I) and Low-Avoidance (RLA-I) rat strains and the genetic heterogeneous rat stock (NIH-HS, "National Institutes of Health Genetically Heterogeneous Rat Stock"; derived from crossing the MR/N, WN/N, WKY/N, M520/N, F344/N, ACI/N, BN/SsN, and BUF/N strains; Hansen and Spuhler, 1984), from the permanent colonies maintained at our laboratory (Medical Psychology Unit, Dept. Psychiatry, and Forensic Medicine, School of Medicine, Autonomous University of Barcelona) since 1996 (RHA-I, RLA-I) and 2004 (NIH-HS), respectively. They were approximately 4 months old at the beginning of the experiments (weight range 320–420 g), and were housed in same-sexed pairs in standard ($50 \times 25 \times 14$ cm) macrolon cages. They were maintained under a 12:12 h light-dark cycle (lights on at 08:00 a.m.), with controlled temperature ($22 \pm 2°$C) and humidity (50–70%) and with free access to food and water.

In Experiment 1 subjects were male rats, from the RHA-I ($n = 16$) and RLA-I ($n = 19$) strains and from the NIH-HS genetically heterogeneous rat stock ($n = 30$), which were submitted to PPI testing and to the working memory tasks (see below). The same RHA-I and RLA-I rats were tested in the cued leaning task, jointly with 17 NIH-HS rats that were randomly selected from the initial sample of 30 animals.

In Experiment 2 subjects were 78 NIH-HS rats which were tested for PPI. From these, 33 NIH-HS rats were randomly selected to be evaluated in the working memory task. From these, 5–6 randomly selected rats from each of the three subgroups formed (see details below) underwent the cued learning task.

Rats in the RHA-I and RLA-I groups came from at least 10 different litters/strain, while NIH-HS rats were from 30 litters in each experiment.

The experiments were performed from 9:00 to 18:00 h. and were approved by the committee of Ethics of the Autonomous University of Barcelona in accordance with the European Communities Council Directive (86/609/EEC) regarding the care and use of animals for experimental procedures.

Prepulse Inhibition of the Acoustic Startle Response

Four sound-attenuated boxes (SR-Lab Startle Response System, San Diego Inst., San Diego, USA) were used. Each box consists of a Plexiglas cylinder situated on the top of a platform with a sensor that detects the strength made by the rat in each trial. Two speakers situated 15 cm from each side of the cylinder deliver the acoustic stimuli and a white noise generator provides the background noise. Each box is constantly lit by a 10 W lamp. The data are transduced by an accelerometer into a voltage which is amplified, digitized, and saved into a computer for further analysis.

The startle session starts with a 5 min habituation period in the startle chambers. Then, 10 "pulse-alone" trials (105 dB, 40 ms) are delivered in order to obtain a basal measure of the ASR (BASELINE 1). After this, each one of the six different types of trials are randomly administered 10 times (60 trials in total):

(1) Pulse-alone trials (105 dB 40 ms, BASELINE 2, this was the variable used to calculate the %PPI; see the formula below).
(2) Prepulses of 65/70/75/80 dB (20 ms) followed by the startle stimulus (105 dB, 40 ms), with an inter-stimulus interval of 100 ms.
(3) No stimulus trials (background noise 55 dB).

At the end, in order to measure the habituation to the startle stimulus, five "pulse-alone" trials were delivered (BASELINE 3).

The interval between trials was 10–20 s with a mean of 15 s. The startle magnitude was recorded during 200 ms after the onset of the pulse.

The degree of PPI (in percentage) is calculated according to the formula:

$$\%PPI = 100 - \left(\frac{\textit{startle amplitude on prepulse trials}}{\textit{startle amplitude on pulse trials}} \times 100 \right)$$

Spatial Working Memory (Delayed Matching-to-place Task; DMP) and Cued Learning in the Morris Water Maze

The Morris water maze test was performed in a circular water tank (140 cm in diameter and 30 cm deep) filled with water (24°C) made opaque with white paint.

The animals were tested on 3 consecutive days. They were allowed to swim for 90 s or until they located a platform (diameter 16 cm; height 28 cm) submerged (2 cm) in a fixed position each day. Each rat went through 2 trials per day: a sample/acquisition trial and a retention trial. The two trials were separated by 30 s and the rat was allowed to stay on the platform for 15 s and then spent another 15 s in an individual cage before the second trial started.

Three platform positions were defined: the first day the platform was located in the center of the NW quadrant, the second day it was located at a distance of 15 cm in the S direction and the third day the platform was at the center of the tank. Three starting positions were also defined: S, E, and W, respectively. The starting point and the location of the platform were pseudo-randomly varied each day.

Several room cues were constantly visible from the pool. Escape latencies, path lengths, and swimming speed from each rat and trial were provided by a tracking system (Smart v.2.5.14; PANLAB, Barcelona, Spain) connected to a video camera placed above the pool. Two variables (highly associated to each other) were computed as indexes of spatial working memory: "Mean T1-T2," distance savings in T2 vs. T1 (i.e., subtraction T1-T2) averaged for the 3 days. "Mean %DP T1-T2," difference of percentage of distance traveled in the periphery between T1 and T2, averaged for the 3 days.

Cued Learning

This task consisted of four consecutive trials at 15 min intervals on 2 consecutive days (i.e., 8 trials in total). For this test, the platform protruded 1 cm above the surface of the water and was cued with a small striped (black and white) flag. Black curtains were drawn to minimize the availability of extra-maze cues. There was one platform position (center of the SW quadrant) for the 2 days and 4 starting positions (N, S, E, W). The trials began with the rat facing the wall at the starting point and, if after the maximum allocated time (90 s) the animal had not found the platform, it was gently guided to its position by the experimenter. The parameter used in this task was the distance to reach the platform in each trial. This task is used to see if the animals had any visual, motor, or motivational problems.

Statistical Analysis

Statistical analysis was performed using the "Statistical Package for Social Science" (SPSS, version 17).

Pearson's correlation coefficients were performed among the main variables of both experiments. Multiple linear regression and factorial (direct oblimin; oblique rotation) analyses were applied to data from Experiment 2.

Repeated measures ANOVAs, with the 4 prepulse intensities as a within-subject factor ("3 strains" × "4 prepulse intensities" ANOVA) or with the 3 baseline startle trial blocks as within-subject factor ("3 strains" × "3 baseline startle blocks" ANOVA), were used to evaluate the results from the PPI session.

As differences in navigation speed were observed among the experimental groups (data not shown) in the working memory and cued learning tasks, we have taken the "distance traveled" to reach the platform as the main dependent variable from both experiments. For spatial working memory measures, repeated measures ANOVAs with "T1-T2" (mean distance traveled in the 3 first trials –T1- and three second trials –T2- of each trial pair) as within-subject factor ("3 groups" × "2 trial means" ANOVA) were applied. One-Way ANOVAs were then separately applied to T1 and T2 results (Experiment 1).

Analysis of "distance traveled through the periphery" along the 6 training trials of the working memory task and analysis of performance along the 8 trials of the cued learning task were carried out with the appropriate repeated measures ANOVAs with 6 or 8 trials as within-subject factors (i.e., "3 groups" × "6 trials" or "3 groups" × "8 trials"). *Post-hoc* LSD tests following significant ANOVA effects were applied for comparisons between groups.

Results

Experiment 1

Pearson's correlation coefficients between the main variables and for the three groups pooled are shown in **Table 1**. As expected, there are high within-test (or within-phase) correlations, ranging from $r = 0.73$ to $r = 0.83$ for baseline startle (and habituation) variables, and ranging from $r = 0.72$ to $r = 0.94$ for PPI parameters. There are also moderate correlations between the distance traveled in "T1" and "T2" and in the cued learning task (ranging from $r = 0.40$ to $r = 0.45$), but very low or no correlation between performance in the cued task and the working memory ("Mean T1-T2") index ($r = -0.02$ and $r = -0.29$). With regard to between-test associations, there are only moderate correlations among %PPI variables and "T2" performance in the working memory task (from $r = -0.31$ to $r = -0.38$).

The %PPI for each experimental group and prepulse intensity is represented in **Figure 1A**. The repeated measures ANOVA revealed a significant "strain" effect [$F_{(2, 62)} = 5.43$ $p < 0.007$; **Figure 1A**]. There was also the expected "prepulse intensity" effect [Huynh-Feldt $F_{(2.56, 166.54)} = 92.1$, $p < 0.001$], as %PPI increased with prepulse intensity (**Figure 1A**). One-Way ANOVA of the "Mean %PPI" for the 4 prepulse intensities revealed a significant "strain" effect [$F_{(2, 62)} = 5.43$ $p = 0.007$], and the LSD *post-hoc* tests showed that the RHA-I rats display lower mean %PPI than the other two strains (see **Figure 1B**).

A significant "strain" effect was also observed for baseline startle measures [$F_{(2, 62)} = 3.35$ $p = 0.041$; **Figure 1C**], which is apparently due to the fact that RLA-I rats display increased baseline startle along the three trial blocks in which the session was divided (in agreement with previous studies; López-Aumatell et al., 2009a,c). There was also a "trial block" effect [Huynh-Feldt $F_{(1.44, 93.5)} = 16.27$ $p < 0.001$], reflecting the habituation of the startle response along the session in the three experimental groups (**Figure 1C**].

In **Figure 2A** the average distance traveled in the first trials (T1) and in the second trials (T2) is represented. The repeated measures ANOVA analysis showed a "trial" effect [$F_{(1, 62)}$ =13.9, $p < 0.001$] and a significant "strain" effect [$F_{(2, 62)} = 5.74$ $p = 0.005$], mainly because RHA-I were overall worse than the other two groups (**Figure 2A**). Moreover, in order to control for the possible influence of "Mean DIST T1" we conducted a one-way analysis of covariance (ANCOVA), taking the mean distance saved between the first and second trials (Mean T1-T2) as the dependent variable and the "distance traveled in the first trials" (Mean DIST T1) as a covariate. This analysis yielded a significant "strain" effect [$F_{(2, 61)} = 6.39$ $p = 0.003$; **Figure 2B**], which is apparently due to a relative impairment of RHA-I rats in that measure.

We conducted repeated measures ANOVA on swimming speed data for each trial. A significant "strain" effect [$F_{(2, 62)} = 13.95$ $p < 0.001$] was observed, due to the fact that NIH-HS rats

TABLE 1 | Pearson's correlations among the main variables.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1-Baseline 1	1																
2-Baseline 2	**0.76****	1															
3-Baseline 3	**0.73****	**0.83****	1														
4-Habituation	**0.82****	**0.43****	**0.30***	1													
5-%PPI65	**0.26***	0.24	0.18	0.22	1												
6%PPI 70	**0.32****	**0.36****	**0.27***	0.23	**0.72****	1											
7-%PPI75	**0.25***	**0.27***	0.22	0.19	**0.87****	**0.79****	1										
8-%PPI80	0.18	0.19	0.11	0.16	**0.75****	**0.79****	**0.81****	1									
9-PPI Mean	0.29	**0.30***	0.23	0.23	**0.92****	**0.90****	**0.94****	**0.89****	1								
10-Mean DIST T1	−0.08	−0.05	−0.15	0.02	−0.18	−0.15	−0.11	−0.05	−0.15	1							
11-Mean DIST T2	−0.10	−0.08	−0.15	−0.03	**−0.38****	**−0.31***	**−0.35****	**−0.33****	**−0.38****	0.23	1						
12-Mean T1-T2	0.01	0.02	−0.02	0.03	0.13	0.10	0.16	0.20	0.15	**0.69****	**−0.55****	1					
13-Mean %DP T1	0.01	0.07	−0.09	0.05	−0.22	−0.10	−0.12	−0.12	−0.16	**0.64****	**0.38****	**0.27***	1				
14-Mean %DP T2	−0.00	0.05	0.00	−0.02	**−0.30***	−0.19	−0.23	**−0.29***	**−0.27***	**0.31***	**0.61***	−0.20	**0.59****	1			
15-Mean %DP T1-T2	0.01	0.02	−0.10	0.07	0.11	0.12	0.13	0.20	0.15	**0.32****	**−0.30***	**0.50****	**0.39****	**−0.52****	1		
(#)16-Cue day 1	−0.11	0.06	0.05	−0.08	**−0.42****	**−0.32***	**−0.29***	**−0.44****	**−0.41****	**0.40****	**0.44****	−0.02	**0.54****	**0.63****	−0.17	1	
(#)17-Cue day 2	0.06	**0.33***	−0.02	0.16	−0.22	−0.05	−0.12	−0.12	−0.15	0.09	**0.45****	**−0.29***	**0.45****	**0.53****	−0.15	**0.52****	1

*"Baseline 1," corresponds to the 10 first pulse-alone trials. "Baseline 2," corresponds to the 10 pulse-alone trials that are presented pseudorandomly in combination with the prepulse-pulse trials. "Baseline 3," refers to the last 5 pulse-alone trials. "Habituation" refers to the difference between the mean of the 5 pulse-alone trials presented in the beginning of the session and the mean of the last 5 pulse-alone trials. "%PPI65-%PPI80," percentage of response inhibition in the prepulse-pulse trials for each prepulse intensity. "PPI Mean," the global percentage inhibition averaged for the 4 prepulse intensities. "Mean DIST T1" and "Mean DistT2," mean distance traveled in the three first and the three second trials, respectively. "Mean T1-T2," distance savings in T2 vs. T1 (i.e., subtraction T1-T2) averaged for the 3 days. "Mean %DP T1" and "Mean %DP T2," mean of the percentage of the distance traveled in the periphery in the three first (T1) and second (T2) trials, respectively. "Mean %DP T1-T2," difference of percentage of distance traveled in the periphery between T1 and T2, averaged for the 3 days. "Cue day 1," mean distance traveled in the 4 trials of cued learning in the first day. "Cue day 2," mean distance traveled in the 4 trials of cued learning in the second day. Bold letter means significant Pearson's coefficient *p < 0.05 and **p < 0.01. n = 65. (#) n = 41.*

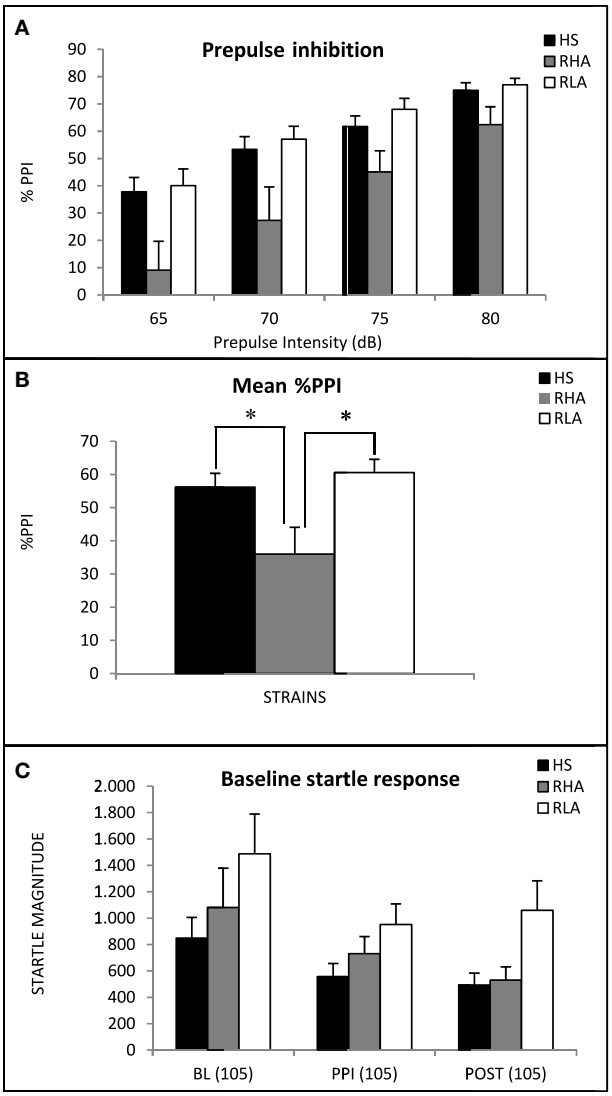

FIGURE 1 | (A) Mean prepulse inhibition (± S.E.M.) is shown for the three strains. **(B)** Mean ± S.E.M. of the "Mean %PPI" averaged for the four prepulse intensities. **(C)** Mean ± S.E.M. of the startle response in the three blocks of pulse-alone trials: BL(105), initial pulse-alone 10-trial block; PPI(105), second pulse-alone 10-trial block, pseudorandomly administered in combination with presentation of the prepulse-pulse trials; POST(105), the final pulse-alone 5-trial block. *$p < 0.05$ between the indicated groups (LSD tests).

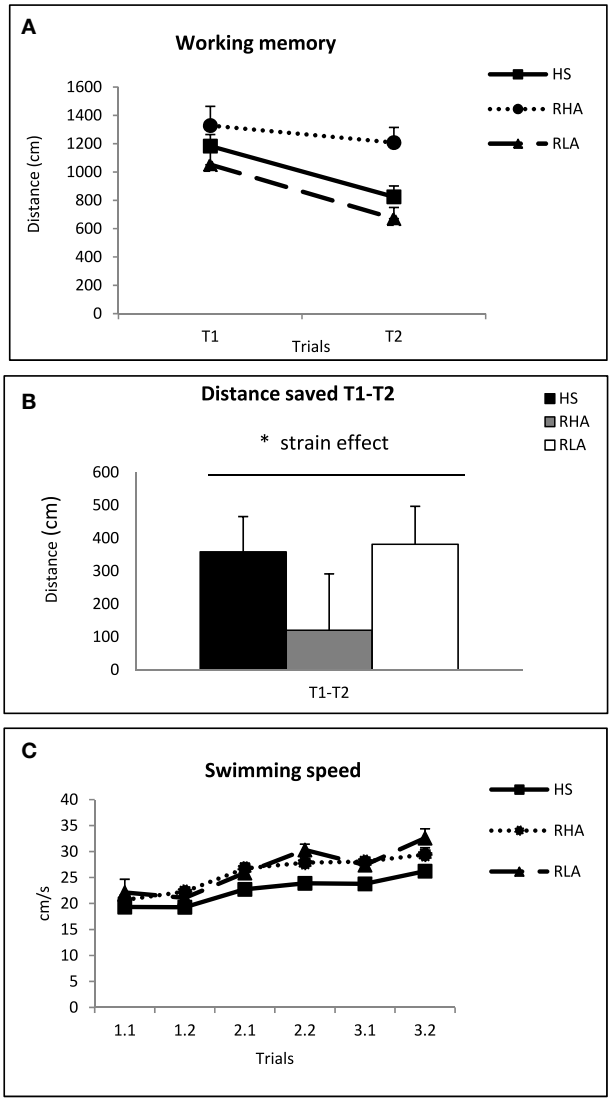

FIGURE 2 | (A) Mean ± S.E.M. of the distance (cm) traveled by the rats of the three strains in the first (T1) and second (T2) trials, averaged for the 3 days. **(B)** Mean ± S.E.M. of the three differences (i.e., subtractions T1-T2), corresponding to the 3 days, between the first (T1) and the second trials (T2) of the working memory task. **(C)** Mean ± S.E.M. swimming speed for each trial and strain. *$p < 0.05$, "Strain" effect (One-Way ANCOVA; see text).

swam apparently slower than both RHA-I and RLA-I groups (see **Figure 2C**).

The percentage of distance swam in the periphery, in each trial is shown in **Figure 3A**. In the repeated measures ANOVA we found a significant "strain" effect [$F_{(2, 62)} = 12.91$ $p < 0.001$]. "Trial" and "trial × strain" effects were also found [$F_{(5, 325)} = 15.17$, $p < 0.001$ and $F_{(10, 325)} = 5.04$, $p < 0.001$, respectively]. Further separate One-Way ANOVAs showed group effects in all trials except one [second trial in the first day, 1.2; $F_{(2, 62)} \geq 4.11$ $p \leq 0.021$ for the remaining five trials; **Figure 3A**]. *Post-hoc* LSD tests indicated that RHA-I rats swam longer distances in the periphery than the other two groups (trials 2.1, 2.2, and 3.2; **Figure 3A**) or than RLA-I rats (trial 3.1; see other LSD differences in **Figure 3A**). In accordance with the results observed for "Mean T1-T2" (**Figures 2A–C**), an ANOVA on the "difference of percentage of distance traveled in the periphery between T1 and T2 (averaged for the 3 days)" ("Mean %DP T1-T2") yielded a significant "strain" effect [$F_{(2, 62)}$ =3.95 $p < 0.024$; **Figure 3B**], and the LSD *post-hoc* tests revealed that RHA-I rats showed significantly lower values in that variable than NIH-HS rats (**Figure 3B**). Given that the "Mean %DP T1-T2" variable is highly and positively correlated/associated with "Mean T1-T2" (in both Experiments 1 and 2; see correlations between both variables in **Table 1** -r = 0.50-, **Table 4** -r = 0.69-, and the

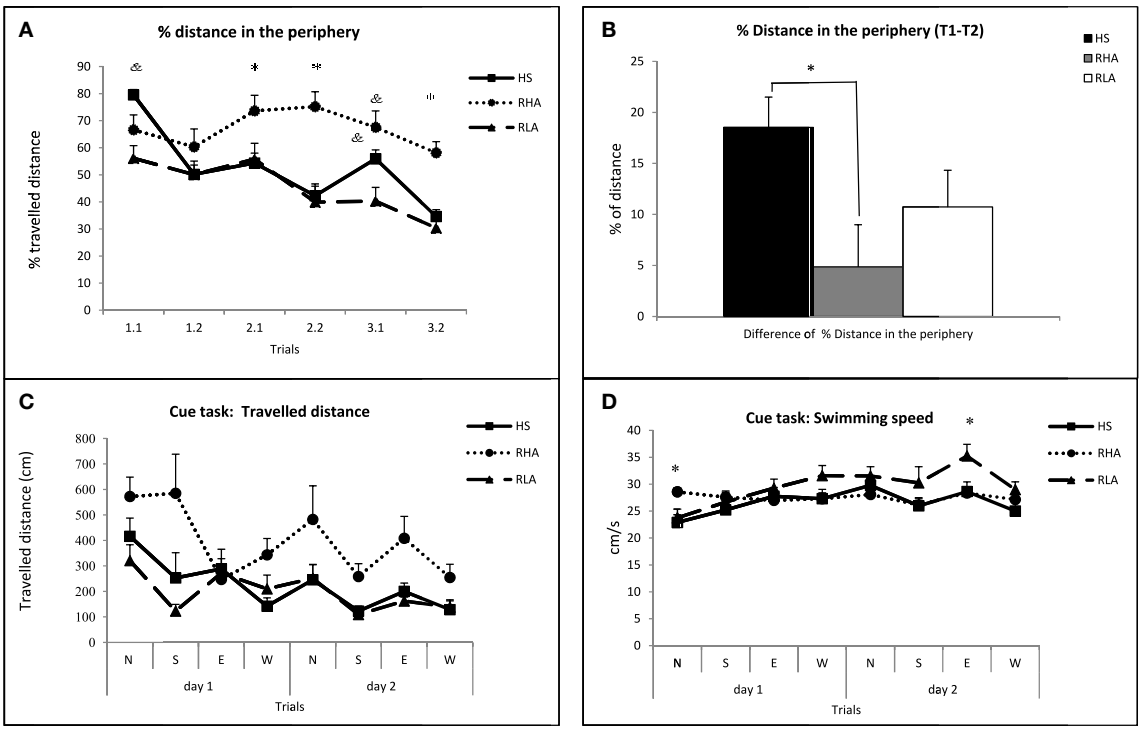

FIGURE 3 | (A) Mean ± S.E.M. of the percentage of distance traveled in the periphery in each trial for the three groups. **(B)** Mean ± S.E.M. of the difference of "percentage of distance traveled in the periphery between T1 and T2 (averaged for the 3 days)" ("Mean %DP T1-T2"). **(C)** Mean ± S.E.M. of the distance traveled by the rats in each of the 8 trials of the cued task. **(D)** Mean ± S.E.M. swimming speed in the cued task for each trial. *$p < 0.05$ vs. the other two strains **(A,D)** or between the groups indicated **(B)**; &, $p < 0.05$ vs. the RLA-I group (LSD tests following the corresponding significant ANOVA effects).

loadings of both variables −0.79 and 0.81- in the factor analysis from **Table 6B**), the impairment of RHA-I rats in the former variable suggest a relative deficit of RHA-I rats in a working memory-related process.

The repeated measures ANOVA of the distance traveled in the cued task (**Figure 3C**) revealed significant "strain" [$F_{(2, 38)} = 11.18$ $p < 0.001$] and "trial" [Huynh-Feldt $F_{(4.62, 175.38)} = 6.44$ $p < 0.001$] effects, apparently due to the longer distances traveled by RHA-I rats in some trials. Analysis of the swimming speed of the rats during the cued task (repeated measures ANOVA) indicated a significant "trial × strain" interaction [$F_{(14, 252)} = 2.01$ $p = 0.018$]. Further LSD *post-hoc* tests revealed significant differences between RHA-I rats and the other two strains in the first trial and between the RLA-I and the other two strains in the seventh trial (see LSD tests in **Figure 3D**).

Experiment 2

Table 2 shows the mean, S.E.M., and Standard Deviation (S.D.) %PPI of the 78 NIH-HS rats tested in the PPI session.

Table 3 shows the mean ± S.E.M of the three sub-groups of NIH-HS rats stratified on the basis of their mean %PPI performance with the 4 prepulse intensities. The 3 sub-groups were formed as follows: HIGH-PPI group ($n = 9$), consisting of rats showing total PPI scores 1 SD above the mean of the whole group ($n = 78$), i.e., PPI scores > 78%; LOW-PPI group ($n = 14$), consisting of rats showing total PPI scores 1 SD below the mean of the whole group, i.e., PPI scores < 42%; MEDIUM-PPI group ($n = 10$), consisting of randomly selected rats with total PPI scores falling within the mean ± 1 SD (see descriptives of the 3 subgroups in **Table 3**). The results listed in **Table 3** show that the 2 groups with extreme values of %PPI also diverge in the magnitude of the baseline startle response.

The correlation matrix in **Table 4** shows a pattern of high within-test correlations which is similar to that observed in **Table 1** (Experiment 1). Apart from these expected correlations it is remarkable that the "Mean T1-T2" is positively and significantly correlated with the PPI variables (i.e., %PPI70, %PPI75, %PPI80, and PPI Mean), with correlations ranging from 0.39 to 0.53. So, PPI is correlated with a typical measure of working memory such as the "Mean T1–T2." Another related correlation, also supporting the main hypothesis of the present study, is the one between the mean distance traveled in the second trial (Mean DIST T2) and the performance in the PPI when the prepulse had an intensity of 75 dB (−0.36). In the case of the other prepulse intensities the correlation with "Mean DIST T2" is not significant but there is a tendency, with Pearson's coefficients ranging from −0.25 to −0.33. Remarkably, the "difference of percentage of distance traveled in the periphery between T1 and T2 averaged for the 3 days" ("Mean % DP T1-T2"), which is highly correlated with the working memory

TABLE 2 | Mean, S.E.M. and Standard Deviation (S.D.) of the 78 NIH-HS rats tested in the PPI.

	Mean	*S.E.M*	*S.D.*
STARTLE RESPONSE			
Baseline 1	899.93	114.92	1014.96
Baseline 2	590.66	74.61	658.98
Baseline 3	474.84	58.74	518.76
Habituation	350.74	72.19	637.54
PPI			
%PPI65	41.29	3.20	28.24
%PP70	56.34	2.26	19.95
%PPI75	67.71	2.03	17.92
%PPI80	78.50	1.41	12.43
PPI Mean	60.96	2.01	17.78

*Symbols/abbreviations as in **Table 1**.*

TABLE 3 | Mean ± S.E.M. of the PPI and startle response variables of the 3 NIH-HS subgroups.

	Medium-PPI (n: 10)	High-PPI (n: 9)	Low-PPI (n: 14)
Baseline 1	606.24 ± 263.68	1550.98 ± 392.36*	571.63 ± 122.74
Baseline 2	256.84 ± 64.03	1194.12 ± 225.94*	361.22 ± 88.58
Baseline 3	348.54 ± 117.13	825.42 ± 254.31	381.64 ± 91.00
Habituation	352.66 ± 250.32	807.18 ± 349.05	303.04 ± 112.32
%PPI65	42.88 ± 4.74[a]	74.98 ± 2.97[a]	10.30 ± 4.26[a]
%PP70	62.66 ± 4.39[a]	83.77 ± 1.11[a]	27.14 ± 3.88[a]
%PPI75	68.84 ± 3.69[a]	87.79 ± 0.91[a]	39.91 ± 2.91[a]
%PPI80	79.62 ± 3.82[a]	91.85 ± 1, 00[a]	60.88 ± 2.50[a]
PPI Mean	63.50 ± 3.42[a]	84.60 ± 1.11[a]	34.56 ± 1.48[a]

*Symbols/abbreviations as in **Table 1**. *$P < 0.05$ vs. the other 2 groups; [a]$p < 0.05$ between groups with the same letter (LSD tests following significant One-Way ANOVAs; all [$Fs_{(2, 30)} \geq 4.57$ and $p \leq 0.019$].*

measure "Mean T1-T2" ($r = 0.69$), is also positively correlated with %PPI (i.e., %PPI75, %PPI80 and PPI Mean), with Pearson's coefficients ranging from 0.41 to 0.57. Moreover, correlations between measures of (total) distance (i.e., Mean DIST T1, Mean DIST T2) and "distance traveled in the periphery" (Mean %DP T1, Mean %DP T2) are significant, with coefficients ranging from 0.61 (between Mean DIST T2 and Mean %DP T2) to 0.68 (between Mean DIST T1 and Mean % DP T2; **Table 4**).

Forward stepwise multiple regression, with PPI variables as predictors and "Mean T1-T2" as the dependent variable revealed 2 significant models, with the first model showing that the variable %PPI75 is a significant predictor of "Mean T1-T2" (see **Figure 4A**, **Table 5**). %PPI75 correlated significantly with "Mean T1-T2," explaining 28% of its variability ($p = 0.002$; **Table 5**). In addition, a second significant regression model, with %PPI75 and %PPI65 as predictors (correlation of $r = 0.66$ with "Mean T1-T2") accounted for 44% of the variability ($p < 0.001$; **Table 5**). We also conducted the multiple regression analysis for the "Mean %DP T1-T2" and we observed that %PPI80 predicted "Mean %DP T1-T2" (**Figure 4B**; **Table 5**).

Obliquely-rotated factor analyses (direct oblimin) were performed with the 14 main variables of the two tests/tasks (**Table 6(A)**). The first result we obtained was a 4-factor structure (**Table 6(A)**). In the first factor we can see an association between PPI and working memory, with loadings of PPI variables ranging from 0.89 to 0.95 and with 0.43 and 0.41 loadings for "Mean T1-T2" and "Mean %DP T1-T2," respectively. The second factor is based on the startle response magnitude, particularly baseline startle, with loadings from 0.78 to 0.99. The third and fourth factors are related to the DMP task (loadings ranging from 0.41 to 0.91), with the main difference between the two factors being that in the third the distances in the first trials (Mean DIST T1 and Mean %DP T1) have the highest loadings, while in the fourth factor the distances in the second trials (Mean DIST T2 and Mean %DP T2) are the most relevant. Another important difference between these two factors is that "Mean T1-T2" has a higher loading in the third factor (0.74) while "Mean %DP T1-T2" has a -0.82 loading in the fourth factor. So, in this analysis we found that, with the exception of the first factor, the other three are predominantly task-related (or phase-related) factors (**Table 6(A)**).

In order to obtain a reduced number of theoretically meaningful factors the same factor analysis was reduced to a 2-factor solution (see **Table 6(B)**). In the first factor the PPI variables and the DMP task variables have the highest loadings, with 0.60–0.83 for %PPI65–%PPI80 and, remarkably, with 0.79 for "Mean T1-T2" and 0.81 for "Mean %DP T1-T2" (**Table 6(B)**). The second factor is essentially composed by the baseline startle variables (0.73–0.91) plus moderate loadings of %PPI65–%PPI75 variables (0.48–0.59). Thus, this factor analysis clearly links PPI and the working memory task in the first factor, while the second factor is mainly related to baseline startle response.

In **Figure 5A** we show the average distance traveled in the first (T1) and second (T2) trials by the three groups of NIH-HS rats. The repeated measures ANOVA showed that there is a significant interaction between "trial type" (T1 or T2) and NIH-HS rat "sub-group" [$F_{(2, 30)} = 3.44$ $p = 0.045$]. This interaction is important because it shows that the "LOW-PPI" group had a very small saving between T1 and in T2, while "HIGH-PPI" and "MEDIUM-PPI" groups show a clear cut decrease in the distance traveled between the two trials (**Figure 5A**). Further One-Way ANOVAs for each trial type revealed no significant differences between sub-groups. In **Figure 5B** the differences between T1 and T2 are shown for the three groups. One-Way ANOVA revealed significant differences between the three groups [$F_{(2, 30)} = 3.44$ $p = 0.045$], and *post-hoc* LSD tests evidenced that these differences were between the "LOW-PPI" group and the other two groups ($p < 0.05$; **Figure 5B**), indicating that the "LOW-PPI" sub-group displays a poorer working memory performance. Analysis of the swimming speed with a repeated measures ANOVA revealed a significant "sub-group" effect [$F_{(2, 30)} = 7.77$ $p = 0.002$], which is due to the fact that the LOW-PPI group was apparently slower than the other two sub-groups in some trials (**Figure 5C**).

The repeated measures ANOVA for the percentage of distance traveled in the periphery by the three NIH-HS sub-groups revealed no significant group effect [$F_{(2, 30)} = 0.70$ $p = 0.50$] and

TABLE 4 | Pearson's correlations among the main variables, pooling the three NIH-HS subgroups (*n* = 33).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1-Baseline 1	1																
2-Baseline 2	**0.83****	1															
3-Baseline 3	**0.86****	**0.79****	1														
4-Habituation	**0.81****	**0.51****	**0.46****	1													
5-%PPI65	**0.45****	**0.53****	**0.37***	0.33	1												
6-%PPI 70	**0.35***	**0.44***	0.23	0.23	**0.86****	1											
7-%PPI75	0.29	**0.41***	0.23	0.16	**0.83****	**0.85****	1										
8-%PPI80	0.18	**0.37***	0.14	0.10	**0.73****	**0.78****	**0.85****	1									
9-PPI Mean	**0.36***	**0.48****	0.28	0.24	**0.94****	**0.95****	**0.94****	**0.88****	1								
10-Mean DIST T1	0.21	0.23	0.13	0.23	0.04	0.21	**0.36***	**0.37***	0.23	1							
11-Mean DIST T2	0.09	0.01	0.10	0.15	−0.25	−0.32	**−0.36***	−0.33	−0.33	0.08	1						
12-Mean T1-T2	0.10	0.17	0.03	0.07	0.21	**0.39***	**0.53****	**0.52****	**0.42***	**0.70****	**−0.65****	1					
13-Mean %DP T1	**0.36***	0.34	0.27	0.29	0.20	0.31	**0.37***	**0.38***	0.32	**0.68****	0.14	**0.42***	1				
14-Mean %DP T2	0.09	0.02	0.05	0.13	−0.09	−0.08	−0.15	−0.23	−0.13	0.18	**0.61****	−0.30	**0.50****	1			
15-Mean %DP T1-T2	0.20	0.26	0.18	0.01	0.26	0.34	**0.48****	**0.57****	**0.41***	**0.38***	**−0.56****	**0.69****	0.28	**−0.69****	1		
(#) 16-Cue Day 1	0.36	0.21	0.32	0.37	0.17	0.13	0.22	−0.01	0.15	0.32	0.18	0.13	**0.66****	**0.66****	−0.27	1	
(#) 17-Cue Day 2	−0.01	0.32	−0.15	0.01	**0.47***	0.41	0.41	0.29	0.44	−0.12	0.18	−0.20	0.15	0.19	−0.11	0.13	1

*Symbols/abbreviations as in **Table 1**. Bold letter means significant Pearson's coefficient *p < 0.05 and **p < 0.01. (#) n = 17.*

a significant trial effect [$F_{(5,\ 150)} = 28.6\ p < 0.001$] (**Figure 6A**). On the other hand, One-Way ANOVA of the "Mean %DP T1-T2" showed a significant "sub-group" effect [$F_{(2,\ 30)} = 4.51\ p < 0.019$], and the LSD *post-hoc* tests revealed that the differences were between LOW-PPI and HIGH-PPI groups, with the former traveling longer distances in the periphery (**Figure 6B**).

In **Figure 6C** we show the distance traveled in the 8 trials of the cued task. The results of the repeated measures ANOVA showed a significant group effect [$F_{(2,\ 14)} = 4.37\ p = 0.033$] and a trial effect [Huynh-Feldt $F_{(3.62,\ 50.78)} = 2.88\ p = 0.036$; **Figure 6C**], which is apparently due to HIGH-PPI group traveling longer distances in some trials (see **Figure 6C**). Swimming speed along the 8 trials was analyzed with a repeated measures ANOVA, that only revealed a "trial" effect [$F_{(7,\ 98)} = 3.96\ p = 0.001$; **Figure 6D**].

Discussion

Experiment 1 represents the first joint characterization of PPI and spatial working memory in RHA-I/RLA-I and NIH-HS rats. In keeping with the hypothesis that RHA-I rats could be a tool to discern cognitive peculiarities linked to some schizophrenia-related symptoms, we have found that this rat strain displays deficits in PPI and in spatial working memory compared with RLA-I and NIH-HS rats. In addition, it is also shown for the first time that the genetically heterogeneous NIH-HS rat stock displays relatively high PPI levels as well as efficient spatial working memory, as their values in both processes/measures are similar to those shown by RLA-I rats (see Experiment 1). The results from Experiment 1 and Experiment 2 also point out an apparent positive association between PPI and working memory, as the strains (i.e., RLA-I, NIH-HS; Experiment 1) or sub-groups (i.e., the HIGH-PPI and MEDIUM-PPI sub-groups of NIH-HS rats; Experiment 2) displaying the highest PPI levels also present the best working memory performance.

RLA rats from the Swiss outbred line have already been shown to outperform their RHA counterparts in spatial learning/memory tasks (e.g., Driscoll et al., 1995; Escorihuela et al., 1995b; Aguilar et al., 2002). Accordingly, we recently found that inbred RHA-I rats performed worse than RLA-I animals in a spatial place task (i.e., reference memory) in the Morris Water maze (Martínez-Membrives et al., 2015). But the present is the first time in which (spatial) working memory has been assessed in the inbred Roman rat strains, showing that the RHA-I strain displays impaired memory ability in this task as compared with both RLA-I and NIH-HS rats. Remarkably, as we have reported for anxiety/stress-related behavioral/neuroendocrine traits (e.g., López-Aumatell et al., 2009a,b; Díaz-Morán et al., 2012, 2013c; Estanislau et al., 2013), PPI and working memory performance of the heterogeneous NIH-HS rat stock very closely resemble the profile of RLA-I rats in these tests/tasks. These similar profiles, in both a pre-attentive process (PPI) and spatial working memory, jointly with their comparable performance in cued learning, in spatial place learning and in two-way avoidance acquisition (e.g., López-Aumatell et al., 2009a,b; Díaz-Morán et al., 2012, 2013c; Estanislau et al., 2013; Martínez-Membrives et al., 2015), suggest that inbred RLA-I and heterogeneous NIH-HS rats may also present similarities in some other cognitive traits or profiles. Further studies aimed at comparing the performance of RLA-I/RHA-I and NIH-HS rats in other cognitive/executive tasks are therefore warranted.

The RHA-I rat strain also showed a poorer performance in the cue learning task, a test that is commonly used to rule out possible sensorial, motivational, or motor deficits. This result was unexpected, because we have recently found that RHA-I and RLA-I rats present identical performance in a cue

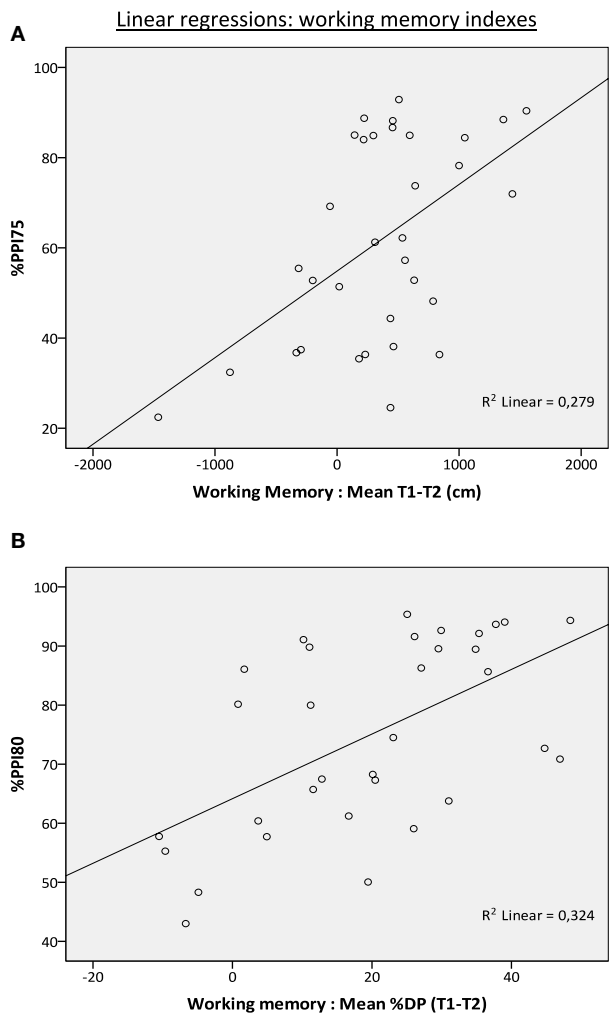

FIGURE 4 | Linear regressions between %PPI and "working memory" measures (all rats pooled, *n* = 33). (A) Regression between %PPI75 (predictor variable) and "Mean T1-T2" (dependent variable). **(B)** Regression between %PPI80 (predictor variable) and "Mean %DP T1-T2" (dependent variable).

task administered after a place task in the MWM (Martínez-Membrives et al., 2015). Apart from that recent work, several studies performed at our laboratory and others allow us to rule out the possibility of any visual, motivational, or motor deficit in RHA-I rats while, on the contrary, it is well-known that they are characterized by: (i) enhanced exploratory drive (motivation) and a novelty seeker profile, as observed in many different novelty situations (e.g., reviews by Fernández-Teruel et al., 1992; Escorihuela et al., 1999; Driscoll et al., 2009); (ii) augmented impulsivity and tendency to stereotyped behavior (Zeier et al., 1978; Moreno et al., 2010; Klein et al., 2014) and (iii) enhanced perseverative responses and/or lack of behavioral flexibility (e.g., Zeier et al., 1978; Nil and Bättig, 1981; Escorihuela et al., 1995b; Moreno et al., 2010). Finally, the "strain × trial" effect (and the absence of "strain" effects) on navigation speed in the cued task (Experiment 1) do not suggest any motivational deficit which could account for the performance results observed. Thus, although it is not the only possible interpretation (see below), the impairment of RHA-I rats in the cue task might be partly due to the fact that they perseverate in (spatial or/and non-spatial) swimming strategies acquired during the working memory task.

In that context, it is worth noting that in both the working memory and cued tasks RHA-I rats display increased circular navigation along the periphery of the pool. This may suggest enhanced thigmotaxis in that rat strain. Nevertheless, we know, from a number of studies using open field-like tests (e.g., open field, activity cages, hole-board), that RHA-I rats spend the same time close to the walls (i.e., in the periphery) and travel the same percentage of distance along the periphery as RLA-I rats (e.g., Estanislau et al., 2013, and unpublished results from our laboratory). Hence, thigmotactic-like behavior is neither an inherent nor a general trait in RHA-I rats but, still, they display an excessive amount of peripheral navigation in the present MWM tasks. Thus, there is the possibility that a working memory deficit in the RHA-I rats may not be the only interpretation of the present results, and that a non-optimal navigation/orientation strategy (which would be in agreement with RHA vs. RLA results in other aquatic spatial tasks; Nil and Bättig, 1981) could have a role in the increased total distance and "% distance in the periphery" traveled by the RHA-I strain.

The results of Experiment 1 also highlight that the RHA-I strain fulfills some criteria for being considered as a convenient model for studying some schizophrenia-relevant symptoms, since it displays clear PPI and (possibly) working memory deficits along with a relative deficit in latent inhibition (Fernández-Teruel et al., 2006), an impaired performance in the five-choice serial reaction time task (5-CSRTT; which reflects executive function and sustained attention; Moreno et al., 2010; Klein et al., 2014) and an increased sensitivity to acutely administered DA receptor agonists as well as to psychostimulant (DA agonist)-induced sensitization (e.g., Corda et al., 2005; Giménez-Llort et al., 2005; Giorgi et al., 2007; Guitart-Masip et al., 2008; Del Río et al., 2014).

Experiment 2 was devoted to further investigate such a PPI-working memory association by evaluating whether PPI levels would statistically predict memory performance in a sample of the most genetically heterogeneous laboratory rat in existence, i.e., the NIH-HS rats (e.g., Baud et al., 2013, 2014a,b; Johannesson et al., 2009). Thus, the expected advantage of addressing that issue in NIH-HS rats would be that their enhanced genetic variability could make results more generalizable than those obtained using typical laboratory rodent strains. The results of Experiment 2 clearly support a positive relationship between PPI and working memory, as shown by the memory performance of HIGH-PPI as compared with MEDIUM-PPI and LOW-PPI rats, as well as by the positive associations between PPI (at different prepulse intensities) and the working memory measures revealed by correlational, factorial, and regression analyses (see **Tables 4–6** and **Figure 4**). In fact, Pearson's correlations between working memory ("Mean T1-T2" index) and %PPI70, %PPI75, %PPI80, and mean %PPI range from $r = 0.39$ to $r = 0.53$ (**Table 4**). Likewise, the stepwise multiple regression analysis

TABLE 5 | Forward stepwise multiple regression performed with "Mean T1-T2" and "Mean %DP T1-T2" as the dependent variables, and including all the variables recorded in the PPI session (i.e., Baseline 1–3, Habituation and %PPI65–%PPI80) as predictors in the model.

Dependent variable	Method	Model	Predictor variables	*R*	R^2	*p*
Mean T1-T2	Forward Stepwise	1	%PPI75	0.53	0.28	0.002
Mean T1-T2	Forward Stepwise	2	%PPI75; %PPI65	0.66	0.44	< 0.001
Mean %DP T1-T2	Forward stepwise	1	%PPI80	0.57	0.32	0.001

Mean T1-T2 is predicted by the PPI variables %PPI75 and %PPI65. Mean %DP T1-T2 is predicted by %PPI80. The three NIH-HS subgroups (n = 33) are pooled for analysis.

TABLE 6 | Loadings ≥ 0.40 are shown.

(A)	Factor 1	Factor 2	Factor 3	Factor 4	(B)	Factor 1	Factor 2
Baseline 1	–	0.99	–	–	Baseline 1	–	0.91
Baseline 2	0.47	0.87	–	–	Baseline 2	–	0.84
Baseline 3	–	0.89	–	–	Baseline 3	–	0.78
Habituation	–	0.78	–	–	Habituation	–	0.73
%PPI65	0.92	0.45	–	–	%PPI65	0.60	0.59
%PPI70	0.95	–	–	–	%PPI70	0.71	0.52
%PPI75	0.94	–	–	–	%PPI75	0.81	0.48
%PPI80	0.89	–	0.41	–	%PPI80	0.83	–
Mean DIST T1	–	–	0.94	–	Mean DIST T1	–	0.41
Mean DIST T2	–	–	–	0.84	Mean DIST T2	−0.72	–
Mean T1-T2	0.43	–	0.74	−0.64	Mean T1-T2	0.79	–
Mean %DP T1	–	–	0.85	–	Mean %DP T1	–	0.60
Mean %DP T2	–	–	–	0.91	Mean %DP T2	−0.55	–
Mean %DP T1-T2	0.41	–	0.44	−0.82	Mean %DP T1-T2	0.81	–
% of variance (cumulative)	39.40	61.01	74.52	85.50	% of variance (cumulative)	39.40	61.01
Correlations	1				Correlation	0.17	
	0.31	1			N =	33	
	0.27	0.18	1				
	−0.27	0.07	−0.07	1			
N =	33						

(A) Oblique four-factor solution (direct oblimin) with the main behavioral variables and correlations between factors. Only factors with eigenvalues greater than 1 are considered. (B) Two-factor solution and correlation between factors, showing that both factors are almost orthogonal/independent. Symbols/abbreviations as in ***Table 1****. n = 33 (the three NIH-HS subgroups are pooled for the analyses).*

supports this correlational pattern, as the %PPI75 and %PPI65 are significant predictors of both working memory measures, "Mean T1-T2" and "Mean %DP T1-T2" (see **Table 5** and **Figures 4A,B**). Further supporting these results, the factor analysis (oblimin direct; unforced rotation) shows an initial four-fold solution in which, (1) the first factor combines high loadings of %PPI variables (0.89–0.95) with moderate loadings of the working memory variables (i.e., "Mean T1-T2," loading 0.43; "Mean %DP T1-T2," loading 0.41) (**Table 6(A)**); (2) a second factor related to the startle response variables and (3) the last two factors related to the distance variables measured in the MWM. Likewise, when forcing this factor analysis to a two-fold solution (direct oblimin), a very similar first factor is observed, with high loadings of %PPI variables and working memory indexes ("Mean T1-T2"and "Mean %DP T1-T2"), and also high loading of distance traveled in the second trials ("Mean DIST T2" and "Mean %DP T2"). The second and almost orthogonal factor (as the between-factor correlation is low) is mainly grouping loadings of baseline startle, habituation, and moderate loadings in the PPI variables and the distance traveled in the first trials in the MWM (**Table 6(B)**).

Thus, correlational, regression, and factor analyses confirm that: (1) the differences of % distance traveled in the periphery between T1 and T2 ("Mean %DP T1-T2") are strongly associated to "Mean T1-T2," so both of them are working memory indexes; and (2) both working memory variables are positively predicted by %PPI.

As concerns to animal research, to the best of our knowledge the PPI-working memory association has only been addressed in one study in mice and no study has evaluated it in untreated/undisturbed genetically heterogeneous rats. Singer et al. (2013) reported, using a cohort of 23 male C57BL/6 mice, that PPI levels positively predicted (with $r = 0.50$) spatial working memory in the Morris Water maze (delayed matching-to-place task). The authors showed that such an effect was only present when taking as predictor the %PPI levels at the lowest pre-pulse intensity (i.e., 69 dB). Our present

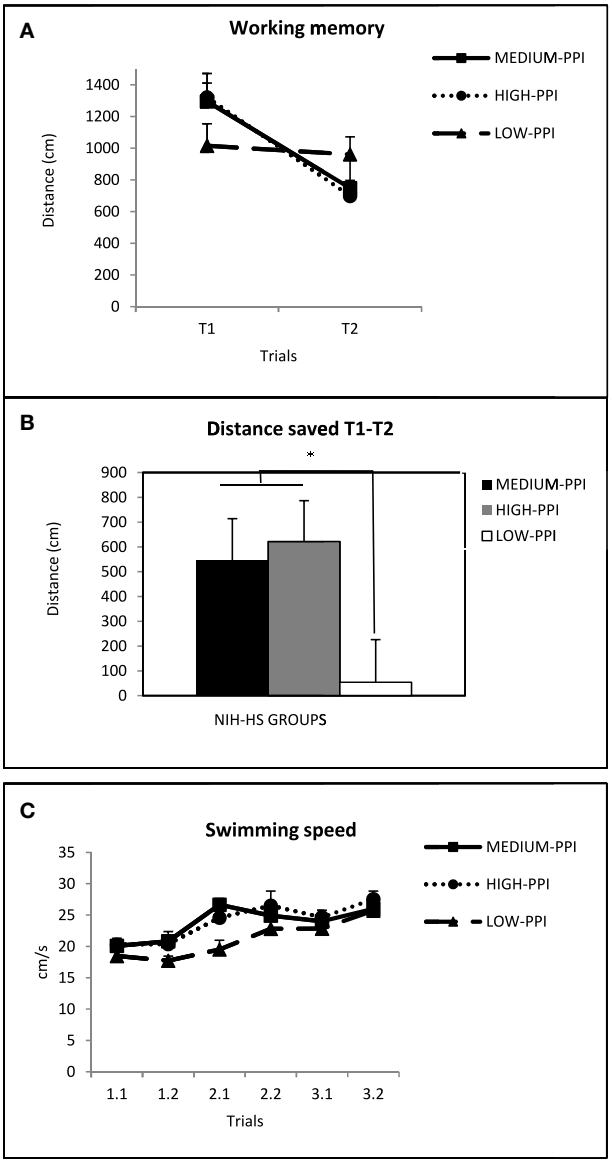

FIGURE 5 | (A) Mean ± S.E.M. of the distance (cm) traveled by the three subgroups of NIH-HS rats (selected for their extreme performance in the PPI test) in the first (T1) and second trials (T2), averaged for the 3 days (see text for the criteria followed to build these 3 subgroups). **(B)** Mean ± S.E.M. of the three differences (i.e., "Mean T1-T2"), corresponding to the 3 days, between the first (T1) and second (T2) trials of the working memory task. **(C)** Mean ± S.E.M. swimming speed of the NIH-HS sub-groups for each trial.*$p < 0.05$ between the indicated groups (LSD test following significant One-Way ANOVA).

results confirm and extend the previous study in mice to different rat types and statistical approaches, as we also found a positive association between %PPI and spatial working memory by comparing the RHA-I, RLA-I, and NIH-HS strains/stocks and by studying PPI-working memory associations in NIH-HS rats.

Remarkably, Experiment 2 shows that cue learning is neither associated with %PPI nor with working memory performance in NIH-HS rats (see **Table 4** and **Figure 6B**). Finally, the extreme LOW-PPI rats from such stock presented a pattern of results both across the different intensities of the prepulse inhibition study and on working memory measures at the DMP test, which paralleled those of the Roman High-Avoidance rats in Experiment 1. This suggests that either by psychogenetic selection or by focusing on extremes from a heterogeneous rats stock, it is possible to detect a useful (perhaps "at risk") phenotype to study cognitive peculiarities linked to some schizophrenia anomalies.

It has been proposed that PPI may be associated to, and modulated by, higher cognitive processes. This contention is supported by the frequent co-existence of PPI deficits and cognitive impairments in clinical samples, including schizophrenic patients, as well as by some studies in healthy volunteers which have shown that PPI is positively correlated with performance in several cognitive tasks (e.g., Bitsios and Giakoumaki, 2005; Hagan and Jones, 2005; Bitsios et al., 2006; Giakoumaki et al., 2006; Csomor et al., 2009; for reviews see Young et al., 2009; Singer et al., 2013). In particular, in human volunteers, PPI has been found to be positively associated to proper searching strategies in the CANTAB (*"Cambridge Neuropsychological Test Automated Battery")* spatial working memory task, Csomor et al., 2009). In spite of these positive results, the possibility that PPI predicts cognitive function in humans remains to be established (Young et al., 2009; Singer et al., 2013), given the small number of studies that have addressed that issue.

Koch and Schnitzler (1997) have proposed that the essential circuit underlying PPI involves the midbrain inferior colliculus, the superior colliculus, the pedunculopontine tegmental nucleus, and the caudal pontine reticular nucleus, which regulates the activity of motor neurons and the motor response (Koch and Schnitzler, 1997; Kohl et al., 2013). Importantly, however, cortical, and limbic areas, such as the orbitofrontal cortex, anterior cingulate, medial prefrontal cortex, nucleus accumbens, basolateral amygdala, and the hippocampus are known to modulate PPI or to affect its regulation in different ways, as reflected by disruption of PPI following manipulations of these structures (for review see Kohl et al., 2013). Spatial working memory, as assessed in the DMP task, is known to be hippocampus dependent (Whishaw, 1985; Morris et al., 1986a,b; Wible, 2013). Thus, the finding that PPI and spatial working memory can be modulated by hippocampal function, albeit to a different extent or in different ways, together with the important role attributed to the hippocampus in other schizophrenia-relevant cognitive processes and schizophrenic symptoms (e.g., Gray et al., 1991; Sawa and Snyder, 2002; Wible, 2013), suggests that the hippocampus represents a prime candidate structure to investigate neurobiological processes underlying particular symptom clusters. In this connection, it is remarkable that the RLA-I rat strain has a more functional hippocampus and higher hippocampal neuronal density than the RHA-I strain (Meyza et al., 2009; Garcia-Falgueras et al., 2012), which may underlie the better performance of RLA-I rats in both PPI and spatial working memory.

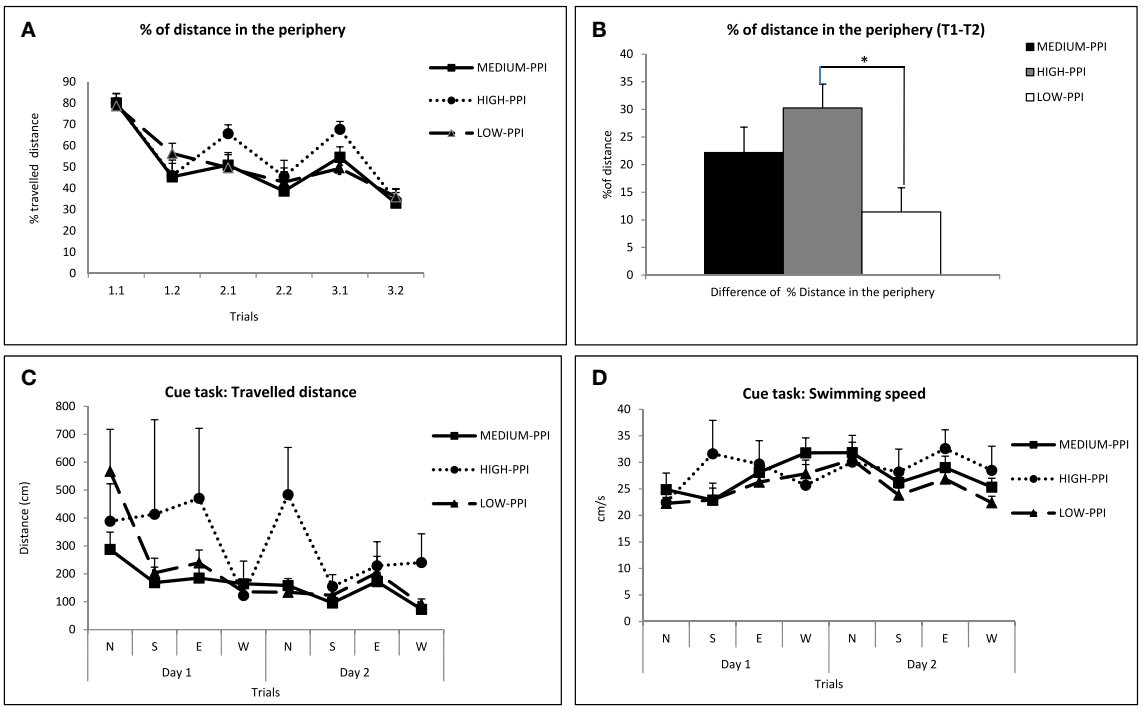

FIGURE 6 | (A) Mean ± S.E.M. of the percentage of distance traveled in the periphery in each trial for the 3 groups of NIH-HS rats. **(B)** Mean ± S.E.M. of the "difference of percentage of distance traveled in the periphery between T1 and T2 (averaged for the 3 days)" (i.e., "Mean %DP T1-T2"). **(C)** Mean ± S.E.M. of the distance (cm) traveled by the rats of the three groups in each of the 8 trials of the cued task. **(D)** Mean ± S.E.M. swimming speed of the NIH-HS sub-groups for each trial. *$p < 0.05$ between the groups indicated (LSD test following significant One-Way ANOVA).

In summary, the present study demonstrates a consistent and positive PPI-working memory association using three different strategies: (1) comparing three strains/stocks of rats which show differential PPI levels (Experiment 1); (2) evaluating working memory in subsamples of NIH-HS rats displaying extreme scores in PPI; and (3) performing correlational, regression, and factor analysis of PPI and working memory assessed in a sample of genetically heterogeneous NIH-HS rats. The results of the present study, together with those from Singer et al. (2013) in mice, support the idea that PPI and working memory are positively associated in untreated animals, thus paving the path for the study of possible common neurobiological mechanisms of pre-attentive (sensorimotor gating) and higher cognitive processes in rodents that can illuminate routes for abnormal functioning in schizophrenias.

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Event-related brain potential correlates of prospective memory in symptomatically remitted male patients with schizophrenia

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Prospective memory (PM) refers to the ability to remember to perform intended actions in the future. Although PM deficits are a prominent impairment in schizophrenia, little is still known about the nature of PM in symptomatically remitted patients with schizophrenia. To address this issue, event-related brain potentials (ERPs) were recorded from 20 symptomatically remitted patients with schizophrenia and 20 healthy controls during an event-based PM paradigm. Behavioral results showed that symptomatically remitted patients with schizophrenia performed poorly on the PM task compared with healthy controls. On the neural level, the N300, a component of the ERPs related to PM cue detection, was reliable across these two groups, suggesting a degree of functional recovery of processes supporting cue detection in patients with symptomatically remitted schizophrenia. By contrast, the amplitude of the prospective positivity, a component of the ERPs related to PM intention retrieval, was significantly attenuated in symptomatically remitted schizophrenia patients relative to healthy controls. Furthermore, a significant positive correlation between the amplitude of the prospective positivity and accuracy on the PM task was found in those patients, indicating that patients' poor performance on this task may result from the failure to recover PM cue-induced intention from memory. These results provide evidence for the existence of altered PM processing in patients with symptomatically remitted schizophrenia, which is characterized by a selective deficit in retrospective component (intention retrieval) of PM. Therefore, these findings shed new light on the neurophysiological processes underlying PM in schizophrenia patients during clinical remission.

Keywords: prospective memory, ERPs, symptomatically remitted patients with schizophrenia, N300, prospective positivity

Introduction

Patients with schizophrenia are characterized by cognitive deficits (Heinrichs and Zakzanis, 1998; Elvevåg and Goldberg, 2000). Specifically, previous research has shown that schizophrenia patients show deficits in a variety of cognitive domains, including attention, executive function and memory (Chan et al., 2004; Mesholam-Gately et al., 2009). In the context of

such generalized cognitive deficits, some researchers argue that memory deficit is one of the core impairments in those patients (Chan et al., 2004; Reichenberg and Harvey, 2007).

Prospective memory (PM) is thought to be an important aspect of episodic memory, which refers to remembering to perform intended actions in the future such as remembering to put the trash in a garbage bin at 9 am or to post a letter after seeing a mailbox (Meacham and Singer, 1977; Kliegel et al., 2008). Given that PM is so highly prevalent in our daily life, understanding its underlying mechanisms has gradually become a focus of experimental inquiry in cognitive psychology over the past decades (Graf and Uttl, 2001; Brandimonte et al., 2014). According to a popular conceptual framework, the realization of delayed intentions entails both prospective (cue detection) and retrospective (intention retrieval) components (McDaniel and Einstein, 1992; Smith and Bayen, 2004; Brandimonte et al., 2014). The prospective component refers to processes that support the detection of prospective cues (e.g., the post office) signaling that an intended action (e.g., buying a stamp) should be performed and the retrospective component involves processes that support subsequent recovery of that intention (e.g., buying a stamp) from memory (Einstein and McDaniel, 1990; Brandimonte et al., 2014). Moreover, work using event-related brain potentials (ERPs) has revealed that two ERP components (N300 and prospective positivity) are differentially related to the prospective and retrospective components of PM in humans (West et al., 2001; West and Ross-Munroe, 2002; West and Krompinger, 2005; West, 2011). The theoretical and empirical advances on PM also have clinical implications for exploring the nature of PM in patients with mental disorders (Rendell and Henry, 2009; Zogg et al., 2012).

Particularly, PM deficits in schizophrenia patients have been gaining increasing attention over the past decade. Earlier studies have revealed that schizophrenia patients performed more poorly than healthy controls across different types of PM tasks (event-based, time-based and activity-based PM; Elvevåg et al., 2003; Shum et al., 2004; Kumar et al., 2005, 2008; Altgassen et al., 2008; Ungvari et al., 2008). This is corroborated by recent meta-analytic studies describing that such deficits in schizophrenia patients can be observed with moderate to large effect sizes (Wang et al., 2009). Meanwhile, other recent research contributes to clarifying the nature and extent of PM deficits by controlling for other cognitive functions such as working memory (Zhuo et al., 2013), retrospective memory (Shum et al., 2004; Xiang et al., 2010), IQ (Lui et al., 2011), executive function (Henry et al., 2007; Lui et al., 2011) as well as clinical variables such as duration of illness (Zhou et al., 2012) and medication (Zhuo et al., 2013). These findings suggest that PM deficits reflect a primary impairment of schizophrenia rather than a secondary to other cognitive impairments (Henry et al., 2007; Wang et al., 2008; Ordemann et al., 2014). In addition, some researchers have begun to elucidate whether PM deficits are driven by impairments in specific components of PM in schizophrenia patients. The existing evidence suggests that schizophrenia-associated PM deficits are due to failures of both prospective (cue detection) and retrospective (intention retrieval) components (Woods et al., 2007; Wang et al., 2008).

Despite such evidence, little is still known about PM in symptomatically remitted patients with schizophrenia, although prior studies have shown the persistence of neuropsychological deficits in some cognitive domains including theory of mind (Mo et al., 2008) and emotional reaction (Yalcin-Siedentopf et al., 2014) in those patients. To address this question, we combined an event-based PM paradigm with ERPs in 20 symptomatically remitted patients with schizophrenia and 20 healthy controls. In this event-based PM task, participants were required to remember to respond to a word in blue (PM task), which was embedded in a simple categorization task (ongoing task). It is recognized that ERP-based biomarkers provide sensitive and reliable measures of the neural events underlying cognition. In particular, the promise of ERP-based biomarkers of cognitive dysfunction in schizophrenia has recently been demonstrated (Luck et al., 2011), thereby providing a unique opportunity to distinguish and examine the influence of processes associated with prospective (PM cue detection, N300) and retrospective (PM intention retrieval, prospective positivity) components of PM on task performance in symptomatically remitted patients with schizophrenia.

Materials and Methods

Participants

We recruited 20 symptomatically remitted patients with schizophrenia. All were right-handed males, because recruiting males could avoid the potential effect of the menstrual cycle known to have an impact on memory in females (Phillips and Sherwin, 1992; Wizeman, 2012). All patients had an earlier acute episode of schizophrenia according to DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders (fourth edition, text revision)) criteria for schizophrenia diagnosis (Association, 2000). Diagnosis was determined by the Structured Clinical Interview for DSM-IV-TR AXIS I Disorders (SCID; First et al., 2001). Patients with a history of any past or present major medical or neurological illnesses, brain injury, drug dependence and mental retardation were excluded (according to medical records, information collected from family members and interview with the patients). Furthermore, those with schizoaffective, anxiety and depression disorders were excluded. We used the Chinese version of Positive and Negative Symptom Scale (PANSS; Kay et al., 1987) to measure severity of symptoms (**Table 1**). Symptomatic remission were defined according to a criteria (both severity of core symptoms and their time criteria) proposed by the Remission in Schizophrenia Working Group (RSWG; Andreasen et al., 2005). We also assessed the patients' intellectual functioning using the Chinese version of the Wechsler Adult Intelligence Scale-Revised (WAIS-R; Gong, 1992), which has been employed to rate this function of Chinese schizophrenia patients in previous studies (Wang et al., 2008; Zhou et al., 2012).

We also recruited 20 healthy controls reporting no prior history of psychiatric illness and drug abuse. Healthy controls

TABLE 1 | Demographic and clinical characteristics of the study sample.

	Remitted schizophrenia (n = 20) (M ± SEM)	Healthy controls (n = 20) (M ± SEM)	Group comparison
Age (years)	23.65 ± 0.58	22.55 ± 0.80	$t_{(38)} = 1.11, p > 0.05$
Education (years)	10.35 ± 0.57	10.65 ± 0.43	$t_{(38)} = 0.42, p > 0.05$
IQ	89.95 ± 2.78	98.05 ± 2.65	$t_{(38)} = 2.11, p < 0.05$
Duration of illness (months)	27.15 ± 4.01		
Medication[a]	250.00 ± 28.89		
PANSS[b]			
Positive symptoms	8.80 ± 0.41		
Negative symptoms	13.65 ± 0.77		
General psychopathology	26.00 ± 1.07		
Total score	48.45 ± 1.42		

M, mean; SEM, standard error of mean; [a]Chlorpromazine equivalence mg/d; [b]PANSS, Positive and Negative Symptom Scale.

also underwent psychiatric evaluation. Both groups were matched in age, gender and education (**Table 1**). A diagnosis of schizophrenia or any other mental disorders in first-degree relatives was also an exclusion criterion.

The study was carried out in according with the guidelines approved by the ethics committee of PLA general hospital. All participants in this study gave written informed consent to participate in the experiment.

Stimuli and Event-Based PM Paradigm

The event-based PM paradigm followed the task used in a previous study (Zhuo et al., 2013). The ongoing task consisted of a simple categorization task. On ongoing activity trials, a Chinese word in green was presented in the center of a computer screen, while four different Chinese words in black referring to four different categories (animal, plant, commodity and people) were shown below the center of the screen. The participants' task was to decide which category the word in green belongs to. In the PM task, the participants were asked to suspend the ongoing task and switch to the PM task by pressing a corresponding button on the Joystick when a word in blue (PM cue) was presented.

There were a total of 1000 trials which were equally divided into 10 blocks. Each block included 94 ongoing activity trials and 6 PM cue trials. Within each block, a stimulus (a word in green or blue), together with the words in black referring to four categories which maintained below the center of the screen throughout the entire block, was shown in the center of the screen and the participants had a maximum of 4000 ms to make a response indicating which category this word belongs to. After the response, the stimulus was replaced by a fixation cross lasting randomly for 500–800 ms. It was followed by the next stimulus within the block. The PM and ongoing trials were randomly interspersed within each block. Before the experimental task began, the participants performed a practice block containing 20 ongoing activity trials and 3 PM cue trials to ensure that they understood the instructions.

Words were drawn from text books and reading materials used in Chinese primary schools (People's Education Press) and were matched for written frequency, familiarity, and concreteness. Words from four categories (animal, plant, commodity and people) were included. There were a total of 500 words and each category included 125 words. In each block, 25 words were randomly selected from each category.

ERP Data Recording and Analysis

EEG was recorded (SynAmps amplifier, NeuroScan) with a quick cap carrying 32 Ag/AgCl electrodes (Fp1, Fp2, F3, Fz, F4, Fc3, Fcz, Fc4, C3, Cz, C4, CP3, CPz, CP4, P3, Pz, P4, F7, F8, Ft7, Ft8, T3, T4, Tp7, Tp8, T5, T6, O1, Oz, O2) placed at standard locations covering the whole scalp (the extended international 10–20 system). The reference electrode was attached to the right mastoid (A2), and the ground electrode was placed on the forehead. The vertical electrooculogram (VEOG) was recorded with electrodes placed above and below the left eye. The horizontal electrooculogram (HEOG) was recorded with electrodes placed beside the two eyes. The impedance was kept below 5 kΩ. The electrophysiological data were continuously recorded with a bandwidth 0.05–100 Hz and sampled at a rate of 1000 Hz.

Offline data analysis was conducted using EEGLAB (Delorme and Makeig, 2004) and ERPLAB (Lopez-Calderon and Luck, 2014). Data were first re-referenced to linked mastoid (A1 and A2). An independent component analysis (ICA)-based artifact correction was achieved by using the ICA function of EEGLAB. Independent components with topographies representing saccades, blinks, and heart rate artifact were thus removed according to published guidelines (Jung et al., 2000). The resultant EEG data were then epoched from 200 ms pre-stimulus to 1000 ms post-stimulus and digitally low pass filtered by 30 Hz (24 dB/octave). The 200 ms pre-stimulus period was used for baseline correction. In order to remove movement artifacts, epochs were rejected when fluctuations in potential values exceeded ±75μV at any channels except the EOG channel. The ERPs evoked by PM cue trials and ongoing activity trials were calculated by averaging individual artifact-free trials in each participant. Finally, the grand-averaged ERPs were computed and averaged for correctly performed PM cue trials and correctly performed ongoing activity trials in each group.

Statistical Analysis

For statistical analysis on the behavioral data, both response times and accuracy were analyzed using a two-way mixed Analysis of Covariance (ANCOVA) with group (symptomatically remitted patients with schizophrenia vs. healthy controls) as a between-subject factor, task type (PM vs. ongoing task) as a within-subject factor. Because there was a significant between-group difference in IQ, we also included IQ in our mixed model as a covariate.

With regard to statistical analysis on electrophysiological data, our data were analyzed according to the topographical distribution of grand averaged ERP activity as well as the methods of previous ERP studies (West et al., 2001; West and Ross-Munroe, 2002). Our ERP statistical analysis involved two PM-related ERP components: N300 and prospective positivity. The N300 referred to the maximum negative voltage over the occipital region between 190 and 400 ms and the prospective positivity represented the maximum positive voltage over the parietal region between 400 and 1000 ms. Based on the methods of previous ERP studies (West et al., 2001; West and Ross-Munroe, 2002), the amplitudes of both ERP components were quantified as the mean voltages across 50 ms window that centered on the peaks so that the measurements would have similar levels of stability. Then, amplitudes of these two ERP components were analyzed using a three-way mixed ANCOVA with task type (PM vs. ongoing task) and electrode sites as within-subject factors, group (symptomatically remitted patients with schizophrenia vs. healthy controls) as a between-subject factor and with IQ as a covariate. The selection of electrodes characterizing N300 and prospective positivity was based on previous ERP findings (West and Ross-Munroe, 2002; West and Krompinger, 2005). Specifically, the amplitude of N300 over the occipital region (electrodes: O1/Oz/O2) was quantified and prospective positivity over the parietal region (electrodes: P3/Pz/P4) was measured.

Statistical comparisons were made at p-values of $p < 0.05$, with the Greenhouse–Geisser correction when violations of sphericity occurred.

Results

Behavioral Performance

Response times and accuracy in the two groups are presented in **Table 2**.

For response times, there was a significant main effect of group ($F_{(1,75)} = 14.90$, $p < 0.001$), revealing that symptomatically remitted patients with schizophrenia responded more slowly than healthy controls (**Figure 1A**). Moreover, there was also a significant main effect of task type ($F_{(1,75)} = 42.91$, $p < 0.001$), with response times being longer for ongoing activity trials than for PM cue trials. However, the interaction between group and task type was not significant ($F_{(1,75)} = 1.53$, $p > 0.05$).

With regard to accuracy, there was a significant main effect of group ($F_{(1,75)} = 5.16$, $p < 0.05$; **Figure 1B**). The symptomatically remitted patients with schizophrenia were less accurate than healthy controls, consistent with findings from a prior study using the similar task (Zhuo et al., 2013). Furthermore, accuracy was also significantly higher for ongoing activity trials than for PM cue trials ($F_{(1,75)} = 14.71$, $p < 0.001$). More importantly, we found a robust group × task type interaction ($F_{(1,75)} = 7.10$, $p < 0.01$). An analysis of simple effects showed that this interaction was driven by low accuracy on PM cue trials in symptomatically remitted patients with schizophrenia compared with healthy controls ($p < 0.01$; **Figure 1C**).

TABLE 2 | Accuracy (%) and response times (ms) for symptomatically remitted patients with schizophrenia and healthy controls.

	Remitted schizophrenia (n = 20) (M ± SEM)	Healthy controls (n = 20) (M ± SEM)
Accuracy (%)		
PM cue	80.10 ± 4.60	93.95 ± 1.47
Ongoing activity	96.15 ± 0.82	96.85 ± 0.95
Response times (ms)		
PM cue	916.66 ± 47.18	780.89 ± 21.22
Ongoing activity	1235.18 ± 51.81	998.20 ± 37.58

M, mean; SEM, standard error of mean.

ERP Results

N300

Regarding the N300, there was a main effect of task type ($F_{(1,227)} = 6.43$, $p < 0.05$), with the N300 amplitude being more negativity for PM cue trials than for ongoing activity trials (**Figures 2A,B**). There was also a main effect of group ($F_{(1,227)} = 89.24$, $p < 0.001$), with the amplitude of the N300 being larger in healthy controls than in symptomatically remitted patients with schizophrenia. In addition, the main effect of electrode sites was also significant ($F_{(2,227)} = 3.30$, $p < 0.05$). The *post hoc* analysis revealed a significant difference between O2 and O1 ($p < 0.05$), with the amplitude of the N300 being larger in the electrode O2 than in the electrode O1. However, no significant interaction effects among these three factors were found.

Prospective Positivity

For the prospective positivity, there was a significant main effect of group ($F_{(1,227)} = 4.62$, $p < 0.05$), with the amplitude of prospective positivity being larger in healthy controls than in symptomatically remitted patients with schizophrenia (**Figure 3A**). There was also a significant main effect of task type ($F_{(1,227)} = 250.61$, $p < 0.001$), with the amplitude being larger for the PM cue trials than for ongoing activity trials. In addition, there was a main effect of electrode sites ($F_{(2,227)} = 5.44$, $p < 0.01$). The *post hoc* analysis revealed a significant difference between Pz and two other electrode sites (P3 and P4; $p < 0.05$ and $p < 0.005$), indicating that prospective positivity was mainly distributed over the electrode (Pz) which was placed in the central location of the parietal region. More importantly, group × task type interaction was significant ($F_{(1,227)} = 10.19$, $p < 0.01$). An analysis of simple effects revealed that this interaction was due to smaller amplitude of the prospective positivity for PM cue trials in symptomatically remitted patients with schizophrenia than in healthy controls

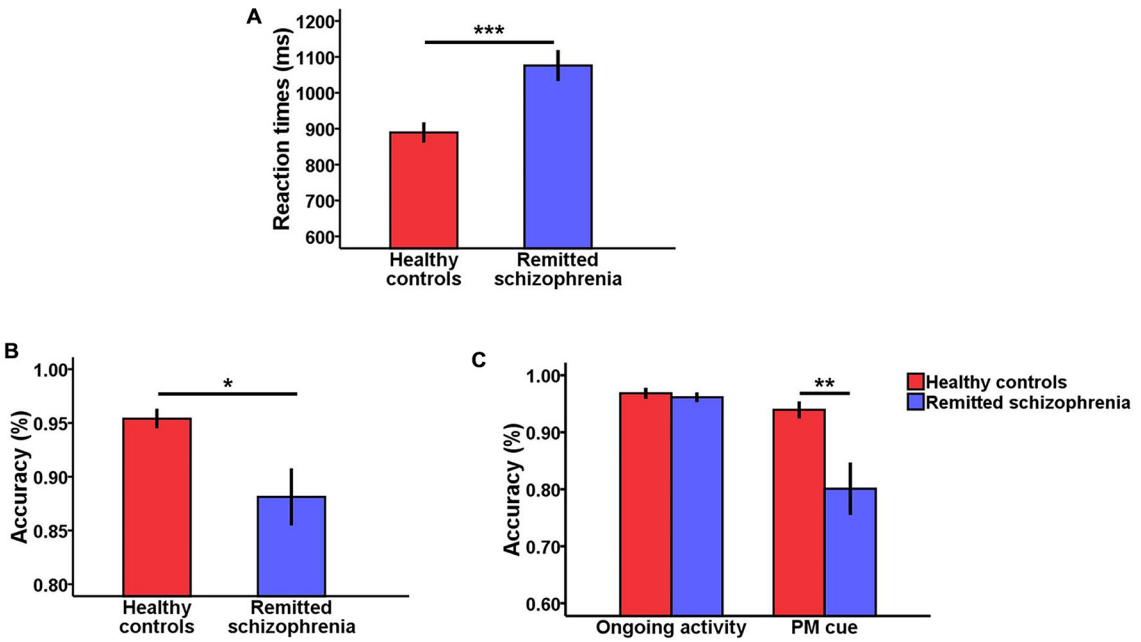

FIGURE 1 | Behavioral results. (A) Bar plots of response times according to group (symptomatically remitted patients with schizophrenia vs. healthy controls). **(B)** Bar plots of accuracy according to group (symptomatically remitted patients with schizophrenia vs. healthy controls). **(C)** Bar plots of group × task type interaction effects on accuracy. Error bars indicate standard error of mean (SEM), (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

($p < 0.001$; **Figures 3B,C**). Given the existence of interaction between group and task type for both accuracy and prospective positivity over the parietal region, it is interesting to investigate whether there is a link between accuracy on PM cue trials and PM cue-elicited prospective positivity in symptomatically remitted patients with schizophrenia. A partial correlation analysis, with IQ statistically controlled, revealed a significant positive correlation between them ($r = 0.51$, $p < 0.05$), suggesting that larger amplitude of prospective positivity was associated with better accuracy on the PM cue trials in symptomatically remitted patients with schizophrenia (**Figure 3D**).

Discussion

The present study was to examine the ability to perform an intended action in symptomatically remitted patients with schizophrenia. The behavioral data revealed that symptomatically remitted patients with schizophrenia performed more poorly during the event-based PM task than healthy controls, which was driven by the decreased accuracy on PM cue trials in those remitted patients relative to healthy controls. This finding suggests that symptomatically remitted patients with schizophrenia are associated with PM deficits. Since there was a significant between-group difference in IQ and a number of studies revealed that intelligence was associated with PM performance in schizophrenia patients (Henry et al., 2007; Wang et al., 2009), this seemed to suggest that the observed PM deficits may be due to discrepancy in IQ between these two groups. However, the observed PM deficits in symptomatically remitted patients with schizophrenia were still maintained when IQ was statistically controlled, suggesting that PM deficits in symptomatically remitted patients with schizophrenia may be a primary deficit not secondary to other cognitive functions such as IQ. Despite such evidence, the causes of PM deficits in these remitted schizophrenia patients is not readily apparent merely on the basis of behavioral findings. It may arise from difference in the processes supporting the prospective component of PM between these two groups, differences in the processes supporting the retrospective component between these two groups or both. Our electrophysiological data may help shed important insights on it.

ERP Related to Cue Detection

N300 over the occipital region has been demonstrated to be preferentially related to prospective component (cue detection) of PM (West et al., 2001; West and Ross-Munroe, 2002; West and Krompinger, 2005; West, 2011). It has been proposed that prospective component of PM reflects the biasing of attention between external events (e.g., identifying a cue amid distracting stimuli; Smith and Bayen, 2004). Our electrophysiological data showed that symptomatically remitted patients with schizophrenia were able to distinguish PM cues from the ongoing task as reflected by the significant main effect of task type across these two groups and the non-significant interaction between task type and group. As a consequence, this observation may indicate a degree of functional recovery of preparatory attentional processes that facilitates the processing of PM cues in these patients during clinical remission, thereby providing further evidence for recent studies showing that symptomatic remission in schizophrenia is associated with a degree of

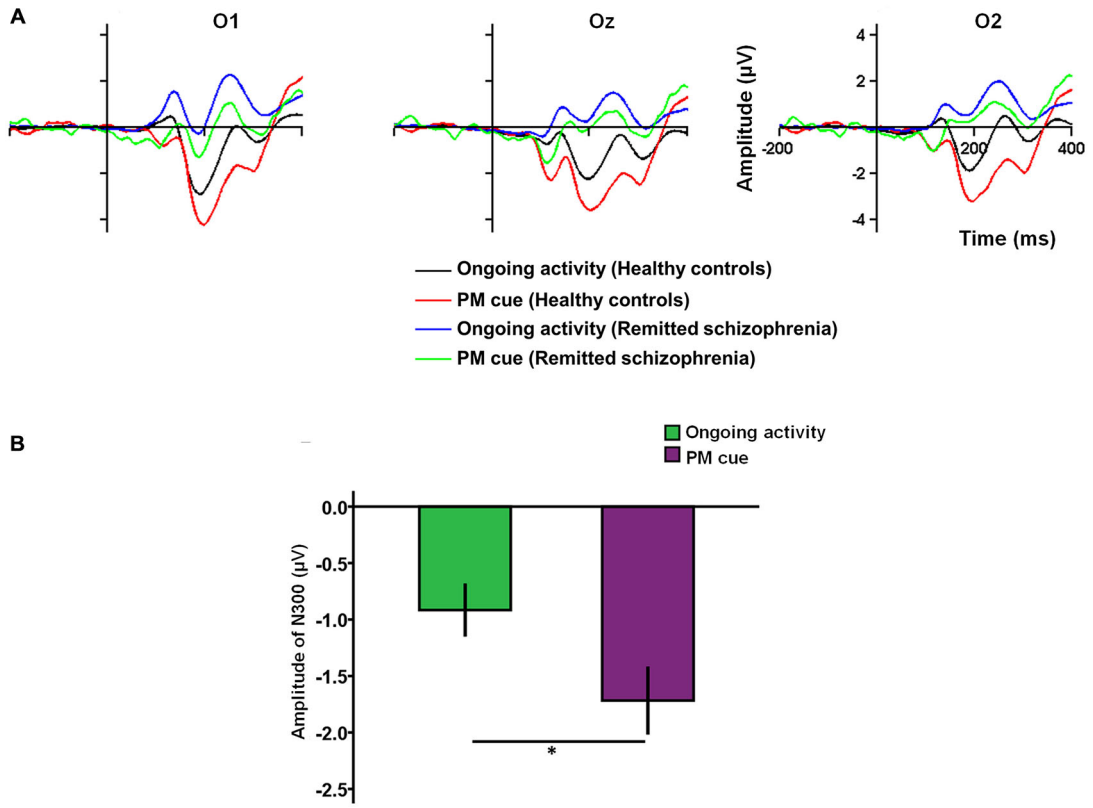

FIGURE 2 | The N300 related to prospective memory (PM) cue detection. (A) The N300 over the occipital region (O1/Oz/O2) for PM cue trials and ongoing activity trials in symptomatically remitted patients with schizophrenia and healthy controls. **(B)** Bar plots illustrating the amplitudes of the N300 according to task type (PM vs. ongoing task). Error bars indicate SEM, (*$p < 0.05$).

functional recovery of attentional processes (Fukumoto et al., 2014).

ERP Related to Intention Retrieval

In contrast, our electrophysiological data related to retrospective component (intention retrieval) of PM showed that the amplitude of PM cue-elicited prospective positivity elicited was significantly decreased in symptomatically remitted patients with schizophrenia relative to healthy controls. This is corroborated by our subsequent observation about the positive correlation between such reduced prospective positivity and reduced accuracy on the PM task in symptomatically remitted patients with schizophrenia. Thus, these findings clearly demonstrate that PM deficits are characterized by the impairment in retrieving intentions from memory in remitted schizophrenia patients, which is distinct from acute episode of schizophrenia patients characterized by impairments in both cue detection and intention retrieval (Woods et al., 2007; Wang et al., 2008). This may have several important clinical implications. Our preliminary evidence about the selective deficit in PM intention retrieval in symptomatically remitted schizophrenia patients may have an impact on evaluation and improvement of the effectiveness of schizophrenia therapeutics during clinical remission because PM-based effective treatment interventions rely on precise characterization of PM deficits in symptomatically remitted patients with schizophrenia. As such, our finding may help direct clinicians to the selective deficit in retrospective component of PM and develop targeted psychological treatments and PM-based remediation strategies to improve functional outcomes in schizophrenia during clinical remission. Furthermore, there is recent evidence showing that PM can act as an important predictor of medication management ability and have an impact on medication adherence in acute phases of schizophrenia (Zogg et al., 2012; Lam et al., 2013), our observation in this study may indicate that the effective treatment interventions on the selective deficit in the retrospective component of PM may be one of the effective means of improving adherence in schizophrenia during clinical remission.

In addition, given that the selective deficit in retrospective component of PM in remitted schizophrenia patients is still observed in the present study and PM is thought to be a unique form of episodic memory (an important category of declarative memory; Kliegel et al., 2008), this seems to indicate that episodic memory deficit is one of the core impairments in schizophrenia patients and thus, may serve as one of stable markers of clinical remission in schizophrenia patients.

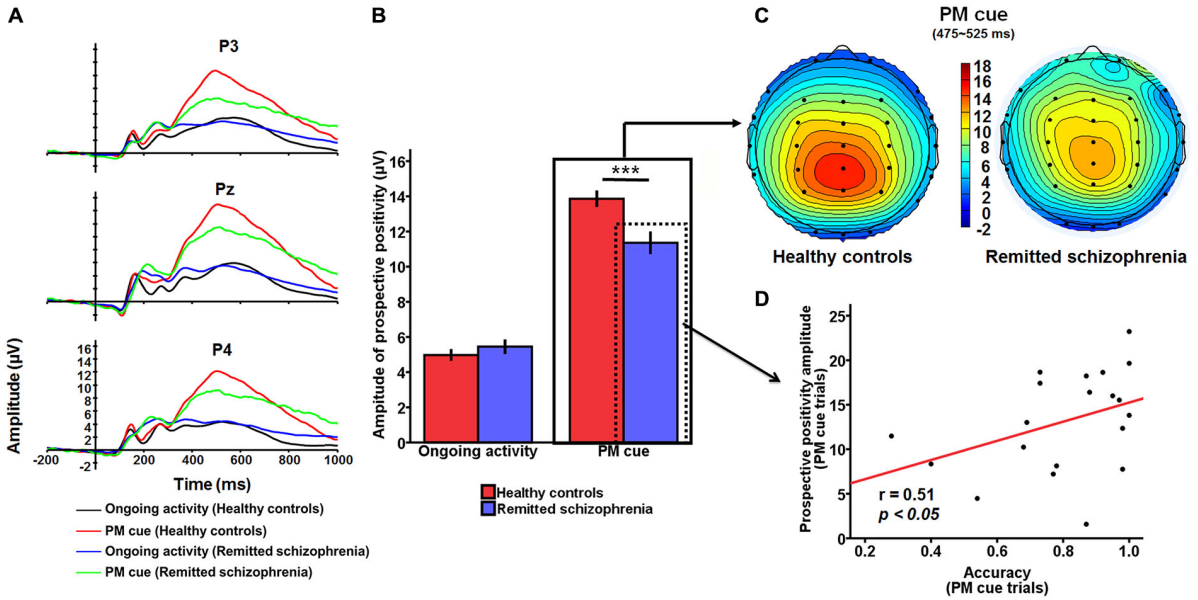

FIGURE 3 | The prospective positivity related to PM intention retrieval. (A) The prospective positivity over the parietal region (P3/Pz/P4) for PM cue trials and ongoing activity trials in symptomatically remitted patients with schizophrenia and healthy controls. **(B)** Bar plots illustrating the group × task type interaction effect on the amplitude of the prospective positivity over the parietal region. As illustrated in the figure, this interaction effect is driven by the reduced amplitude of prospective positivity elicited by PM cues in symptomatically remitted patients with schizophrenia compared with healthy controls. Error bars indicate SEM, $^{***}p < 0.001$. **(C)** Topographical voltage distributions within 475–525 ms centered on the peak of prospective positivity elicited by PM cue for healthy controls (left) and symptomatically remitted patients with schizophrenia (right). Positive isopotential lines are in red and negative isopotential lines are in blue. As shown in the figure, there is reduced amplitude of prospective positivity over the parietal region in those patients compared with healthy controls. **(D)** Correlation between accuracy and amplitude of prospective activity on PM cue trials over the parietal region in remitted patients with schizophrenia. As illustrated in this scatter plot, amplitude of PM cue-elicited prospective amplitude over the parietal region is positively correlated with accuracy for PM cue trials in those patients, ($p < 0.05$).

Taken together, these findings suggest a degree of functional recovery of processes associated with PM cue detection in symptomatically remitted schizophrenia patients, while the impairment in processes associated with PM intention retrieval still maintains.

Potential Limitations

The present study is not without any limitations. First, only male patients with symptomatically remitted schizophrenia were involved in the current study. It remains unclear whether these findings could be generalized to female patients with remitted schizophrenia. This is important because it has been shown that gender differences exist in several aspects of schizophrenia (Ochoa et al., 2012). Second, one type of PM (event-based PM) was only involved in the present study. It is unknown whether these findings could be generalized to the other two types of PM (time-based and activity-based PM). This is important because recent research suggests that PM tasks cannot be viewed as being interchangeable and there are key differences among different types of PM tasks (Ordemann et al., 2014). Third, given that prior studies have shown that clinical remission can be predicted by the onset conditions of the schizophrenia patients (Marchesi et al., 2014), it should be interesting to link the deficits in cognitive functioning to the onset conditions of those remitted schizophrenia patients in the follow-up studies. Despite these limitations, we think that our current findings are still robust and may foster further research on neuropsychological characteristics of PM in symptomatically remitted patients with schizophrenia.

Conclusion

In conclusion, the present study aimed at characterizing event-related potential correlates of PM in symptomatically remitted patients with schizophrenia. Our main finding is that on the neural level, the amplitude of the prospective positivity, a component of the ERPs related to PM intention retrieval, was significantly attenuated in symptomatically remitted schizophrenia patients relative to healthy controls. Moreover, the amplitude of this component was positively correlated with the accuracy on PM task in those patients. Therefore, this study demonstrates PM deficits in symptomatically remitted patients with schizophrenia characterized by a selective deficit in retrospective component of PM. This is distinct from PM deficits in acute phase of schizophrenia patients showing impairments in both prospective and retrospective components of PM. Our results may have important implications for further research charactering PM alterations in patients with symptomatically remitted schizophrenia.

Author Contributions

GC, HC and YL designed the experiment; GC, LZ, WD, PX, SL, ZJ, LS, HL and HC performed the experiment; WD, PX, SL, ZJ,

LS, HL analyzed behavioral data; GC, RZ and YL analyzed data; GC and YL wrote the manuscript.

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Mouse hippocampal GABAB1 but not GABAB2 subunit-containing receptor complex levels are paralleling retrieval in the multiple-T-maze

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GABAB receptors are heterodimeric G-protein coupled receptors known to be involved in learning and memory. Although a role for GABAB receptors in cognitive processes is evident, there is no information on hippocampal GABAB receptor complexes in a multiple T maze (MTM) task, a robust paradigm for evaluation of spatial learning. Trained or untrained (yoked control) C57BL/6J male mice ($n = 10$/group) were subjected to the MTM task and sacrificed 6 h following their performance. Hippocampi were taken, membrane proteins extracted and run on blue native PAGE followed by immunoblotting with specific antibodies against GABAB1, GABAB1a, and GABAB2. Immunoprecipitation with subsequent mass spectrometric identification of co-precipitates was carried out to show if GABAB1 and GABAB2 as well as other interacting proteins co-precipitate. An antibody shift assay (ASA) and a proximity ligation assay (PLA) were also used to see if the two GABAB subunits are present in the receptor complex. Single bands were observed on Western blots, each representing GABAB1, GABAB1a, or GABAB2 at an apparent molecular weight of approximately 100 kDa. Subsequently, densitometric analysis revealed that levels of GABAB1 and GABAB1a but not GABAB2- containing receptor complexes were significantly higher in trained than untrained groups. Immunoprecipitation followed by mass spectrometric studies confirmed the presence of GABAB1, GABAB2, calcium calmodulin kinases I and II, GluA1 and GluA2 as constituents of the complex. ASA and PLA also showed the presence of the two subunits of GABAB receptor within the complex. It is shown that increased levels of GABAB1 subunit-containing complexes are paralleling performance in a land maze.

Keywords: spatial learning, mice, multiple T-maze, GABAB receptor, receptor complex

INTRODUCTION

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system and mediates its effect through two distinct classes of GABA receptors: the ionotropic (GABAA and GABAC) receptors, which, when activated produce a rapid and very short-lived inhibition via chloride currents (Watanabe et al., 2002) and the metabotropic GABAB receptor, which is responsible for slow but sustained inhibitory currents (Couve et al., 2000; Bettler et al., 2004).

GABAB receptors are obligatory heterodimers composed of GABAB1 and GABAB2 subunits, which are required for agonist binding and G-protein coupling (Robbins et al., 2001). The receptors are implicated in numerous behaviors and central nervous system disorders, including epilepsy, depression, anxiety, panic, and sleep disorders (Bettler et al., 2004). Molecular diversity of GABAB receptors arise from the two pharmacologically indistinguishable GABAB1 isoforms (1a and 1b) (Bettler et al., 2004; Pinard et al., 2010) and from potassium channel tetramerization domain-containing (KCTD) auxiliary subunits (Schwenk et al., 2010).

Both, GABAB1a and GABAB1b receptor subunits are expressed in the hippocampus (Liang et al., 2000), where they can modulate neuronal excitability. It is suggested that GABAB receptors containing the GABAB1a subunit are primarily located presynaptically and those with GABAB1b subunits are primarily postsynaptic (Vigot et al., 2006). However, this distribution may vary with the brain region and whether the presynaptic neuron is GABAergic or glutamatergic (Waldmeier et al., 2008). Heteroreceptors are predominantly found at glutamatergic boutons, mediating presynaptic inhibition of glutamate release largely via suppression of Ca^{2+} currents (Bussières and El Manira, 1999; Barral et al., 2000; Vigot et al., 2006; Waldmeier et al., 2008), while postsynaptic receptors activate slow inhibitory postsynaptic potentials through activation of inwardly rectifying K^+ channels (Fernández-Alacid et al., 2009; Liu et al., 2012). In addition, GABAB receptors can function as autoreceptors in interneurons (Waldmeier et al., 2008) and also modulate adenylyl cyclase, through which they influence downstream molecular pathways (Couve et al., 2000; Bettler et al., 2004).

A substantial body of evidence exists indicating the modulatory role of GABAB receptors in learning and memory. Stimulation of GABAB receptors impaired working memory in the basal forebrain (DeSousa et al., 1994) and medial septum (Stackman and Walsh, 1994) using double Y- and radial maze. Conversely, a series of centrally active GABAB receptor blockers have been shown to improve performance in a host of learning paradigms, including passive avoidance (Bianchi and Panerai, 1993; Mondadori et al., 1993), active avoidance (Getova and Bowery, 1998), and radial maze (Helm et al., 2005). However, Brucato et al. (1996) reported that the antagonist CGP 46381 showed no effect in radial maze and decreased performance in a water maze (Brucato et al., 1996).

Crosstalk among the neurotransmitter repertoire is common in the central nervous system. This is made possible through formation of signaling complexes as a result of receptor assembly with other receptors or regulatory proteins. As studies so far have not been addressing GABAB receptor containing complexes as actors in the MTM, it was the aim of the current study to show which GABAB-containing receptor complex(es) are involved in a MTM task, a paradigm for evaluation of spatial learning.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6J ($n = 20$, male, aged 10–12 weeks, 10 Trained which are quick learners and were selected from 20 animals and 10 Untrained) mice were used for the study. C57BL/6J mice were obtained from JANVIER SAS laboratory (France). All mice were kept and maintained in polycarbonate cages Type II (207 × 140 × 265 mm, Ehret, Austria) filled with autoclaved wood chips (Ligncell select, Rettenmaier, Austria) in the core unit of Biomedical Research, Division of Laboratory Animal Science and Genetics, Medical University of Vienna. The animals were housed in groups with access to autoclaved Altromin standard rodent diet (Altromin, Germany) and water *ad libitum,* and maintained under standard conditions (ambient temperature of 22 ± 1°C, relative humidity of 50 ± 10%, light/dark cycle of 14:10, and ventilation with 100% fresh air that resulted in an air change rate of 15 times per hour). The room was illuminated with artificial light at an intensity of about 200 l× in 2 m from 5 a.m. to 7 p.m. The experiments were carried out under a license obtained from the Federal Ministry of Education, Science and Culture, which includes an ethical evaluation of the project (Project: BMWF-66.009/0240-II/10b/2009). Housing and maintenance of animals were in compliance with European and national regulations (Ghafari et al., 2012a).

Multiple-T-Maze

MTM is one of the spatial learning tasks in which animals learn to find the goal box based on their memory of previously visited arms and carried out as described elsewhere (Patil et al., 2009), with minor modifications. The MTM was constructed of wood and consisted of a platform with seven choice points, and dimensions of 150 × 130 × 15 cm and a path width of 8 cm. Before testing, mice were deprived of food for 16 h to motivate food searching. Ten mice were then placed in a start box (diameter: 10.5 cm) and allowed to search the reward. The trial was considered to be completed when the mice either reached the goal box or failed to find it within 5 min. In the goal box, mice were allowed to consume a small piece of food pellet provided as a reward. Immediately after each trial, the entire maze was cleaned with 1% incidin solution (Incidin extraN, Lohmann and Rauscher, Austria) to remove possible cues. Another 10 mice were used as untrained controls (yoked controls) that spent the same time in the MTM without learning performance as there was no reward in the goal box. After testing, animals were returned to their cage and given food based on their body weight (120 g/kg) so as to preserve their weight and keep them starved for the following day task. Mice were trained for 4 days, with three trials per day having 20 min interval between each trial. Trials were recorded using computerized tracking/image analyzer system (video camcorder: 1/3 in. SSAMHR EX VIEW

HAD coupled to the computational tracking system: TiBe-Split). The system provides the following parameters: correct or wrong decision, path length, speed, and latency to reach the goal box. Six hours following the last training on day 4, animals were euthanized by neck dislocation. Hippocampi were exstirpated from the brain within 1 min and kept at −80°C until analyses (Falsafi et al., 2012).

Sample Preparation

A total of 20 samples (obtained from 10 trained and 10 untrained mice) were homogenized in ice-cold homogenization buffer (10 mM HEPES, pH 7.5, 300 mM sucrose and 1 complete protease inhibitor tablet [Roche Molecular Biochemicals, Mannheim, Germany] per 50 mL) using an Ultra-Turrax homogenizer (IKA, Staufen, Germany). The homogenate was then centrifuged for 10 min at 1000 × g, and the pellet was discarded. The supernatant was subsequently centrifuged at 50,000 × g for 30 min in an ultracentrifuge (Beckman Coulter Optima L-90K), and the resulting pellet was homogenized in 5 mL washing buffer (homogenization buffer without sucrose), incubated on ice for 30 min and centrifuged at 50,000 × g for an additional 30 min (Falsafi et al., 2012).

Sucrose Gradient Ultracentrifugation

The plasma membrane purification procedures for the obtained pellets were performed as previously described with slight modifications (Ghafari et al., 2012a). Sucrose density gradients were formed by carefully layering solutions of 700 μL of each of 69, 54, 45, 41, and 37% (w/v) on top of each other. Membrane pellets were re-suspended in the homogenizing buffer, and 500 μL were layered on top of the tubes that were then finally filled with the homogenizing buffer. Samples were ultracentrifuged at 4°C and 70,000 × g for 3 h. After centrifugation, the 41% fraction was collected from the sucrose interface, diluted 10 times with the homogenizing buffer, and then ultracentrifuged at 4°C and 100,000 × g for 30 min. After discarding the supernatant, the pellet was stored at −80°C until use.

Blue Native-Polyacrylamide Gel Electrophoresis (BN-PAGE)

Membrane pellets obtained from the 41% fraction of sucrose gradient ultracentrifugation were solubilized in extraction buffer [1.5 M 6-aminocaproic acid, 300 mM Bis–Tris, pH 7.0] and 10% Triton X-100 stock solution was added, at a ratio of 1:4, to achieve a final concentration of 2% Triton X-100, with vortexing every 10 min for 1 h. Following solubilization, samples were cleared by centrifugation at 4°C and 20,000 × g for 60 min. The protein content was estimated using the BCA protein assay kit (Pierce, Rockford, IL, USA).

Following mixing of 100 μL of the membrane protein preparation with 16 μL BN-PAGE loading buffer [5% (w/v) Coomassie G250 in 750 mM 6-aminocaproic acid], 50 μg of the membrane protein preparations were loaded onto gels. BN-PAGE was performed in a PROTEAN II xi Cell (BioRad, Germany) using 4% stacking and 5–18% separating gels. The BN-PAGE gel buffer contained 500 mM 6-aminocaproic acid, 50 mM Bis–Tris, pH 7.0; the cathode buffer contained 50 mM Tricine, 15 mM Bis–Tris, 0.05% (w/v) Coomassie G250, pH 7.0; and the anode buffer contained 50 mM Bis–Tris, pH 7.0. The voltage was set to 50 V for 1 h followed by 75 V for 6 h, and then sequentially increased to 400 V (maximum current 15 mA/gel, maximum voltage 500 V) until the dye front reached to the bottom of the gel (Ghafari et al., 2012a). Native high molecular mass markers were obtained from Invitrogen (Carlsbad, CA, USA).

Immunoblotting

Membrane proteins were transferred from BN-PAGE to PVDF membranes. After blocking of the membranes for 1 h with 10% non-fat dry milk in 0.1% TBST (100 mM Tris–HCL, 150 mM NaCl, pH 7.5, 0.1% Tween 20), mouse anti-GABAB1 (Abcam, ab55051, Cambridge, UK; 1/5000). Goat anti-GABAB1a (sc-73401/2500), goat anti-MuscarinicM2 (Abcam, ab140473, Cambridge, UK; 1/3000) and rabbit anti-GABAB2 (Abcam, ab75838, Cambridge, UK; 1/5000). The antibodies were detected using horseradish peroxidase-conjugated secondary anti-rabbit IgG antibodies (Abcam, ab6721, Cambridge, UK; 1/10,000), anti-Goat IgG antibodies (Abcam, ab6741, Cambridge, UK; 1/5000), Anti-Mouse IgG antibodies (Abcam, 97035, Cambridge, UK; 1/10,000) Membranes were then developed with the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). All membranes were stained with Coomassie blue R-350 (GE Healthcare, Buckinghamshire, UK, Falsafi et al., 2012). Arbitrary optical densities of immunoreactive bands were measured by the Image J software program (http://rsb.info.nih.gov/ij/). Loading controls for the BN-PAGE western blot were carried out according to Welinder and Ekblad (Welinder and Ekblad, 2011).

Immunoprecipitation of the GABAB1 Receptor

Total hippocampal membrane fractions were suspended in lysis buffer containing 1% Triton X100, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 8.0), 10 mM NaF, 10 mM Na3VO4 and protease inhibitor cocktail (Roche, Mannheim, Germany) on a rotating shaker for 1 h at 4°C. After centrifugation at 15,300 × g and 4°C for 10 min, the supernatant was incubated with affinity purified rabbit antibody against GABAB1 and subsequently incubated with protein G agarose beads (GE Healthcare, Uppsala, Sweden) for 4 h at 4°C with gentle rotation. After washing five times with the same lysis buffer, proteins bound were denatured with sample buffer containing 125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 10% beta-mercaptoethanol, and 0.02% bromophenol blue at 95°C for 3 min. Samples were then loaded onto 10% SDS-polyacrylamide gels, electrophoresed and subsequently transferred to PVDF membranes and analyzed by the western blotting as described in the previous subsection (Ghafari et al., 2012b).

In-gel Digestion of Proteins and Peptides

Bands that were recognized by the corresponding antibodies against the GABAB1 receptor subunit were picked from the SDS gel and put into a 1.5 mL tube. Gel pieces were initially washed with 50 mM ammonium bicarbonate and then two times

with washing buffer (50% 100 mM ammonium bicarbonate/50% acetonitrile) each time for 30 min with vortexing. An aliquot of 100 μL of 100% acetonitrile was added to the tubes to cover the gel pieces completely and the mixture was incubated for 10 min. Gel pieces were dried using a SpeedVac concentrator. Reduction of cysteine residues was carried out with 10 mM dithiothreitol (DTT) solution in 100 mM ammonium bicarbonate (pH 8.6) for 60 min at 56°C. After discarding the DTT solution, the same volume of 55 mM iodoacetamide (IAA) solution in 100 mM ammonium bicarbonate buffer (pH 8.6) was added and incubated in darkness for 45 min at 25°C to achieve alkylation of cysteine residues. The IAA solution was replaced by washing buffer (50% 100 mM ammonium bicarbonate/50% acetonitrile) and washed twice for 15 min each time with vortexing. Gel pieces were washed and dried in 100% acetonitrile followed by dryness in a SpeedVac. Dried gel pieces were re-swollen with 12.5 ng/μL trypsin (Promega, Germany) solution reconstituted with 25 mM ammonium bicarbonate. Gel pieces were incubated for 16 h (overnight) at 37°C (trypsin). The supernatant was transferred to new 0.5 mL tubes, and peptides were extracted with 50 μL of 0.5% formic acid / 20% acetonitrile for 20 min in a sonication bath. This step was repeated two times. Samples in extraction buffer were pooled in 0.5 mL tubes and evaporated in a SpeedVac concentrator. The volume was reduced to approximately 20 μL and an equal volume of HPLC grade water (Sigma, Germany) was then added for spectrometric analysis (Ghafari et al., 2012b).

Nano-LC–ESI–CID/ETD-MS/MS

The analysis utilized an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA) equipped with a PepMap100 C-18 trap column (300 μm × 5 mm) and a PepMap100 C-18 analytic column (75 μm × 150 mm). The gradient applied was as follows: A = 0.1% FA in water and B = 0.08% FA in acetonitrile: 4–30% B from 0 to 105 min, 80% B from 105 to 110 min, and 4% B from 110 to 125 min. An HCT ultra ETD II (Bruker Daltonics, Bremen, Germany) was used to record the peptide spectra over a mass range of m/z 350–1500 and MS/MS spectra via information-dependent data acquisition over a mass range of m/z 100–2800. The MS spectra were repeatedly recorded, followed by four data-dependent CID MS/MS spectra and four ETD MS/MS spectra generated from the four highest intensity precursor ions. Active exclusion of 0.4 min following 2 spectra was used to detect low-abundance peptides. The voltage between the ion spray tip and spray shield was set to 1500 V. Drying nitrogen gas was heated to 150°C, and the flow rate was set to 10 L/min. The collision energy was set automatically according to the mass and the charge state of the peptides chosen for fragmentation. Multiple charged peptides were chosen for the MS/MS experiments due to their good fragmentation characteristics. The obtained MS/MS spectra were interpreted, and peak lists were generated with DataAnalysis 4.0 (Bruker Daltonics, Bremen, Germany).

MASCOT searches against the most recent version of the UniProtKB database were performed using MASCOT 2.2.06 (Matrix Science, London, UK) for protein identification. The search parameters were set as follows: enzymes selected as trypsin with a maximum of 2 missing cleavage sites; species taxonomy limited to mouse; a mass tolerance of 0.2 Da for peptide tolerance, and 0.2 Da for MS/MS tolerance; an ion score cutoff below 15; fixed modification of carbamidomethyl (C); and variable modification of oxidation (M), deamidation (N, Q), and phosphorylation (S, T, Y). Proteins were positively identified based on a significant MOWSE score. Following protein identification, an error-tolerant search was performed to detect non-specific cleavage and unassigned modifications. The returned protein identification information was manually inspected and filtered to confirm the protein identification lists.

Higher sequence coverage was obtained using Modiro® software with the following parameters: the selected enzymes exhibited two maximum missing cleavage sites; a peptide mass tolerance of 0.2 Da was selected for peptide tolerance; 0.2 Da was used as the fragment mass tolerance; and modification 1 of carbamidomethyl (C) and modification 2 of methionine oxidation were applied. Positive protein identification was first based on the obtained spectra, and each identified peptide was subsequently considered significant based on its ion charge status, b- and y-ion fragmentation quality, ion score (>200) and significance scores (>80). The protein identifications were manually inspected and filtered to obtain the confirmed lists of identified proteins (Ghafari et al., 2012b).

Antibody Shift Assay

For the antibody shift assay (ASA), samples were pre-incubated for an hour with mouse anti-GABAB1 (Abcam, ab55051, Cambridge, UK; 1/5000) and rabbit anti-GABAB2 (Abcam, ab75838, Cambridge, UK; 1/5000), with gentle shaking at 4°C, followed by loading the mixture onto the BN gel. The lanes from the BN gel were cut out and the gel strips were equilibrated for 30 min in equilibration buffer [1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol] with gentle shaking and then briefly rinsed twice with SDS-PAGE electrophoresis buffer [25 mM Tris–HCl, 192 mM glycine and 0.1% (w/v) SDS; pH 8.3] and subsequently by Milli-Q water. SDS-PAGE was performed in a PROTEAN II xi Cell using 4% stacking gel and 10% separating gel. The gel strips were run at 12°C with an initial current of 50 V (during the 1st h). The voltage was subsequently increased to 100 V for the next 12 h (overnight) and then increased to 150 V until the dye front reached the bottom of the gel. The gel was then further subjected to a western blotting analysis as described earlier.

Proximity Ligation Assay

Three C57BL/6J mice were anesthetized with 0.3 ml/kg intraperitoneal injection of sodium penthobarbital (Release, 300 mg/ml, Wirtschaftsgenossenschaft deutscher Tierärzte eG, Grabsen, Germany) and perfused intracardially with ice-cold PBS (0.1M phosphate buffered saline, pH 7.2), containing 0.2% heparin followed by 4% paraformaldehyde at a pH of 7.4 in PBS. Brain samples were post-fixed in 4% paraformaldehyde for 24 h at 4°C then transferred into a 30% sucrose solution (in PBS) for 48 h. Brain tissues were embedded with Tissue-Tek media (OCT compound, Sakura Finetek Europe, The Netherlands) then immersed in isopentane cooled with dry ice

and sectioned at 30 μm with a cryostat (Leica CM 3050S, Wetzlar, Germany).

The Proximity Ligation Assay (PLA) was performed according to the manufacturer's protocol (O-LINK Bioscience, Uppsala, Sweden) with slight modifications. Free floating brain slices were blocked for 30 min at room temperature using blocking buffer supplied with the kit. After blocking, brain slices were incubated with diluted mouse monoclonal anti-GABAB1 (1:50, Abcam, Cambridge, UK) and rabbit polyclonal anti-GABAB2 receptor (1:25 Abcam, Cambridge, UK) primary antibodies for 72 h at 4°C on a rocking platform. Following incubation with primary antibodies, slices were washed with wash buffer A (O-LINK Bioscience, Uppsala, Sweden) and then incubated with rabbit PLUS and mouse MINUS probes (1:40, O-LINK Bioscience, Uppsala, Sweden) for 2 h at 37°C with gentle orbital shaking. Ligation was performed according to the manufacturer's protocol with the exception of the incubation time (45 min were used instead of 30 min mentioned in the protocol). DNA polymerase was diluted (1:20) and incubated for 120 min at 37°C. The rest of the amplification steps remained unchanged. After amplification, slices were washed with 1× then 0.01× wash buffer B prepared according to the recipe supplied in the kit manual. Finally, brain slices were transferred to glass slides, mounted with Duolink *in Situ* Mounting Medium with DAPI (O-LINK Bioscience, Uppsala, Sweden). Images were acquired with a Zeiss LSM 780 confocal laser scanning microscope (Carl Zeiss GmbH, Jena, Germany) at 100× magnification keeping all acquisition settings even through all samples.

Statistical Analyses

Data obtained from MTM and western blotting are presented as mean ± SD. Data from western blot were analyzed by unpaired Student's *t*-test, while those of MTM were analyzed using ANOVA with repeated measure. Statistical analysis and calculations were performed using SPSS for Windows 19.0 and GraphPad Prism (San Diego, CA, USA).

RESULTS

Behavioral Studies

Mice learned the maze quickly and efficiently Schematic of the Multiple T maze (MTM) (**Figure 1A**). Training enabled the animals to decrease their path length (**Figure 1B**) across training days. Latency was relatively unchanged in the first 2 days of training but was reduced remarkably thereafter (**Figure 1C**). Animals also refined their path with each training day as

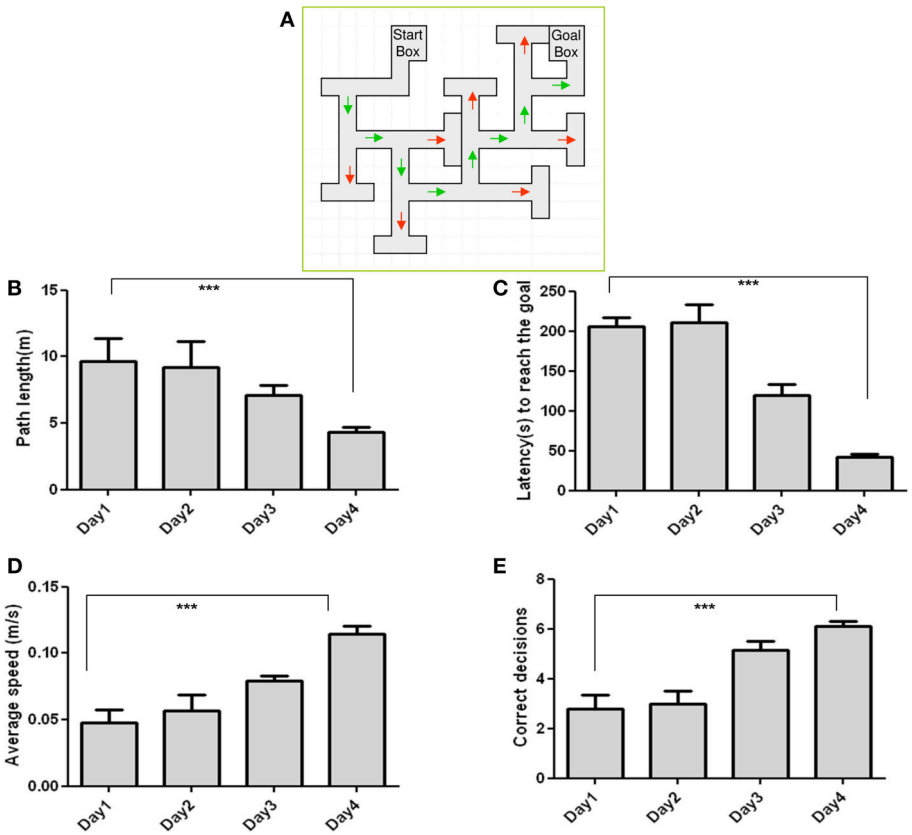

FIGURE 1 | Performance of C57BL/6J in the Multiple-T maze task: (A) Measured parameters include Path length (B), Latency (C), Speed (D), and Correct decisions (E). Latency and path length decreased, while speed and correct decision increased across the training days. $n = 10$; Data are expressed as mean ± SD and were analyzed by ANOVA repeated measures; ***$p < 0.001$.

demonstrated by an increase in the average speed to reach the goal box (**Figure 1D**). Errors appear to be left for chance in the first 2 days of the training but started to decrease thereafter, as revealed by a significantly increased number of correct decisions on day 4 (**Figure 1E**).

Protein-based Studies

To assess the role of GABAB receptors in the MTM task, exstirpated hippocampi from trained and untrained mice were subjected to blue native-PAGE followed by western blotting (BN-PAGE-WB). The analysis revealed the presence of a complex containing GABAB1 and GABAB2 subunits at an apparent molecular weight of approximately 600 kDa (**Figure 2A**) and 500 kDa (**Figure 2B**), (range 480–720 kDa). Subsequent quantitative analysis was made to see the effect of training on receptor levels. It was found out that a complex containing the GABAB1 (**Figure 2A**), and GABAB1a (**Figure 2C**), subunit containing receptor complex levels were significantly increased ($p < 0.01$) in trained compared to untrained mice. By contrast, there was no apparent difference between the two groups when GABAB2 subunit containing receptor complex levels were considered (**Figure 2B**). In addition, based on reports demonstrating direct interaction between GABAB2 and muscarinic subtype 2 (M2) receptors through receptor assembly at the plasma membrane (Boyer et al., 2009), an attempt was also made to detect and quantify M2 containing receptor complex levels using BN-PAGE-WB. M2 was detected as having an apparent molecular weight of approximately 600 kDa, but its levels did not exhibit any significant difference between trained and untrained animals (**Figure 3**).

Assays used for analysis of proteins showed the presence of the two subunits within the complex. The ASA is often used to verify the co-existence of a protein in a complex. The binding of specific antibodies to a target protein causes a shift of the whole target protein complex on the BN-PAGE by the added mass of the antibody. A corresponding mass shift of a protein complex, therefore, is indicative of the presence of the target protein in the complex. In this assay, antibodies specific for B1 and B2 subunits shifted the complex to higher apparent molecular weights, with the discrete increments most likely reflecting assembly of these subunits into GABAB receptors.

ASA was performed to provide additional evidence for co-assembly of the two subunits in order to form functional

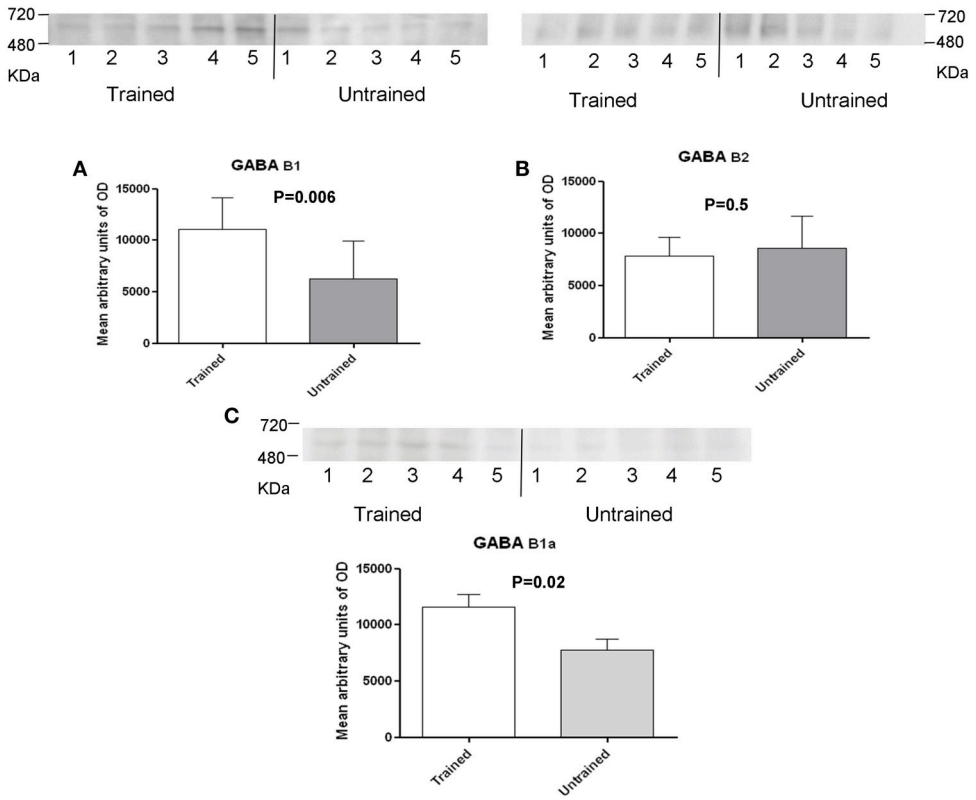

FIGURE 2 | Western blot analysis of training-induced changes in levels of GABAB1 and GABAB1a (A,C) and GABAB2 (B) subunits containing receptor complex in C57BL/6J mice, the number of mice are shown in each bar (A) there were samples from 9 untrained and 10 trained animals: membrane preparations obtained as described in the method section were subjected to BN-SDS-PAGE followed by Western blotting. Subunits migrated in a blue native gel as a GABAB receptor complex at anapparent molecular weight of 500 and 600 kDa (Range, 480–720 kDa). Levels were determined measuring arbitrary optical densities of immunoreactive bands using the Image G software; data are expressed as mean ± SD and analyzed by unpaired student's *t*-test; $^*p < 0.01$ compared to untrained mice.

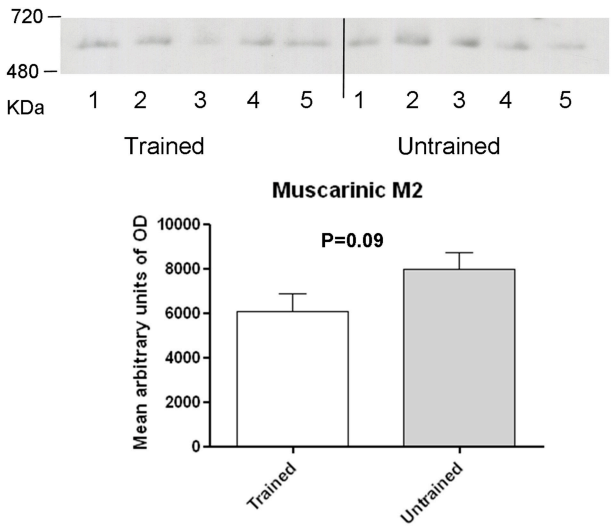

FIGURE 3 | Western blot analysis of training-induced changes in levels of muscarinic type 2 receptor containing complex in C57BL/6J mice: membrane preparations obtained as described in the method section were subjected to BN-PAGE followed by western blotting. Complexes migrated in a blue native gel as a muscarinic type 2 containing receptor complex at an apparent molecular weight of 600 kDa (Range, 480–720 kDa). Levels were determined measuring arbitrary optical densities of immunoreactive bands using the Image G software. Data are expressed as mean ± SD and analyzed by student's *t*-test.

receptors (**Figure 4**). The assay revealed that the band in the control (without incubation of an antibody) (upper band) was shifted toward a high molecular weight when incubated with anti-GABAB1 antibody (central band) and with anti-GABAB2 antibody (lower band).

Immunoprecipitation was carried out using an antibody against the GABAB1 subunit (also used for Western blotting, see above) and the subunit was shown to co-precipitate with GABAB2. the GABAB1 receptor subunit was detected at an apparent molecular weight of approximately 100 kDa (**Figure 5**). Mass spectrometry following in-gel digestion of bands from the corresponding SDS-PAGE showed the presence of GABAB1, GABAB2, Calcium Calmodulin kinase II (CaMKII α and β subunits), Glutamate receptor 1 (GluA1), and Glutamate receptor 2 (GluA2). Data from mass spectrometry indicating the total sequence coverage for the individual proteins in the immunoprecipitate are provided in **Table 1**. Peptides Peptides used for the protein identifications are given in the Supplementary Table 1.

PLA

Evidence for the presence of the two GABAB subunits within the complex was provided by the PLA (**Figure 6**). The assay produced a typical number of discrete fluorescent spots, demonstrating the co-localization of both GABAB1 and GABAB2 subunits within the complex in the hippocampal formation.

DISCUSSION

Spatial learning and memory is largely dependent on the hippocampus and is mediated by persistent changes in neuronal structure and activity. GABAB receptors are localized in the perisynaptic areas of dendritic spines as well as in the dendritic shafts, which enable them to regulate synaptic inputs (Bettler and Tiao, 2006). As a result, changes in the activity of GABAB receptors are critical for controlling neuronal activity and cognitive functioning (Bowery et al., 2002).

The use of BN-PAGE allows subsequent immunoblotting of strongly hydrophobic membrane proteins as receptors and native receptor complexes including high molecular weight material (Kang et al., 2009). The GABAB1 and GABAB2 complexes could therefore be detected in their native forms. The present study alluded to the fact that trained mice displayed an enhanced performance in MTM, a spatial learning task, (**Figure 1**) and this performance was paralleled by an increased expression of a GABAB1/1a containing receptor complex.

Most of the evidence relating GABAB receptors to learning and memory came out of studies using pharmacological ligands. Accordingly, antagonists appear to enhance cognition in majority of the cases (Bianchi and Panerai, 1993; Mondadori et al., 1993; Staubli et al., 1999; Helm et al., 2005), but have either neutral or inhibitory effect in a few cases (Brucato et al., 1996). The pattern for agonists is variable, ranging from impairment (DeSousa et al., 1994; Nakagawa and Takashima, 1997; Cryan et al., 2004; Zarrindast et al., 2004) through facilitation (Georgiev et al., 1988; Saha et al., 1993) to no effect (Cryan et al., 2004; Li et al., 2013). Possible explanations for these discrepancies could be that: (i) behavioral paradigms that assess learning and memory differentially involve stress- or anxiety-related behavior; therefore, the underlying neurobiology and pharmacology are not identical (Sharma et al., 2010) large species and strain differences exist in these paradigms (Sharma et al., 2010) dose administered (Li et al., 2013) and outcomes assessed (learning or retention) (Cryan et al., 2004) could be different: or (iv) as selectivity of these ligands for the two isoforms of GABAB1 is unclear, they can differentially bind to one of the two isoforms, thereby producing different effects. As no antibody against GABAB1b was available, herein the GABAB1a isoform was determined which was significantly increased in the GABAB1-containing complex paralleling retrieval in the MTM.

Subunit phosphorylation appears to regulate the surface dynamics of GABAB receptors. Phosphorylation at S867 by calcium/calmodulin-dependent protein kinase II (CaMKII) triggers endocytosis (Guetg et al., 2010), whereas protein kinase A (PKA) mediated phosphorylation at S892 stabilizes the receptor at the cell surface (Couve et al., 2002). The degree of activation of NMDA receptors, however, appears to control phosphorylation status of S783. Whilst transient NMDA activation leads to phosphorylation by AMP kinase and stabilization of the receptor, prolonged stimulation causes dephosphorylation by PP2A, which serves as a signal to promote degradation (Terunuma et al., 2010, 2014). Phosphorylation of S867 by CaMKII is detectable only in the GABAB1b subunit (Guetg et al., 2010), which mostly resides in the dendrites, in

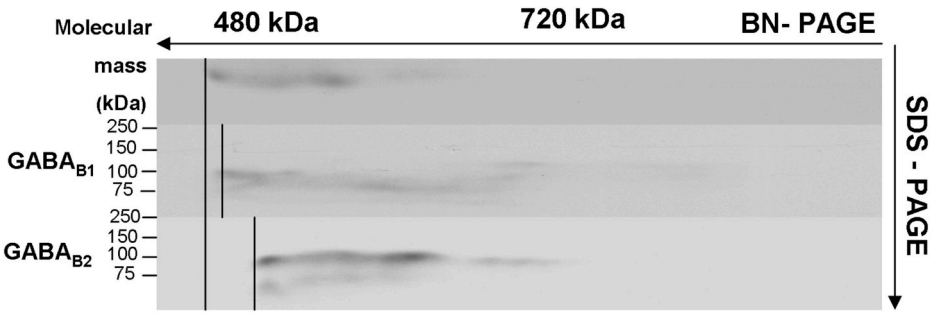

FIGURE 4 | Antibody shift assay to show the presence of GABAB subunits in receptor complexes: membrane preparations were incubated with and without an antibody against the respective subunits of GABAB1 and GABAB2 and subjected to BN-SDS-PAGE. Complexes incubated with the respective antibody shifted toward the high molecular weight (middle and lower bands) compared to controls (upper band).

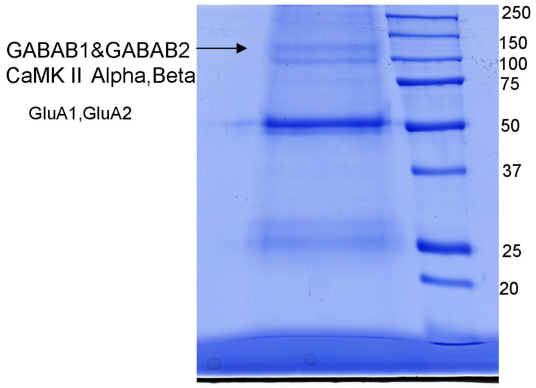

FIGURE 5 | Immunoprecipitation using an antibody against the GABABI subunit run on an SDS-PAGE. The resulting gel showed the pattern of probable receptor complex composition containing GABAB1, GABAB2, Calcium/calmodulin-dependent protein kinase [CaMKII α and β isoforms, Glutamate receptor type1 (GluA1), and 2 (GluA2)]. Mass spectrometry was carried out for identification of receptor proteins picking the bands shown by arrows.

TABLE 1 | Mass spectrometric identification of the GABAB1 subunit containing receptor complex.

Receptor	Accession number	Length	Mass (Da)	Sequence coverage (%)
GABAB1	Q9WV18	960	108	23
GABAB2	Q80T41	940	105	17
CaMKII Alpha	P11798	478	54	21
CaMKII Beta	P28652	542	60	12
GRIA1	P23818	907	101	3
GRIA2	P23819	883	98	10

Bands picked from SDS-PAGE and subjected to MS/MS analysis.

contrast to the GABAB1a subunit, which efficiently penetrates spines (Vigot et al., 2006). In addition, S783A mutation increased the expression of postsynaptic GABAB receptors by blocking receptor degradation, and this was accompanied by an increase in excitatory synapses and elevated surface AMPA receptors (Terunuma et al., 2014). And indeed, CaMKII and AMPA receptors GluA1 and GluA2 were co-immunoprecipitating with GABAB1.

Mice with S783A mutation displayed significant alterations in contextual fear learning and reduced long-term spatial memory in Barnes's maze, effects thought to result from enhanced expression of postsynaptic GABAB receptors (Terunuma et al., 2014). In the hippocampus, GABAB1a/2 receptors are located mainly presynaptically, whereas GABAB1b/GABAB2 receptors are located primarily postsynaptically (Vigot et al., 2006), and CGP 35348, a GABAB receptor antagonist with significantly higher affinity for post- vs. presynaptic receptors, is shown to enhance cognition (Olpe et al., 1993; Staubli et al., 1999). In the present study, an increase in GABAB1 containing, but not GABAB2 containing receptor complexes and this finding was paralleling performance in the MTM task. Findings from the current study suggest that the increased GABAB1a isoform-containing subunit involves a presynaptic mechanism. Since increased complex levels were noted following training of mice in an MTM task, the observation is consistent with the physiological role of GABAB1a containing receptors in ensuring that plasticity processes are maintained in the dynamic range (Gassmann and Bettler, 2012). Training did not have any appreciable effect on levels of GABAB2 containing receptor complexes as well as other receptors that interact with this subunit such as the M2 receptors. This suggests that although the GABAB2 subunit of the complex is required for G-protein coupling, receptor-mediated effects are largely dependent on GABAB1 subunit of the complex.

The maximal distance between the secondary antibodies in PLA is slightly larger than the distance between flurophores in energy transfer technologies, allowing validation of the molecular proximity of endogenous proteins *in vitro* or *ex vivo* (Schwenk et al., 2009; Navarro et al., 2013). The PLA produced a signal representing co-localization of the two subunits within the hippocampus providing further evidence for the presence of both subunits in a complex.

It is known that the GABAB receptor is a heterodimer receptor, but so far mass spectrometric evidence for a

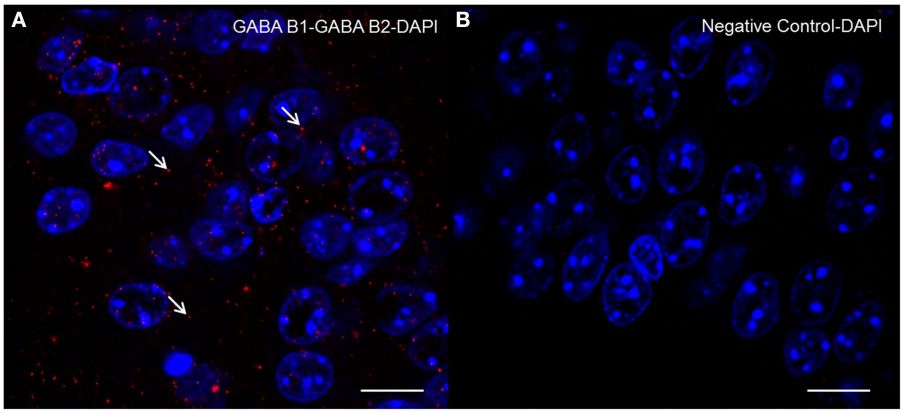

FIGURE 6 | Detection of GABAB1-GABAB2 receptor complexes by "*in situ*" Proximity Ligation Assay: Brain slices were incubated with primary antibodies for the two subunits. Slices were then incubated with plus and minus probes, ligated, and amplified. After that, slices were mounted and images were acquired through confocal microscopy. Representative images showing the GABAB1-GABAB2 receptor complex **(A)** negative control with no primary antibody **(B)** counterstained nuclei with DAPI in mouse hippocampus. Each signal represents the proximity/probable interaction of GABAB1 with GABAB2 receptors. The arrows indicate examples of signals. Confocal microscope images acquired at 100× magnification. Scale bar 10 μm.

complex containing the subunits from mouse brain is missing. Immunoprecipitation followed by mass spectrometry produced evidence for the presence of a protein complex containing both subunits along with proteins involved in the glutamatergic signaling system. Methodologically, nano-LC–ESI–MS/MS was applied for identification of receptor subunits and unambiguous identification with high sequence coverage was obtained. This was achieved through in-gel digestion with trypsin and the use of two mass spectrometric principles, the combination of CID and ETD.

The co-precipitation of the GABAB1 receptor with proteins involved in glutamatergic signaling indicates that the two systems interact with each other through formation of a signaling complex. Indeed, previous co-immunoprecipitation followed by pull down assay showed that CaMKII associates with the GABAB1 receptor in mouse brain. This association may enable CaMKII to phosphorylate GABAB receptor at S867 and trigger endocytosis (Guetg et al., 2010). This observation is consistent with our finding of detecting CaMKII within the receptor complex.

Taken together, data presented herein demonstrate that training leads to increased levels of GABAB1/1a—containing receptor complexes but not GABAB2—containing receptor complexes in mouse hippocampus. The presence of these subunits in the complex is unequivocally confirmed using several biochemical methods including BN-PAGE, ASA, PLA, and immunoprecipitation followed by mass spectrometry. The findings are relevant for previous studies and the design of future studies on GABAB-receptors and spatial memory.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnbeh.2015.00276

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ORIGINAL RESEARCH
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Comparison of Visuospatial and Verbal Abilities in First Psychotic Episode of Schizophrenia Spectrum Disorder: Impact on Global Functioning and Quality of Life

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Objectives: Deficit in visuospatial functions can influence both simple and complex daily life activities. Despite the fact that visuospatial deficit was reported in schizophrenia, research on visuospatial functions as an independent entity is limited. Our study aims to elucidate the impact of visuospatial deficit in comparison with verbal deficit on global functioning and quality of life in the first psychotic episode of schizophrenia spectrum disorder (FES). The significance of clinical symptoms and antipsychotic medication was also studied.

Methods: Thirty-six FES patients and a matched group of healthy controls (HC group) were assessed with a neuropsychological battery focused on visuospatial (VIS) and verbal (VERB) functions. Using multiple regression analysis, we evaluated the cumulative effect of VERB and VIS functions, psychiatric symptoms (PANSS) and antipsychotic medication on global functioning (GAF) and quality of life (WHOQOL-BREF) in the FES group.

Results: The FES group demonstrated significant impairment both in VIS and VERB cognitive abilities compared to the HC group. Antipsychotic medication did not significantly affect either VIS or VERB functioning. PANSS was not related to cognitive functioning, apart from the Trail Making Test B. In the FES group, the GAF score was significantly affected by the severity of positive symptoms and VERB functioning, explaining together 60% of GAF variability. The severity of negative and positive symptoms affected only the Physical health domain of WHOQOL-BREF. The degree of VERB deficit was associated with both Physical and Psychological health. Although we did not find any relation between VIS functioning, GAF, and WHOQOL-BREF, a paradoxical finding emerged in the Environment quality domain, where a worse quality of the environment was associated with better VIS functioning.

Conclusions: Our results suggest that the deficit in VIS functions is an integral part of cognitive deficit in schizophrenia spectrum disorders, rather than a side effect of

symptomatology or antipsychotic medication. Moreover, VERB functioning was a better predictor of GAF and WHOQOL-BREF than VIS functioning. Given the findings of negative or missing effect of VIS deficit on WHOQOL-BREF and GAF, the accuracy of these measures in evaluating the impact of global cognitive deficit on everyday life in schizophrenia could be questioned.

Keywords: cognitive deficit, first psychotic episode, schizophrenia spectrum disorder, global functioning, quality of life, visuospatial functions, verbal functions, antipsychotic medication

INTRODUCTION

Abnormalities in cognitive functions are considered to be one of the key components in Schizophrenia (SZ). Neurocognitive deficit represents a reliable feature, with moderate to large effect size on global functioning across all cognitive domains (Milev et al., 2005). An important amount of research in SZ has examined the relationship between cognition and variety of clinical factors including age of onset, symptomatology, severity, duration, medication, functional outcome, and Quality of life (QOL) (Heinrichs and Zakzanis, 1998; Bilder et al., 2000; Mesholam-Gately et al., 2009). However, studies focusing on specific relationships among cognitive domains or relationships inside the domains and their influence on daily functioning are limited (Harvey et al., 2012). One of the most neglected areas is the comparison of the impact that visuospatial (VIS) and verbal (VERB) abilities have on global functioning and QOL. Focused research of VIS functions can help provide a better understanding of neuropsychological patterns of heterogeneity in SZ. In addition, since VIS functions are less biased by language skills, research in this area can enlarge the neuropsychiatric field (Paradis, 2008). Furthermore, VIS functions are an important tool for a comparative research on animal models of SZ (Fajnerová et al., 2014).

One of the main reasons why visuospatial functions did not receive much attention as an independent entity in the research of SZ was the negative impact of the first-generation (typical) antipsychotic treatment on some motor and visuospatial functions (e.g., psychomotor retardation; Spohn and Strauss, 1989; Bilder et al., 1992; Meltzer and McGurk, 1999; Arana, 2000). With the development of second generation (atypical) antipsychotics (AP), the risk of neurological side effects seemed reduced. Moreover, findings of slightly positive effects of some atypical AP on cognitive deficit were reported in SZ (e.g., Peuskens et al., 2005; Houthoofd et al., 2008) and these findings were supported by results in an animal model of SZ (Bubeníková et al., 2005). However, when atypical antipsychotics were directly compared with the typical ones, no differences were found in either psychomotor functions or other cognitive areas (Jones et al., 2006; Keefe et al., 2007; Lewis and Lieberman, 2008). Another limitation related to the study of VIS functions is that not all studies use a separate model of verbal and visuospatial functions. Usually, both VERB and VIS functions are included in the same cognitive domain (e.g., memory domain) or in the total IQ score; alternatively, they are studied as isolated variables. The question how much (if at all) the AP medication affects the motor and VIS functions when compared with the verbal functions, and to what extent the visuospatial functions impact daily functioning and the quality of life requires more research.

Visuospatial impairment can negatively affect various daily activities from the most common, such as watching TV or reading a book, to the most complex, including social interactions (visual recognition of social signals), and recognition of territorial boundaries (interpersonal space; Cummings and Mega, 2003). SZ subjects exhibit impaired performance in a wide range of VIS functions, from the most basic level of visual perception to more complex visuospatial processing and navigation abilities (e.g., Stuve et al., 1997; Doniger et al., 2001; Butler et al., 2005; Hanlon et al., 2006; Piskulic et al., 2007; Weniger and Irle, 2008; Cocchi et al., 2009; Folley et al., 2010; Landgraf et al., 2010; Fajnerová et al., 2014). This decline in VIS performance is already present in the first episode of schizophrenia and performance further deteriorates over time, predicting poor outcome (Stirling et al., 2003). Cross-sectional studies in subjects with late-life schizophrenia report the impairment in visuospatial ability, alongside with the executive and verbal fluency deficit. Moreover, longitudinal studies suggest that the cognitive decline in late-life schizophrenia may first affect VIS abilities (Rajji and Mulsant, 2008). It was also demonstrated that VIS tasks related to attention, memory, and planning predict improvements on psychosocial functions, such as autonomy in daily living, treatment compliance, and social competence in subjects with psychosis (Prouteau et al., 2005). Given the significance of VIS functions in our daily life, it is expected that visuospatial tests would be good predictors of functional outcome in SZ.

Functional capacity and quality of life play a key role in the study of the course, treatment efficacy, and other factors related to functional outcome in SZ. Both functional capacity and QOL are negatively associated with clinical symptoms (Gaite et al., 2005; Malla and Payne, 2005; Milev et al., 2005; Makara-Studziñska et al., 2011). Negative symptoms are more strongly related to poor QOL and psychosocial functioning in SZ outpatients (Eack and Newhill, 2007; Rocca et al., 2009), whereas general psychopathology shows a consistent negative relationship with QOL across all study samples and treatment settings (Eack and Newhill, 2007; Rocca et al., 2009). Findings about the influence of positive symptoms are heterogenous, with the relationship toward negative and general symptomatology being more evident, varying only in the extent of impact (Eack and Newhill, 2007; Rocca et al., 2009).

In terms of cognition, the current state of the literature did not enable drawing any conclusions about specific cognitive constructs related to global functioning and QOL. Some studies have documented a relationship between measured general cognitive ability (IQ index) and global functioning or QOL

(Tzeng et al., 2004; Chaplin et al., 2006; Leeson et al., 2009), while other studies also point out the importance of specific neuropsychological domains. Results of these studies are very heterogeneous, depending on the measures used in the assessment. Despite the heterogeneity of measures, the deficit in executive functions appears to be the most evident and burdensome, and is most related to the impairment of global functioning and QOL in SZ (Bilder et al., 2000; Reed et al., 2002). In addition, lower QOL is related to the deficit in verbal memory (Ritsner, 2007; Fiszdon et al., 2008; Matsui et al., 2008). As was stated previously, VIS and VERB functions are usually combined in a single domain or IQ score. Thus, the role of VIS functions in global functioning and QOL remains unclear.

To our knowledge, no study to date has described the extent to which visuospatial functions affect everyday life of SZ patients, in contrast to the effect of verbal abilities. Our study aimed to answer the following questions:

(1) Are visuospatial abilities impaired in the first-episode schizophrenia spectrum (FES) patients in comparison with the matched group of healthy controls? If that is the case, is the degree of the deficit the same as in verbal functions?
(2) Are the VIS functions in FES patients affected by the antipsychotic medication and the actual psychiatric symptomatology (measured with PANSS)? Is similar effect visible in the VERB functions?
(3) Is the global functioning and the quality of life in FES patients affected by VIS functioning when analyzed in the presence of VERB functions and clinical characteristics (symptoms and medication dose)? If so, is the effect of visuospatial and verbal functioning the same?

MATERIALS AND METHODS

Subjects

Thirty-six subjects (22 males and 14 females, FES group) who met ICD-10 criteria for the first psychotic episode of schizophrenia spectrum disorder [F20.X ($N = 4$) and F23.1/F23.2 ($N = 32$)] were recruited at the National Institute of Mental Health (NIMH). Patients were evaluated once they were stabilized at the end of their first psychiatric hospitalization in partial symptomatic remission state, according to Andreasen's remission criteria (2005). The group was considered in partial remission state rather than in complete remission, as they did not fulfill the criterion of asymptomatic 6-month period. Study subjects were diagnosed in a routine clinical process by two experienced psychiatrists. In case of diagnostic disagreements (e.g., comorbidity) the specific case was excluded from the study.

In order to compare the cognitive performance in FES subjects with the healthy population, a group of healthy control subjects ($N = 36$, group HC) was recruited from the same socio-demographic background via a local advertisement. The inclusion criteria for both groups were: (a) 17–35 years of age; (b) no history of neurological disease or loss of consciousness longer than 10 min; (c) native in Czech/Slovak language; and (d) additionally for the FES group to meet ICD-10 diagnostic criteria (dg F20.X or F23.1, F23.2) and to be first admitted to psychiatric care. The main exclusion criterion for the control subjects was personal history of any psychiatric disorder; for the FES group it was the fulfilled diagnostic criterions for another psychiatric disorder. Both groups were carefully matched in terms of sex, age (max 2 years difference tolerance), and level of education (for details and statistical comparison of the matching parameters, see **Table 1**). In each group there were 16 participants with a higher education level (university studies) and 20 with a lower level of education.

Prior to the study, all participants signed a written informed consent in accordance with the Declaration of Helsinki, approved by the Ethics Committee of NIMH.

Clinical and Neuropsychological Assessment

Two psychiatric scales were used to evaluate clinical characteristics in the FES subjects. Current symptomatology

TABLE 1 | Demographic data, clinical assessment, and QOL questionnaire.

Demographic variables	Group mean ± SD		Group differences	
	FES	HC	Mann–Whitney *U*	*p*-value
N	36	36		
Sex (M:F)	22:14	22:14		
Age	26.3 ± 5.6	25.7 ± 5.2	614	0.697
Education level (1–6)	3.7 ± 1.3	4.0 ± 1.2	556	0.261

Clinical assessment	FES subjects (mean ± SD)
PANSS total score	50.8 ± 17
PANSS-positive	12.5 ± 5.2
PANSS-negative	16.0 ± 7.3
PANSS-general	26.4 ± 6
AP medication—CPZ equivalents (mg)	391.2 ± 122
GAF	64.5 ± 18.3

WHOQOL-BREF	FES subjects (mean ± SD)	Normative data (mean ± SD) (Dragomirecká and Bartoňová, 2006b)
Physical health (domain 1)	14.4 ± 2.4	15.5 ± 2.6
Psychological health (domain 2)	14.1 ± 2.5	14.8 ± 2.4
Social (domain 3)	13.2 ± 3.1	15.0 ± 2.9
Environmental (domain 4)	14.3 ± 2.1	13.3 ± 2.1

First psychotic episodes of schizophrenia spectrum disorder subjects (FES) and healthy controls (HC) individually matched by sex, age (within 2 years), and education level (see demographic variables). All values are present as mean ± SD. Clinical scales PANSS (Positive and Negative Symptoms Scale) and GAF (Global Assessment of Functioning), antipsychotic medication level in CPZ (chlorpromazine) equivalents, and Quality of life questionnaire WHOQOL-BREF assessed in FES subjects. Education level: 1, less than high school; 2, started high school; 3, completed high school; 4, started university; 5, completed university; 6, started postgraduate studies.

was assessed using the Positive and Negative Syndrome Scale (PANSS; Kay et al., 1987). The Global Assessment of Functioning (GAF; Jones et al., 1995) was used in order to objectively evaluate general psychosocial functioning of the FES group. The GAF scale is used to address general functioning (score 0–100) in daily activities of individual FES subjects. All FES subjects were medicated by different dose and type of atypical antipsychotics or their combination (olanzapin, amisulpirid, and risperidon), that is why chlorpromazine equivalents (CPZ; Woods, 2003; Andreasen et al., 2010) were used to evaluate the effect of medication dosage on cognitive functioning. For details on the clinical parameters see **Table 1**.

The quality of life was subjectively evaluated by FES subjects using the Quality of life questionnaire WHOQOL-BREF (WHO group, 1998), a short-form quality of life assessment that calculates four domain profiles (Physical health, Psychological health, Social relationships, and Environment), and was validated for FES population (Mas-Expósito et al., 2011). The questionnaire was translated and validated for a Czech population (Dragomirecká and Bartoňová, 2006b).

Regarding the neuropsychological assessment, the used measures were chosen in accordance with the evidence of related articles (mentioned in introduction) and suggested also by the MATRICS initiative (Green et al., 2004; Nuechterlein et al., 2008). Some additional measures not commonly used and standardized in schizophrenia population were used in order to assess VIS functions in greater detail. The final neuropsychological battery consisted of 11 tests focused on both the visuospatial and the verbal functions (see **Table 2**). All tests were assessed by trained clinicians, according to the cited administration protocols. Detailed information about all test methods is provided in **Table 2**. In order to compare cognitive

TABLE 2 | Description of (A) Visuospatial and (B) Verbal neuropsychological tests.

Test	Monitored cognitive function	Test outputs	References	Test description
(A) VISUOSPATIAL NEUROPSYCHOLOGICAL TESTS				
Trail Making Test (TMT A and B)	Psychomotor speed (A); visuospatial working memory (B); mental flexibility (B/A)	Time A (s); Time B (s); Ratio B/A	Reitan and Wolfson, 1985; Preiss and Preiss, 2006	Chaining a sequence of numbers (A) or alternatively numbers and letters (B) that are randomly distributed on a single paper
Rey-Osterrieth (Taylor) Complex Figure Test (RCFT)	Visuospatial organization, constructional functions and visual memory	Raw score for copy trial (RCFT-copy), reproduction after 3 (RCFT-3) and 30 min (RCFT-30)	Osterrieth, 1944; Preiss et al., 2012	Copy and reconstruction of figure after 3 and 30 min
Key Search Test (KST)	Executive functions	Raw scores of strategy	BADS (Wilson et al., 1996)	Strategy of exploration of 2-dimensional space (2D square shape)
Money Road-Map Test (RMT)	Spatial orientation	Raw scores for number or errors/32; A, B and C error types	Money et al., 1965	Ability to determine right/left turns on crossroads in 2D view of a simple maze/city
Spatial Span (SS)	Visuospatial working memory	Raw scores: total (forward + backward)	PC version adjusted from the Corsi block test in (PEBL, 2012) according to WMS-III (Wechsler, 1997)	Repeating a sequence of spatial positions presented in 2D plane, forward or backward
PEBL Perceptual Vigilance Task (PVT)	Vigilance and attention	Number of lapses [Reaction time (RT) over 500 ms], average RT speed	PEBL battery (PEBL, 2012; Dinges et al., 1985; Loh et al., 2004)	Response to stimulus appearing in the variable time interval (1–9 s) during 10 min
(B) VERBAL NEUROPSYCHOLOGICAL TESTS				
Auditory Verbal Learning Test (AVLT)	Verbal learning and memory	Learning curve and total number of words (AVLT-I-V); immediate recall (AVLT-VI); delayed recall (AVLT-30); number of confabulations and repetitions	Rey, 1964; Preiss et al., 2012	Repeated recall of 15 words with interference trial (B) and delayed recall after 30 min
Verbal Fluency Test (VFT)	Psychomotor speed and mental flexibility	Number of words for phonemic (total of three trials) and semantic fluency	Preiss et al., 2012	Speaking aloud words beginning with letters N, K, P or naming category of animals during 1 min
Digit Span (DS)	Attention (forward), verbal working memory (backward)	Raw scores: total (forward + backward)	WAIS-III (Wechsler, 1997); Czech version (Černochová et al., 2010)	Repeating list of numbers forward and backward
Similarities (Sim)	Verbal conceptualization	Raw score–correct responses		Describe similarities between pair of words

performance in the FES subjects with the healthy population, the same test battery was administered to a HC subjects. Below is a description of the three visuospatial methods that have some specific characteristics.

The PEBL (PEBL, 2012) version of the Perceptual Vigilance Task (PVT) was used in its 10-min-long alternative (Loh et al., 2004) in order to test attention and vigilance. A simple circle stimulus appears in the PVT at intervals ranging between 2 and 12 s, and the participant is required to press the spacebar as quickly as possible.

A computerized version of the Spatial Span (SS) test was used in order to test spatial attention and working memory without uncontrolled examiner effects (such as prolonged presentation of the longer spatial sequences). We adjusted the original protocol of the Corsi block-tapping test (Kessels et al., 2000) applied in the PEBL battery (PEBL, 2012) to match individual positions and spatial sequences of the SS in the WMS-III (Wechsler, 1997; Černochová et al., 2010).

The Money Road-Map-Test (Money et al., 1965) is not traditionally used in standard test batteries such as MATRICS; this test was selected in order to test specific visuospatial functions, such as mental rotation and perspective taking strategy. For this reason, the total number of errors (out of a total of 32 turns) was divided into three categories (according to Marková et al., 2015) by the angle of the route before each turn relative to the subject's heading: (A) rotation of <70° (9 turns), (B) rotation of 90° (13 turns), and (C) rotation of more than 110° (10 turns). Another specifically selected test, the Key Search Test, was chosen as a sensitive method testing dysexecutive syndrome in schizophrenia (Evans et al., 1997), using spatial planning abilities.

Data Analysis

Statistical analysis was performed using the SPSS software (version 15.0). The significance level of all statistical analysis was set to 0.05. The group differences in demographic variables (age, education) were analyzed using non-parametric Mann–Whitney *U*-test. Identical method was used to compare the raw scores obtained in the visuospatial tests. Non-parametric Spearman Rank Order Correlations were used in order to detect correlations between variables.

The raw scores of neurocognitive tests were used to compare performance between the FES subjects and the HC. Raw scores were transformed to z-scores in order to calculate cumulative scores of VERB and VIS scores. Z-scores were calculated as the difference among raw scores of the individual FES subjects and the HC group mean, divided by the HC standard deviation. The cumulative scores (VERB and VIS) were computed as a sum of the standardized z-scores divided by the number of applied measures from the relevant variables list as follows (for explanation of individual abbreviations see **Table 2**): VERB score (AVLT_I-V, AVLT-VI, AVLT-30, VFT-semantic, VFT-phonemic, DS-backward, Similarities) and VIS score (RCFT-copy, RCFT-3, RCFT-30, TMT-A, TMT-B, SS-backward, RMT-total errors). PVT test results were not included in calculation of the cumulative scores, as performance on this test is purely attentional. In addition, to assure the accuracy of input variables to the cumulative scores and their consistency, reliability analysis and factor analysis were performed (Cronbach's Alpha for VERB = 0.73; Cronbach's Alpha for VIS = 0.75). Multiple linear regression analysis (stepwise method criteria as follows: probability-of-F-to-enter ≤ 0.05; probability-of-F-to-remove ≥ 0.10) was used to assess the effect of performance in individual visuospatial and verbal tests (dependent variables) of (A) the clinical characteristics (independent variables): PANSS (scores divided into three sub-scores: general symptoms–G, positive symptoms–P, negative symptoms–N), and (B) the antipsychotic medication calculated in CPZ. In additional stepwise multiple linear regression analysis an overall effect of PANSS scores, CPZ level, and cumulative VERB and VIS scores was assessed on (A) global functioning measured by GAF and (B) four individual domains of quality of life measured by WHOQOL-BREF (WHO group, 1998).

RESULTS

Differences in Cognitive Performance Between FES and HC Group

As a result of matching the participants on an individual basis, no significant group differences in age and education were observed (**Figure 1** and **Table 1**). In each group, there were 16 participants with a higher education level (university studies) and 20 with lower level of education.

Group differences in the results of neuropsychological tests were significant in most of the applied VIS and VERB measures, when analyzed from raw scores using the non-parametric Mann–Whitney *U*-test (for more details see **Tables 3A,B**).

The group of FES subjects showed significantly lower VIS performance on all three parts of the Rey-Osterrieth Complex Figure Test (RCFT-copy, RCFT-3, RCFT-30). The FES group was also slower in the Trail Making Test part A and part B. The average speed of FES subject responses in the PVT was lower and they also had a higher number of lapses (with reaction times above 500 ms). The FES subjects also made more errors on the Road Map Test (RMT). In addition, splitting of individual turns in the RMT (according to Marková et al., 2015) to three possible error types showed that schizophrenia subjects tend to fail more in the turns demanding mental rotation of the spatial scene (turns B and C) compared to the turns that demand no or very small mental rotation (see **Figure 2**). We found no significant differences in the raw scores of the KST from the BADS battery. In contrast to the Digit Span (DS) test, the SS test did not show any significant group differences. However, the difference observed in the backward score was approaching the significance level ($p = 0.063$).

VERB performance was significantly impaired in most of the VERB measures as follows: verbal learning and delayed recall in the Verbal Learning Test (AVLT); the phonemic and semantic Verbal Fluency (VFT), abstract executive functions in the Similarities (Sim) and working memory in the DS task. No

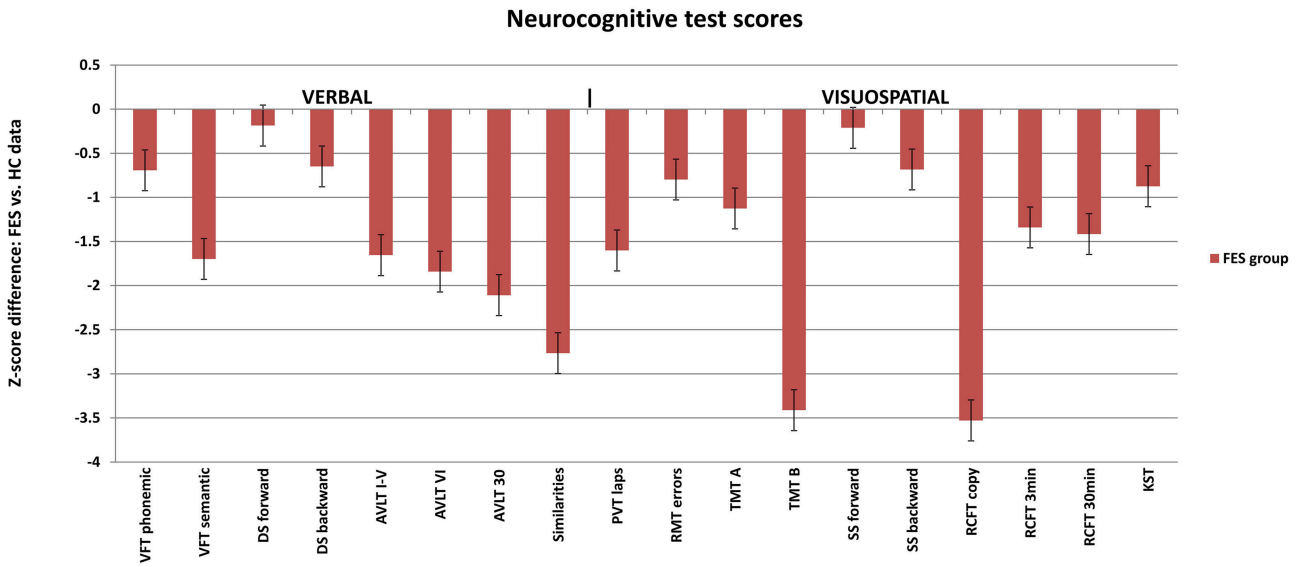

FIGURE 1 | Neuropsychological profile of FES subjects calculated in z-scores for individual test parameters relative to the healthy controls (average Z-score = 0). Selected test results are divided into verbal (left) and visuospatial (right) tests. FES, first psychotic episodes of schizophrenia spectrum disorder; HC, healthy controls; VFT, Verbal Fluency Test; AVLT, Auditory Verbal Learning Test; DS, Digit span; PVT, Perceptual Vigilance Test; TMT, Trail Making Test; RMT, Money Road-Map Test; RCFT, Rey-Osterrieth Complex Figure; SS, Spatial span; KST, Key Search Test.

difference was observed in the immediate recall and attention performance in DS forward.

Regression Model of PANSS Effect on Performance in Visuospatial and Verbal Tests in FES

First set of stepwise multiple regression analysis models of psychiatric symptomatology measured using PANSS (predictors: PANSS-P, PANSS-N, PANSS-G) showed no effect of symptomatology on the performance in individual visuospatial and verbal tests (dependent variables), except for the performance in one single test—the Trail Making Test part B. Two of the three predictors from the regression model performed by stepwise method were significant: PANSS-N and PANSS-G. They explained more than 40% of performance variability in the TMT-B test (see **Table 4**). No effect of individual PANSS scores was identified on cumulative VERB and VIS scores.

Regression Model of CPZ Level Effect on Performance in Visuospatial and Verbal Tests in FES

We did not observe any significant influence of medication (CPZ) on the performance in any of the visuospatial tests using the linear regression analysis. We identified negative effect of medication dosage ($B = -0.040$, $SE = 0.017$, Beta $= -0.373$) only in one verbal test—the phonemic Verbal Fluency Test ($N = 35$). CPZ explained 11% of performance variability ($SE = 12.253$, Durbin-Watson $= 2.247$; $F = 5.338$, $p < 0.05$). This observation would not survive Bonferroni adjustments ($p = 0.027$). No effect of CPZ level was identified on cumulative VERB and VIS scores.

Regression Model of Clinical and Neuropsychological Factors on Global Functioning in FES

In order to analyze the possible effect of verbal and nonverbal cognitive performance on global functioning (measured on the GAF scale), we performed a multiple regression analysis with the following predictors: PANSS-P, PANSS-N, PANSS-G, CPZ level, and cumulative VIS and VERB scores (see **Table 5**). Only two of the independent variables, the severity of positive symptoms (PANSS-P score) and verbal functioning (cumulative VERB score), showed a significant effect on global functioning. Together, these variables explained more than 60% of GAF variability.

Regression Model of Clinical and Neuropsychological Factors on Quality of Life (QOL) in FES

The same model of clinical and neuropsychological parameters was applied in the stepwise multiple regression analysis that identified significant effect of severity of negative symptoms (PANSS-N score), verbal functioning (cumulative VERB score) and positive symptoms (PANSS-P score) on perceived quality of Physical health (domain 1) in FES. These predictors together explained nearly 50 % of the observed variability (for details see **Table 6**). The QOL domain Psychological health (domain 2) was found to be affected by the overall VERB functioning (see **Table 7**).

No significant effect of clinical and neuropsychological parameters was observed in the evaluated quality of Social relationships (domain 3). In contrast, the Environment quality (domain 4) appeared to be affected mostly by one significant

TABLE 3 | Group differences in (A) Visuospatial and (B) Verbal neurocognitive tests.

Neurocognitive assessment	FES raw scores (*N* = 36)				HC raw scores (*N* = 36)				Mann–Whitney *U*	*p*-value
	mean	SE	median	SD	mean	SE	median	SD		
(A) VISUOSPATIAL PERFORMANCE										
TMT–*A*	38.3	2.1	35.5	12.5	27.8	1.6	26.5	9.4	306.5	< 0.001***
TMT–*B*	93.5	7.6	84.5	45.4	50.6	2.1	49.5	12.8	181	< 0.001***
Ratio B/A	2.5	0.1	2.3	0.8	1.9	0.1	1.9	0.5	417	< 0.01**
RCFT–copy	32.5	0.5	33.5	3.0	35.6	0.1	36	0.9	201	< 0.001***
RCFT–3 min	18.8	1.1	22.0	6.5	25.7	0.9	26	5.2	234	< 0.001***
RCFT–30 min	19.3	1.1	19.8	6.4	25.8	0.8	26	4.6	237	< 0.001***
Recognition (errors)	4.6	0.4	4.5	2.1	3.6	3.0	0.3	1.9	360	0.073
KST	11.9	0.6	12.5	3.4	13.2	0.2	13	1.5	532.5	0.188
RMT–number of errors/32	3.1	0.6	2	3.6	1.2	0.4	0	2.4	394	0.003**
RMT A	0.3	0.1	0	0.8	0.1	0.1	0	0.5	628	0.661
RMT B	1.6	0.3	1	1.8	0.8	0.3	0	1.7	457.5	0.021*
RMT C	1.2	0.3	1	1.7	0.3	0.1	0	0.8	385	< 0.001***
SS total	16.3	0.5	17	3.1	17.6	0.4	18	2.3	460	0.147
SS forward	8.6	0.3	9	1.6	8.9	0.2	9	1.4	535	0.592
SS backward	7.8	0.4	8	2.1	8.7	0.2	9	1.5	429	0.063
PVT–average response speed	337.3	8.7	321	44.5	305.5	8.0	294.5	40.8	189.5	0.007**
PVT–correct	66.3	1.8	70	10.0	70.7	0.8	72	4.9	415	0.068
PVT–comissions	1.3	0.4	0	2.5	0.9	0.2	0	1.3	541	0.791
PVT–lapses	7.9	1.6	4	9.1	2.1	0.6	1	3.6	279.5	< 0.001***
(B) VERBAL PERFORMANCE										
AVLT—I-V	47.8	1.8	48.0	10.8	59.9	1.2	61.5	7.5	231.5	< 0.001***
AVLT—3 min	9.3	0.4	9.0	2.5	13.1	0.3	14.0	2.1	157.5	< 0.001***
AVLT—30 min	8.6	0.5	8.0	3.0	13.1	0.4	14.0	2.2	137.5	< 0.001***
AVLT–repetitions	6.0	0.8	4.5	5.0	1.9	0.4	1.0	2.2	302.5	< 0.001***
AVLT–confabulations	2.0	0.4	1.0	2.5	1.1	0.3	1.0	1.6	543.5	0.214
VFT phonemic	41.8	2.2	41	12.9	48.9	1.7	48.5	10.4	395.5	0.004**
VFT semantic	19.7	1.0	18.5	5.6	28.9	0.9	28.0	5.5	120	< 0.001***
DS total	14.8	0.7	14.5	4.5	17.0	0.7	17.0	3.9	450	0.025*
DS forward	9.3	0.4	8.5	2.2	9.7	0.4	9	2.3	573	0.392
DS backward	5.2	0.4	6	2.1	7.4	0.4	7.5	2.3	397	0.004**
Similarities	22.9	0.8	23	4.9	28.8	0.4	29	2.2	165	< 0.001***

SE, standard error of the mean; SD, standard deviation. For abbreviations of individual methods see ***Table 1****. TMT, Trail Making Test; RCFT, Rey-Osterrieth Complex Figure; KST, Key Search Test; RMT, Money Road-Map Test (A, B, C—type of errors); SS, Spatial Span; PVT, Perceptual Vigilance Test; AVLT, Auditory Verbal Learning Test; VFT, Verbal Fluency Test; DS, Digit Span. Significance level: *p < 0.05, **p < 0.01, ***p < 0.001.*

parameter, the cumulative VIS score performance. This factor explained together with CPZ level around 27% of the observed variability in domain 4 (see **Table 8**). However, worse Environment QOL was predicted by better VIS functioning and higher AP dosage.

DISCUSSION

Differences in Cognitive Performance Between FES and Control Group

Our study confirmed the presence of a deficit in visuospatial cognitive abilities in a sample of first psychotic episode of schizophrenia spectrum disorder subjects, when compared with matched group of healthy controls. Similar pattern of deficit was also observed in verbal functions. This result is in accordance with previous studies, both in the first episodes and chronic SZ subjects (e.g., Green et al., 2004; Fioravanti et al., 2005). First, we selected the PVT as an indicator of vigilance and response speed, as deficits in these abilities could affect performance in other applied measures. Our sample of FES subjects demonstrated deficit in vigilance and response speed on the PVT task. However, this deficit ranged between none and moderate (SD 0–1.5), with more severe deficits observed in other cognitive measures. Nevertheless, we suggest future visuospatial studies apply such attentional measure as a covariate factor in order to clarify how vigilance and response speed may affect tested visuospatial abilities.

Deficit on visuospatial functions was manifested in perceptual organization abilities (copy in the Rey-Osterrieth Complex

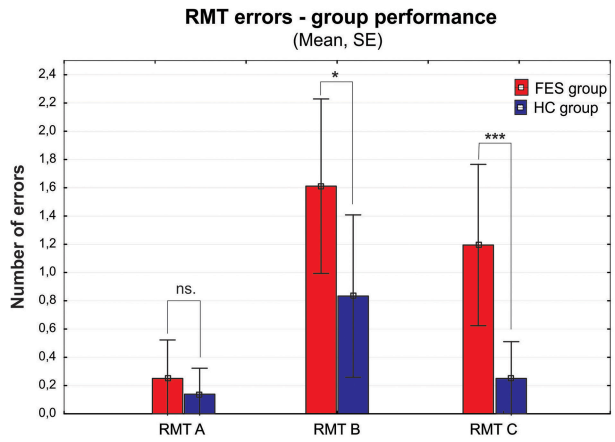

FIGURE 2 | Group performance presented as number of errors on the Money Road Map Test (RMT) (mean ± standard error). Errors are divided into three different types according to the rotation toward subjective perspective of the tested subject (A–less than 70° deviation from subject's heading; B–90°; C–more than 110°). FES group, first psychotic episodes of schizophrenia spectrum disorders; HC group, healthy controls. Significance; ns, non-significant; $^{*}p < 0.05$, $^{***}p < 0.001$.

Figure Test- RCFT) and in the delayed recall of visuospatial information (RCFT after 3 and 30 min), while the recognition (RCFT recognition) of visuospatial material was comparable with the performance of HC. This finding was characterized by a reduced number of recalled details but not by an increased rate of forgetting. This is consistent with a disturbance in encoding or retrieval from memory but no deficit in information storage (Rodriguez, 2012). The problems in recall might be further compounded by a primary deficit in visual scanning abilities or limited analysis of the visual fields, leading to the omission of significant details and even whole sections of the figure (Golden et al., 2002).

Trail Making Test (TMT-A and B) also showed significantly slower processing speed, impaired visuomotor tracking and switching ability. Although the performance on the TMT might be perceived as partly language dependent, since the sequence of numbers or numbers and letters has a verbal component, participants' visuospatial tracking ability is the main cognitive domain assessed by this task. The higher ratio of TMT B/A also pointed out a deficit in executive control function (Lezak et al., 2012). The repeated finding of significantly impaired TMT A and B performance in SZ (Heinrichs and Zakzanis, 1998), complemented by the TMT ratio B/A deficit, suggests an independent deficit in both processing speed and switching ability. Similar to the RCFT, the problems on both these functions might be further compounded by a primary deficit in visual scanning abilities. Future research in this area is needed.

Interesting results were found in the Money Road Map Test (RMT), which to our knowledge has not been previously used in FES patients. The higher number of total errors shows deficit in left/right direction sense, and additionally in perspective taking abilities (Schultz, 1991; Marková et al., 2015). These abilities are suggested as the main solution strategy in RMT. The lower RMT performance in FES group is therefore even more apparent, if the individual intersections are divided into three types according to their perspective taking demands, which is essential in order to respond in the left/right condition. While the number of errors in condition A (no change in perspective) is not significantly disrupted in FES, both conditions B and C (90 and more degrees deviations requiring perspective taking) showed significant impairment. On top of that, the number of errors increased with the growing degree of deviation (from condition A–C), similarly to the mental rotation abilities described previously (e.g., de Vignemont et al., 2006). This finding deserves further investigation and standardization of the method that could lead to a wider usage of this test in SZ clinical research. Moreover, due to its spatial characteristic the test could also be very useful in comparative studies of SZ.

The visuospatial executive planning ability, measured with the Key Search Test (KST), failed to show a deficit in FES subjects. Impairment in KST was previously demonstrated in studies of chronic SZ (Evans et al., 1997; Ihara et al., 2003; Vargas et al., 2009). The fact that we evaluated first psychotic episode of schizophrenia spectrum patients in early remission could explain this contradictory finding. Ihara et al. (2003) showed connection between the KST performance and the severity of negative symptoms in chronic SZ subjects. The mild severity of negative symptoms in our FES group could be responsible for the lack of significance found in KST. The lack of information about the symptomatology in the other two cited studies does not allow us to properly compare our findings. In addition, the fact that the differences were analyzed using the raw scores (0–16) could cause smaller sensitivity of the KST measure, as the recalculated profile scores (range 0–4) separate the performance more strictly into five categories. To test this presumption, we did an ex post facto analysis with raw scores transposed to profile scores, which indeed led to significant disadvantage of the FES group ($U = 445$; $p = 0.023$).

Verbal abilities showed a deficit in conceptualization and executive functions (semantic Verbal Fluency Test and Similarities), and in verbal learning and delayed recall (AVLT). It would be interesting to also analyze the verbal recognition pattern and compare it to the results of visuospatial recognition. Studies addressing this topic in SZ showed that the verbal recognition is preserved (see Fiszdon et al., 2008). However, because of some missing data in verbal recognition of HC group we were unable to complete this comparison. We are aware that this is a limitation of the present study. The significant increase in the number of repetitions suggests that SZ subjects have difficulties in self-monitoring and tracking abilities that are the key in the retrieval process (Lezak et al., 2012).

The verbal and visuospatial measures (DS and SS) of immediate recall and working memory (WM) showed similar patterns in FES (see **Figure 1**). No deficit was observed on either the verbal or visuospatial tasks assessing immediate recall and attention (DS and SS forward). Even though the deficit in verbal WM (DS backward) was stronger than in the spatial WM, there was a trend of significance in SS backward ($p = 0.06$). Moreover, the pattern of deviation in both tests was similar (see **Figure 1**). One of the reasons for the lack of significance

TABLE 4 | Regression model of PANSS effect (predictor) on performance in TMT B (dependent variable).

Dependent variable *TMT–B*	Adjusted R^2	SE of the estimate	Durbin-Watson	*B*	*SE*	*Beta*	*F*	*p*
Predictor variable (Model):	**0.409**	**35.1850**	**1.850**				**12.4**	**<0.001**
(Constant)				112,244	29,361			0.001
PANSS-N				3.766***	0.850	0.599		<0.001
PANSS-G				−2.968**	1.034	−0.388		0.007
Excluded:								
PANSS-P						0.006		0.968

*Stepwise multimodal linear regression in FES subjects (N = 34); PANSS, Positive and Negative Symptoms Scale (N, negative; G, general; P, positive); TMT-B, Trail Making Test –part 2; Adjusted R^2, explained variability; B, Unstandardized Coefficients beta; SE, Standard error; Beta, Standardized beta coefficient; Significance: **p < 0.01, ***p < 0.001.*

TABLE 5 | Regression model of clinical and neuropsychological variables (predictors) on global functioning measured by GAF (dependent variable).

Dependent variable: *GAF*	Adjusted R^2	SE of the estimate	Durbin-Watson	*B*	*SE*	*Beta*	*F*	*p*
Predictor variable (Model):	**0.587**	**11.407**	**1.954**				**18.775**	**<0.001**
(Constant)				104.853	6.971			<0.001
PANSS–P				−2.153***	0.389	−0.712		<0.001
VERB				0.898*	0.388	0.298		0.030
Excluded:								
PANSS–N						−0.168		0.248
PANSS–G						−0.105		0.461
CPZ						0.044		0.754
VIS						0.093		0.603

*Stepwise multimodal linear regression in FES subjects (N = 26); GAF, Global Assessment of Functioning; PANSS, Positive and Negative Syndrome Scale (P, positive; N, negative; G, general); CPZ, chlorpromazine equivalents; VERB, cumulative score for verbal tests; VIS, cumulative score for visuospatial tests; Adjusted R^2, explained variability; B, Unstandardized Coefficients beta; SE, Standard error; Beta, Standardized beta coefficient; Significance: *p < 0.05, ***p < 0.001.*

in SS backward could be the smaller size of the study sample producing reduced power size. Other reason might be the use of a computerized version of the task. Because the computerized version has not been validated, its sensitivity can be questioned. However, computerized version of SS and its alternative, the Corsi block tapping test, are commonly used in clinical studies (e.g., Kessels et al., 2000; or studies reported in Lezak et al., 2012), and they bring the advantage of administration reliability independent of examiner bias. Despite the lack of significance in the SS backward, the finding of impaired TMT-B (switching) supports the assumption of visuospatial WM deficit in SZ.

Effect of Symptomatology on Cognitive Functioning in FES

In agreement with more recent reviews and empirical reports (Andreasen et al., 2005; Keefe and Fenton, 2007; Ventura et al., 2009), we confirm the absence of relations between the symptoms severity and standard cognitive measures, except for performance on the Trail Making Test (TMT-B). TMT-B performance was negatively affected by negative symptoms and positively affected by general symptomatology. No effect of positive symptoms was identified. Current literature describes a strong to moderate association of cognitive functioning and negative symptoms (O'Leary et al., 2000), whereas positive symptoms and cognitive performance are usually independent in SZ (Addington et al., 1991; Rossi et al., 1997; Andreasen et al., 2005). The finding that better TMT-B performance was predicted by worse general symptomatology could be explained by the fact that the PANSS scale had higher inter-rater variability, particularly in the negative and general symptomatology, which could generate distortion in our findings. Indeed, our FES group showed higher scores especially in some general symptoms (not reported in detail), such as the item G4 (tension), which was previously identified as more difficult for some raters (Khan et al., 2013). Despite the single observation in TMT-B, in general, the cognitive performance showed to be independent of clinical symptoms.

Effect of Pharmacological Treatment on Cognitive Functioning in FES

In agreement with other studies (Jones et al., 2006; Keefe et al., 2007; Lewis and Lieberman, 2008), we found no effect of atypical antipsychotic medication (antipsychotic dosage calculated in CPZ equivalents) on visuospatial or verbal performance, except the phonemic verbal fluency performance. There was a negative effect of CPZ on phonemic Verbal Fluency Test; however, this result became non-significant after Bonferroni correction. Our

TABLE 6 | Regression model of clinical and neuropsychological variables (predictors) on perceived quality of Physical health (dependent variable).

Dependent variable: *Physical health*	Adjusted R^2	SE of the estimate	Durbin-Watson	*B*	*SE*	*Beta*	*F*	*p*
Predictor variable (Model):	**0.485**	**1.8198**	**1.753**				**8.234**	**0.001**
(Constant)				17.064	1.202			<0.001
PANSS–N				−0.162**	0.050	−0.520		0.004
VERB				0.173*	0.067	0.405		0.017
PANSS–P				0.150*	0.066	0.353		0.033
Excluded:								
PANSS–G						−0.079		0.628
CPZ						−0.172		0.354
VIS						−0.206		0.385

*Stepwise multimodal linear regression (N = 24); Physical health, domain 1 in WHOQOL-BREF; PANSS, Positive and Negative Syndrome Scale (P, positive; N, negative; G, general); CPZ, chlorpromazine equivalents; VERB, cumulative score for verbal tests; VIS, cumulative score for visuospatial tests; Adjusted R^2, explained variability; B, Unstandardized Coefficients beta; SE, Standard error; Beta, Standardized beta coefficient; Significance: *$p < 0.05$, **$p < 0.01$.*

TABLE 7 | Regression model of clinical and neuropsychological variables (predictors) on perceived quality of Psychological health (dependent variable).

Dependent variable: *Psychological health*	Adjusted R^2	SE of the estimate	Durbin-Watson	*B*	*SE*	*Beta*	*F*	*p*
Predictor variable (Model):	**0.144**	**2.235**	**1.803**				**4.859**	**0.038**
(Constant)				16.214	1.013			<0.001
VERB				0.173*	0.079	0.425		0.038
Excluded:								
PANSS–P						0.031		0.879
PANSS–N						−0.235		0.250
PANSS–G						0.016		0.934
CPZ						−0.087		0.691
VIS						−0.107		0.728

*Stepwise multimodal linear regression (N = 27); Psychological health, domain 2 in WHOQOL-BREF; PANSS, Positive and Negative Syndrome Scale (P, positive; N, negative; G, general); CPZ, chlorpromazine equivalents; VERB, cumulative score for verbal tests; VIS, cumulative score for visuospatial tests; Adjusted R^2, explained variability; B, Unstandardized Coefficients beta; SE, Standard error; Beta, Standardized beta coefficient; Significance: *$p < 0.05$.*

study implies that the impairment in visuospatial functions is independent of the dosage of neuroleptic medication.

Clinical Factors and Neurocognition, and their Effect on Global Functioning in Schizophrenia Spectrum Disorder

In our FES group we did not find any associations between GAF and VIS functions. The fact that the only visuospatial measure that correlated with the GAF score was the Trail Making Test B[1] could be responsible for this negative finding. Moreover, the strong association between the negative and general symptoms toward TMT-B performance described earlier (see Section Effect of Symptomatology on Cognitive Functioning in FES) suggests that VIS performance, moderated by the symptomatology, might not survive the regression analysis as an independent predictor. Another possible explanation for this finding is the fact that GAF scale was constructed as a measure of psychosocial disability in relation to symptomatology, rather than neurocognition (Jones et al., 1995; Roy-Byrne et al., 1996). Thus, more specific neurocognitive functions, such as VIS, might not be captured. On the other hand, GAF was positively affected by VERB functions (cumulative VERB score). However, VERB functioning was only an accompanying factor of the main negative effect produced by positive symptoms. The effect of negative symptoms reported in previous studies (Gaite et al., 2005) was not identified as significant in our FES sample. We assume that positive symptoms might have a more pronounced negative effect on the functioning of individuals' in our FES sample than negative and general symptoms, due to the early remission state.

In order to compare our results with previous studies, we used the GAF as a scale of functioning recommended as a mandatory control assessment by EGOFORS (European Group On Functional Outcomes and Remission in Schizophrenia) initiative (Peuskens and Gorwood, 2012). However, in our opinion, a more ecologically valid scale to measure functioning in relationship to individual neurocognitive domains is needed.

[1]Ex post facto analysis revealed that the GAF scale is significantly correlated only with two neurocognitive measures. Negative correlation was found in visual Trail Making Test B ($r = -0.43$, $p < 0.001$) and positive correlation in the semantic Verbal Fluency Test ($r = 0.36$, $p < 0.01$).

TABLE 8 | Regression model of clinical and neuropsychological variables (predictors) on perceived quality of Environment (dependent variable).

Dependent variable: *Environment*	Adjusted R^2	SE of the estimate	Durbin-Watson	*B*	*SE*	*Beta*	*F*	*p*
Predictor variable (Model):	**0.278**	**1.9072**	**1.986**				**5.425**	**0.013**
(Constant)				15.946	1.404			<0.001
VIS				−0.107**	0.037	−0.533		0.008
CPZ				−0.008*	0.004	−0.392		0.043
Excluded:								
PANSS—P						−0.068		0.711
PANSS—N						−0.141		0.495
PANSS—G						0.224		0.251
VERB						−0.109		0.720

*Stepwise multimodal linear regression (N = 24); Environment, domain 4 in WHOQOL-BREF; PANSS, Positive and Negative Syndrome Scale (P, positive, N, negative, G, general); CPZ, chlorpromazine equivalents; VERB, cumulative score for verbal tests; VIS, cumulative score for visuospatial tests; Adjusted R^2, explained variability; B, Unstandardized Coefficients beta; SE, Standard error; Beta, Standardized beta coefficient; Significance: *$p < 0.05$.*

Clinical Factors and Neurocognition, and their Effect on Quality of Life in Schizophrenia Spectrum Disorder

According to our results, the quality of life seems to be related more to verbal than visuospatial cognitive measures. Two of the four domains of WHOQOL-BREF (Physical and Psychological health) were positively associated with overall VERB performance, whereas only one domain (Environment) was related to overall VIS functioning and this association was negative. Better cumulative VIS score was associated with worse environment quality (health services, leisure time activities, etc.). Some previous studies have reported similar counterintuitive negative correlations (Prouteau et al., 2005; Fiszdon et al., 2008; Narvaez et al., 2008). This negative relation between QOL and neurocognition is often explained with a lack of insight (Prouteau et al., 2005; Narvaez et al., 2008) or with an overestimation of the level of disability due to present depressive symptoms (Bowie et al., 2007). We do not attempt to interpret this negative relation in terms of insight, as some other possible moderators might attenuate the relationship between neurocognition and QOL. However, we are aware of this discrepancy and we suggest that future research is needed in order to clarify the character of such puzzling results.

The WHOQOL-BREF domain of Social relationships was not associated with any of the clinical or neurocognitive measures. We believe that this domain might not fully reflect quality of social relationships. This domain includes only three questions that report on the quality of social relations, sexual life, and social support. These items do not cover all aspects of interpersonal relationships. Moreover, the item "Friend's support" was reported to be less relevant for the younger population assessed also in our study (Dragomirecká and Bartoňová, 2006a). WHOQOL-BREF might therefore not be a suitable tool for the measurement of social QOL in such a specific population.

Our choice of QOL measure, the WHOQOL-BREF, likely also played a role in the obtained results. In general, research findings on the relationship of neurocognition and QOL are very heterogeneous and often report weak associations between these two constructs (Heslegrave et al., 1997; Aksaray et al., 2002; Fiszdon et al., 2008). One issue that has to be considered is how other QOL questionnaires address cognition in individual items. For example, only one question of the WHOQOL-BREF specifically concentrates on the cognitive functioning. When we ex post facto analyzed the correlation between this item (Q7, quality of concentration in Psychological health domain) and individual cognitive measures, we found a strong relationship toward several cognitive tests, both VIS and VERB (mostly related to processing speed, memory and executive functions)[2]. This is in agreement with previous studies that highlighted the role of executive functions (e.g., Fiszdon et al., 2008; Matsui et al., 2008) and memory domains as the most representative measures related to QOL. If QOL questionnaires were more focused on cognitive functioning, we believe that the contradictory findings could be reduced.

In terms of clinical symptomatology, out of the four WHOQOL-BREF domains only Physical health appeared to be significantly affected by psychiatric symptoms. As expected, cognitive performance was not the only factor affecting subjective quality of physical health; the severity of the negative and positive symptoms obviously had some impact as well. Meta-analysis by Eack and Newhill (2007) described the strongest, but still small, association of Physical health QOL to general symptoms. However, our study found no such association. An explanation for the differences between our results and the previous findings can be the fact that we assessed first psychotic episode in schizophrenia spectrum patients and that the length of illness might moderate the relationship between symptoms and QOL (Eack and Newhill, 2007).

[2]Ex post facto analyzed relationship of Psychological health item Q7 (concentration) with individual test methods, showed positive correlation with the AVLT test in terms of learning ($r = 0.572$) and delayed memory ($r = 0.508$), Verbal Fluency (phonemic: $r = 0.519$; semantic: $r = 0.457$) and Spatial Span performance (total score: $r = 0.479$; backwards: $r = 0.445$), and similarly negative correlation with TMT processing time (part A: $r = -0.432$; B: $r = -0.602$).

Concluding Remarks

The studies addressing effect of cognitive deficit on global functioning or QOL are common in current research of SZ. However, studies that address these findings in first psychotic episode of schizophrenia spectrum disorder and which include complex verbal and visuospatial cognitive assessment are quite rare. We addressed the need for such research in the present study.

The results of the present study confirmed a deficit of visuospatial functions in FES. This deficit is independent of antipsychotic medication and clinical symptoms. Both global functioning and quality of life were shown to be more related to verbal than to visuospatial functions. Given the findings of negative or missing effect of visuospatial deficit on WHOQOL-BREF and GAF, the accuracy of these measures to evaluate the impact of global cognitive deficit on everyday life in schizophrenia could be questioned. We suggest the need for further investigation of the association of QOL questionnaires and GAF scale to cognitive functioning. Finally, according to our findings, the deficit in executive and memory domains is the most pronounced in the FES group. We suggest that these two domains may contribute to the cumulative cognitive performance affecting QOL and GAF scores. Further research needs to clarify this assumption.

Limitations of the Study and Future Directions

There are several limitations of the present study that warrant discussion. First, the results of the study could be tempered by the small size of the sample and consequent reduction in power size. Despite the smaller number of participants, we were able to demonstrate the deficit in both visuospatial and verbal cognitive functioning in schizophrenia, and the relationship of these abilities toward global functioning and quality of life. In order to reveal possible effects of other variables on these relationships (such as demography and subtypes of schizophrenia) and to identify individual cognitive domains affecting QOL and GAF, a larger sample size is needed.

Second, the issue of cross-sectional vs. longitudinal studies in this area is important. The present study, although cross-sectional, has identified some specific effects that can be examined over a longer time period. The next step in this research is, therefore, to track the longitudinal effect of visuospatial functions on global functioning and QOL in schizophrenia. We are currently conducting a follow-up assessment in our study group 1 year after their first hospitalization to measure the cognitive functioning in full remission state. Moreover, studies comparing FES with chronic schizophrenia patients are limited. To address this limitation we are assessing a chronic SZ sample in order to analyze the influence of the illness duration on relations between neurocognition, QOL, and GAF.

Third, the fact that the verbal and visuospatial neuropsychological tests were not always matched in terms of the measured cognitive domain, and for psychometric parameters, might be another possible limiting factor. In addition, not all test methods are validated in schizophrenia and some of them are not standardized for Czech population either. Measures validated in schizophrenia population might be expected to be more sensitive when capturing a degree of deficit. We are also aware of the fact that we don't cover all the functions of each cognitive domain. For example we did not include verbal recognition in our analysis; therefore the encoding ability could not be assessed as clearly as in the visuospatial domain. More specific and detailed visuospatial assessment is necessary in order to cover all domains that can be related to functional outcome and QOL in schizophrenia spectrum disorders.

Fourth, measures of QOL and global functioning applied in this study could be limited in terms of their association with cognitive functioning in SZ. It would be very helpful to compare them to other methods that might be more related to neurocognition. For example, the Social and Occupational Functional Assessment Scale (SOFAS) could provide a better measurement of functional outcome that is not tied to symptomatology. In addition, only subjective QOL was measured in this study. Objective measures of QOL are needed in order to understand the complex relationships of psychosocial functioning and neurocognition in SZ. According to our results about relation of specific domains of QOL and GAF obtained in the ex post facto measures, we suggest that these associations should be further investigated in future research.

Finally, several neurotransmitter functions are affected by atypical antipsychotics and our study applied only the CPZ equivalent. Future study should also address other factors in order to analyze the effects of medication on cognitive abilities in greater detail.

AUTHOR CONTRIBUTIONS

MR, KV, and IF designed the study and together with KD, FS, and ZK wrote the original protocol. IF, DL, LK, and KS recruited the participants, and they performed the neuropsychological assessment. IF and KV pre-processed the data and JP undertook the statistical analysis. MR supervised the study. MR and IF wrote the first draft of the manuscript. FS, KV, and KD contributed to data interpretation. All of the authors discussed the results and contributed to the final version of the paper and have approved it.

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ORIGINAL RESEARCH
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Abnormal Sense of Agency in Patients with Schizophrenia: Evidence from Bimanual Coupling Paradigm

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A fruitful approach to the understanding the human awareness of action is the study of those pathologies in which some aspects of it are altered. Previous evidences showed that patients with schizophrenia tend to attribute someone else' actions to their own, as internally, rather than externally, generated. Here, we asked whether schizophrenics have an "excessive" sense of agency, while observing others' movements. We took advantage from the circles-lines task, known to show bimanual interferences. Twenty schizophrenics and 20 age-matched healthy controls were administered: (a) the bimanual version of the task: drawing lines with one hand and circles with the other; and (b) a modified version: drawing lines while observing the examiner drawing circles. In the bimanual version, patients and controls showed a comparable interference effect. In the observation version, schizophrenics, compared to controls, showed a significantly greater interference effect of the examiners' hand drawing circles on the own hand drawing lines. This effect was significantly correlated to the strength of the positive symptoms (hallucinations and delusions) and to the alteration of the sense of agency, reported during the task. These findings suggest that an altered sense of agency, as shown by schizophrenics, can induce objective consequences on the motor system.

Keywords: schizophrenia, positive symptoms, sense of body-ownership, sense of agency, bimanual coupling effect

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INTRODUCTION

Despite the subjective experience of willed actions having a unitary nature, converging evidence in cognitive neuroscience have clearly pinpointed that conscious awareness of actions has a rich and multifaceted signature, and can be dissociated in different components. To this respect, a fundamental approach to the understanding of human awareness of action is the study of those pathological conditions, such as schizophrenia, in which some aspects of it are altered.

Schizophrenia is a well-known widespread mental disease characterized by a variety of signs and symptoms, which are differentially expressed across patients and through the course of the illness.

Symptoms include characteristic distortions of thinking and perception, cognitive impairments, motor abnormalities, avolition and apathy, difficulties in communication, and restricted affective expression. These abnormalities are generally classified into positive, negative, cognitive, disorganization, mood, and motor symptom dimensions (Tandon et al., 2009). Within positive symptoms dimension, Schneider conceptualized First Rank Symptoms which are self-disturbances including passivity experiences or delusions of controls, in which the individual has the experience of the mind or body being under the influence or control of some kind of external force or agency (Schneider, 1959). This altered conscious awareness of action has been explained within the well-established forward model of motor control (Wolpert et al., 1995; Blakemore and Frith, 2003). The model states that when motor predictions match sensory consequences, the movement is perceived as one's own. On the contrary, when there is a mismatch, the sense of being the agent of that action (i.e., sense of agency) decreases. According to this model, patients with schizophrenia are unable to create predictions of willed actions and, hence, experience movements as generated by external forces (Frith, 1992; Frith et al., 2000a,b; Blakemore et al., 2002; Lindner et al., 2005).

Such interpretation of a reduced sense of agency based on clinical symptoms, however, cannot easily explain those evidences showing that, in some experimental contexts, schizophrenics show, on the opposite, a hyper-sense of agency. Within this line of research, a first approach consists in asking participants to explicitly indicate how much they sense a feeling of control and of authorship over an externally generated action they can see on a screen. Previous literature employed these kinds of tasks in patients with schizophrenia, introducing, with a variety of techniques, temporal or spatial distortion of the visual feedbacks (i.e., body movements). Essentially, those studies showed that patients are inaccurate and tend to attribute what is seen to them, a sort of over-attribution of agency (Daprati et al., 1997; Franck et al., 2001; Synofzik et al., 2010). The second experimental approach to the study of agency in schizophrenics, involves implicit measures as, for instance, the temporal binding paradigm known to show in healthy participants that voluntary action and its sensory consequences bind each other in time (Haggard and Eimer, 1999; Haggard et al., 2002). The studies which administered this paradigm to patients affected by schizophrenia (Haggard et al., 2003; Voss et al., 2010) showed that they have stronger binding effects respect to the healthy population. In other words, their altered sense of agency consists in perceiving their own actions as having exaggerated causal effects on the external world.

In the present study, we proposed a new approach for studying the sense of agency in schizophrenia, asking whether, in these patients, an altered sense of agency may have consequences on the patients' motor parameters. This working hypothesis was based on previous data recently obtained on neurological patients showing a monothematic delusion of body ownership (Garbarini and Pia, 2013; Garbarini et al., 2013b, 2014, 2015a; Pia et al., 2013). Specifically, whenever someone else's hand is located in a 1st person perspective and in a body-congruent position, these patients falsely misattribute to themselves this latter hand as well as its movements. In other words, this means that when the sense of body ownership is altered, so that the alien hand is embodied, the sense of agency is altered as well. Moreover, this altered sense of agency is so deeply embedded within patients' motor system as to affect even their motor performance. To study this delusion of body ownership, Garbarini et al. (2014) employed a modified version of circles-lines task known to show bimanual interferences in healthy subjects. When people have to draw lines with one hand while drawing circles with the other hand, each movement interferes with the other one and both trajectories tend to become ovals (curved lines or line-like circles); i.e., bimanual coupling effect (Franz, 1997; Garbarini et al., 2012, 2013a, 2015b; Piedimonte et al., 2014). Patients with delusion had to draw lines with their intact hand while watching an alien hand (the examiner's one) performing circles (Garbarini et al., 2013b). When the alien hand drew circles, patients misidentified the hand as their own (i.e., altered sense of body ownership), misattributed its movements to themselves (i.e., altered sense of agency) and displayed a clear coupling effect (comparable to that found in normal individuals actually performing the bimanual task). It is important to note that, in the same condition, neither healthy controls nor hemiplegic patients without embodiment showed any coupling effect. This suggests that simply looking at a hand drawing circles is not sufficient to induce line ovalization.

Based on this previous evidence, in the present study we reasoned that this modified version of the circles-lines task (drawing lines while observing the examiner's hand drawing circles) would have allowed us to make clear predictions respect to our main question in patients with schizophrenia. Does, in those patients, an altered sense of agency over the observed movement induces objective consequences on the patients' motor performance? If patients with schizophrenia, particularly those with first-rank symptoms such as hallucinations and delusions (Daprati et al., 1997; Voss et al., 2010), have an "excessive" sense of agency, they would be more likely to perceive externally generated circles (drown by the examiner) as internally generated, as if they had to perform the bimanual task with both hands. Therefore, compared to controls, they should show a greater interference effect of someone else's hand drawing circles on the own hand drawing lines.

MATERIALS AND METHODS

Participants

The study has been conducted at the Department of Neuroscience, University of Turin, Struttura Semplice di Coordinamento a Valenza Dipartimentale (SSCVD) and at the Psychiatric Emergency Service (PES), Department of Neuroscience and Mental Health, A.O. Città della Salute e della Scienza di Torino—Presidio Molinette, Turin, during the period between January and June 2015.

For the study 40 participants were recruited, 20 patients with schizophrenia (10 females/10 males; mean age= 46.7, SD= 14.7) and 20 age-matched healthy controls (10 females/10 males; mean

TABLE 1 | Patients' demographic (a) and clinical (b) features.

	Patients	Acute patients	Stable patients	Controls
(a) Demographic characteristics				
Sample (N)	20	10	10	20
Gender	10 M; 10 F	6 M; 4 F	4 M; 6 F	10 M; 10 F
Age (years)	46.7 ± 14.7	49 ± 12.6	44.4 ± 16.9	45.2 ± 12.4
Education (years)	12 ± 3.9	11.3 ± 3.7	12.6 ± 4.1	13 ± 3.4
(b) Psychiatric assessment				
Onset of schizophrenia (years)	26.4 ± 12.3	28.8 ± 16.1	23.9 ± 6.7	–
Duration of illness (years)	20.3 ± 13.6	20.2 ± 8	20.4 ± 18.1	–
CPZ equivalent (mg/die)	551.8 ± 464.5	707.4 ± 536.6	396.2 ± 340.7	–
SAPS global*	31.3 ± 27.4	51.4 ± 24.7	11.2 ± 8.9	–
(a) Hallucinations*	5.3 ± 7.9	9.3 ± 9.4	1.2 ± 2.7	–
(b) Delusions*	11.2 ± 10.8	19.7 ± 8.5	2.6 ± 3.7	–
(c) Bizarre Behavior*	5.3 ± 4.1	7.6 ± 2.9	2.9 ± 3.7	–
(d) Positive Formal Thought Disorder*	9.7 ± 8	14.8 ± 7.1	4.5 ± 5.3	–
SANS global	51.3 ± 23.7	59.8 ± 17	42.7 ± 27.1	–
(a) Affective Flattening or Blunting	15.4 ± 6.6	17 ± 4.3	13.7 ± 8.1	–
(b) Alogia	9 ± 5.8	9.9 ± 4.6	8 ± 6.9	–
(c) Avolition—Apathy*	8.4 ± 5.1	10.7 ± 3.8	6 ± 5.3	–
(d) Anhedonia—Asociality	12.5 ± 6.3	13.8 ± 5.4	11.1 ± 7.2	–
(e) Attention*	6.2 ± 3.9	8.4 ± 2.5	3.9 ± 3.8	–

**Indicate a significant difference ($p < 0.05$, Kruskal-Wallis non-parametric test) between acute and stable patients.*

age = 45.2; SD = 12.4). All participants were right-handed according to the Edinburgh Inventory (Oldfield, 1971). Within the patients' group, 10 acute inpatients, were recruited from PES; 10 stable outpatients, were recruited from SSCVD.

Patients all fulfilled formal Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR; American Psychiatric Association, 2000) diagnostic criteria for schizophrenia. The diagnosis of schizophrenia was determined by treating psychiatrists and was confirmed by two expert clinicians (P.R., M.S.) using the Structured Clinical Interview for DSM-IV disorders (SCID; First, 2015). Patients' demographic and clinical features are shown in **Table 1**. Subjects were excluded if they had a current disorder other than schizophrenia, a current or past co-diagnosis of autistic disorder or another pervasive developmental disorder, a history of severe head injury (coma ≥48 h), or a diagnosis of a psychiatric disorder due to a general medical condition. All the patients were receiving antipsychotic medication at the time of assessment. All the patients included in the study were treated with second-generation antipsychotics (SGAs; **Table 2**). None of the patients were receiving anticholinergic drugs at the time of assessment. In order to evaluate and exclude the effects of drug-induced EPS, patients were evaluated with the Modified Simpson-Angus Scale (MSAS; Simpson and Angus, 1970). The mean rating (± SD) of MSAS in our sample of patients was 2.6 ± 3.61, showing absence or minimal degree of movement disorder.

Twenty healthy control subjects with no history of neurological or psychiatric disease volunteered for this study. The two groups, patients with schizophrenia and healthy control, were matched for age, sex and handedness. Written informed consent was obtained from all subjects after a complete description of the study. The study was performed in accordance to the ethical standards of the Declaration of Helsinki and was approved by our Local Research Ethics Commitee (LREC; AOU Città della Salute e della Scienza, Turin, Italy).

TABLE 2 | Patients drug intake.

		Daily dose	
N patients	Drug (active principle)	Range (mg)	Mean (mg)
6	Aripiprazole	10–30	20
2	Clozapine	400–600	500
3	Olanzapine	2.5–20; 10–20	15
4	Paliperidone	9–12	11.25
2	Quetiapine	200–800	500
6	Risperidone	1–5	3
1	Ziprasidone	120	120

Psychiatric Assessment

Current levels of psychopathological symptoms were assessed using the Scale for the Assessment of Negative Symptoms (SANS; Andreasen, 1984a) and the Scale for the Assessment of Positive Symptoms (SAPS; Andreasen, 1984b). We decided to use SAPS and SANS for the assessment of psychopathology because we decided to focus on positive and negative symptoms of schizophrenia. Furthermore, this type of choice would allow us to a better comparison with previous results in the literature.

The SANS measures negative symptoms and consists of 22 items divided into five subscales (Affective Flattening or Blunting, Alogia, Avolition-Apathy, Anhedonia-Asociality, and Attention). A global score for each subscale intended to summarize all of the symptoms within a subscale category is also included. A semi-structured interview is used to make some

of the item ratings, with additional ratings based on direct behavioral observation.

The SAPS consists of 34 items divided into four positive symptom subscales: hallucinations, delusions, bizarre behavior, and positive formal thought disorder. As with the SANS, each subscale also includes a global rating scale.

SANS and SAPS global and subscales scores are shown in **Table 1**.

Circles-Lines Coupling Task

Participants were asked to perform unimanual and bimanual movements, continuously drawing vertical lines and/or circles without interruptions for 12 s for each trial. The subjects sat on a chair with both hands lying on the table on which a tablet PC was positioned to the right of the subject's sagittal midline. The view of the right hand was blocked with a cardboard panel and subjects were explicitly requested to focalize the attention on the left side. In all conditions, the right hand trajectories were automatically recorded by the tablet PC whereas, in bimanual or observation conditions (see below), the left hand had to draw on a sheet of paper. In the alien conditions, also an alien hand (the examiner's one) was on the table. It was a left alien hand aligned with the patient's left shoulder, that is in a congruent position with respect to the patient's trunk midline, between the patient's body and the patient's left hand (observation circle condition). A white sheet was used to cover the patients' and the examiner's arms. It is worth noting that in all conditions the subject's left hand was visible on the table. As shown in **Figure 1**, in the experimental design, there were one baseline condition and two experimental conditions:

1. Baseline Unimanual Lines (UL): participants were asked to draw lines with the unseen right hand;
2. Bimanual Circles and Lines (BCL): participants were asked to simultaneously draw lines with the unseen right hand and circles with the left hand;
3. Observation Circles (OC): participants were asked to draw lines with the unseen right hand and to observe the alien left hand drawing circles.

In the experimental procedure there were 10 trials for each condition, for a total of 30 trials. We used the following balanced sequence: UL; BCL; OC; OC; BCL; UL.

Instrumented Analysis of Bimanual Coupling During Drawing

An Ovalization Index (OI) was calculated, according to previous studies (see Garbarini et al., 2012, 2013a, 2015b; Piedimonte et al., 2014), as the standard deviation (SD) of the right-hand trajectories in relation to an absolute vertical line. For a throughout description of the steps involved in calculating the OI refer to Garbarini et al. (2012). Briefly, OI index ranges between a value of 0 for straight trajectories without any sign of ovalization and a value of 100 for circular trajectories. The amount of interference of the left (own or alien) hand executing circles on the right hand executing lines is shown as an increase of the OI (bimanual coupling/interference effect) compared to the baseline UL.

Ownership and Agency "Online" Evaluation in Patients With Schizophrenia

In order to evaluate the patients' sense of body-ownership and sense of agency, during the OC condition in which the examiner's hand drew circles, at the end of each block of five trials, an ownership/agency questionnaire was administered. Patients were asked to answer using a 7-point Likert scale from −3 (i.e., I don't agree at all) to 3 (i.e., I totally agree) if they were in agreement/disagreement with the following statements:

1. "*I felt as if the alien hand was my hand*" (Body-ownership statement)

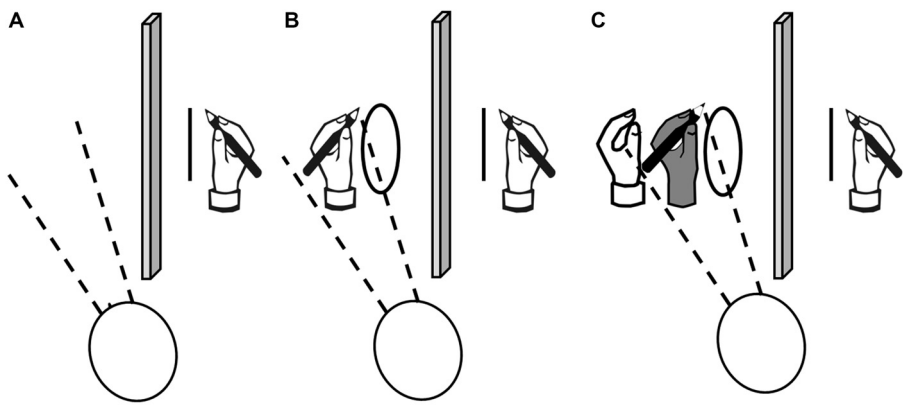

FIGURE 1 | Graphical representation of the experimental paradigm. From left to right: **(A)** baseline condition where patients performed lines with their right hand (UL); **(B)** bimanual circles-lines condition where patients performed line with their right hand and simultaneously performed circles with their left hand (BCL); **(C)** observation condition where patients performed lines with their right hand while simultaneously watching circles performed by an alien hand (in dark gray in the figure) in their right egocentric peri-personal space (OC). To be noted that in all three conditions, participants had to watch on their left peri-personal egocentric space and vision of their right hand was precluded by a paper separator.

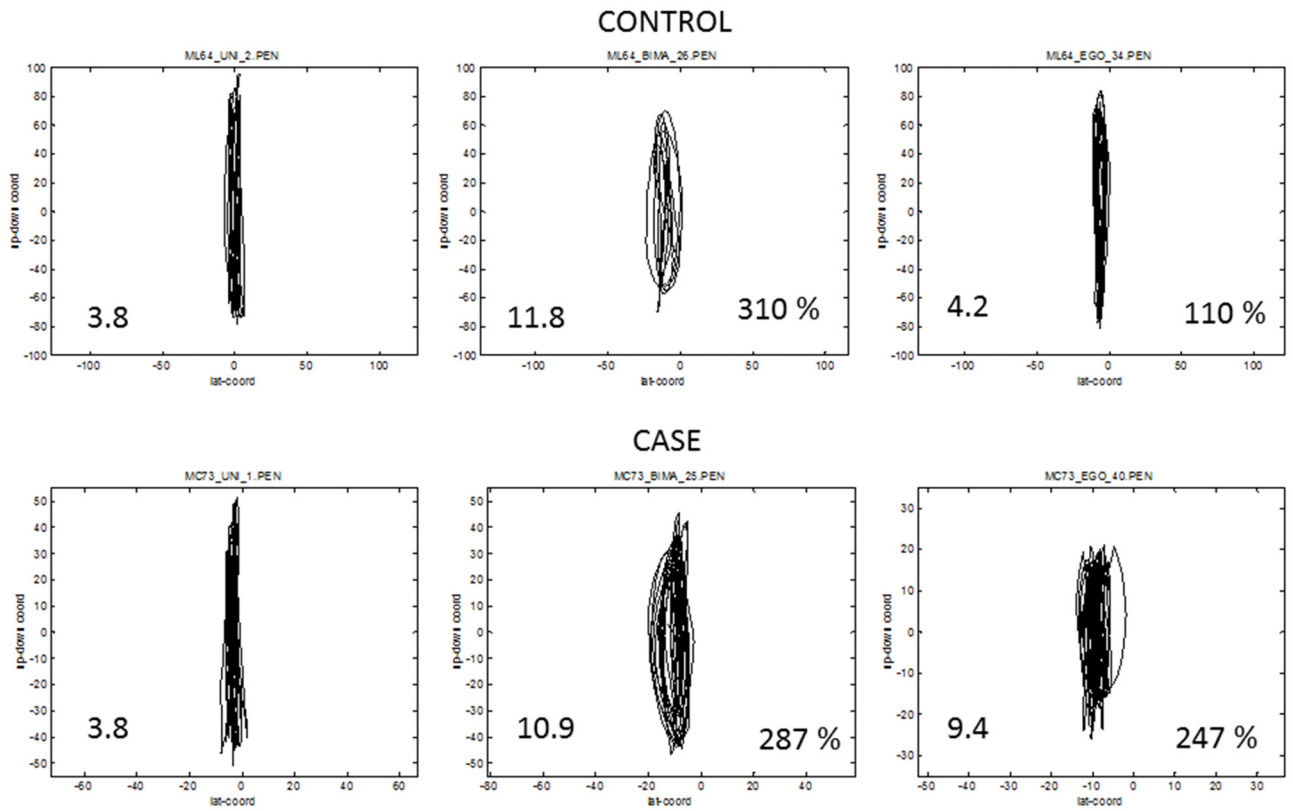

FIGURE 2 | Examples of the right hand trajectories in schizophrenic case (below) and a control subject (above) in Unimanual Lines (UL), Bimanual Circles and Lines (BCL) and Observation Circles (OC) conditions. The Ovalization Index (OI) is reported inside each of the plots, at bottom left, and in BCL and OC conditions it is reported, at bottom right, also the percent ratio relative to the UL condition. Both control and case show the expected ovalization in the BCL condition, but, when considering the Observative condition, only the case shows a residual ovalization.

2. "*The alien hand moved just like I wanted it to, as if it was obeying my will*" (Agency statement).

These statements were adopted from existing questionnaires used in traditional rubber hand illusion experiments (Kalckert and Ehrsson, 2012).

Statistical Analysis

For each participant, the OI mean value for the experimental BCL and OC conditions was expressed as percentage of the baseline UL condition (BCL% = $[OI_{BCL}/OI_{UL}]*100$; OC% = $[OI_{OC}/OI_{UL}]*100$). The obtained values were analyzed by using unpaired *t*-tests (two tailed), to compare patients and controls during both BCL and OC conditions. Furthermore, in patients with schizophrenia, unpaired *t*-tests (two tailed) were used to compare acute and stable patients in both BCL and OC conditions. A paired *t*-test (two tailed) was used to compare the patients' performance in BCL and OC conditions. Finally, in patients, we estimated the correlations (Pearson's r) between the BCL/OC% of the baseline, SAPS and SANS global and subscales scores and the ratings obtained from the ownership/agency questionnaire.

RESULTS

Comparison Between Patients and Controls During the Bimanual Task

When controls and schizophrenics were asked to perform the bimanual circles-lines task, the expected coupling effect was evident as a clear ovalization of the line trajectories, performed by the right hand, due to the interference of the circles simultaneously performed by the left hand. See examples in **Figure 2**.

The statistical comparison between patients and controls during BCL condition indicates no significant difference between the two groups (BCL% of baseline mean ± SD; Controls: 265.09 ± 102.38; Patients: 287.06 ± 147.95; $T = -0.54$, $p = 0.58$, Unpaired *t*-test, two tailed; see **Figure 3**).

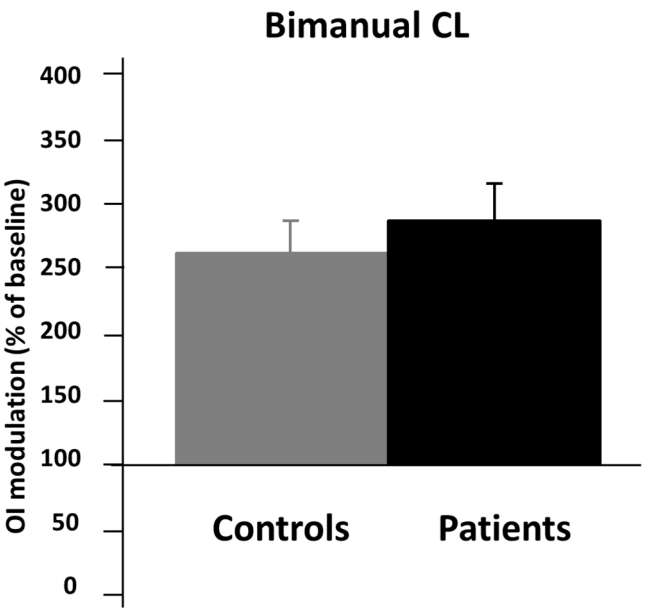

FIGURE 3 | Results from the comparison between schizophrenics and controls during BCL condition. On the *y* axis, the OI of the BCL condition (bimanual circles and lines expressed as % of the baseline condition (unimanual lines condition). Black bars represent patients' conditions while gray bars represent controls conditions. Error bars represent standard errors of the mean (SEMs).

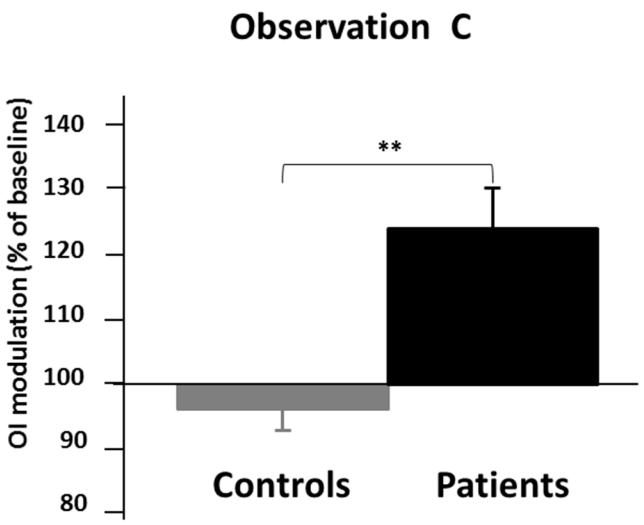

FIGURE 4 | Results from the comparison between schizophrenics and controls during OC condition. On the *y* axis, the OI of the OC condition (observation of circles performed by an alien hand while performing lines) expressed as % of the baseline condition (UL condition). Black bars represent patients' conditions while gray bars represent controls conditions. Error bars represent standard errors of the mean (SEMs). $^{**}p < 0.01$.

When comparing acute and stable patients, no difference was found during BCL condition (BCL% of baseline mean ± SD; Acute Patients: 257.36 ± 118.85; Stable Patients: 272.82 ± 89.1; $T = -0.32$, $p = 0.74$, Unpaired *t*-test, two tailed).

Comparison Between Patients and Controls During the Observation Task

During the observation version of the circles-lines task, a stronger coupling effect was evident in patients than in controls, as a greater ovalization of the line trajectories, performed by the right hand, due to the interference of the circles performed by the examiner's left hand. See examples in **Figure 2**.

The statistical comparison between patients and controls during OC condition indicates significant difference between the two groups (OC% of baseline mean ± SD; Controls: 97.34 ± 16.94; Patients: 113.82 ± 25.46; $T = -2.75$, $p < 0.01$, Unpaired *t*-test, two tailed; see **Figure 4**).

When comparing acute and stable patients, no difference was found during OC condition (OC% of baseline mean ± SD; Acute Patients: 118.21 ± 28.04; Stable Patients: 109.43 ± 23.22; $T = 0.76$, $p = 0.45$, Unpaired *t*-test, two tailed).

In both patients and controls, when comparing the obtained values in BCL and OC conditions, a significant difference was found ($p < 0.001$ for each comparison; Paired *t*-test, two tailed).

Correlations Results in Patients

We examined whether, in patients with schizophrenia, a relation exists between the values obtained in BCL and OC conditions and both SAPS and SANS global and subscales scores and the Ownership/Agency ratings. The significant differences between acute and stable patients in SAPS and SANS global and subscales scores are reported in **Table 1**. With respect to the Ownership/Agency ratings, no significant differences were found between acute and stable patients (Ownership ratings ± SD; Acute Patients: −1.95 ± 2.14; Stable Patients: −1.05 ± 2.2; Agency ratings ± SD; Acute Patients: −0.55 ± 2.45; Stable Patients: 0.1 ± 2.5; $p > 0.3$ for each comparison; Unpaired *t*-test, two tailed). Correlation analysis showed that the OC value was significantly related with SAPS global score and with hallucinations and delusions subscales scores (SAPS global score: $r = 0.43$, $p = 0.03$; SAPS Hallucinations subscale score: $r = 0.56$, $p = 0.01$; SAPS Delusions subscale score $r = 0.46$, $p = 0.04$; see **Figure 5**). Furthermore, OC values significantly correlated with the ratings given by the patients at the Agency questionnaire: "*The alien hand moved just like I wanted it to, as if it was obeying my will*" ($r = 0.43$, $p = 0.05$; see **Figure 6**). Finally, a significant correlation existed between SAPS Hallucination subscale scores and the Agency ratings ($r = 0.44$, $p = 0.05$; see **Figure 7**). There were no significant correlation with SANS global and subscales scores and with the ratings at the Ownership questionnaire.

DISCUSSION

The present study is related to one of the most important component of the human self-awareness; i.e., the sense of agency, that is the sense of being the agent of a willed action (Gallagher,

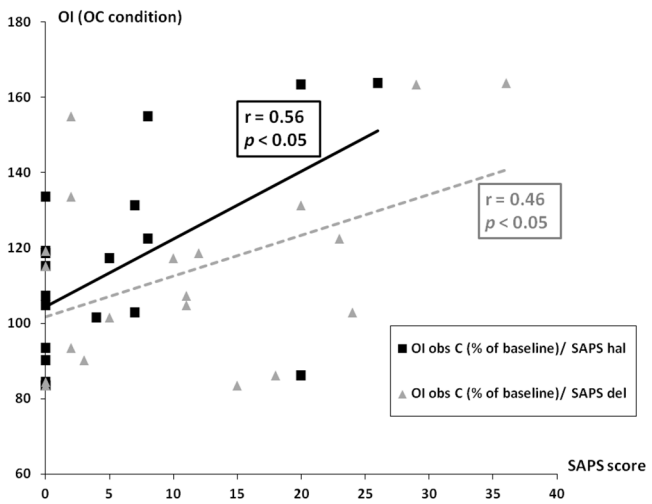

FIGURE 5 | Correlation between OC and scale for the assessment of positive symptoms (SAPS) global and subscales scores. On the *y* axis, the OI of the OC condition (observation of circles performed by an alien hand while performing lines) expressed as % of the baseline condition (UL condition). On the *x* axis, SAPS scores. Black squares represent comparison of Observation C with SAPS hallucinations subscale scores (SAPS hal) while gray triangles represent comparison of Observation C with SAPS delusion subscale scores (SAPS del).

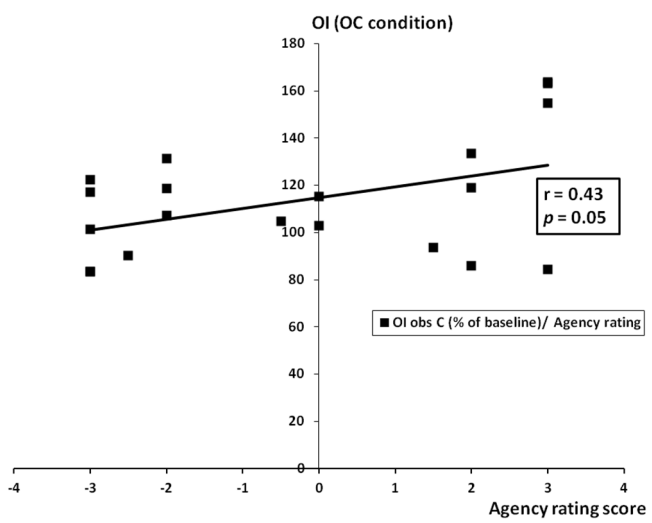

FIGURE 6 | Correlation between OC condition and agency rating scores. On the *y* axis, the OI of the OC condition (observation of circles performed by an alien hand while performing lines) expressed as % of the baseline condition (UL condition). On the *x* axis, the agency rating scores.

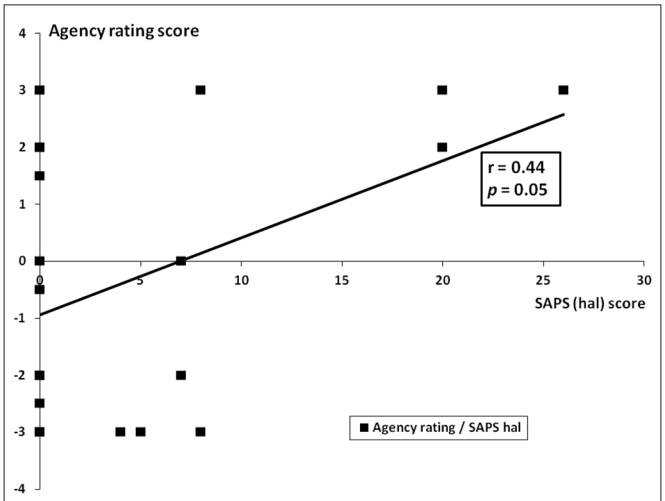

FIGURE 7 | Correlation between agency rating and SAPS hallucinations subscale scores. On the *y* axis, the agency rating scores while on the *x* axis, the SAPS hallucinations subscale scores (SAPS hal).

2000; Jeannerod, 2003). In particular, we sought for evidence that, in schizophrenia, an excessive sense of agency, for externally generated actions, affects the patients' motor parameters during a bimanual circles-lines task.

The bimanual interference is a highly reproducible effect, always present across different ages (Piedimonte et al., 2014), induced by different tasks, in both spatial (e.g., Franz et al., 1991) and temporal (e.g., Pia et al., 2013) domain. The neural mechanism subserving this phenomenon is supposed to be the default coupling of homologous muscles, promoted by the neural crosstalk. Converging neuroimaging evidence show that, during non-congruent bimanual movements (as those performed in the circles-lines task), the pre-supplementary motor area (pre-SMA) exerts an inhibitory function on this default coupling, thus allowing the execution of non-congruent bimanual movements (e.g., Sadato et al., 1997; Wenderoth et al., 2004; Garbarini et al., 2013a).

In the present study, patients and controls showed a comparable interference effect during the "classical" bimanual version of the circles-lines task (i.e., no difference was found between patients and controls for the OI enhancement in BCL condition respect to UL condition). This suggest that, at least in our schizophrenics sample, no motor abnormalities, specific for bimanual tasks, can be described (neither in acute nor in stable patients, showing a comparable interference effect in BCL condition). On the contrary, in the observation version of the task, the results showed a significant difference between schizophrenics and controls. In particular, in schizophrenics, respect to controls, we found a greater interference effect of the examiner's left hand movements, performing circles, on the actual movements of the patients' right hand, performing lines. No significant difference in OC condition was found between acute and stable patients, suggesting that the effect we found does not depend on the phase of the illness. Crucially, in patients, the interference enhancement was related to the strength of the sense of agency reported during the task (the patients' rating at the Agency statement: "*The alien hand moved just like I wanted it to, as if it was obeying my will*").

Human's sense of agency can be measured with at least two different paradigms. An explicit paradigm consists in asking participants to verbally indicate how much they sense a feeling of control and of authorship over an action. An

example of implicit paradigm to evaluate the sense of agency is the "intentional binding" effect in subjective time experience during an intentional action (Haggard and Eimer, 1999; Haggard et al., 2002). As before mentioned (see "Introduction" Section), both explicit and implicit paradigms have been employed in schizophrenia. Daprati et al. (1997), asked the patients to execute simple hand movements (without visual control) while showing, in real time, a video of either own hand or an alien hand, executing the same or a different movement. The patients' sense of agency was explicitly evaluated by asking the patients if they saw on the screen their own hand performing the movement. They found that, hallucinating and deluded patients with schizophrenia were more impaired in discriminating their own hand from the alien one than the non-hallucinating ones, and tended to misattribute the alien hand to themselves. Franck et al. (2001) asked the patients to hold a joystick and to perform discrete movements in different directions while showing them the image of a virtual right hand holding a joystick. Angular biases and temporal delays were randomly introduced in some trials, such that the movement of the virtual hand departed from the movement executed by the subjects. The patients' sense of agency was explicitly evaluated by asking the patients whether the movement they saw was their own. They found that, compared with normal subjects, deluded schizophrenics made significantly more recognition errors. On the other side, studies employing the intentional binding, as an implicit measure of the sense of agency, showed that patients feel both forward (when the external events follow their intentional actions; Haggard et al., 2003; Voss et al., 2010) and backward (when the external events precede their intentional actions; Maeda et al., 2012) exaggerated causal efficacy (i.e., an hyper-sense of agency) in the temporal event sequence during the intentional action. In the present study, we investigated the sense of agency by jointly employing both explicit (Agency rating) and implicit (OI value in OC condition) measures, also showing a significant correlation between them.

It is interesting to note that in the reviewed papers, employing either explicit or implicit measure of the sense of agency, a correlation with the positive symptoms, in particular hallucinations and delusions, has been reported as crucial in order for the effects of interest to occur (Daprati et al., 1997; Franck et al., 2001; Voss et al., 2010; Maeda et al., 2012). Also in our schizophrenics' sample, a significant correlation was found between positive symptoms (in particular hallucinations and delusions as identified by SAPS hallucinations and delusions subscales scores) and both the explicit and the implicit measure of the sense of agency: the higher the positive symptoms, the higher both the patients' agency rating and the OI value in OC condition. Positive symptoms could be defined as an excess or a distortion of normal functions (thoughts, emotions or behavior). Within the positive symptomatology, the patients may attribute their own action or thought to an external source, or they may experience the reverse as well; i.e., they may perceive the others' action as a consequence of their own intentions and they may have the strong tendency to "bind" external events to their actions and feel they cause all external phenomena around them. According to previous studies (Daprati et al., 1997; Franck et al., 2001; Voss et al., 2010; Maeda et al., 2012), our data provide experimental evidence supporting the hypothesis that positive symptoms seem to be relevant to a dysfunction of the awareness of action in schizophrenia.

Positive symptoms are typical of the acute stage of the disease but can be present throughout the different phases of the disorder. In our schizophrenics' sample, although the positive symptomatology was stronger in acute than in stable patients, the correlations with positive symptoms (hallucinations and delusions) were still significant also when the phases of the illness (acute/stable) was controlled for.

The sense of agency has to be distinguished from a sense of body-ownership, which is the perceptual condition that allows the subject to ascribe body parts to oneself (Tsakiris et al., 2007). The sense of agency and the sense of body-ownership are fundamental components of self-awareness and they interact in determining a conscious feeling of the self as an acting body. Previous studies showed that patients with schizophrenia have a more flexible body representation and weakened sense of self. Thakkar et al. (2011) demonstrated that watching a rubber hand being stroked, while one's unseen hand is stroked synchronously (i.e., the phenomenon known as the Rubber Hand Illusion), can lead to qualitative (the reported sense of ownership over the rubber hand) and quantitative (a shift in perceived position of the real hand and a limb-specific drop in stimulated hand temperature) stronger effects in schizophrenics compared to controls. Ferri et al. (2012) investigated the schizophrenics' performance during an implicit self-recognition task (visual matching), employing both participant's own and others' body-effectors. Schizophrenics did not show the typical self-advantage described in healthy subjects (i.e., when the visual matching task involves the own body-effectors, the healthy subjects' accuracy is greater than when it involves the others' body-effectors). In our study, we did not find any significant correlation with the patients' rating at the Body-ownership statement: "*I felt as if the alien hand was my hand*". This suggests that the interference effect we found in OC conditions can be better explained by the sense of agency (showing a significant correlation with the OI parameter) than by the sense of ownership the patients experienced during the task for the examiner's hand drawing circles.

In this balance between agency and ownership, the difference between the results found in psychiatric and neurological patients can be addressed. In schizophrenics, tested here, the effect we found in the observation version of the circles-lines task was not comparable to that found in the bimanual version of the task (i.e., a significantly greater effect was found in BCL condition then in OC condition). On the contrary, in brain-damaged patients with pathological embodiment the interference effect found in the observation task was very similar to that found in healthy subjects actually performing bimanual Circles-Lines task (i.e., no difference between BCL condition and OC condition was found; see "Introduction" Section and for a full description Garbarini et al., 2013b). During the observation task, neurological patients with pathological embodiment showed a profoundly altered sense of body ownership, claiming that the

examiner's hand drawing circles was their own hand. This primary body-ownership deficit, in turn, affected the patients' motor awareness and sense of agency (i.e., the patients ascribed the examiner's movement, drawing circles, to themselves). On the contrary, schizophrenics were always conscious that, in the experimental setting, the hand drawing circles was not their own, although hallucinated and deluded patients reported to feel *as if* "the alien hand moved just like they wanted it to, as if it was obeying their will". However, what these data tell us is that, both in neurological and in psychiatric patients, the delusional beliefs are not mere verbal confabulations, but instead have objective consequences on the patients' motor performance. In particular, in schizophrenics, we found that the greater the patients' agreement over the agency statement ("*The alien hand moved just like I wanted it to, as if it was obeying my will*"), the greater the modulation of the patients' motor performance. We can speculate that, in schizophrenics, the delusion of agency can automatically trigger the intention-programming processes for the own hand, as if the alien hand was actually "obeying to the patients will", so that, when the examiner's hand drew circles, the lines drawn by the patients' intact hand become ovalized.

In normal conditions, the feeling of being the agent of a willed action is so self-evident and obvious that people think there is nothing to explain about it. In fact, the study of pathological conditions can reveal that the awareness of willed action is build up in a complex way and that its coherence can be severely and unexpectedly affected. The data discussed here show that, in schizophrenia, an excessive sense of agency, for externally generated actions, affects the patients' motor parameters during a bimanual circles-lines task. Compared to controls, schizophrenics showed a significantly greater interference effect of the examiners' hand drawing circles on the own hand drawing lines. This interference enhancement was related to the strength of positive symptoms (SAPS global score and with hallucinations and delusions subscales scores) and to the sense of agency reported by the patients during the task (Agency rating). These findings suggest that an altered sense of agency, as shown by schizophrenics, can induce objective consequences on the motor system.

AUTHOR CONTRIBUTIONS

FG, LP, MS, AP and PR, designed the study; MS and PR selected patients; MR carried out the software to acquire data; AM acquired data; FG, MR and AM analyzed data; AP carried out the figures; MS and AM carried out the tables; FG, LP, MS and AM wrote the manuscript.

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