PARKINSON'S DISEASE: CELL VULNERABILITY AND DISEASE PROGRESSION

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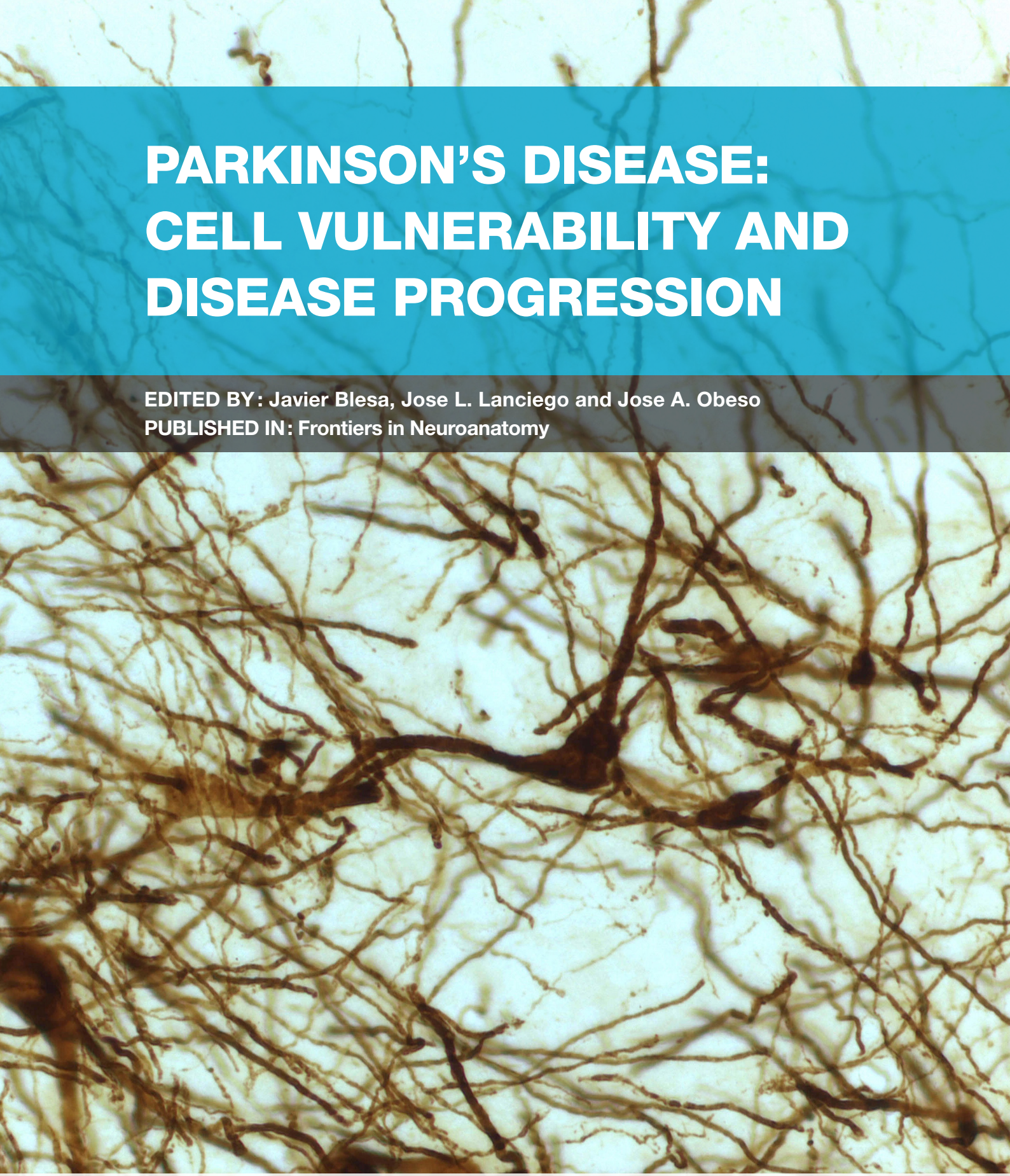

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PARKINSON'S DISEASE: CELL VULNERABILITY AND DISEASE PROGRESSION

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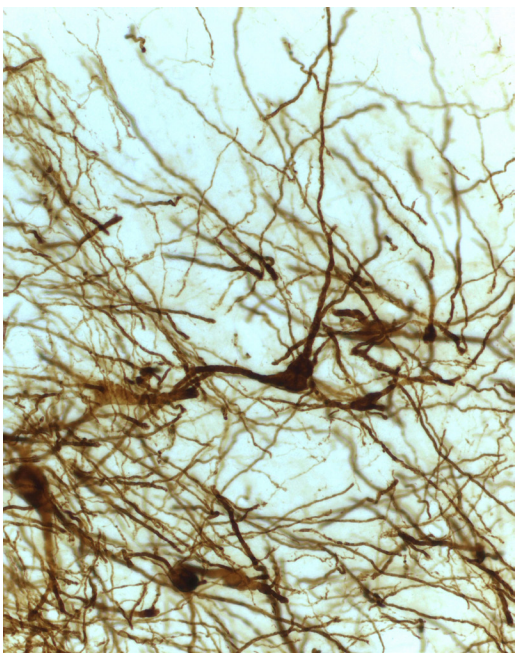

The image represents adult macaque monkey dopaminergic neurons from the substantia nigra. The tissue was immunostained for tyrosine hydroxylase.
Image courtesy of Dr. Carmen Cavada for this issue.

Parkinson's disease is a neurodegenerative disorder that affects 1.5% of the global population over 65 years of age. The hallmark feature of this disease is the degeneration of dopamine neurons in the substantia nigra pars compacta and a consequent striatal dopamine deficiency. The pathogenesis of Parkinson' Disease remains unclear. Despite tremendous growth in recent years in our knowledge of the molecular basis of Parkinson's Disease and the molecular pathways of cell death important questions remain regarding why are substantia nigra cells especially vulnerable, which mechanisms underlie progressive cell loss or what do Lewy bodies or alpha-synuclein reveal about disease progression. Understanding the different vulnerability of the dopaminergic neurons from midbrain regions and the mechanisms whereby pathology becomes widespread are primary objectives of basic and clinical research in Parkinson's Disease.

This e-Book discuss the etiopathogenesis of Parkinson's Disease, presenting a series of papers that provide up-to-date, state-of-the-art information on molecular and cellular mechanisms involved in the neurodegeneration process in the disease, the role of activation of functional anatomical organization of the basal ganglia and in particular habitual vs goal directed systems as a factor of neuronal vulnerability, the possibility that Parkinson's Disease coulb be a prion disease and how genetic factors linked to familial and sporadic forms of PD.

We hope that this eBook will stimulate the continuing efforts to understand the cell and physiological mechanisms underlying the origin of Parkinson Disease.

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EDITORIAL
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Editorial: Parkinson's disease: cell vulnerability and disease progression

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Keywords: Parkinson disease, synuclein, dopamine, vulnerability, substantia nigra, striatum

The hallmark of Parkinson Disease (PD) is the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the consequent striatal dopamine (DA) deficiency, although it is well recognized that neurodegeneration in PD goes beyond the SNc. Major advances have occurred in recent years on the molecular and pathophysiological basis of PD, however there remain many questions and unknowns regarding SNc cells vulnerability, and the exact significance of Lewy bodies and alpha-synuclein (α-syn) aggregation process regarding disease onset and progression. This Research Topic discuss the etiopathogenesis of PD, presenting a series of papers that provide up-to-date, state-of-the-art information on molecular and cellular mechanisms involved in the neurodegeneration process, neuroimmune pathways, the role of functional and anatomical organization of the basal ganglia as a factor of neuronal vulnerability, the possibility that PD is a prion disease and the cellular response to α-syn aggregation. Understanding the mechanisms underlying vulnerability of dopaminergic midbrain neurons and how pathology becomes widespread are primary objectives of basic and clinical research in PD.

Are dopaminergic and other neurons dying by the same pathogenic mechanisms? Do they all die to the same extent or at the same rate? What are the molecular determinants of susceptibility to the disease? To gain insights into these questions, researchers mainly rely in animal models. Blesa and Przedborski (2014) provide a summary of the current knowledge of *in vivo* models of PD. Whereas PD can be sporadic, genetic or possibly related with toxic/infectious agents, a differential pattern of cell loss among midbrain dopaminergic neurons is observed regardless of disease etiology suggesting that differential dopaminergic neuron vulnerability does not depend on the factor triggering PD "*per se*" but on intrinsic properties of these specific cell groups. Here, Brichta and Greengard (2014) provides an update review on the molecular basis underlying differential vulnerability of midbrain dopaminergic neurons in PD. For example, for many years many studies have suggested calbindin (CB) as a marker to distinguish between midbrain dopaminergic neurons with different susceptibility to degeneration in PD. Although CB dopaminergic neurons seem to be less prone to MPTP-induced degeneration, Dopeso-Reyes et al. (2014) clearly demonstrated that these neurons are not giving rise to nigro-striatal projections and indeed CB-ir/TH-ir neurons only originate nigro-extrastriatal projections. This data sustain the presence of a potential imbalance between the nigro-striatal and nigroextrastriatal systems in advanced diseases states. Also, Afonso-Oramas et al. (2014) revealed that midbrain dopaminergic axons are in close apposition to striatal vessels and perivascular astrocytes in rats and monkeys. The relative weight of this "vascular component" within the meso-striatal pathway suggests a role in the pathophysiology of PD.

Aging is another major risk factor for developing PD. Rodriguez et al. (2014) reviewed similarities between neurodegeneration in PD and aging. The progressive course of aging and PD could be induced by the same multi-factorial etiology, including astrocytic and microglia alterations, anomalous action of different proteins, mitochondrial disturbances, alterations of the mitophagy or the ubiquitin-proteasome system and oxidative stress. Proteins involved in PD such as α-syn, PINK1 or DJ-1, are also involved in aging. All these mechanisms of degeneration are

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review here giving an update of the classical pathways, the biochemical and molecular events that mediate DA neuronal vulnerability, and the role of PD-associated gene products in modulating cellular responses to oxidative stress (Blesa et al., 2015). Additionally, Labandeira-García et al. (2014) discuss the role of renin-angiotensin system in oxidative stress, aging and inflammation in the nigrostriatal dopaminergic system. Inflammation is indeed a major characteristic feature of the SNc in PD mainly as a consequence of neuronal death. Herrero et al. (2015) review the role of inflammation and glucocorticoids in PD while Cebrián et al. (2014) review the neuronal MHC-I expression in the SNc and its implications in synaptic function, axonal regeneration in PD and other brain diseases.

The dopaminergic neurons of the SNc project primarily to the striatum, but also provide significant innervation of other basal ganglia nuclei and the thalamus. Villalba et al. (2015) discuss evidence for synaptic glutamatergic dysfunction and pathology of cortical and thalamic inputs to the striatum and subthalamic nucleus in models of PD. The altered neuronal firing activity of the basal ganglia and other nuclei contribute largely to parkinsonisms. Galvan et al. (2015) reviewed the current knowledge of the electrophysiologic changes at the single cell level, the level of local populations of neural elements, and the entire basal ganglia-thalamocortical network in PD, and discuss the possible use of this information to optimize treatment approaches. Neuroprotection by endogenous glial cell-derived neurotrophic factor (GDNF) stimulation has been suggested as one of the potential preventive therapies in PD for many years. In this issue d'Anglemont de Tassigny et al. (2015) summarize current knowledge on brain GDNF delivery, homeostasis, and its effects on SNc neurons and discuss the therapeutic potential of endogenous GDNF stimulation in PD.

Formation and accumulation of misfolded α-syn aggregates are a central and very hot topic of PD research currently. Several authors review and discuss here the importance of this protein, it's role in different cellular domains (Guardia-Laguarta et al., 2015), the pathophysiological mechanisms connecting α-syn and lysosomal dysfunction in neuronal cell death (Bourdenx et al., 2014), how this protein can undergo a toxic conformational change, spread from cell to cell and from region to region, and initiate the formation of aggregates (Recasens and Dehay, 2014), and its possible relation to other neurodegenerative diseases like progressive supranuclear palsy (Erro Aguirre et al., 2015).

While all the features summarized above play a significant role in nigro-striatal neurodegeneration, it is unlikely that the origin of neurodegeneration in PD could be tight to a single pathogenic mechanism, hence the importance of defining markers and features of neuronal vulnerability. Obeso's group introduces here the interesting hypothesis that Parkinson's disease could be related and ultimately be the consequence of human multi-tasking behavior (Hernandez et al., 2015). Thus, the caudal region of the striatum has been associated with habitual behavior, consequently the differential loss of DA from this region provides the pathophysiological substrate for the early impairment of automatic movements (walking, writing…) and probably increased functional demand during multiple and simultaneous tasks performance.

In sum, understanding the mechanisms responsible for intrinsic SNc neuronal vulnerability is mandatory to progress in stopping neurodegeneration in PD. We trust that this Research Topic will spark new ideas and foster further advances in PD.

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Parkinson's disease: animal models and dopaminergic cell vulnerability

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Parkinson's disease (PD) is a neurodegenerative disorder that affects about 1.5% of the global population over 65 years of age. A hallmark feature of PD is the degeneration of the dopamine (DA) neurons in the substantia nigra pars compacta (SNc) and the consequent striatal DA deficiency. Yet, the pathogenesis of PD remains unclear. Despite tremendous growth in recent years in our knowledge of the molecular basis of PD and the molecular pathways of cell death, important questions remain, such as: (1) why are SNc cells especially vulnerable; (2) which mechanisms underlie progressive SNc cell loss; and (3) what do Lewy bodies or α-synuclein reveal about disease progression. Understanding the variable vulnerability of the dopaminergic neurons from the midbrain and the mechanisms whereby pathology becomes widespread are some of the primary objectives of research in PD. Animal models are the best tools to study the pathogenesis of PD. The identification of PD-related genes has led to the development of genetic PD models as an alternative to the classical toxin-based ones, but does the dopaminergic neuronal loss in actual animal models adequately recapitulate that of the human disease? The selection of a particular animal model is very important for the specific goals of the different experiments. In this review, we provide a summary of our current knowledge about the different *in vivo* models of PD that are used in relation to the vulnerability of the dopaminergic neurons in the midbrain in the pathogenesis of PD.

Keywords: MPTP, 6-OHDA, rotenone, synuclein, LRRK2, parkin, DJ1, ATP13A2

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder whose prevalence increases with age (Pringsheim et al., 2014). The cardinal features of PD include tremor, rigidity and slowness of movements, albeit non-motor manifestations such as depression and sleep disturbances are increasingly recognized in these patients (Rodriguez-Oroz et al., 2009). Over the past decade, more attention has also been paid to the broader nature of the neurodegenerative changes in the brains of PD patients. Indeed, for many years, the neuropathological focus has been on the striking neurodegeneration of the nigrostriatal dopaminergic pathway, however, nowadays, disturbances of the serotonergic, noradrenergic, glutamatergic, GABAergic, and cholinergic systems (Brichta et al., 2013) as well as alterations in neural circuits are now being intensively investigated from the angle of the pathophysiology of PD (Obeso et al., 2014), with the underlying expectation of acquiring a better understanding of the neurobiology of this disabling disorder and of identifying new targets for therapeutic purposes. From a molecular biology point of view, the accepted opinion that the PD neurodegenerative process affects much more than the dopaminergic neurons of the substantia nigra pars compacta (SNc), has triggered a set of fascinating questions such as: are dopaminergic and non-dopaminergic neurons in PD dying by the same pathogenic mechanisms; and, given the fact that within a given subtype of neurons, not all die to the same extent nor at the same rate [e.g., dopaminergic neurons in the SNc vs. ventral tegmental area (VTA)], what are the molecular determinants of susceptibly/and resistance to disease?

To gain insights into these types of critical questions, a brief review of the literature demonstrates that the enthusiasm for experimental models of PD, both *in vitro* and *in vivo*, has greatly increased, in part, thanks to new strategies for producing sophisticated models, such as the temporal- and/or cell-specific expression of mutated genes in mice (Dawson et al., 2010), human pluripotent cells coaxed into a specific type of neurons (Berg et al., 2014), and a host of invertebrate organisms like *Drosophila* (Guo, 2012), *Caenorhabditis elegans* (Chege and McColl, 2014), or Medaka fish (Matsui et al., 2014). Thus far, however, all of these experimental models continue to be categorized into two main flavors: toxic and genetic (and sometimes, both approaches are combined). But, more importantly, none of the currently available models phenocopy PD, mainly because they lack some specific neuropathological and/or behavioral feature of PD. Some PD experts see this as fatal flaws, while others tend to ignore the shortcomings. It has always been our personal view that models are just models and, as such, given the large collection of models the field of PD possesses, the prerequisite resides in not using just any model but in selecting the optimal *in vitro* or *in vivo* model whose strengths are appropriate for investigating the question being asked and whose weaknesses will not invalidate the interpretation of an experiment.

Based on our above premise, herein, we discuss the experimental models of PD, with a deliberate emphasis on *in vivo* mammalian

Table 1 | Animal models of Parkinson disease.

	Animal model	Motor behavior	SNc neuron loss	Striatal DA loss	Lewy body/Syn pathology
Toxin-based	MPTP Mice	Reduced locomotion, bradykinesia	↑ ↑ ↑	↑ ↑ ↑	NO
	MPTP Monkeys	Reduced locomotion, altered behavior, tremor, and rigidity	↑ ↑ ↑	↑ ↑ ↑	NO
	6-OHDA rat	Reduced locomotion, altered behavior	↑ ↑ ↑	↑ ↑ ↑	NO
	Rotenone	Reduced locomotion	↑ ↑	↑ ↑ ↑	YES
	Paraquat/maneb	Reduced locomotion	↑ ↑	↑ ↑ ↑	YES
	MET/MDMA	Reduced locomotion	↑ ↑	↑ ↑ ↑	NO
Genetic mutations*	α-Synuclein	Altered behavior, reduced or increased motor activity	↑ Not consistent	↑	↑ (in old animals)
	LRKK2	Mild behavioral alteration	NO	NO	NO
	PINK1	No obvious alterations or reduced locomotion	NO	NO	NO
	PARKIN	No obvious locomotion or reduced locomotion	NO	↑	NO
	DJ-1	Decreased locomotor activity	NO	NO	NO
	ATP13A2	Late onset sensorimotor deficits	NO	NO	NO
Others	SHH	Reduced locomotion	↑ ↑	↑ ↑	NO
	Nurr1	Reduced locomotion	↑ ↑	↑ ↑	NO
	Engrailed 1	Reduced locomotion	↑ ↑	↑	NO
	Pitx3	Reduced locomotion	↑ ↑ ↑	↑ ↑ ↑	NO
	C-Rel-NFKB	Gait, bradykinesia, rigidity	↑ ↑	↑ ↑	YES
	MitoPark	Reduced locomotion, tremor, and rigidity	↑ ↑	↑ ↑	YES
	Atg7	Late onset locomotor deficits	↑ ↑	↑ ↑	YES
	VMAT2	Reduced locomotion and altered behavior	↑ ↑	↑ ↑	YES

↑ ↑ ↑, Severe loss; ↑ ↑, Moderate loss; ↑, Mild loss.
**This table summarizes general observations for each model. See the main text for full and specific description of different animal models for each genetic mutation.*

models induced by reproducible means. Over the years, a constellation of uncommon strategies and organisms have been used to produce models of PD. However, in this review, we have decided not to discuss these cases, because we have limited space and because we are missing sufficient independent information to assessment the reproducibility and reliability of these models, which, to us, is critical for distinguishing between interesting "case reports" and useful tools to model human diseases.

TOXIN MODELS

A number of pharmacological and toxic agents including reserpine, haloperidol, and inflammogens like lipopolysaccharide have been used over the years to model PD, although the two most widely used are still the classical 6-OHDA in rats and MPTP in mice and monkeys. Although the neurotoxic models appear to be the best ones for testing degeneration of the nigrostriatal pathway, some striking departures from PD need to be mentioned: the degeneration of dopaminergic neurons progress rapidly, i.e., days not years, lesions are primarily if not exclusively dopaminergic, and animals lack the typical PD proteinaceous inclusions called Lewy bodies (LBs). In addition, behavioral abnormalities in these animal models are also a challenging question (see below; **Table 1**).

MPTP

MPTP is the tool of choice for investigations into the mechanisms involved in the death of DA neurons in PD. MPTP has been shown to be toxic in a large range of species (Tieu, 2011). The most popular species, besides primates, is the mouse, as rats were found to be resistant to this toxin (Chiueh et al., 1984). A number of intoxication regimens or administration methods have been used over the years in mouse (Jackson-Lewis and Przedborski, 2007; Meredith et al., 2008) and in primates (Bezard et al., 1997; Blesa et al., 2012; Porras et al., 2012). In both species, MPTP primarily causes damage to the nigrostriatal DA pathway with a profound loss of DA in the striatum and SNc (Dauer and Przedborski, 2003).

This specific and reproducible neurotoxic effect on the nigrostriatal system is the strength of this model. Neuropathological data show that MPTP administration causes damage to the nigrostriatal DA pathway that is identical to that seen in PD (Langston

et al., 1983), yet there is a resemblance that goes beyond the loss of SNc DA neurons. Like in PD, MPTP causes greater loss of DA neurons in SNc than in VTA or retrorubral field (Seniuk et al., 1990; Muthane et al., 1994; Blesa et al., 2011, 2012) and, at least in monkeys treated with low doses of MPTP, greater degeneration of DA nerve terminals in the putamen than in the caudate nucleus (Moratalla et al., 1992; Snow et al., 2000; Blesa et al., 2010).

A often raised weakness with this model is the lack of LB (Shimoji et al., 2005; Halliday et al., 2009). Although no LBs have been observed in these models so far, a few reports have investigated the expression, regulation or pattern of α-syn after MPTP exposure (Vila et al., 2000; Dauer et al., 2002; Purisai et al., 2005). Only, in MPTP-injected monkeys, have intraneuronal inclusions, reminiscent of LBs, been described (Forno et al., 1986; Kowall et al., 2000). Behavior is also an issue, and except for the monkeys, features reminiscent of PD are lacking especially in mice. Yet, using a battery of tests, some motor alterations in mice with profound dopaminergic deficit may be detected (Taylor et al., 2010).

6-OHDA

Like MPTP, 6-OHDA is a selective catecholaminergic neurotoxin that is used, mainly, to generate lesions in the nigrostriatal DA neurons in rats (Ungerstedt, 1968). Since 6-OHDA cannot cross the blood-brain barrier, systemic administration fails to induce parkinsonism. So, this induction model requires that 6-OHDA be injected (typically as a unilateral injection) into the SNc, medial forebrain bundle or striatum (Blandini et al., 2008). Intraventricular administration has also been achieved (Rodríguez Díaz et al., 2001). The effects resemble those in the acute MPTP model, causing neuronal death over a brief time course (12 h to 2–3 days). The intrastriatal injection of 6-OHDA causes progressive retrograde neuronal degeneration in the SNc and VTA (Sauer and Oertel, 1994; Przedborski et al., 1995). The pattern of DA loss in animals bearing a full lesion (>90%) again mirrors seen that in PD, with the SNc showing more cells loss compared to the VTA (Przedborski et al., 1995). As in PD, DA neurons are killed, and the non-DA neurons are preserved. However, like in the MPTP model, 6-OHDA does not produce LB-like inclusions in the nigrostriatal pathway. Traditionally, behavioral assessments of motor impairments in the unilateral 6-OHDA model are done by drug-induced rotation tests (Dunnett and Lelos, 2010). However, drug-free sensorimotor behavioral tests have been developed in both rat and mice that may be helpful for the preclinical testing of new symptomatic strategies (Schallert et al., 2000; Glajch et al., 2012).

ROTENONE

Chronic systemic exposure to rotenone in rats causes many features of PD, including nigrostriatal DA degeneration (Betarbet et al., 2000). The rotenone-administered animal model also reproduces all of the behavioral features reminiscent of human PD. Importantly, many of the degenerating neurons have intracellular inclusions that resemble LB morphologically. These inclusions show immunoreactivity for α-syn and ubiquitin as did the original LB (Sherer et al., 2003). Usually, rotenone is administered by daily intraperitoneal injection (Cannon et al., 2009), intravenously or subcutaneously (Fleming et al., 2004). Recently, rotenone has been tested in mice through chronic intragastric administration, (Pan-Montojo et al., 2010) or as a stereotaxic injection or infusion directly in the brain (Alam et al., 2004; Xiong et al., 2009) recapitulating the slow and specific loss of DA neurons. However, administration of rotenone in rats causes high mortality and, somehow, is difficult to replicate.

PARAQUAT/MANEB

Although the idea that the herbicide paraquat (N,N′-dimethyl-4-4-4′-bypiridinium), may cause parkinsonism in humans has attracted some interest, at this time, as pointed out by Berry and collaborators, epidemiological and clinical evidence that paraquat may cause PD is inconclusive (Berry et al., 2010). And, the same view seems to apply to the fungicide maneb (manganese ethylenebisdithiocarbamate; Berry et al., 2010). Moreover, effects of this compound in the nigrostriatal DA system is somewhat ambiguous (Freire and Koifman, 2012). Regarding animal models, some researchers report that, following the systemic application of paraquat, mice exhibit reduced motor activity and a dose-dependent loss of striatal tyrosine hydroxylase (TH) fibers and SNc neurons with relative sparing of the VTA (Brooks et al., 1999; Day et al., 1999; McCormack et al., 2002; Rappold et al., 2011). Like rotenone, paraquat may be useful in the laboratory because of its presumed ability to induce LB in DA neurons (Manning-Bog et al., 2002). Maneb has been shown to decrease locomotor activity and produce SNc neurons loss (Thiruchelvam et al., 2003) and potentiate both the MPTP and the paraquat effects (Takahashi et al., 1989; Thiruchelvam et al., 2000; Bastías-Candia et al., 2013). However, as with rotenone, this model shows contradictory results, variable cell death and loss of striatal DA content (Miller, 2007).

AMPHETAMINE-TYPE PSYCHOSTIMULANTS

Some amphetamine derivatives such as methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA) also have neurotoxic effects on the nervous system causing not only functional deficits but also structural alterations (Cadet et al., 2007; Thrash et al., 2009). The first study to show DA depletion in rats following repeated, high-dose exposure to METH was conducted by Kogan et al. (1976). Hess et al. (1990) and Sonsalla et al. (1996) showed that high-dose treatment with METH in mice resulted in a loss of DA cells in the SNc. Since then, several studies have reported selective DA or serotonergic nerve terminal as well as SNc neuronal loss in rodents, primates or even guinea pig following the administration of very high doses of METH (Wagner et al., 1979; Trulson et al., 1985; Howard et al., 2011; Morrow et al., 2011).

3,4-Methylenedioxymethamphetamine can also elicit significant neurobehavioral adverse effects. Although MDMA toxicity mainly affects the serotonergic system, DA system can also be affected to a lesser extent (Jensen et al., 1993; Capela et al., 2009). In mice, repeated administration of MDMA produces degeneration of DA terminals in the striatum (O'Callaghan and Miller, 1994; Granado et al., 2008a,b) and TH+ neuronal loss in the SNc (Granado et al., 2008b).

Exposure to low concentrations of METH results in a decrease of the vulnerability of the SNc DA cells to toxins like 6-OHDA or

MPTP (Sziráki et al., 1994; El Ayadi and Zigmond, 2011). On the other hand, chronic exposure to MDMA of adolescent mice exacerbates DA neurotoxicity elicited by MPTP in the SNc and striatum at adulthood (Costa et al., 2013). Hence, a METH or MDMA-treated animal model could be useful to study the mechanisms of DA neurodegeneration (Thrash et al., 2009).

GENETIC MODELS

Genetic models may better simulate the mechanisms underlying the genetic forms of PD, even though their pathological and behavioral phenotypes are often quite different from the human condition. A number of cellular and molecular dysfunctions have been shown to result from these gene defects like fragmented and dysfunctional mitochondria (Exner et al., 2012; Matsui et al., 2014; Morais et al., 2014), altered mitophagy (Lachenmayer and Yue, 2012; Zhang et al., 2014), ubiquitin–proteasome dysfunction (Dantuma and Bott, 2014), and altered reactive oxygen species production and calcium handling (Gandhi et al., 2009; Joselin et al., 2012; Ottolini et al., 2013). Some studies have reported alterations in motor function and behavior in these mice (Hinkle et al., 2012; Hennis et al., 2013; Vincow et al., 2013), and sensitivities to complex I toxins, like MPTP, different from wild type (WT) mice (Dauer et al., 2002; Nieto et al., 2006; Haque et al., 2012) although this latter finding is not always consistent (Rathke-Hartlieb et al., 2001; Dong et al., 2002). However, almost all of the studies evaluating the integrity of the nigrostriatal DA system in these genetic models failed to find significant loss of DA neurons (Goldberg et al., 2003; Andres-Mateos et al., 2007; Hinkle et al., 2012; Sanchez et al., 2014). Thus, recapitulation of the genetic alterations in mice is insufficient to reproduce the final neuropathological feature of PD. Below, we describe transgenic mice or rat models which recapitulate the most known mutations observed in familial PD patients (**Table 1**).

α-SYNUCLEIN

α-syn was the first gene linked to a dominant-type, familial PD, called Park1, and is the main component of LB which are observed in the PD brain (Goedert et al., 2013). Three missense mutations of α-syn, encoding the substitutions A30P, A53T, and E46K, have been identified in familial PD so far (Vekrellis et al., 2011; Schapira et al., 2014). Furthermore, the duplication or triplication of α-syn is sufficient to cause PD, suggesting that the level of α-syn expression is a critical determinant of PD progression (Singleton et al., 2003; Kara et al., 2014).

To date, various α-syn transgenic mice have been developed. Although, in some of these mice, decreased striatal levels of TH or DA and behavioral impairments indicate that the accumulation of α-syn can significantly alter the functioning of DA neurons, no significant nigrostriatal degeneration has been found in most of them. The models of α-syn overexpression in mice recapitulate the neurodegeneration, depending primarily on the promoter used to drive the expression of the transgene, whether the transgene codes for the WT or the mutated protein, and the level of expression.

Although a lot of behavioral alterations have been described in both the A30P and A53T mice (Sotiriou et al., 2010; Oaks et al., 2013; Paumier et al., 2013), the mouse prion protein promoter failed to reproduce the cell loss in the SNc or locus coeruleus (LC; van der Putten et al., 2000; Giasson et al., 2002; Gispert et al., 2003). The same phenotype was found with the hamster prion promoter (Gomez-Isla et al., 2003). Mice based on the PDGF-β promoter showed loss of terminals and DA in the striatum but no TH+ cell loss (Masliah et al., 2000). The TH promoter led to TH+ cell loss only in a few studies (Thiruchelvam et al., 2004; Wakamatsu et al., 2008) but did not replicate the α-syn neuropathology as did the Thy-1 promoter (Matsuoka et al., 2001; Chen et al., 2006; Miller et al., 2007; Su et al., 2009). However, the use of the murine Thy-1 promoter often causes loss of DA levels in the striatum but only moderate nigral DA cell loss in the SNc, with α-syn pathology (van der Putten et al., 2000; Rockenstein et al., 2002; Ikeda et al., 2009; Ono et al., 2009; Lam et al., 2011). A new line of tetracycline-regulated inducible transgenic mice that overexpressed α-syn A53T under control of the promoter of Pitx3 in the DA neurons developed profound motor disabilities and robust midbrain neurons neurodegeneration, profound decrease of DA release, the fragmentation of Golgi apparatus, and the impairments of autophagy/lysosome degradation pathways (Lin et al., 2012). Janezic et al. (2013) generated BAC transgenic mice (SNCA-OVX) that express WT human α-syn and which display an age-dependent loss of SNc DA neurons preceded by early deficits in DA release from terminals in the dorsal striatum, protein aggregation and reduced firing of SNc DA neurons. Regarding the transgene expressed, the A53T seems to be more effective than the A30P, in general.

Several viral vectors, primarily lentiviruses and adeno-associated viruses (AAVs), have been used to drive exogenous α-syn. Rats are usually used for these studies because viral vector delivery requires stereotactic injections within or near the site of the neuronal cell bodies in the SNc (Kirik et al., 2002; Klein et al., 2002; Lo Bianco et al., 2002; Lauwers et al., 2003, 2007). In contrast to all of the α-syn transgenic mice, viral vector-mediated α-syn models display α-syn pathology and clear dopaminergic neurodegeneration. The injection of human WT or A53T mutant α-syn by AAVs into the SNc neurons of rats induces a progressive, age-dependent loss of DA neurons, motor impairment, and α-syn cytoplasmic inclusions (Kirik et al., 2002; Klein et al., 2002; Lo Bianco et al., 2002; Decressac et al., 2012). This cell loss was preceded by degenerative changes in striatal axons and terminals, and the presence of α-syn positive inclusions in axons and dendrites (Kirik et al., 2003; Decressac et al., 2012). These results have been replicated in mice (Lauwers et al., 2003; Oliveras-Salvá et al., 2013). Although these models still suffer from a certain degree of variability, they can be of great value for further development and testing of neuroprotective strategies.

Recently, several studies have demonstrated that α-syn may be transmissible from cell to cell (Luk and Lee, 2014). In WT mice, a single intrastriatal inoculation of synthetic α-syn fibrils or pathological α-syn purified from postmortem PD brains led to the cell-to-cell transmission of pathologic α-syn and LB pathology in anatomically interconnected regions and was accompanied by a progressive loss of dopaminergic neurons in the SNc and reduced DA levels in the striatum, culminating in motor deficits (Luk et al., 2012a,b; Masuda-Suzukake et al., 2014; Recasens et al., 2014). Moreover, the hind limb intramuscular injection of α-syn

can induce pathology in the central nervous system in transgenic mouse models (Sacino et al., 2014).

LRKK2

Mutations in LRRK2 are known to cause a late-onset autosomal dominant inherited form of PD (Healy et al., 2008). Several mutations have been identified in LRRK2, the most frequent being the G2019S mutation, a point mutation in the kinase domain, whereas R1441C, a mutation in the guanosine triphosphatase domain, is the second most common (Rudenko and Cookson, 2014). Overall, LRRK2 mice models display mild or not functional disruption of the nigrostriatal DA neurons of the SNc.

LRRK2 KO mice are viable and have an intact nigrostriatal DA pathway up to 2 years of age. Neuropathological features associated with neurodegeneration or altered neuronal structure were absent, but α-syn or ubiquitin accumulation has been reported in these mice (Andres-Mateos et al., 2009; Lin et al., 2009; Tong et al., 2010; Hinkle et al., 2012). To date, two LRRK2 KO rat models have been developed, although the consequences of LRRK2 deficiency in the brain are still unknown (Baptista et al., 2013; Ness et al., 2013).

Both G2019S and R1441C LRRK2 KI mice are viable, fertile, and appear grossly normal. This mutation had no impact on DA neuron number or morphology in the SNc, or on noradrenergic neurons in the LC. Striatal DA levels and DA turnover are also normal in these mice (Tong et al., 2009; Herzig et al., 2011).

Overexpression of G2019S LRRK2 leads to a mild progressive and selective degeneration of SNc DA neurons (20%) up to 2 years of age. Furthermore, no alteration in striatal DA levels or locomotor activity could be detected in older G2019S LRRK2 mice (Ramonet et al., 2011; Chen et al., 2012). Also, Maekawa et al. (2012) generated transgenic mice constitutively expressing V5-tagged human I2020T LRRK2 from a CMV promoter with no influence on SNc DA neuronal number or striatal DA fiber density. Zhou et al. (2011) developed a transgenic rat model expressing G2019S LRRK2. Despite a mild behavioral alteration, LRRK2 expression had no effect on the number of DA neurons or on striatal DA content. Recently, conditional expression of R1441C LRRK2 in midbrain dopaminergic neurons of mice results in nuclear abnormalities but, without neurodegeneration (Tsika et al., 2014).

Additional LRRK2 BAC transgenic mouse models have also been developed. These mice displayed age-dependent and progressive motor deficits at 10–12 months of age, accompanied by a mild reduction of striatal DA release. Adult neurogenesis and neurite outgrowth are impaired. No DA neurons loss or degeneration of striatal nerve terminals where observed in mice at 9–10 months of age (Li et al., 2009b, 2010; Melrose et al., 2010; Winner et al., 2011).

Regarding the viral vector-based models, Lee et al. (2010) developed a herpes simplex virus (HSV) amplicon-based mouse model of G2019S LRRK2-induced DA neurotoxicity. The nigrostriatal expression of WT LRRK2 induced modest nigral DA neurodegeneration (10–20%), whereas expression of the kinase-hyperactive G2019S LRRK2 resulted in a 50% neuronal loss in the ipsilateral SNc associated with reduced striatal DA fiber density at 3 weeks post-injection. In another study, a model based on the unilateral injection of recombinant, second-generation human serotype 5 adenoviral (rAd) vectors expressing FLAG-tagged human WT or G2019S LRRK2 driven by a neuronal-specific human synapsin-1 promoter in rats induced the progressive loss (20%) of DA neurons in the ipsilateral SNc over 42 days, but with no reduction of striatal DA fiber density (Dusonchet et al., 2011).

PINK1

Mutations in the gene PINK1 cause another form of PD called PARK6 (Scarffe et al., 2014). PINK1 KO mice have an age-dependent, moderate reduction in striatal DA levels accompanied by low locomotor activity, but do not exhibit major abnormalities in the DA neurons or striatal DA levels (Gautier et al., 2008; Gispert et al., 2009). These mice showed no LB formation or nigrostriatal degeneration for up to 18 months of age. However, in PINK1 KO mice, overexpression of α-syn in the SNc resulted in enhanced dopaminergic neuron degeneration as well as significantly higher levels of α-syn phosphorylation at serine 129 at 4 weeks post-injection (Oliveras-Salvá et al., 2014). Recently, a PINK1 null mouse with an exon 4–5 deletion displayed a progressive loss of DA in the striatum, but there was no degeneration in the SNc (Akundi et al., 2011). The phenotypes of these mice are very similar to those of Parkin KO and DJ-1 KO mice.

PARKIN

Parkin is an E3 ubiquitin ligase that functions in the ubiquitin–proteasome system. Mutations in parkin are a cause of familial PD and are also seen in some young-onset sporadic PD cases (Lücking et al., 2000; Periquet et al., 2003). Several parkin KO mice have been generated, typically produced by deletion at exon 3, exon 7, or exon 2 in the PRKN gene (Goldberg et al., 2003; Itier et al., 2003; Palacino et al., 2004; Von Coelln et al., 2004; Perez and Palmiter, 2005; Zhu et al., 2007; Martella et al., 2009). However, they show no substantial DA-related behavioral abnormalities. Some of these KO mice exhibit slightly impaired DA release (Itier et al., 2003; Kitada et al., 2009a) and reduced norepinephrine levels in the olfactory bulb and spinal cord with an abnormal nigrostriatal region but without loss of SNc neurons (Goldberg et al., 2003; Von Coelln et al., 2004).

Only the Parkin-Q311X-DAT-BAC mice exhibit multiple late onsets and progressive hypokinetic motor deficits, age-dependent DA neuron degeneration in the SNc and a significant reduction in striatal DA and dopaminergic terminals in the striatum (Lu et al., 2009). Recently, overexpression of T240R-parkin and of human WT parkin induced progressive and dose-dependent DA cell death in rats (Van Rompuy et al., 2014).

DJ-1

DJ-1 mutations are linked to an autosomal recessive, early onset PD (Puschmann, 2013). KO models of DJ-1 mice with a targeted deletion of exon 2 or insertion of a premature stop codon in exon 1 show decreased locomotor activity, a reduction in the release of evoked DA in the striatum but no loss of SNc DA neurons and no change of the DA levels (Goldberg et al., 2005; Kim et al., 2005). However, one line of DJ-1 KO mice shows loss of DA neurons in the VTA (Pham et al., 2010).

Interestingly, a recently described DJ-1 KO mouse, backcrossed on a C57/BL6 background, displayed a dramatic early onset unilateral loss of DA neurons in the SNc, progressing to bilateral degeneration of the nigrostriatal axis, with aging. In addition, these mice exhibit age-dependent bilateral degeneration in the LC and display, with aging, a mild motor behavioral deficit at specific time points (Rousseaux et al., 2012). Therefore, if confirmed, this new mouse model would provide a tool to study the preclinical aspects of PD.

ATP13A2

Mutations in ATP13A2 (PARK9), encoding a lysosomal P-type ATPase, are associated with both Kufor–Rakeb syndrome (KRS) and neuronal ceroid lipofuscinosis. KRS has recently been classified as a rare genetic form of PD (Heinzen et al., 2014; Yang and Xu, 2014). Despite the accumulation of lipofuscin deposits in the SNc and late-onset sensorimotor deficits, there was no change in the number of DA neurons in the SNc or in striatal DA levels in aged Atp13a2 KO mice (Schultheis et al., 2013).

OTHER MODELS

Inactivation of multiple PD genes has been shown to be insufficient to cause significant nigral degeneration within the lifespan of mice (Hennis et al., 2014). Triple KO mice lacking Parkin, DJ-1, or PINK1 have normal morphology and normal numbers of dopaminergic and noradrenergic neurons in the SNc and LC. Also, levels of striatal DA in these triple KO mice were normal at 16 months, but increased at 24 months of age (Kitada et al., 2009b).

Sonic hedgehog (SHH), nuclear receptor related protein-1 (Nurr1), pituitary homeobox3 (Pitx3), and engrailed 1 (EN1) are transcription factors important to the development and maintenance of the nigro-striatal system (Jankovic et al., 2005; Jiang et al., 2005; Li et al., 2009a; Gonzalez-Reyes et al., 2012; Zhang et al., 2012). Both SHH and Nurr1 KO mice show a progressive loss of DA neurons without LB formation (Jiang et al., 2005; Kadkhodaei et al., 2009; Gonzalez-Reyes et al., 2012). Also, Pitx3 gene mutations cause a complete loss of SNc and VTA DA neurons and altered locomotor activity in mice (Hwang et al., 2003; van den Munckhof et al., 2003). Recently, engrailed 1 heterozygous mice (En1+/–) showed a significant and progressive retrograde degeneration of SNc neurons and dystrophic and swollen striatal TH+ terminals (Nordström et al., 2014). c-Rel (a subunit of the NFκB complex) KO mice also develop a PD-like neuropathology on aging. At 18 months of age, c-rel (–/–) mice exhibit a significant loss of DA neurons in the SNc, loss of dopaminergic terminals and a significant reduction of DA and HVA levels in the striatum. In addition, these mice show age-dependent deficits in locomotor activity and a marked immunoreactivity for fibrillary α-syn in the SNc (Baiguera et al., 2012).

Conditional disruption of the gene for mitochondrial transcription factor A in DA neurons (MitoPark) results in a parkinsonism phenotype in mice that includes an adult-onset, slowly progressive impairment of motor function, DA neuron death, degeneration of nigrostriatal pathways and intraneuronal inclusions (Ekstrand et al., 2007; Good et al., 2011). Also, cell-specific deletion of the essential autophagy gene Atg7 in midbrain DA neurons causes DA neuron loss in the SNc at 9 months, accompanied by late-onset locomotor deficits. Atg7-deficient DA neurons in the midbrain also exhibit early dendritic and axonal dystrophy, reduced striatal DA content, and the formation of somatic and dendritic ubiquitinated inclusions (Friedman et al., 2012).

Recently, it has been suggested that a vesicular monoamine tranporter (VMAT2) defect may be an early abnormality promoting mechanisms leading to nigrostriatal DA neuron death in PD (Pifl et al., 2014). VMAT2-deficient mice display a progressive loss of nigral DA and LC cells, loss of striatal DA and α-syn accumulation (Taylor et al., 2011, 2014). Neuroprotection from MPTP toxicity in VMAT2-overexpressors and enhanced MPTP toxicity in VMAT2-KO mice suggest that interventions aimed at enhancing vesicular capacity may be of therapeutic benefit in PD (Takahashi et al., 1997; Lohr et al., 2014).

CONCLUDING REMARKS

Despite the significant contribution of all of these animal models to our understanding of PD, none of these models reproduce the human condition. If we consider toxic models, significant nigrostriatal degeneration is generally obtained with some motor deficits (particularly in MPTP-treated monkeys). Although no consistent LB-like formation is detected, this issue in the study of PD pathogenesis remains to be demonstrated. On the other hand, although transgenic models offer insights into the causes of PD pathogenesis or LB-like formation, the absence of consistent neuronal loss in the SNc remains a major limitation for these models. Another troubling observation in genetic models is the often inconsistent phenotypes among the lines with the same mutations. Whether or not this is related to an artifact of insertion of the transgene or to the actual genetic background, it would be advisable to test these in more than one line.

In addition to the classical motor abnormalities observed in PD, animal models are increasingly used to study non-motor symptoms (sleep disturbances, neuropsychiatric and cognitive deficits; Campos et al., 2013; Drui et al., 2014). Both toxin-based and genetic models are suitable for studying these non-motor symptoms that are increasingly recognized as relevant in disease-state (McDowell and Chesselet, 2012). Toxins-based models have been mostly used to seek the mechanisms involved in levodopa induced dyskinesias (LID) thus far (Morin et al., 2014). However, recently viral vector-mediated silencing of TH was used to induce striatal DA depletion without affecting the anatomical integrity of the presynaptic terminals and study LID (Ulusoy et al., 2010). And more recently, for the first time, a genetic mouse model overexpressing A53T α-syn in nigrostriatal and corticostriatal projection neurons shows involuntary movements and increased post-synaptic sensitivity to apomorphine (Brehm et al., 2014). It seems unlikely that a single model can fully recapitulate the complexity of the human disease. Future models should involve a combination of neurotoxin and genetic animal models in order to study the progressive neurodegeneration associated with PD. Understanding the mechanisms responsible for this progressive and intrinsic SNc neuronal loss is completely necessary at this point.

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Molecular determinants of selective dopaminergic vulnerability in Parkinson's disease: an update

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Numerous disorders of the central nervous system (CNS) are attributed to the selective death of distinct neuronal cell populations. Interestingly, in many of these conditions, a specific subset of neurons is extremely prone to degeneration while other, very similar neurons are less affected or even spared for many years. In Parkinson's disease (PD), the motor manifestations are primarily linked to the selective, progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). In contrast, the very similar DA neurons in the ventral tegmental area (VTA) demonstrate a much lower degree of degeneration. Elucidating the molecular mechanisms underlying the phenomenon of differential DA vulnerability in PD has proven extremely challenging. Moreover, an increasing number of studies demonstrate that considerable molecular and electrophysiologic heterogeneity exists among the DA neurons within the SNpc as well as those within the VTA, adding yet another layer of complexity to the selective DA vulnerability observed in PD. The discovery of key pathways that regulate this differential susceptibility of DA neurons to degeneration holds great potential for the discovery of novel drug targets and the development of promising neuroprotective treatment strategies. This review provides an update on the molecular basis of the differential vulnerability of midbrain DA neurons in PD and highlights the most recent developments in this field.

Keywords: Parkinson's disease, substantia nigra, ventral tegmental area, dopamine, selective vulnerability, differential vulnerability

INTRODUCTION

The selective death of distinct neuronal cell populations is the key feature of many disorders of the central nervous system (CNS). The identity of the affected neurons as well as the pattern of neuronal degeneration are specific to the respective disorder and determine the clinical signs that are typically associated with the condition. Examples of diseases that are characterized by the selective degeneration of neurons in the CNS include Alzheimer's disease, Parkinson's disease (PD), Amyotrophic Lateral Sclerosis (ALS) and autosomal recessive proximal Spinal Muscular Atrophy. It is largely unknown why different groups of neurons are highly vulnerable to degeneration in different diseases. Moreover, it is unclear why, in a particular condition, some neurons are extremely prone to degeneration while other, very similar neurons are spared over years. In early Alzheimer's disease, distinct subgroups of neurons in layer II of the entorhinal cortex, the subiculum, and the CA1 region of the hippocampus are particularly vulnerable to degeneration, while many other cortical and hippocampal regions do not show pathological signs at this disease stage (Morrison and Hof, 2002, 2007; Stranahan and Mattson, 2010). Amyotrophic Lateral Sclerosis affects both upper and lower motor neurons (Boillée et al., 2006; Talbot, 2014). However, cortical, spinal and lower cranial nerve motor neurons undergo degeneration early in the disease, while the motor neurons of Onuf's nucleus, as well as the oculomotor, trochlear and abducens nerves remain largely unaffected from cell loss even at late disease stages (Alexianu et al., 1994; Kihira et al., 1997; von Lewinski and Keller, 2005). Autosomal recessive proximal spinal muscular atrophy is characterized by the progressive selective loss in particular of the lower motor neurons in the anterior horns of the spinal cord, while upper motor neurons are spared (Talbot and Davies, 2001). In PD, the motor manifestations are primarily linked to the selective loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc; Brichta et al., 2013). In contrast, the very similar DA neurons in the ventral tegmental area (VTA) demonstrate a higher degree of resistance to degeneration (Dauer and Przedborski, 2003). Our current knowledge about the determinants of the differential vulnerability of SNpc and VTA DA neurons in PD is still extremely limited. Undoubtedly, discovery of the factors that mediate this differential vulnerability would have far-reaching implications for the development of promising neuroprotective strategies and the treatment of PD. This review provides an update on the molecular basis of the differential vulnerability of midbrain DA neurons in PD. We will focus on the most recent developments in this field and discuss these data in the light of previous findings and also regarding their relevance in explaining different aspects of the pattern of DA cell loss in PD.

MIDBRAIN DA NEURONS AND THEIR DIFFERENTIAL VULNERABILITY IN PD

The largest groups of DA neurons in the midbrain are located in very close proximity to each other in the SNpc (A9 group) and in the VTA (A10 group). SNpc and VTA DA neurons represent two of the nine major DA neuron groups in the mammalian brain as identified by staining for tyrosine hydroxylase (TH), the enzyme that catalyzes the rate-limiting step in the synthesis of dopamine (Björklund and Dunnett, 2007). In mice and rats, considerably fewer DA neurons are found in the midbrain than in monkeys and humans (**Figure 1**). Moreover, while SNpc and VTA contain comparable numbers of DA neurons in mice and rats, SNpc DA neurons outnumber VTA DA neurons in monkeys and humans. The variations among the reported counts of TH-positive neurons in the SNpc and the VTA for a specific species may at least in part be due to interindividual differences between study subjects, but are most likely the result of different statistical methods that were applied to calculate the total number of DA neurons in the two midbrain nuclei. In addition to the DA neurons, both SNpc and VTA also contain a considerable number of GABAergic neurons, while only a very few glutamatergic neurons are present whose location is mainly restricted to the VTA (Nair-Roberts et al., 2008).

SNpc DA neurons are heavily involved in the control of movement, whereas VTA DA neurons are responsible for the regulation of reward, emotional behavior and addiction. Both groups of neurons are characterized by distinct but overlapping projection patterns that have been extensively investigated in numerous tracing studies (comprehensively reviewed in (Bentivoglio and Morelli, 2005)). The majority of projections that originate from SNpc DA neurons innervate the dorsal striatum, and only some nigral fibers project to the ventral striatum and the cortex. In contrast, VTA DA neurons mainly project to the ventral striatum as well as cortical areas, while significantly fewer projections innervate the dorsal striatum. Both SNpc and VTA DA neurons send minor projections to additional brain regions including the globus pallidus, the subthalamic nucleus, and the habenula. Moreover, a minority of DA projections originating in the VTA projects to the amygdala. DA neurons located in the midbrain also sparsely innervate distinct hippocampal regions. The density of the DA innervation of a distinct brain region often varies significantly between different species. Interestingly, anatomic differences in the DA projection sites correlate with several dissimilarities observed in DA neuron function. A combination of retrograde tracing, electrophysiological and basic molecular studies in mice demonstrated that distinct subgroups of VTA and SNpc DA neurons in confined midbrain territories project to specific striatal, cortical and limbic target regions, and these neurons can be distinguished by their expression levels of dopamine transporter (DAT), their electrophysiological properties and their capacities for dopamine D_2 autoreceptor signaling (Lammel et al., 2008).

Despite the fact that SNpc and VTA DA neurons generate, store and release the same neurotransmitter, that their cell bodies in the midbrain are localized in close proximity to each other, and that significant overlap exists in the brain areas that are innervated by the projections of these neurons, SNpc and VTA DA neurons exhibit a different susceptibility to degeneration in PD. The DA neurons in the SNpc are highly vulnerable to the fatal molecular mechanisms associated with the disease. Along with the formation of alpha-synuclein-rich intraneuronal protein aggregates termed Lewy Bodies in various brain regions (Spillantini et al., 1997; Braak et al., 2003; Burke et al., 2008), the progressive loss of pigmented SNpc DA neurons is a pathological hallmark of PD (Brichta et al., 2013). Calculations of the number of DA neurons (as identified by the expression of TH) in post-mortem tissue from patients with advanced idiopathic (non-genetic) PD and control subjects revealed that, on average, almost 80% of all SNpc DA neurons undergo degeneration in PD (Hirsch et al., 1988; Damier et al., 1999b). DA cell loss is most severe in the ventrolateral part of the SNpc (Halliday et al., 1996; Damier et al., 1999b; Braak et al., 2003), which was also unequivocally

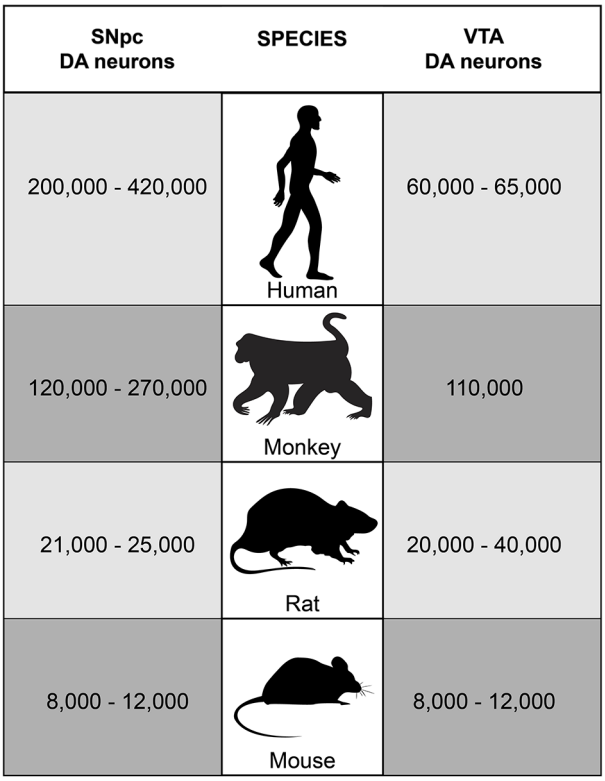

FIGURE 1 | Number of SNpc and VTA DA neurons in humans, monkeys, rats and mice. In adult C57BL/6 mice, approximately 8,000–12,000 TH-positive neurons are located in each of the two neuron groups bilaterally (German et al., 1996; Nelson et al., 1996; Jackson-Lewis and Przedborski, 2007). Studies in rats revealed about 21,000–25,000 TH-positive neurons in the SNpc and about 20,000–40,000 TH-positive neurons in the VTA bilaterally (German and Manaye, 1993; Nair-Roberts et al., 2008). In monkeys and humans, much higher numbers of DA neurons are found in the midbrain. Stereology studies counted approximately 120,000–270,000 TH-positive neurons bilaterally in the SNpc and about 110,000 TH-positive neurons bilaterally in the VTA in rhesus and squirrel monkeys (Emborg et al., 1998; McCormack et al., 2004; Collier et al., 2007; Kanaan et al., 2007). In humans, about 200,000–420,000 TH-positive neurons bilaterally were reported for the SNpc in adults and about 60,000–65,000 TH-positive neurons for the VTA (Hirsch et al., 1988; McRitchie et al., 1997; Damier et al., 1999a; Chu et al., 2002).

demonstrated in a more current study that investigated a large set of post-mortem brain samples obtained from patients with PD (Kordower et al., 2013). These data show that DA cell loss in the SNpc follows a specific pattern and suggest that subtle molecular differences exist among subgroups of SNpc DA neurons. In contrast to SNpc DA neurons, the very similar DA neurons in the VTA are more resistant to degeneration. In their studies, Damier et al. and Hirsch et al. analyzed the number of both SNpc and VTA DA neurons, providing cell counts that can be directly compared with each other due to the investigation of the same midbrain tissue samples and the application of the same statistical methods. The average loss of VTA DA neurons observed in patients with advanced idiopathic PD as compared to unaffected controls was estimated to be only about 50% which is a much lower percentage of DA neuron loss than in the SNpc (Hirsch et al., 1988; Damier et al., 1999b). These data strongly suggest that specific modifiers partially protect VTA DA neurons from degeneration as compared to SNpc DA neurons, and/or that specific modifiers increase the vulnerability of SNpc DA neurons to PD-associated cell loss as compared to VTA DA neurons.

PD can be caused by non-genetic or genetic factors (Cookson and Bandmann, 2010; Houlden and Singleton, 2012; Hirsch et al., 2013; Singleton et al., 2013). However, differential vulnerability among midbrain DA neurons is observed regardless of the disease etiology. Neuropathological findings in published cases with various genetic forms of PD include loss of SNpc DA neurons without describing VTA DA neuron degeneration (Mori et al., 1998; Spira et al., 2001; Gouider-Khouja et al., 2003; Farrer et al., 2004; Zarranz et al., 2004; Zimprich et al., 2004; Pramstaller et al., 2005; Hasegawa et al., 2009). Thus, it is reasonable to conclude that differential DA neuron vulnerability does not depend on the factor triggering PD, but is based on slightly varying intrinsic properties of subgroups of these neurons.

COMPARATIVE EXPRESSION PROFILING OF SNPC AND VTA DA NEURONS

A highly promising approach to identify intrinsic factors that distinguish SNpc from VTA DA neurons and that mediate the differential vulnerability of these neurons to degeneration in PD is the comparative expression profiling of SNpc and VTA DA neurons. Technologies such as laser-capture microdissection (LCM), microarray and next-generation sequencing facilitate the isolation of specific types of DA neurons and the characterization of their gene expression profiles. Comparative expression profiling studies in human brain samples are surprisingly rare. An early study in post-mortem tissue samples from control subjects described the isolation of DA neurons from several different brain regions including the SNpc and the VTA and the subsequent generation of cell-specific RNA expression profiles using a fingerprinting approach (Lu et al., 2004). However, this investigation did not go beyond the description of a methodological protocol and did not provide a list of differentially expressed transcripts or any functional analyses. Since then, several studies have focused on the analysis of the gene expression profiles of laser-captured SNpc DA neurons in patients with PD and control subjects, but a comparison between SNpc and VTA DA neurons was not pursued (Cantuti-Castelvetri et al., 2007; Simunovic et al., 2009; Zheng et al., 2010). The lack of such comparative expression profiling studies may be due to the fact that many brain banks have difficulties providing tissue samples containing VTA DA neurons. It is often standard protocol that donated brains are crudely divided into the left and right hemispheres which are then processed separately. Since VTA DA neurons are located along or close to the midline, the tissue containing these neurons is often destroyed.

In contrast to the lack of human studies, three different studies have applied LCM in combination with microarray analysis to compare the expression profiles of SNpc and VTA DA neurons in rodents (Grimm et al., 2004; Chung et al., 2005; Greene et al., 2005). Another comparative study was carried out by manually dissecting whole SNpc and VTA followed by serial analysis of gene expression (SAGE). However, the main goal of this analysis was not the elucidation of transcripts that are differentially expressed between SNpc and VTA, but rather the overall comparison of the gene expression patterns of 11 different mouse brain regions (Brochier et al., 2008). The rationale for these investigations was that both SNpc and VTA are conserved between humans, mice and rats. Moreover, the differential susceptibility of these two DA neuron groups to degeneration in PD can be reproduced with the neurotoxin MPTP in various animal models for PD (Seniuk et al., 1990; Sirinathsinghji et al., 1992; Muthane et al., 1994; Varastet et al., 1994; Jackson-Lewis et al., 1995). All three comparative microarray analyses demonstrated that SNpc and VTA DA neurons are closely related and only <1% (Grimm et al., 2004) or <3% (Greene et al., 2005) of all detected genes were differentially expressed. A functional analysis of the gene expression patterns of SNpc and VTA DA neurons suggested that many transcripts related to metabolism, transcripts encoding mitochondrial proteins, transcripts related to lipid, protein and vesicle-mediated transport and transcripts related to kinase/phosphatase signaling are more highly expressed in SNpc neurons, whereas transcripts implicated in axon guidance or in neuropeptide signaling were mainly enriched in VTA neurons (Chung et al., 2005; Greene et al., 2005). Grimm et al. found that many genes encoding for proteins involved in synaptic plasticity, cell survival (neuroprotection, detoxification) or axonal pathfinding and neuronal migration are more highly expressed in the VTA (Grimm et al., 2004). Several of the differentially expressed gene candidates were confirmed by all three microarray studies and a small selection of interesting candidates was discussed elsewhere (Greene, 2006). However, the overall overlap observed between these three studies is limited which, at least in part, might be due to the facts that two studies were carried out in rats and one study in mice, and that different microarray chips with comparably small numbers of probe sets were used for sample analysis. Consequently, even with these expression data at hand, the identification of specific key molecular pathways that clearly mediate the selective DA neuron vulnerability in PD has remained challenging. Chung et al. investigated two VTA DA neuron-enriched transcripts in more detail: G-substrate and Rab3b (Chung et al., 2007, 2009). G-substrate is an endogenous inhibitor of Serine/Threonine protein phosphatases, while Rab3b is an isoform belonging to the Rab3 GTPase protein family that is enriched in synaptic vesicles in neurons and involved in synaptic vesicle trafficking. Interestingly,

the virus-mediated overexpression of either candidate in SNpc DA neurons in rats protected these neurons from 6-OHDA-mediated neurodegeneration. However, protein overexpression in a different animal model that more closely resembles the slow, progressive DA neuron loss in PD, such as the subacute MPTP-induced mouse model, was not pursued. Moreover, convincing data indicating that boosting G-substrate or Rab3b function may attenuate SNpc DA neuron degeneration in PD are lacking thus far. Additionally, the development of small molecules that are able to specifically activate Rab3b or G-substrate in the SNpc might be hampered by the expression of both proteins in brain regions other than the midbrain and their apparent involvement in various different signaling pathways whose manipulation could potentially interfere with normal brain function (Endo et al., 2009; Tsetsenis et al., 2011).

Surprisingly, the elucidation of the molecular determinants of the differential vulnerability of SNpc and VTA DA neurons in PD has received little attention since. One study investigated the transcriptional changes in SNpc and VTA DA neurons in mice after the injection of saline or 4 mg MPTP/kg/day for two days or 10 days (Phani et al., 2010). This injection schedule is unconventional and has not been described in the literature. The authors observed a 30% loss of SNpc DA neurons in MPTP-treated animals after two days and a 70% loss after 10 days. In the VTA, only 10% of DA neurons degenerated and this loss did not progress over time. Mice were sacrificed seven days after the final injection. Following the isolation of DA neurons by LCM and RNA purification, expression analysis was carried out using microarrays. The conclusions of this investigation remained very general, stating that a number of genes were up-regulated in the VTA as compared to the SNpc after exposure to MPTP. Almost no overlap existed between the genes up-regulated before and after MPTP treatment, and only very few genes were consistently up-regulated at two days and at 10 days after MPTP treatment, which is somewhat in contrast to the fact that no progressive VTA DA neuron loss was observed. These results suggest that the expression signature of VTA DA neurons may be very variable depending on the respective experimental conditions. However, independent confirmation of this finding using the same or a similar animal model is desired. In a follow-up study, Phani et al. suggested that the exogenous addition of the peptide gremlin, whose mRNA levels were elevated in the VTA DA neurons after MPTP treatment of mice, protects SNpc DA neurons from MPP^+-induced degeneration, possibly via vascular endothelial growth factor receptor 2 (Phani et al., 2013). Data from additional animal models or human data were not presented, and due to its peptide nature, the delivery of gremlin to the SNpc could be problematic for the development of a therapy. Another study laser-captured DA neurons from rats infused with rotenone for 28 days or from mice acutely treated with MPTP and investigated the levels of a set of transcripts related to energy metabolism using quantitative real-time PCR (Greene et al., 2010). SNpc DA neurons demonstrated higher baseline levels and more pronounced changes in the levels of these transcripts than VTA DA neurons. These findings are in agreement with those from Chung et al. and are interesting particularly in light of the predicted higher metabolic demand of SNpc DA neurons which is discussed below. The mechanisms underlying these DA neuron subtype-specific observations remain to be elucidated.

Future studies addressing the gene expression differences between SNpc and VTA DA neurons would be of great interest, in particular if next-generation sequencing is used as an unbiased method to unequivocally identify and more accurately quantify the entirety of all transcripts that are expressed at different stages of DA neuron degeneration. In addition, the preferred application of the more sophisticated bacTRAP methodology over LCM would most likely result in a significant refinement of the available data sets that describe the gene expression profiles of midbrain DA neurons, as bacTRAP focuses on the specific analysis of translated messages and does not include translationally silent RNAs (Doyle et al., 2008; Heiman et al., 2008).

G-PROTEIN-ACTIVATED INWARDLY RECTIFYING POTASSIUM CHANNEL 2 (GIRK2)

GIRK2 has long been considered a protein whose expression pattern may potentially correlate with the differential vulnerability of subgroups of midbrain DA neurons. It represents one family member of a group of ion channels and is involved in the regulation of neuronal activity (Kobayashi and Ikeda, 2006). Weaver mice carry a homozygous missense mutation in the gene encoding for GIRK2 (Patil et al., 1995) which leads to the progressive loss of SNpc DA neurons that starts after postnatal day 7 and reaches a 69% loss on postnatal day 90 (Triarhou et al., 1988; Verney et al., 1995). Ventral SNpc DA neurons are most severely affected by the degeneration (Graybiel et al., 1990). In contrast, the loss of DA neurons in the VTA is delayed and less severe than in the SNpc (Triarhou et al., 1988; Graybiel et al., 1990; Martí et al., 2000, 2007). Although the conclusions presented in these studies are convincing, it is noteworthy that these investigations did not apply state-of-the-art stereological methods to determine the number of surviving DA neurons. Therefore, the presented percentages and cell numbers may slightly deviate from the actual values. Also, caution is required when interpreting these findings in the context of PD, as degeneration in weaver mice starts before the complete maturation of SNpc DA neurons, which may introduce a developmental component that is not present in PD.

Initial *in-situ* hybridization and immunohistochemistry studies in adult control mice showed that most SNpc DA neurons and lateral VTA DA neurons express *Girk2*/GIRK2, while the majority of medial VTA DA neurons seemed to lack detectable *Girk2*/GIRK2 levels (Schein et al., 1998). Schein et al. also suggested that the fraction of VTA DA neurons that are *Girk2*/GIRK2-positive express lower levels than *Girk2*/GIRK2-positive SNpc DA neurons (Schein et al., 1998). These findings implied an overall enrichment of *Girk2*/GIRK2 in the SNpc as compared to the VTA, although the absence or presence of *Girk2*/GIRK2 could not serve as a qualitative marker to clearly distinguish between these two DA nuclei.

Several subsequent studies in humans, mice and rats then suggested that the expression of GIRK2 distinguishes between the DA neurons in the dorsal and ventral tier of the SNpc, as GIRK2 was detected particularly in ventral tier SNpc DA neurons (Mendez et al., 2005; Thompson et al., 2005; Björklund and Dunnett, 2007). These data gave rise to speculations that

GIRK2 may not only differentiate between SNpc and VTA DA neurons, but it may also serve as a molecular marker for ventral SNpc DA neurons which show the highest vulnerability in PD. However, a functional explanation for this observation was not provided.

In an extensive immunohistochemistry study in tissue samples from five human brain donors and six wild-type mice, Reyes et al. recently revisited the expression of GIRK2 protein in midbrain DA neurons (Reyes et al., 2012). This study revealed that some level of GIRK2 is expressed in almost all TH-positive neurons in the SNpc and the VTA in mice and in almost all neuromelanin-positive neurons in human midbrain. In both species, most GIRK2-positive SNpc DA neurons showed strong expression of GIRK2, while few SNpc DA neurons were characterized by weak GIRK2 expression. Importantly, no significant difference was observed between the GIRK2 expression pattern in dorsal and ventral tier SNpc DA neurons. In both tiers, the vast majority of SNpc DA neurons were positive for GIRK2, and similarly high numbers of these neurons showed high GIRK2 expression levels. These findings suggest that GIRK2 expression does not distinguish between dorsal and ventral tier SNpc DA neurons. Most VTA TH-positive neurons in mice and humans also expressed some level of GIRK2. Therefore, the qualitative assessment of GIRK2 expression cannot serve as a tool to discriminate between SNpc and VTA DA neurons. However, strong GIRK2 expression was detected in about half of the VTA DA neurons, which represents a large fraction of VTA DA neurons but is a considerably smaller number than that of DA neurons with high GIRK2 levels in the SNpc. Consistent with these data, Chung et al. recently reported that, overall, *Girk2* mRNA levels are higher in SNpc DA neurons than in VTA DA neurons (Chung et al., 2005). This was demonstrated in adult wild-type mice using LCM of DA neurons followed by quantitative real-time PCR.

Strikingly, another recent immunohistochemistry study in mice further confirmed that GIRK2 is expressed in almost all SNpc TH-positive neurons in both ventral and dorsal tiers, as well as the majority of VTA TH-positive neurons (Fu et al., 2012). Similar results were obtained in an immunohistochemistry study in rats (Eulitz et al., 2007). Fu et al. did not confirm that more SNpc DA neurons express high levels of GIRK2 than VTA DA neurons (Fu et al., 2012).

Taken together, these results indicate that GIRK2 is unlikely to be a marker to distinguish between dorsal and ventral tier SNpc DA neurons, and that the GIRK2 expression pattern does not correlate with the differential vulnerability of these two SNpc DA neuron subgroups in PD. It is unclear at this point if GIRK2 contributes to the selective vulnerability of SNpc and VTA DA neurons. It appears that a larger fraction of SNpc DA neurons than VTA DA neurons express high GIRK2 levels, which is consistent with the overall enrichment of *Girk2* transcript in the SNpc as compared to the VTA. However, the observations that the majority of VTA DA neurons do express some GIRK2 and that a considerable percentage of VTA DA neurons contain GIRK2 levels as high as most SNpc DA neurons complicate matters and weaken the potential relevance of GIRK2 as a therapeutic target, as these findings do not unequivocally correlate with the differential susceptibility of SNpc and VTA DA neurons in PD.

CALBINDIN

Calbindin is a calcium-binding protein that is widely expressed in many brain areas and involved in the regulation of intracellular calcium levels (Liu and Graybiel, 1992). Similar to GIRK2, many studies have suggested calbindin as a marker to distinguish between midbrain DA neurons with different susceptibility to degeneration in PD. Gene expression profiling studies in rats and mice applying LCM in combination with microarray analysis demonstrated that calbindin transcripts are enriched in VTA DA neurons as compared to SNpc DA neurons (Chung et al., 2005; Greene et al., 2005). Immunostaining in mice and rats revealed that TH-positive cells in both SNpc and VTA express calbindin. However, the number of TH-positive VTA neurons that co-express calbindin is higher than that of TH-positive SNpc neurons (Liang et al., 1996; Björklund and Dunnett, 2007). Within the SNpc, TH-positive, calbindin-expressing neurons were mainly found in the dorsal tier. A similar calbindin expression pattern was observed in human midbrain (Mendez et al., 2005).

In their recent immunohistochemistry study in tissue samples derived from five human controls and six wild-type mice, Reyes et al. confirmed many of the previously reported data regarding the expression of calbindin in midbrain DA neurons (Reyes et al., 2012). In the mouse and human SNpc, fewer TH- or neuromelanin-positive neurons contained calbindin than in the VTA. These neurons were mainly localized in the medial and lateral SNpc, and a very small number of calbindin-positive DA neurons were also detected in the dorsal SNpc. However, calbindin-positive neurons were completely absent from the ventral tier of the SNpc. A much larger fraction of TH- or neuromelanin-positive VTA neurons in mice and humans, respectively, contained calbindin. These findings correlate with the overall enrichment of calbindin transcripts in VTA DA neurons over SNpc DA neurons that was reported in the LCM/microarray expression studies discussed above (Chung et al., 2005; Greene et al., 2005). Nevertheless, the expression pattern of calbindin does not accurately reflect the different degrees of DA neuron vulnerability. Many but not all VTA DA neurons express calbindin, and some SNpc DA neurons express calbindin as well, such that calbindin expression cannot serve as a qualitative marker to clearly distinguish between SNpc and VTA. Moreover, calbindin is indeed absent from ventral tier SNpc DA neurons, but not all dorsal tier SNpc DA neurons contain calbindin, which raises the question how closely the expression pattern of calbindin correlates with the differential vulnerability of dorsal and ventral tier SNpc DA neurons.

Fu et al. also revisited the expression of calbindin in mouse midbrain using immunohistochemistry and confirmed that no SNpc DA neurons in the ventral tier express calbindin (Fu et al., 2012). In the dorsal tier, a signal for calbindin was detected in fewer than 2% of SNpc DA neurons, suggesting that some difference exists between the ventral and dorsal tiers of the SNpc. However, many dorsal tier SNpc DA neurons do not follow this expression pattern as they are calbindin-negative, underscoring the view that calbindin does not represent a suitable marker for different degrees of DA neuron vulnerability. Also in agreement with previously published data, it was demonstrated that some TH-positive neurons outside the dorsal tier of the SNpc express

calbindin, particularly in the medial and lateral SNpc, but the number of TH- and calbindin-positive neurons in these SNpc regions was lower than that in the VTA.

Several years ago, it was demonstrated that the presence or absence of calbindin in SNpc and VTA DA neurons correlates with distinct electrophysiological features of these DA neuron subgroups that are mediated by hyperpolarization-activated, cyclic nucleotide-regulated cation (HCN) channels (Neuhoff et al., 2002). Interestingly, another electrophysiological study now identified a novel function for calbindin in midbrain DA neurons expressing this protein (Pan and Ryan, 2012). Using rat midbrain DA neuronal cultures, an inverse correlation was demonstrated between the expression level of calbindin and the probability of vesicle exocytosis, suggesting that calbindin is involved in the control of dopamine release. Based on these results, it appears that calbindin-negative SNpc DA neurons are characterized by a higher dopamine release probability than most VTA DA neurons which are calbindin-positive. However, this study did not address the heterogeneous expression of calbindin that is observed among SNpc DA neurons and also among VTA DA neurons. No experiments were carried out to investigate if and how this mechanism can be linked to the differential vulnerability of DA neurons in PD. Early investigations indicated that calbindin does not directly mediate protection, as the same subgroups of DA neurons were spared from degeneration in MPTP-treated calbindin knockout mice and in their wild-type littermates (Airaksinen et al., 1997). However, some caution is recommended when interpreting these data, as this study did not yet apply the stereological methods that are currently considered state-of-the-art to accurately determine neuronal numbers. In consideration of the available data, and based on the complex expression pattern of calbindin in SNpc and VTA DA neurons, many questions remain regarding the suitability of calbindin as a marker for DA neurons with increased resistance to degeneration in PD and the potential role of calbindin in mediating differential DA vulnerability.

RECEPTORS AND TRANSPORTERS

DA neurons express a variety of cell surface receptors and transporters that may serve as potential binding or entry sites for neurotoxic substances. Providing proof of concept for this hypothesis, it is well established that MPP^+ is taken up specifically by DA neurons via DAT, resulting in mitochondrial damage and ultimately DA neurodegeneration (Dauer and Przedborski, 2003). In the context of differential DA vulnerability, it may be feasible to speculate that in particular the most vulnerable DA neurons express a unique set of transporters and/or receptors that facilitate the uptake of or transmit extracellular signals initiated by PD-related neurotoxins. A number of studies have focused on the expression pattern of various receptors in the midbrain DA system over the years. In one of the most recent investigations, Reyes et al. applied immunohistochemistry and co-staining for TH to analyze the expression patterns of the five dopamine receptors and DAT in post-mortem human midbrain tissue in great detail (Reyes et al., 2013a). The dopamine receptors D1, D2, D3 and D5 were detected in most SNpc and VTA DA neurons, although slightly fewer VTA DA neurons were positive for D1 and D2, respectively, than SNpc DA neurons. Notably, considerably more SNpc DA neurons than VTA DA neurons showed high expression levels of the D1 or D2 receptor. Similarly, while most midbrain DA neurons expressed DAT, significantly more SNpc DA neurons than VTA DA neurons expressed high levels of DAT. Reyes et al. also investigated the levels of glycosylated DAT which is the mature, highly functional version of this transporter (Li et al., 2004). While more than 90% of the analyzed SNpc DA neurons and more than 80% of the analyzed VTA DA neurons expressed some level of glycosylated DAT, high levels of glycosylated DAT were found, in particular, in a fraction of DA neurons located in the most vulnerable ventral tier of the SNpc. This is in agreement with the findings of another immunohistochemistry study in rats and human midbrain (Afonso-Oramas et al., 2009). However, ventral tier SNpc DA neurons did not show uniform expression levels of glycosylated DAT, and fewer than 20% of these neurons were characterized by high levels. If the reported differences in the expression levels of some dopamine receptors and DAT indeed contribute to the differential DA vulnerability, the underlying mechanism remains to be determined. Importantly, D1, D2 and glycosylated DAT are present in almost all midbrain DA neurons, only at slightly different levels, and it is unclear how these rather small expression differences would be able to account for the varying degrees of vulnerability observed between SNpc and VTA DA neurons or between DA neurons in the dorsal and ventral tiers of the SNpc.

In this context, it is noteworthy to mention that SNpc and VTA DA neurons receive input from a considerable number of different brain areas. In an elegant study that was carried out in mice, the Cre/LoxP system was combined with rabies virus-based transsynaptic retrograde tracing to generate a comprehensive list of the brain areas that provide monosynaptic input to SNpc or VTA DA neurons or both (Watabe-Uchida et al., 2012). Not surprisingly, it was demonstrated that each group of midbrain DA neurons is innervated by an overlapping, yet distinct set of brain areas. These data clearly show that SNpc and VTA DA neurons are wired differently in the brain and that each of these two DA neuron groups receives and integrates a distinct set of signals. Although not all of the findings from mice may carry over to the human brain, it can be speculated that SNpc DA neurons may receive toxic inputs that do not reach the VTA, or VTA DA neurons may receive protective signals that do not reach the SNpc. Together, the slightly different connectivity of SNpc and VTA DA neurons within the brain and small differences in the expression levels of specific receptors at the cell surface of SNpc and VTA DA neurons may contribute to the differential vulnerability of these neurons observed in PD. This hypothesis is also interesting in light of a recent investigation in mice which showed that the introduction of preformed fibrils of alpha-synuclein to a particular brain region led to the progressive propagation of Lewy Body/Lewy neurite pathology in other brain regions based on the connectivity of neurons and cell-to-cell transmission (Luk et al., 2012). After the stereotaxic injection of preformed fibrils consisting of recombinant mouse alpha-synuclein into the dorsal striatum, severe alpha-synuclein pathology was observed in the SNpc TH-positive neurons but not in the VTA TH-positive neurons. This is consistent with the fact that the dorsal striatum is heavily innervated by the SNpc DA neurons while VTA DA neurons send significantly

fewer projections to this area, providing an example of how differences in the neuronal connectivity of midbrain DA neurons can lead to the selective damage of distinct DA neuron groups. Importantly, the propagation of alpha-synuclein pathology is a process that very likely also occurs in the human brain, which has been demonstrated by the identification of alpha-synuclein-positive inclusions in DA neuron grafts years after transplantation into the striatum of patients with PD (Kordower et al., 2008a,b; Li et al., 2008).

ELECTROPHYSIOLOGICAL DETERMINANTS

It has long been suggested that SNpc DA neurons are characterized by autonomous pacemaking which is driven by calcium channels (Nedergaard et al., 1993; Mercuri et al., 1994). However, one of the major findings regarding the differential vulnerability of SNpc and VTA DA neurons came from a well-designed electrophysiological study in mice which demonstrated that only adult SNpc DA neurons rely on L-type $Ca_v1.3$ calcium channels for pacemaking, whereas juvenile SNpc DA neurons and VTA DA neurons rely on voltage-dependent sodium channels (Chan et al., 2007). This reliance of adult SNpc DA neurons on calcium for pacemaking seems to result in increased cytosolic calcium levels and elevated levels of oxidative stress, which could explain the higher vulnerability of SNpc DA neurons to damaging insults and degeneration in PD (Guzman et al., 2010). Another study in cultured murine midbrain DA neurons demonstrated that the reliance of SNpc DA neurons on calcium for pacemaking facilitates the L-DOPA-induced increase of dopamine to cytotoxic levels (Mosharov et al., 2009). This L-DOPA-induced increase of intracellular dopamine levels was not observed in VTA DA neurons, which provided further insight into physiological differences between SNpc and VTA DA neurons. However, the molecular basis of these observations remains largely unclear. It is unknown which factors and pathways force maturing SNpc DA neurons into using calcium channels for pacemaking, and why and how VTA neurons escape the switch from sodium to calcium channels. Moreover, it is not understood which endogenous mechanisms SNpc DA neurons possess to counterbalance the continuous, calcium-related oxidative stress in order to survive under control conditions. Clearly, the identification of these SNpc-specific pro-survival mechanisms has the potential to lead to the development of exciting novel therapeutic targets for the treatment of PD.

Electrophysiological differences between SNpc and VTA DA neurons were also revealed in a study focusing on the role of Kir6.2 and SUR1 subunit-containing ATP-sensitive potassium (K-ATP) channels in DA neurodegeneration in mice (Liss et al., 2005). In mouse brain slice cultures treated with the mitochondrial neurotoxins MPP^+ or rotenone, spontaneous electrical activity was abolished exclusively in SNpc DA neurons, while VTA DA neurons remained unaffected. This was explained by the selective activation of K-ATP channels in SNpc DA neurons. Since K-ATP channels in SNpc and VTA DA neurons consist of the same subunits, the molecular composition of the channels did not account for these findings. However, the study suggested that the different responses of SNpc and VTA DA neurons may be due to different degrees of mitochondrial uncoupling, which controls the activation of K-ATP channels, most likely via different levels of reactive oxygen species (ROS). These findings are interesting and warrant further investigation and confirmation of these mechanisms *in vivo*. Interestingly, using LCM and mRNA quantification, a subsequent investigation provided evidence for the increased expression of the K-ATP channel subunit *SUR1* in SNpc DA neurons from patients with PD as compared to controls (Schiemann et al., 2012). Moreover, based on intra-operative measurements in PD patients, it was suggested that K-ATP channel-dependent burst firing is elevated in surviving DA neurons in the SNpc. Although the latter findings in particular need to be interpreted with caution due to the obvious lack of suitable control measurements, these data may further argue for a role of K-ATP channels in the degeneration specifically of SNpc DA neurons.

Bishop et al. established a preliminary link between PD caused by PINK1 mutations, calcium, and the different electrophysiological properties of SNpc and VTA DA neurons (Bishop et al., 2010). A comparison of brain slices from wild-type and *Pink1* knockout mice revealed that PINK1 deficiency in SNpc DA neurons is associated with irregular firing patterns due to the reduced activation of small-conductance calcium-activated potassium channels which was caused by impaired calcium release from the mitochondria and the endoplasmic reticulum. The authors speculated that the observed changes in the SNpc DA neuron firing pattern may eventually contribute to the increased calcium burden of these neurons and further elevate cellular stress. However, direct evidence for this particular hypothesis was not provided in the study. In contrast, VTA DA neurons did not change their firing patterns as a result of PINK1 deficiency, although the activity of small-conductance calcium-activated potassium channels was similarly decreased as in SNpc DA neurons. This was explained by the fact that SK3, the small-conductance calcium-activated potassium channel member whose mRNA is most abundant in midbrain DA neurons, is expressed at lower levels in VTA than in SNpc DA neurons and does not control certain aspects of pacemaking in the VTA as it does in the SNpc (Wolfart et al., 2001). Taken together, these findings are certainly of interest and demonstrate that the different molecular profiles of SNpc and VTA DA neurons may have significant consequences for the physiologic properties of these neurons and their differential response to PD-related changes. However, some of the conclusions of this study are still preliminary and require further investigation to identify all of the molecular components involved in mediating the differential response of SNpc and VTA DA neurons. It would also be of interest to compare the effects of PINK1 deficiency with PINK1 overexpression or to investigate the consequences that other PD-related mutations may have on DA neuron electrophysiology.

DEVELOPMENTAL TRANSCRIPTION FACTORS

The developmental formation of the midbrain, the differentiation of DA progenitor cells, and the induction of DA neuron markers are complex processes that require the spatial and temporal expression of specific sets of transcription factors (reviewed in (Smidt et al., 2003)). It has been demonstrated in mice that a close correlation exists between the developmental position

of different DA progenitors, the expression pattern of specific protein markers that are typically present in subsets of these cells, and the vulnerability of the corresponding adult DA neurons in PD (Smits et al., 2013). Interestingly, some of the transcription factors expressed during neurogenesis remain expressed in the DA neurons during adulthood, and based on their expression patterns in SNpc and VTA DA neurons, a role in mediating the differential vulnerability of midbrain DA neurons has been hypothesized for these proteins.

Pituitary homeobox 3 (PITX3) is a transcription factor that is crucial for the development of SNpc DA neurons. Aphakia mice carry a homozygous null mutation in the *Pitx3* locus which results in the lack of PITX3 expression in the midbrain (Semina et al., 2000; van den Munckhof et al., 2003). While midbrain DA neurons appear normal in aphakia mice until embryonal day E12.5, PITX3 deficiency then interferes with the further development and the maintainance of SNpc DA neurons, resulting in severe DA neuron loss (Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003). In contrast, VTA DA neurons were observed to first develop normally. However, using unbiased stereology to estimate the number of TH-positive neurons, significant loss of VTA DA neurons was found in adult aphakia mice at the age of ∼3 months (van den Munckhof et al., 2003; Luk et al., 2013). Although this loss was less severe than in the SNpc, these data suggest that PITX3 may also play a role for the long-term survival of VTA DA neurons.

For wild-type mice, it was reported that PITX3 is mainly expressed in the DA neurons in the ventral SNpc and in about 50% of VTA DA neurons without demonstrating non-DA neuron expression in the midbrain (van den Munckhof et al., 2003). The DA neurons in the dorsal SNpc were mainly PITX3-negative, which is consistent with the finding that in aphakia mice, some TH-positive cells were spared in the dorsal tier of the SNpc as these neurons may not require PITX3 to survive (van den Munckhof et al., 2003). The expression pattern of PITX3 in the midbrain has been investigated in several studies since and the reported results are controversial. In a study in three- to four-week-old rats, co-immunofluorescence staining for TH and PITX3 revealed that almost all SNpc and VTA DA neurons express PITX3 (Korotkova et al., 2005). A qualitative difference in PITX3 expression between the dorsal and ventral tiers of the SNpc was not observed. Two additional, very recent studies focused on the expression pattern of PITX3 in the midbrain. Luk et al. confirmed the localization of PITX3-positive SNpc DA neurons predominantly to the ventral tier and of PITX3-negative SNpc DA neurons to the dorsal tier in wild-type mice (Luk et al., 2013). It was reported that, in the VTA, many TH-positive neurons contained PITX3, and PITX3-positive and PITX3-negative DA neurons appeared intermixed with each other. However, it is not entirely clear from this study how many VTA TH-positive neurons expressed PITX3. Furthermore, Luk et al. compared the expression pattern of PITX3 with that of calbindin and found that the majority of SNpc DA neurons expressed either PITX3 or calbindin, but not both. In agreement with this observation, it was demonstrated in aphakia mice that the PITX3-negative DA neurons in the dorsal tier of the SNpc which escape degeneration are positive for calbindin. In contrast, the expression of calbindin in PITX3-negative VTA DA neurons was not sufficient to prevent degeneration of all of these neurons, which could point to a potential SNpc-specific survival mechanism of DA neurons expressing calbindin while lacking PITX3. Unfortunately, these findings are currently limited to the description of expression patterns, and a causal relationship between the absence of PITX3, the presence of calbindin and increased resistance to degeneration was not provided. Importantly, the potential function of PITX3 in adult midbrain DA neurons and its relevance for DA neuron vulnerability remains unclear. Immunostaining of brain slices obtained from wild-type mice after treatment with MPTP suggested that PITX3-expressing DA neurons are more vulnerable to the effects of this neurotoxin than PITX3-negative SNpc neurons. While it is well known that ventral SNpc DA neurons are more vulnerable to degeneration than dorsal SNpc DA neurons, convincing evidence for a direct link between this observation and the expression of PITX3 is missing. Interestingly, heterozygous aphakia mice with reduced PITX3 expression showed increased DA neuron loss in the SNpc after MPTP treatment. These results would suggest that PITX3 is neuroprotective, which is in contrast to the higher vulnerability of PITX3-positive SNpc neurons to MPTP than PITX3-negative neurons, and needs to be investigated in more detail to confirm that these observations are indeed due to the presence or absence of PITX3. Moreover, in these studies, MPTP was administered to mice at postnatal day 35, which represents a rather young mouse age at which DA neuron maturation may not entirely be completed, and thus the study results might be influenced by the contribution of developmental factors.

Another immunohistochemistry study in mouse and human tissue samples confirmed the DA neuron-specific expression of PITX3 in the midbrain (Reyes et al., 2013b). However, in humans, PITX3 was found to be expressed in more than 90% of DA neurons in both SNpc and VTA, suggesting that almost all DA neurons in both nuclei express some level of PITX3. In mice, more than 90% of SNpc DA neurons and about 80% of VTA DA neurons expressed PITX3, presenting a similar situation as in the human midbrain. The authors did not observe differences in the PITX3 expression pattern between the dorsal and ventral tiers of the SNpc. Interestingly, an analysis of publicly available human microarray data implied that PITX3 transcripts are enriched in the SNpc as compared to the VTA, possibly pointing to SNpc DA neurons expressing higher PITX3 levels than VTA DA neurons.

Taken together, various studies have described different expression patterns of PITX3 in the midbrain DA neurons even for the same species. The reason for these discrepancies is unknown, although it is possible that different degrees of sensitivity were reached with the different immunostaining protocols that were applied, resulting in the detection of low PITX3 levels in a subset of DA neurons in one study, while another study might have been less sensitive, leaving these DA neurons unlabeled. Therefore, it currently remains unclear if PITX3 can serve as a marker for subsets of midbrain DA neurons with different susceptibility to degeneration. Moreover, the data regarding the enrichment of PITX3 mRNA in the SNpc as compared to the VTA need to be supported by additional studies, as only a very limited number of subjects was investigated by Reyes et al. Furthermore, future investigations should focus on the function of PITX3 in

adult DA neurons to identify the molecular pathways in which PITX3 is involved. These data may provide clues regarding the potential role of PITX3 in regulating the vulnerability of DA neurons to degeneration.

The nuclear receptor NURR1 is another transcription factor that is involved in the specification of midbrain DA neurons. *Nurr1* mRNA is detectable in the mouse midbrain as early as at embryonic day 10.5, and only after this time point, the expression of mRNAs encoding for DA neuron markers such as TH is induced (Zetterström et al., 1997). In *Nurr1* knockout mice, the final differentiation of DA precursors to neurons is inhibited and SNpc and VTA DA neurons are not generated in the midbrain (Zetterström et al., 1997; Castillo et al., 1998; Saucedo-Cardenas et al., 1998). Adult wild-type mice express NURR1 in almost all TH-positive neurons in the SNpc and the VTA, and several studies have demonstrated that NURR1 supports the maintenance and normal function of these neurons (Bäckman et al., 1999; Kadkhodaei et al., 2009, 2013). Differential expression of NURR1 in subgroups of midbrain DA neurons has not been observed, making it unlikely that NURR1 plays a role in selective DA vulnerability. Similarly, the homeobox proteins engrailed-1 and engrailed-2 are expressed in SNpc and VTA DA neurons in mice from early developmental stages into adulthood (Simon et al., 2001). Engrailed-1 seems to be expressed at high levels in all midbrain DA neurons, whereas engrailed-2 is mostly expressed at lower levels and high expression is found only in a subset of DA neurons. In particular, engrailed-1 is of paramount importance for the development of SNpc and VTA DA neurons, and also plays a role in their postnatal maintenance (Simon et al., 2001; Albéri et al., 2004; Sgadò et al., 2006; Sonnier et al., 2007; Nordström et al., 2014). However, based on the published data, the expression pattern of engrailed-1 does not differ between subgroups of DA neurons. Although it has been reported that the expression levels of engrailed-2 are not uniform among midbrain DA neurons in adult mice (Simon et al., 2001), a correlation between engrailed-2 levels and the differential susceptibility of DA neurons has not been carried out.

The neurogenesis of DA neurons in the mouse midbrain is partially controlled by the homeobox protein OTX2 (Omodei et al., 2008). Although OTX2 is expressed in all midbrain DA progenitors, this transcription factor appears to be of varying importance for the development of different subgroups of DA neurons (Di Giovannantonio et al., 2013). Based on data obtained in conditional *Otx2* knockout and *OTX2* overexpressing mice, it was suggested that OTX2 is of paramount importance for the generation of only a specific subgroup of VTA DA neurons (Di Giovannantonio et al., 2013). In adult mice, LCM of SNpc and VTA DA neurons combined with microarray expression analysis revealed that *Otx2* is about 6-fold enriched in the VTA as compared to the SNpc (Chung et al., 2005). This enrichment in the VTA was confirmed by the quantitative real-time PCR analysis of RNA samples collected from mouse or human SNpc and VTA DA neurons, respectively (Chung et al., 2010). Co-immunostaining of adult mouse midbrain for TH and OTX2 revealed OTX2 expression in many VTA DA neurons, but not in SNpc DA neurons (Chung et al., 2010; Di Salvio et al., 2010b). It was demonstrated that the DA neurons in the ventral and central VTA express OTX2. However, OTX2 expression was not exclusively restricted to DA neurons as some non-DA neurons in the VTA were also positive for OTX2 (Di Salvio et al., 2010b). Consistent with the data discussed above, it was observed that OTX2-expressing neurons are often positive for calbindin but only rarely co-express GIRK2. Moreover, the manipulation of OTX2 levels in midbrain DA neurons demonstrated that the depletion of OTX2 in the VTA leads to the expression of GIRK2, increased levels of the functional glycosylated form of DAT and increased neuronal vulnerability to MPTP, which is consistent with a potential neuroprotective role of OTX2 in VTA DA neurons (Di Salvio et al., 2010a). How OTX2 is able to control the expression of glycoslylated DAT and GIRK2 and the molecular pathway connecting altered DA neuron vulnerability with the expression of these proteins currently remains speculation. A neuroprotective role was also suggested by a different study in which OTX2 was either overexpressed or knocked down in primary mouse midbrain cultures using lentivirus technology (Chung et al., 2010). After MPP^+ treatment, OTX2 overexpression resulted in an increased number of TH-positive neurons as compared to control cultures, whereas OTX2 knockdown led to a reduced number of TH-positive neurons, further indicating that OTX2 may decrease the susceptibility of DA neurons to toxin-induced degeneration.

Surprisingly, recent data presented by Reyes et al. imply that the potentially protective role of OTX2 in VTA DA neurons is not conserved between mice and humans (Reyes et al., 2013b). In agreement with previous analyses of adult mouse midbrain tissue, OTX2 immunoreactivity was detected in VTA DA neurons and not in SNpc DA neurons. These findings were consistent in tissue samples obtained from mice at different ages, ranging from four weeks to two years. In contrast, human SNpc and VTA DA neurons did not show any OTX2 expression either in middle-aged (58 ± 16 years old) or in aged control subjects (85 ± 3 years old) although a signal for OTX2 was detected in other brain regions. An analysis of *OTX2* mRNA levels in SNpc and VTA DA neurons in a 24-year-old and a 39-year-old individual suggested that *OTX2* expression is detectable at some point but severely declines with age. The latter data may also explain why Chung et al. were able to detect *OTX2* mRNA in laser-captured human VTA DA neurons (Chung et al., 2010). The findings by Reyes et al. would suggest that OTX2 does not play a neuroprotective role in aged human VTA DA neurons. However, these data need to be considered preliminary as only a very limited number of subjects were included in this analysis.

DELETED IN COLORECTAL CANCER

Besides transcription factors that play a role in the developing midbrain and that have been discussed in the previous section, another developmentally expressed protein has been investigated regarding its differential expression among adult midbrain DA neurons. Deleted in colorectal cancer (DCC), the receptor for the axon guidance molecule netrin, is located at the cell surface and highly expressed in the developing midbrain and at lower levels in the adult midbrain in rodents (Livesey and Hunt, 1997; Stein et al., 2001). The interaction between netrin and the DCC receptor is crucial for the guidance of SNpc and VTA DA axons to their respective striatal target regions (Li et al., 2014).

Interestingly, in the adult rodent midbrain, co-immunostaining experiments revealed that DCC expression is largely restricted to TH-positive neurons, and most DCC-positive DA neurons were located in the ventral part of the SNpc (Osborne et al., 2005). Only few VTA DA neurons expressed DCC, and, for the most part, DCC-positive DA neurons in the SNpc and the VTA were negative for calbindin and vice versa. These data suggest that DCC may be useful as a marker for the population of SNpc DA neurons that are most vulnerable to degeneration. Reyes et al. investigated the DCC expression in human and mouse midbrain and observed that, in humans, more than 90% of DA neurons in the dorsal and ventral SNpc and in the VTA expressed this receptor. However, an overall enrichment of *DCC* mRNA in the SNpc as compared to the VTA was found, implying that DCC is expressed by almost all DA neurons, but the average *DCC* level may be higher in the DA neurons in the SNpc than in the VTA. In mouse midbrain, all ventral SNpc DA neurons expressed DCC, but only ∼85% of dorsal SNpc neurons and ∼62% of VTA DA neurons, somewhat resembling previously published data. Interestingly, in both human and mouse midbrain, ventral SNpc DA neurons showed a stronger signal for DCC than DA neurons in the dorsal SNpc and the VTA, suggesting that the DCC protein level may distinguish between populations of DA neurons with different vulnerability in both species. As with other investigated candidates, it remains unclear if the expression pattern of DCC is directly linked to the selective DA neuron vulnerability and which pathways DCC may use to modulate the susceptibility of DA neurons to degeneration.

MITOCHONDRIA AND ENERGY DEMAND

Several studies have investigated mitochondrial DNA deletions in SNpc DA neurons in human postmortem midbrain tissue samples. High levels of mitochondrial DNA deletions were found in SNpc DA neurons from both aged controls and patients with PD, while other brain regions were characterized by lower deletion levels (Bender et al., 2006, 2008; Kraytsberg et al., 2006). A recent study now compared mitochondrial DNA deletions in aged human control SNpc and VTA DA neurons using LCM followed by quantitative real-time PCR (Elstner et al., 2011). The number of detected deletions was higher in SNpc DA neurons than in VTA DA neurons, however, it is unclear if this difference is a cause or rather a consequence of the differential vulnerability observed among these two groups of neurons. Moreover, although noradrenergic neurons in the locus coeruleus are often more severely affected by degeneration in PD than SNpc DA neurons (Zarow et al., 2003), they showed fewer mitochondrial DNA deletions than SNpc and VTA DA neurons, suggesting that either the number of mitochondrial DNA deletions are unrelated to the selective vulnerability of DA and noradrenergic neurons, or the vulnerability of noradrenergic neurons is regulated by factors different from those that play a role for the vulnerability of SNpc and VTA.

Using immunostaining, Liang et al. studied the intracellular area that is occupied by mitochondria in various neurons in the mouse brain and used these data to draw conclusions regarding the mitochondrial mass (Liang et al., 2007). According to this investigation, SNpc DA neurons have a lower mitochondrial mass than VTA DA neurons, which might contribute to the selective vulnerability of SNpc and VTA. However, SNpc DA neurons appeared to have a similar mitochondrial mass as the much more resistant midline DA neurons in the interfascicular nucleus, and additional neuronal groups with a low mitochondrial mass were identified outside the midbrain which raises the question of how significantly the observed differences correlate with the selective vulnerability of these neurons to degeneration. Moreover, it is entirely unclear if these findings translate to the human brain.

Investigations addressing mitochondrial DNA deletions and the mitochondrial mass are interesting, particularly in the light of hypotheses arguing that the exceptionally high vulnerability of SNpc DA neurons as compared to VTA DA neurons may at least in part be due to their architecture and the resulting metabolic needs. SNpc DA neurons are estimated to form much larger axonal arbors and a higher number of synapses than VTA DA neurons, which may result in the pronounced redistribution of mitochondria to their axonal terminals and the tremendous elevation of their demand for energy as well as their susceptibility to insults jeopardizing the neuronal energy supply (Surmeier et al., 2010; Bolam and Pissadaki, 2012). Moreover, a recent computational analysis proposed that the unique architecture of SNpc DA neurons leads to a higher need of energy for the propagation of action potentials and the maintenance of the resting membrane potential (Pissadaki and Bolam, 2013). Closely related to this topic, a computational model for the degeneration of motor neurons in ALS predicted a link between mitochondrial dysfunction, the lack of ATP, prolonged depolarization and the disruption of intraneuronal calcium levels (Le Masson et al., 2014). Although very compelling, it needs to be pointed out that a significant part of these suggestions is based on computational evidence and not wet lab experiments. Actual comparative measurements of the energy demand of SNpc and VTA DA neurons, studies investigating and comparing the impact of axonal arborization on the distribution of mitochondria between terminals and neuronal cell bodies, and experiments providing direct evidence for a link between the lack of energy and high intracellular calcium levels have not been conducted to date.

AGING

As aging is the major risk factor for developing PD, some investigations focus on the comparison of the changes that occur in SNpc and VTA DA neurons as a function of age, with the goal of identifying factors that mediate the selective vulnerability of midbrain DA neurons. Although interesting, this approach has been characterized by limited success to date. Using immunofluorescence staining of brain tissue obtained from young, middle-aged and old-aged rhesus monkeys, a recent study investigated the degree of nitrative damage in the midbrain DA neurons and concluded that DA neurons in the ventral SNpc show the most severe nitrative damage over time (Kanaan et al., 2008). Although interesting, these data are rather reflective of the differential DA vulnerability than providing an explanation for it. Gao et al. dissected and collected SNpc or VTA from mice of different ages and compared the molecular signature of the two brain regions using microarray (Gao et al., 2013). Differentially expressed genes

were identified and grouped into functional categories. These results can serve as a database for follow-up studies that are required to confirm some of the differentially expressed gene candidates and to link their function to the selective vulnerability of different groups of DA neurons in the midbrain.

ALDEHYDE DEHYDROGENASE 1

Many studies have now provided evidence that molecular differences exist among SNpc DA neurons and among VTA DA neurons, and the most recent investigations addressing this topic have been discussed above. These data demonstrate that, within the SNpc and the VTA, subgroups of DA neurons exist that can be distinguished by subtle variations in their expression profiles. Such findings may potentially explain why not all DA neurons within the SNpc or the VTA demonstrate the same susceptibility to degeneration in PD. Furthermore, on the electrophysiologic level, Lammel et al. have shown that most VTA DA neurons are functionally different from SNpc DA neurons, and that considerable heterogeneity exists, especially among the DA neurons in the VTA (Lammel et al., 2008). However, an increasing body of evidence suggests that electrophysiologic heterogeneity can also be found among SNpc DA neurons. Activity recordings in monkeys demonstrated that functional diversity can be observed among SNpc DA neurons that project to different target regions, as not all of these neurons exhibit the same response to motivational stimuli (Matsumoto and Hikosaka, 2009). A different study in anesthetized mice revealed that burst firing *in vivo* is gated by ATP-sensitive potassium channels in some but not all SNpc DA neurons (Schiemann et al., 2012), and experiments carried out in mouse brain slices showed that some SNpc DA neurons are capable of co-releasing dopamine and GABA, but only a select subset of SNpc DA neurons has the capacity to do so (Tritsch et al., 2012). The molecular determinants of most of these differences and their potential relevance for the selective DA neuron vulnerability have not been identified to date. As discussed above, the higher vulnerability of ventral SNpc DA neurons as compared to dorsal SNpc DA neurons is one of the most well-known examples for heterogeneity among SNpc DA neurons. Interestingly, two studies in mice have demonstrated that aldehyde dehydrogenase 1 (ALDH1A1) is expressed in a subset of DA neurons, most of which are located in the ventrolateral part of the SNpc (McCaffery and Dräger, 1994; Liu et al., 2014). ALDH1A1 belongs to a group of enzymes that catalyze the oxidation of aldehydes to carboxylic acids. Aldehyde dehydrogenases such as ALDH1A1 presumably oxidize and thereby detoxify 3,4-dihydroxyphenylacetaldehyde (DOPAL), a reactive metabolite of dopamine (Marchitti et al., 2007). The inhibition of aldehyde dehydrogenase and the accumulation of DOPAL may contribute to the pathogenesis of PD (Fitzmaurice et al., 2013). Liu et al. now described a connection between the expression of ALDH1A1 and the vulnerability of DA neurons to alpha-synuclein-mediated degeneration. The authors focused their investigations on transgenic mice overexpressing human mutant alpha-synuclein in the midbrain DA neurons, resulting in moderate progressive DA neuron degeneration (Lin et al., 2012). Using these mice, it was shown that ALDH1A1-positive DA neurons in the ventral SNpc are more resistant to degeneration and develop fewer alpha-synuclein aggregates than ALDH1A1-negative SNpc DA neurons which are found in the dorsomedial part of the SNpc. Although very interesting, these findings are somewhat in contrast to the lower resistance of ventral tier SNpc DA neurons than dorsal tier SNpc DA neurons in PD (Kordower et al., 2013). ALDH1A1-positive VTA DA neurons also showed a higher resistance to degeneration than ALDH1A1-negative VTA DA neurons, indicating that ALDH1A1 is not a SNpc-specific susceptibility factor. Knockout of *Aldh1a1* alone was not sufficient to induce DA cell loss, but facilitated DA neuron degeneration in transgenic alpha-synuclein mice. Virus-mediated overexpression of ALDH1A1 in midbrain cultures from transgenic alpha-synuclein mice increased DA neuron survival. ALDH1A1-mediated protection seemed specific to the alpha-synuclein model and was not observed with the use of other neurotoxins *in vitro* which could point to a specific role for ALDH1A1 in diminishing alpha-synuclein-mediated toxicity. The exact mechanism, however, is unclear. The ALDH1A1 expression pattern in the midbrain was conserved between mice and humans. In midbrain tissue from human controls, ALDH1A1 expression was observed mainly in the ventral SNpc DA neurons. Tissue samples from PD patients revealed extensive DA cell loss in the ventral tier of the SNpc which seemed associated with the reduced expression of ALDH1A1 in the remaining ventral tier SNpc DA neurons at various disease stages. These data indicate that ALDH1A1 may potentially have a protective function for human SNpc DA neurons.

LEFT-RIGHT-ASYMMETRY

Last but not least, it is noteworthy that a number of studies discuss a differential vulnerability of SNpc DA neurons in the left and right hemispheres in PD. Many patients with PD demonstrate a pronounced asymmetric manifestation of the cardinal motor signs with one body side being more affected than the other side, and this asymmetry can also be measured in studies that image the DA system in PD patients using tracers (Djaldetti et al., 2006). These findings suggest that the left and right hemispheres contain a different number of SNpc DA neurons in these patients. A correlation of the number of neuromelanin-positive neurons in the SNpc bilaterally in post-mortem brain samples from PD patients with the asymmetric clinical signs that had been documented for these individuals revealed that many but not all cases showed the expected pattern of asymmetric cell loss (Kempster et al., 1989). Additional studies comparing the number of SNpc DA neurons in the left and right hemispheres in patients with PD have not been published. It is not well understood if the observed asymmetry is indeed due to the different vulnerability of left- and right-sided SNpc DA neurons to degeneration, or to other reasons including inborn, side-specific differences in the number of SNpc DA neurons, unbalanced limb exercise, hand dominance or the uneven, localized permeability of the blood brain barrier to toxins (Djaldetti et al., 2006). If differential susceptibility of the left and right SNpc to degeneration indeed plays a role, the molecular basis has yet to be elucidated. Interestingly, several asymmetric animal models have been developed recently, including a monkey model that was generated by a unilateral surgical lesion of the nigrostriatal pathway followed by systemic administration of MPTP, and a genetic DJ1 knockout mouse model

(Blesa et al., 2011; Rousseaux et al., 2012). These models have the potential to serve as tools to further study the molecular basis of asymmetry in PD.

CONCLUSION

The elucidation of the molecular determinants that increase the susceptibility of SNpc DA neurons or decrease the susceptibility of VTA DA neurons to degeneration in PD is a very exciting but challenging research area that holds great potential for the development of novel therapeutic strategies for PD. The gene expression signatures of SNpc and VTA DA neurons are extremely similar to each other. Only a rather limited number of genes within these two groups of neurons demonstrate differences in their expression levels, and the differences are usually small to moderate. Moreover, studies focusing on the identification and characterization of differentially expressed proteins have revealed that the candidate proteins identified to date are not exclusively expressed in either SNpc or VTA DA neurons but rather enriched in either neuronal group at best. Based on these findings, it may be speculated that it is unlikely that only one or two key proteins mediate the selective vulnerability of DA neurons in PD. Instead, it is more likely that a cell type-specific combination of several modest gene expression differences and subtle differences in protein function are responsible for establishing the increased or decreased midbrain DA neuron vulnerability. Although still at the beginning, current research on mitochondria and energy demand, the molecular underpinnings of electrophysiologic differences between subtypes of DA neurons, and developmentally expressed proteins that remain expressed in adult DA neurons appears promising. Emphasis should be placed on the identification of molecular targets that increase the resistance of DA neurons to degeneration. It is likely that multiple toxic factors contribute to DA neuron degeneration, and it will be difficult to slow down or halt cell loss by inhibiting only one or few of the related pathways. However, boosting the function of proteins that support the survival of SNpc DA neurons may provide a powerful tool to improve DA neuron resistance to many of the toxic pathways associated with PD. The non-uniform expression patterns of some proteins among SNpc as well as VTA DA neurons certainly add another layer of complexity to the investigation of differential DA neuron vulnerability. However, the careful consideration of these differences and the correlation of the expression pattern of these gene and protein candidates with the varying vulnerability of DA neuron subgroups may provide additional opportunities for the discovery of genes that play a role in the selective DA vulnerability in PD.

AUTHOR CONTRIBUTIONS

Lars Brichta and Paul Greengard carried out the literature search, developed the concept for the review and wrote the manuscript.

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Calbindin content and differential vulnerability of midbrain efferent dopaminergic neurons in macaques

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Calbindin (CB) is a calcium binding protein reported to protect dopaminergic neurons from degeneration. Although a direct link between CB content and differential vulnerability of dopaminergic neurons has long been accepted, factors other than CB have also been suggested, particularly those related to the dopamine transporter. Indeed, several studies have reported that CB levels are not causally related to the differential vulnerability of dopaminergic neurons against neurotoxins. Here we have used dual stains for tyrosine hydroxylase (TH) and CB in 3 control and 3 MPTP-treated monkeys to visualize dopaminergic neurons in the ventral tegmental area (VTA) and in the dorsal and ventral tiers of the substantia nigra pars compacta (SNcd and SNcv) co-expressing TH and CB. In control animals, the highest percentages of co-localization were found in VTA (58.2%), followed by neurons located in the SNcd (34.7%). As expected, SNcv neurons lacked CB expression. In MPTP-treated animals, the percentage of CB-ir/TH-ir neurons in the VTA was similar to control monkeys (62.1%), whereas most of the few surviving neurons in the SNcd were CB-ir/TH-ir (88.6%). Next, we have elucidated the presence of CB within identified nigrostriatal and nigroextrastriatal midbrain dopaminergic projection neurons. For this purpose, two control monkeys received one injection of Fluoro-Gold into the caudate nucleus and one injection of cholera toxin (CTB) into the postcommissural putamen, whereas two more monkeys were injected with CTB into the internal division of the globus pallidus (GPi). As expected, all the nigrocaudate- and nigroputamen-projecting neurons were TH-ir, although surprisingly, all of these nigrostriatal-projecting neurons were negative for CB. Furthermore, all the nigropallidal-projecting neurons co-expressed both TH and CB. In summary, although CB-ir dopaminergic neurons seem to be less prone to MPTP-induced degeneration, our data clearly demonstrated that these neurons are not giving rise to nigrostriatal projections and indeed CB-ir/TH-ir neurons only originate nigroextrastriatal projections.

Keywords: calbindin, Parkinson's disease, nigroextrastriatal pathway, neuronal tracers, neuroprotection, MPTP

INTRODUCTION

Parkinson's disease (PD) is characterized by a progressive and selective loss of midbrain dopaminergic (DA) neurons. This cell loss follows a heterogeneous pattern as described in PD patients. The greatest loss of DA neurons is found in the substantia nigra pars compacta (SNc, group A9), whereas DA neurons in the ventral tegmental area (VTA, group A10) are known to be less vulnerable (German et al., 1989; Damier et al., 1999a,b; Lu et al., 2006). Within the SNc, neurons in the ventrolateral and caudal regions are more prone to degenerate than those in the rostromedial and dorsal region of the SNc. (German et al., 1989, 1992; Lu et al., 2006).

Animal models for PD showed a similar pattern of midbrain DA neurons loss (German et al., 1988, 1996; Varastet et al., 1994; Liang et al., 1996; Oiwa et al., 2003; Fitzpatrick et al., 2005). Systemic administration of MPTP to non-human primates induces a selective nigrostriatal degeneration mimicking the pattern of differential vulnerability of DA neurons observed in PD patients; the greatest loss being found in ventrolateral territories of the SNc (Schneider et al., 1987; Schneider and Dacko, 1991; Varastet et al., 1994).

It has been suggested that this selective vulnerability of midbrain DA neurons could be related with diverse differentiation routes during embryonic development (Smits et al., 2006; Smidt and Burbach, 2007), originating different DA phenotypes. Although the mechanism responsible for the preferential loss of DA neurons is still under discussion, a number of neuroprotective mechanisms have been suggested. Although several studies appointed the calcium-binding protein known as calbindin (CB; Gerfen et al., 1985, 1987; Yamada et al., 1990; Ng et al., 1996; Yuan et al., 2013) as a putative neuroprotective agent, candidates other than CB such as the vesicular monoamine transporter (VMAT2;

Liu et al., 1992; Harrington et al., 1996; Miller et al., 1999; Caudle et al., 2007; Afonso-Oramas et al., 2009) have also been suggested.

The fact that CB-immunopositive neurons in the SNc are relatively preserved in patients and animal models of PD (Yamada et al., 1990; Lavoie and Parent, 1991; Ito et al., 1992; Damier et al., 1999a; Choi et al., 2008; Yuan et al., 2013), have led to the idea that CB could confer some neuroprotection to DA neurons against degeneration (Gerfen et al., 1985, 1987; Yamada et al., 1990; Ng et al., 1996; Yuan et al., 2013). CB regulates the availability of calcium ions (Ca^{+2}) within the cell, thus buffering the calcium overload and thereby protecting the cell against neurotoxicity (Reisner et al., 1992). In midbrain DA neurons the Ca^{+2} channels are opened much more time than in any other cell types (Wilson and Callaway, 2000), because they show an unusual physiological phenotype; they are autonomously active showing a pacemaking activity (Grace and Bunney, 1983). The need to maintain Ca^{+2} homeostasis includes the coordination of endoplasmatic reticulum pumps, the uptake of Ca^{+2} into mitochondria and lysosome function; these Ca^{+2} pathways interact with the mitochondrial function and oxidative stress both of which appears to be involved in the pathogenesis of PD (Schapira et al., 1990; Selvaraj et al., 2009, 2012; Surmeier et al., 2011; Davey and Bolaños, 2013). Bearing in mind all these data, the CB theory hypothesized that the resilience of CB immunoreactive DA neurons in the midbrain is due to the presence of calcium binding proteins; which effectively sequester Ca^{+2} without using ATP, so CB reduces vulnerability to mitochondrial toxins and seems to confer resistance to the PD-related neurotoxic agents (German et al., 1992; Ito et al., 1992; Damier et al., 1999b; Hurley et al., 2013; Yuan et al., 2013).

Tract-tracing studies in the monkey showed that DA midbrain neurons also differ in their projection patterns (Haber and Fudge, 1997; Smith and Kieval, 2000), nigrostriatal and nigroextrastriatal projections (nigropallidal and the nigrosubthalamic) arise from different groups of midbrain DA neurons (Fallon and Moore, 1978; Lindvall and Björklund, 1979; Lavoie et al., 1989; Smith et al., 1989; Cossette et al., 1999; Hedreen, 1999; François et al., 2000; Jan et al., 2000; Smith and Kieval, 2000; Anaya-Martinez et al., 2006). The nigrostriatal projection arises from the SNc, VTA and retrorubral area (RRA): (1) the postcomissural putamen (sensorimotor striatum) is mainly targeted by DA cells located in the ventral tier of the SNc; (2) the limbic ventral striatum is innervated by DA neurons from the VTA and dorsal tier of the SNc; and (3) the caudate nucleus (associative striatum) is mainly innervated by DA neurons situated in the ventral tier of the SNc (Haber and Fudge, 1997; François et al., 2000; Smith and Kieval, 2000). The external and internal globus pallidus (GPi) as well as the subthalamic nucleus receive sparse collaterals from the nigrostriatal pathway but those nuclei also receive TH input from the nigroextrastriatal projections (Lavoie et al., 1989; Hassani et al., 1997; Cossette et al., 1999; Hedreen, 1999; François et al., 2000; Cragg et al., 2004). The nigropallidal projection is originated in the SNc and RRA (Jan et al., 2000), while the nigrosubthalamic projection arises from the SNc, VTA and RRA (Lavoie et al., 1989; François et al., 2000). The nigroextrastriatal projections also show a topographical organization similar to the one described in the striatum (François et al., 2000; Jan et al., 2000; Rommelfanger and Wichmann, 2010).

Different reports in MPTP-non-human primates have shown that the cells in the midbrain area that are able to resist chronic MPTP treatment are mostly DA neurons that express CB (CB-ir; Lavoie and Parent, 1991; German et al., 1992). The fact that the globus pallidus of MPTP-treated animals showed TH immunoreactive (TH-ir) sparing fibers (Varastet et al., 1994) suggests that the DA surviving neurons in the midbrain are involved in the nigroextrastriatal pathways instead of the nigrostriatal pathway (Parent et al., 1990; Varastet et al., 1994).

The purpose of the present study was to identify the DA midbrain neurons expressing CB in *Macaca fascicularis*, in control and MPTP-treated animals. Once the TH-ir neurons that also express CB-ir were identified in different midbrain areas, neuronal tracers were used to disclose whether these neuronal phenotypes were involved in the nigrostriatal and/or nigroextrastriatal pathways.

MATERIAL AND METHODS

A total of ten naïve adult male *Macaca fascicularis* primates (body weight 3.8–4.5 kg) were used in this study. Animal handling was conducted in accordance with the European Council Directive 2010/63/UE, as well as in agreement with the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research. The experimental design was approved by the Ethical Committee for Animal Testing of the University of Navarra. All animals were captive-bred and supplied by R.C. Hartelust (The Netherlands).

MPTP TREATMENT

The dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Sigma M0896) was administered intravenously to three macaques at a concentration of 0.3 mg/kg (injected weekly) until animals reached a stable parkinsonian syndrome. The severity of the MPTP-induced parkinsonism was evaluated using clinical rating scales (Kurlan et al., 1991) where the highest score was 29. The clinical features used in this scale included: facial expression (0–3), resting tremor (0–3), action or intention tremor (0–3), posture (0–2), gait (0–3), bradykinesia (0–3), balance/coordination (0–3), gross motor skills upper limb (0–3), gross motor skills lower limb (0–3), defense reaction (0–2). The MPTP-treated macaques reached a stable score between 19–23 points that was maintained over a period of 3 months of MPTP washout.

STEREOTAXIC SURGERY, PERFUSION AND TISSUE PROCESSING

Surgical anesthesia was induced by intramuscular injection of ketamine (0.5 mg/kg) and midazolam (5 mg/kg), resulting in deep anesthesia over a period of 2–3 h. Local anesthesia was implemented just before surgery by means of a 10% solution of lidocaine. As prophylaxis they received a single subcutaneous injection of metilprednisolone (10 mg/kg) and dexamethasone (0.1 mg/kg) delivered at the end of the surgical procedure and daily doses of intramuscular injection of enrofloxacin (5 mg/kg) over a period of 7 days. Analgesia was achieved with a single

subcutaneous injection of carprofen (4 mg/kg–0.08 ml/Kg) delivered at the end of the surgical procedure and repeated 24 and 48 h post-surgery. After surgery, animals were kept under constant monitoring in single cages with ad libitum access to food and water.

Stereotaxic coordinates for the putamen, the caudate and GPi nuclei were taken from the atlas by Lanciego and Vázquez (2012). During surgery, target selection was assisted by ventriculography. Selected coordinates: caudate nucleus 1 mm rostral to the anterior commissure, 4.5 mm lateral to the midline and 5 mm dorsal to the intercommisural plane; putamen nucleus 1 mm caudal to the anterior commissure, 11 mm lateral to the midline and 2 mm ventral to the intercommissural plane. GPi 4.5 mm caudal to the anterior commissure, 8 mm lateral to the midline and 1.5 mm ventral to the intercommissural plane.

Two monkeys received a single pressure-injection of 5 μl of unconjugated cholera toxin subunit B (CTB, List Biological Laboratories, Campbell, CA) through a Hamilton syringe (5 mg/ml in 0.01 M phosphate buffer (PB), pH 7.5) in the dorsolateral postcommissural putamen. An additional single pressure-injection of 0.5 μl of Fluorogold (FG) through a Hamilton syringe (10% in 0.01 M PB (pH 7.5) was made in the head of the caudate nucleus. Both injections were made in the same monkey and in the same side of the brain. Two additional monkeys received a single pressure-injection of 5 μl of CTB through a Hamilton syringe (5 mg/ml in 0.01 M PB, pH 7.5) in the internal division of the globus pallidus. Tracer delivery was accomplished in pulses of 1or 0.1 μl every 2 min. Once completed, the microsyringes were left in place for 15 min before withdrawal to minimize tracer uptake through the injection tract.

Two weeks post-surgery, animals were anesthetized with an overdose of 10% chloral hydrate and perfused transcardially. The perfusates consisted of a saline Ringer solution followed by 3,000 ml of a fixative solution containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.125 M PB, pH 7.4. Perfusion was continued with 1,000 ml of a cryoprotectant solution containing 10% glycerin and 2% dimethylsulphoxide (DMSO) in 0.125 M PB, pH 7.4. Once perfusion was completed, the skull was opened, the brain removed and stored for 48 h in a cryoprotectant solution containing 20% of glycerin and 2% DMSO in 0.125 M PB, pH 7.4. Finally, frozen serial sagittal or coronal sections (40 μm-thick) were obtained on a sliding microtome and collected in 0.125 M PB cryoprotectant solution containing 20% of glycerin and 2% DMSO in 0.125 M PB, pH 7.4, as 10 series of adjacent sections.

DETECTION OF TRANSPORTED CTB AND FLUOROGOLD

Immunohistochemical detection of transported CTB and FG was carried out on coronal sections. Sections were incubated with a primary antibody against CTB raised in goat (1:2000; List Biologicals, INC, 703) and rabbit antibody Anti-Fluorescent Gold (1:2000; Chemicon, AB153), diluted in a solution containing 5% of normal donkey serum (NDS) (Jackson inmunoresearch Laboratories, 017-000-121), 5% normal swine serum (Jackson immunoresearch Laboratories, 014-00-121), 0.04% triton X-100 in phosphate buffer (PBS) pH 7.4 overnight. After rinsing in PBS, sections were incubated for 2 h in a solution containing 5% of NSwS, 5% NDS, swine anti-rabbit IgG (1:50, Dako, Z0196) and donkey anti-goat (Jackson Immunoresearch, 705-065-147) diluted in PBS for 90 min; after washes sections were incubated in a solution containing 5% NDS and Goat PAP (1:600 Sigma, P1901) and afterwards washed in PBS and visualized in brown with DAB (Sigma, D5637). Section were washed in PBS and incubated in a solution containing 5% NSwS and Rabbit PAP (1:50 Dako, Z0113 diluted in PBS and visualized using Vector VIP Peroxidase (HRP) Substrate Kit (Vector laboratories, SK-4600). Sections were mounted on gelatin-coated glass slides, dried at RT and coverslip with Dpex (VWR International).

DETECTION OF TRANSPORTED CTB AND FG COMBINED WITH TH AND CB IMMUNOFLUORESCENCE

The following primary antibodies were used in double inmunofluorescence: (1) a mouse anti-calbindin-D-28K (1:2000, Sigma, C9848); (2) a goat anti- TH (1:50, Santa Cruz, sc-7847); (3) a rabbit anti-Fluorescent Gold Antibody (1:2000, Chemicon, AB153); and (4) a rabbit anti-CTB (1:2000, Genway, 18-272-195906).

In the present study we have used the following secondary antibodies: Alexa Fluor® 633 Donkey Anti-Mouse IgG (1:200, Molecular Probes A 21082); Alexa Fluor® 546 donkey anti-goat IgG (1:200, Molecular Probes A 11056), Alexa Fluor® 488 donkey anti-rabbit IgG (1:200, Molecular Probes A 21206), biotinylated Donkey anti-rabbit IgG (1:600, Jackson Laboratories, 711-066-152) and biotinylated Donkey anti-mouse IgG (1:600, Jackson Laboratories, 715-066-150).

For single and triple immunohistochemistry, free-floating sections were incubated in a blocking solution containing 5% of NDS and 0.04% triton X-100 in PBS pH 7.4 for an hour. After that, sections were incubated overnight at room temperature with the appropriate primary antibody or mix of antibodies, diluted in a solution of 5% NDS, 0.04% triton X-100 in PBS.

For single immunohistochemistry, after rinsing in PBS, sections were sequentially incubated with the appropriate secondary antibody diluted in a solution containing 5% of NDS in PBS for 2 h and afterwards washed in PBS. Finally sections were incubated in a solution of HRP-conjugated streptavidin (1:5000, Sigma, E2886) diluted in PBS for 90 min and visualized in brown with DAB (Sigma, D5637).

For triple immunofluorescence, after rinsing with PBS sections were incubated with the appropriated fluorescent secondary antibodies and diluted in a solution containing 5% NDS in PBS for 2 h.

Finally, sections were rinsed in PBS and mounted on Super-Frost Ultra Plus® slides, dried at RT and coverslip with Dpex (VWR International).

SECTIONS SAMPLED AND QUANTIFICATION OF TH^+/CB^+ CO-LOCALIZATION

One series of sections from three control and three MPTP-treated macaques were used for quantifying TH+/CB+ co-localization in double-stained material. Briefly, three equally-spaced coronal sections through the mesencephalon (1.5 mm apart from each

other) were entirely scanned under the confocal microscope. One section was taken at the level of the exit of the third cranial nerve to properly elucidate the boundaries between the SNc and VTA. Next, two more rostral and caudal coronal sections (1.5 mm rostral and caudal to the exit of the third cranial nerve, respectively) were chosen for counting purposes. The percentages of co-localization were gathered from 2,615 +/− 640 TH+ neurons in control macaques and from 1,112 +/− 145 TH+ neurons in MPTP-treated animals.

CONFOCAL VISUALIZATION SETTINGS

Stained samples (immunofluorescence and PLA) were inspected under a Zeiss 510 Meta confocal laser-scanning microscope (CLSM). To ensure appropriate visualization of the labeled elements and to avoid false positive results, the emission from the argon laser at 488 nm was filtered through a band pass filter of 505–530 nm and color-coded in green. The emission following excitation with the helium laser at 543 nm was filtered through a band pass filter of 560–615 nm and color coded in red. Finally, a long-pass filter of 650 nm was used to visualize the emission from the helium laser at 633 nm and color coded in pale blue.

RESULTS

TH AND CB EXPRESSION IN CONTROL AND MPTP-TREATED MONKEYS

All three monkeys intoxicated with MPTP developed a stable parkinsonian syndrome between 5 and 8 months after the initiation of MPTP administration, scoring between 19–23 points in the accumulative Kurlan scale (Kurlan et al., 1991). The immunohistochemistry for TH confirmed the extension of the nigrostriatal damage induced in the three monkeys treated with MPTP, when compared with the TH stain in the control monkeys (**Figure 1**).

Overall, the pattern of degeneration in the ventral midbrain and striatum was similar for the three monkeys. As expected, the caudate and putamen of control monkeys showed numerous and intense TH-ir (**Figure 1A**), whereas MPTP-treated monkeys showed a marked reduction in TH density in both caudate and putamen nuclei (**Figure 1I**). In control monkeys, TH-ir neurons were easily noticed in the dorsal and ventral tiers of the SNc (**Figures 1B–D**). MPTP treatment induced a severe loss of dopaminergic neurons, although few TH-ir cells within the dorsal and ventral tiers of the SNc were still visible. (**Figures 1J–L**). By contrast, only very small differences in TH immunoractivity were observed in the VTA when comparing control and MPTP-treated monkeys (**Figures 1C,K**).

CB immunohistochemistry in control and MPTP-treated monkeys was carried out using sections adjacent to those stained for TH. The striatum, SNc (rostral, medial and caudal), and VTA showed similar levels of CB-immunoreactivity (CB-ir) in both control and MPTP-treated monkeys (**Figure 1**).

Double-labeling stains for TH and CB were carried out in the VTA and SNc in control and MPTP-treated monkeys (**Figure 2**). In control animals, and in keeping with previous studies, the highest percentage of co-localization was found in the VTA, where 58.2% of TH-ir neurons also showed immunoreactivity for CB (**Figure 2**). At the level of the SNc, 34.7% of TH-ir neurons from the dorsal tier (SNcd, medial and lateral territories) also expressed CB-ir, whereas TH-ir neurons in the ventral tier did not colocalized with CB. As expected, TH-ir neurons located in the ventral tier (SNcv) lacked CB immunoreactivity (**Figure 2**). MPTP-treated macaques showed a similar percentage of TH/CB colocalization in the VTA (62.1%). Moreover, most of the surviving TH-ir neurons (88.6%) in the SNcd also expressed CB (**Figure 2**). Similar to what was found in the SNcv for control macaques, TH-ir neurons in the SNcv did not expressed CB in MPTP-treated animals (**Figure 2**).

DISTRIBUTION OF NIGROSTRIATAL-PROJECTING NEURONS WITHIN THE SNc AS SEEN WITH RETROGRADE TRACERS

Control animals received two injections of the retrograde tracers FG and CTB into the caudate and putamen nuclei, respectively. Tracer leakage through the injection tracts was not observed in any of the injected animals (**Figures 3A,B**). In all cases, CTB- and FG -labeled neurons were found throughout rostral, medial and caudal territories of the ipsilateral SNc (**Figures 3C–E**), and in the ipsilateral VTA. Labeled neurons were distributed in clusters across the entire rostrocaudal extent of the SNc. Although both types of projection neurons were intermingled with each other in all clusters, double-labeled neurons were never observed (**Figures 3F–K**). Few scattered cells labeled with either CTB or FG were also found in the VTA.

EXPRESSION OF TH AND CB IN IDENTIFIED NIGROSTRIATAL DOPAMINERGIC NEURONS

To elucidate the presence of CB-ir within identified nigrostriatal-projecting midbrain dopaminergic neurons, triple immunofluoresce stains detecting TH, CB and FG or CTB were conducted in control animals (**Figures 4**, **5**). Following the deposit of FG in the caudate nucleus, labeled neurons were found in both the dorsal and ventral tiers of the SNc, as well as in the VTA (**Figure 4**). All FG immunoreactive neurons (FG-ir) expressed TH. As described above, CB-ir was only found in a subpopulation of TH-ir neurons in the SNcd and in the VTA (**Figure 4**). However, none of the TH-ir/CB-ir neurons in SNcd and VTA showed FG labeling.

Similar results were obtained following the delivery of CTB in the putamen nucleus. All the observed CTB immunoreactive neurons (CTB-ir) were identified as TH-ir in SNcd, SNcv and VTA territories. Furthermore, all CB-ir neurons were also positive for TH. Similarly to what was observed for nigrocaudate projection neurons, none of the nigro-putaminal projection neuron did express CB (**Figure 5**). In other words, our tract-tracing data indicated that although the identified nigrostriatal-projecting neurons (innervating wither the caudate or the putamen nucleus) were all TH-ir, all these neurons completely lacked CB immunoreactivity.

EXPRESSION OF TH AND CB IN NEURONS GIVING RISE TO NIGROEXTRASTRIATAL PROJECTIONS

Although the data gathered from identified nigrostriatal neurons showed that these neurons did not contain CB, the basal ganglia territories innervated by neurons co-expressing TH and

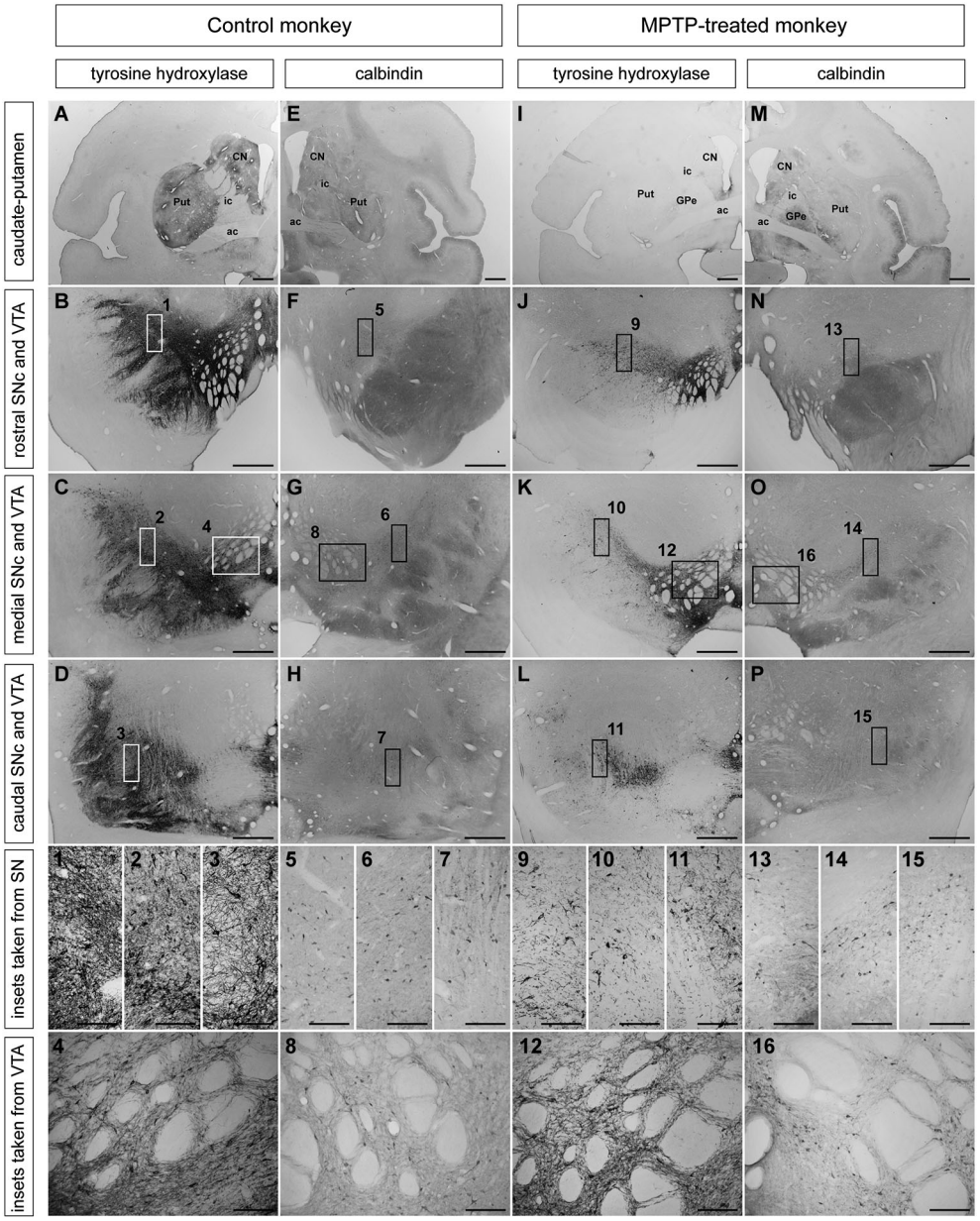

FIGURE 1 | Distribution patterns of TH and CB immunoreactivity in caudate and putamen, SNc and VTA in control and MPTP-treated monkeys. **(A–D)** TH immunohistochemistry in control monkeys in striatum **(A)**, rostral, medial, and caudal SNc **(B–D)** and VTA. **(1–4)** Insets taken at higher magnification of rostral **(1)**, medial **(2)**, and caudal **(3)** SNc and VTA **(4)** showing TH-ir profiles. **(E–H)** CB immunoreactivity in striatum **(E)**, rostral, caudal, and medial **(F–H)** SNc and VTA. **(5–8)** Higher magnification insets of CB-ir cells in rostral **(5)**, medial **(6)** and caudal **(7)** SNc, and VTA **(8)**. **(I–L)** Extent of MPTP-induced dopaminergic depletion. The levels of TH in the striatum and SN that typically characterizes a control primate **(A–D)** are clearly reduced in the MPTP-treated monkeys **(I–L)**. **(9–12)** Higher magnification insets of TH-ir cells in the rostral **(9)**, medial **(10)** and caudal **(11)** SNc as well as in the VTA **(12)**, showing a reduction of the TH immunoreactivity compared with control monkeys **(1–4)**. **(M–P)** calbindin immunohistochemistry in striatum **(M)**, rostral, medial, and caudal SNc **(N–P)** and VTA. **(13–16)** Insets taken a higher magnification from rostral **(13)**, medial **(14)**, and caudal SNc **(15)** and VTA **(16)** showing the presence of CB-ir cells in these areas. Unlike the TH expression, CB immunoreactivity did not changed between control and MPTP-treated monkeys. Scale bars: 1,000 μm in panels **(A, E, I and M)**; 250 μm in panels **(B–D, F–H, J–L and N–P)**; 25 μm in panels **(1–16)**. Abbreviations: substantia nigra, pars compacta (SNc), ventral tegmental area (VTA), putamen (Put), caudate nucleus (CN), external division of the globus pallidus (GPe), internal capsule (ic), anterior commissure (ac).

CB remained to be elucidated. Accordingly, two more control primates were injected with CTB in the internal section of the globus pallidus (GPi) to further identify nigroextrastriatal neurons innervating the GPi (**Figure 6A**). Tracer leakage through the needle tract was not seen in any of the injected animals (**Figure 6A**).

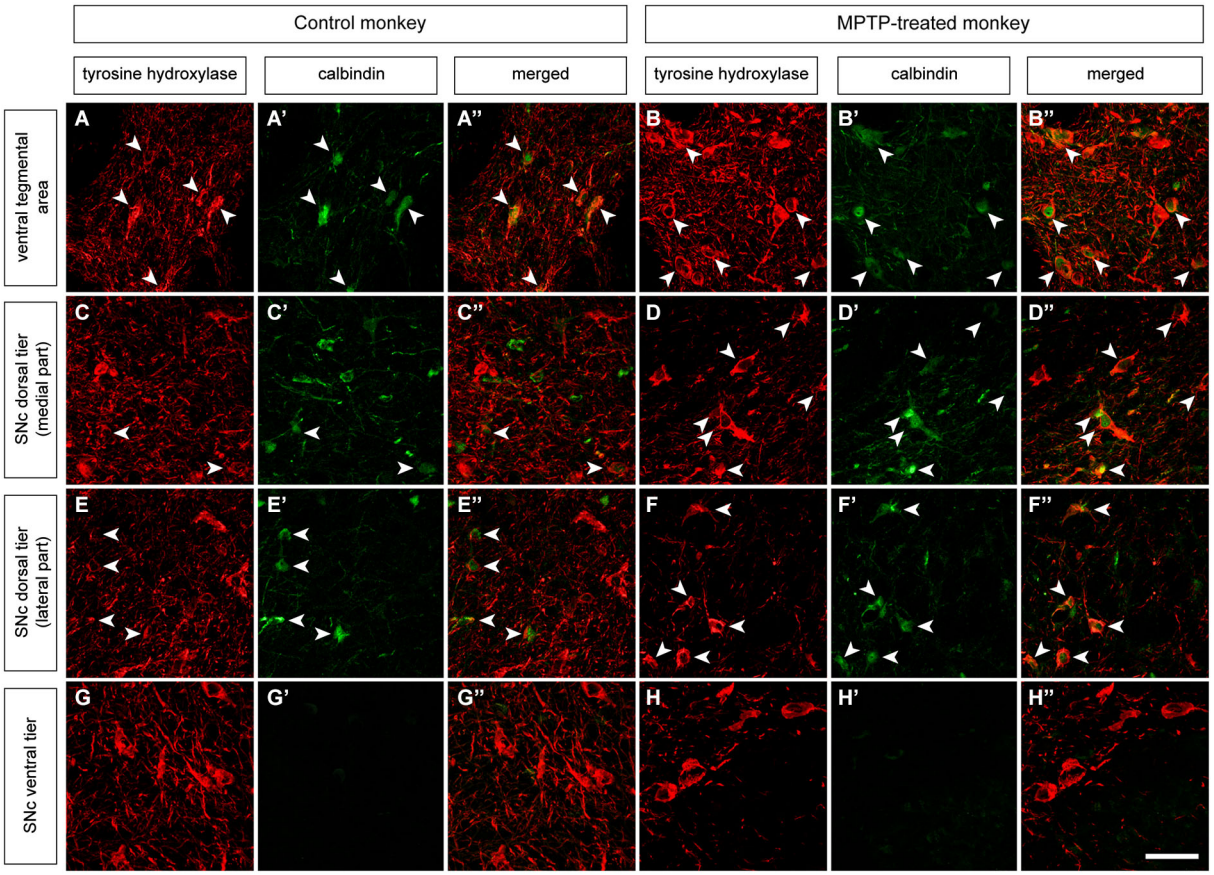

FIGURE 2 | Double immunofluorescent detection of TH and CB in VTA and SNc in control and MPTP-treated monkeys. At the level of the VTA in control macaques **(A–A")**, 58.2% of the CB-ir neurons are also TH-ir, and 62.1% in MPTP-treated animals **(B–B")**. When considering the dorsal tier of the SNc (SNcd, medial and lateral territories) of control animals **(C–C" and E–E")** the percentage of co-localization is estimated as 34.7% (arrowheads). By contrast, in MPTP-treated macaques and besides a substantial loss of TH-ir neurons, most of the surviving dopaminergic neurons expressed CB (arrowheads in **D–D"** and **F–F"**). The percentage of TH-ir/CB-ir neurons increased up to 88.6%. In other words, dopaminergic neurons from the SNcd containing CB are less prone to degenerate following MPTP insult. At the level of the ventral tier of the SNc (SNcv) none of the TH+ neurons expressed CB in control animals **(G–G")**. Panels **(H–H")** are taken from the boundaries between SNcd and SNcv, since most -if not all- of the TH-ir neurons in the SNcv degenerated following MPTP treatment because of the lack of CB in these cells. Scale bar is 25 μm in all panels.

The simultaneous triple stain for TH, CB and CTB showed that TH and CB co-localized in all the observed CTB-ir neurons. In other words, the observed nigro-pallidal projection neurons in the VTA and SNcd were all CB-ir/TH-ir (**Figure 6**).

DISCUSSION

CB have been proposed as a resilience factor in the DA neurons of the midbrain; the presence of this calcium binding protein could efficiently buffer Ca^{+2}, therefore reducing vulnerability to mitochondrial toxins, ultimately conferring resistance to the PD-related toxins (Gerfen et al., 1985, 1987; Yamada et al., 1990; German et al., 1992; Ito et al., 1992; Ng et al., 1996; Damier et al., 1999b; Hurley et al., 2013; Yuan et al., 2013).

In the present work we have studied the content of CB within identified subpopulations of TH-ir cells located in the VTA and the SNc. Our results showed the presence of CB in TH-ir cells located in the VTA and in the SNcd, whereas CB was absent in the TH-ir cells of the SNcv. We also observed the presence of CB within the few TH-ir surviving cells of the SNcd and in the VTA from MPTP-treated monkeys. These results are in keeping with earlier reports (Gerfen et al., 1987; Yamada et al., 1990; Lavoie and Parent, 1991; Varastet et al., 1994; Damier et al., 1999a,b). However, after identifying different subpopulations of nigral efferent neurons innervating the caudate, the putamen or the internal division of the globus pallidus, we have found that nigrostriatal-projecting neurons were all CB negative, whereas co-localization of TH and CB was only found in those nigral neurons giving rise to nigroextrastriatal -nigropallidal- projections.

The highest percentage of co-localization of TH and CB was observed in the VTA (58.2%), followed by the SNcd (34.7%), similarly to previous studies in macaques reporting a co-localization rate of 43% in the VTA and 22% in the SNcd (Lavoie and Parent, 1991). In keeping with Lavoie and Parent (1991), co-localization of TH and CB was only

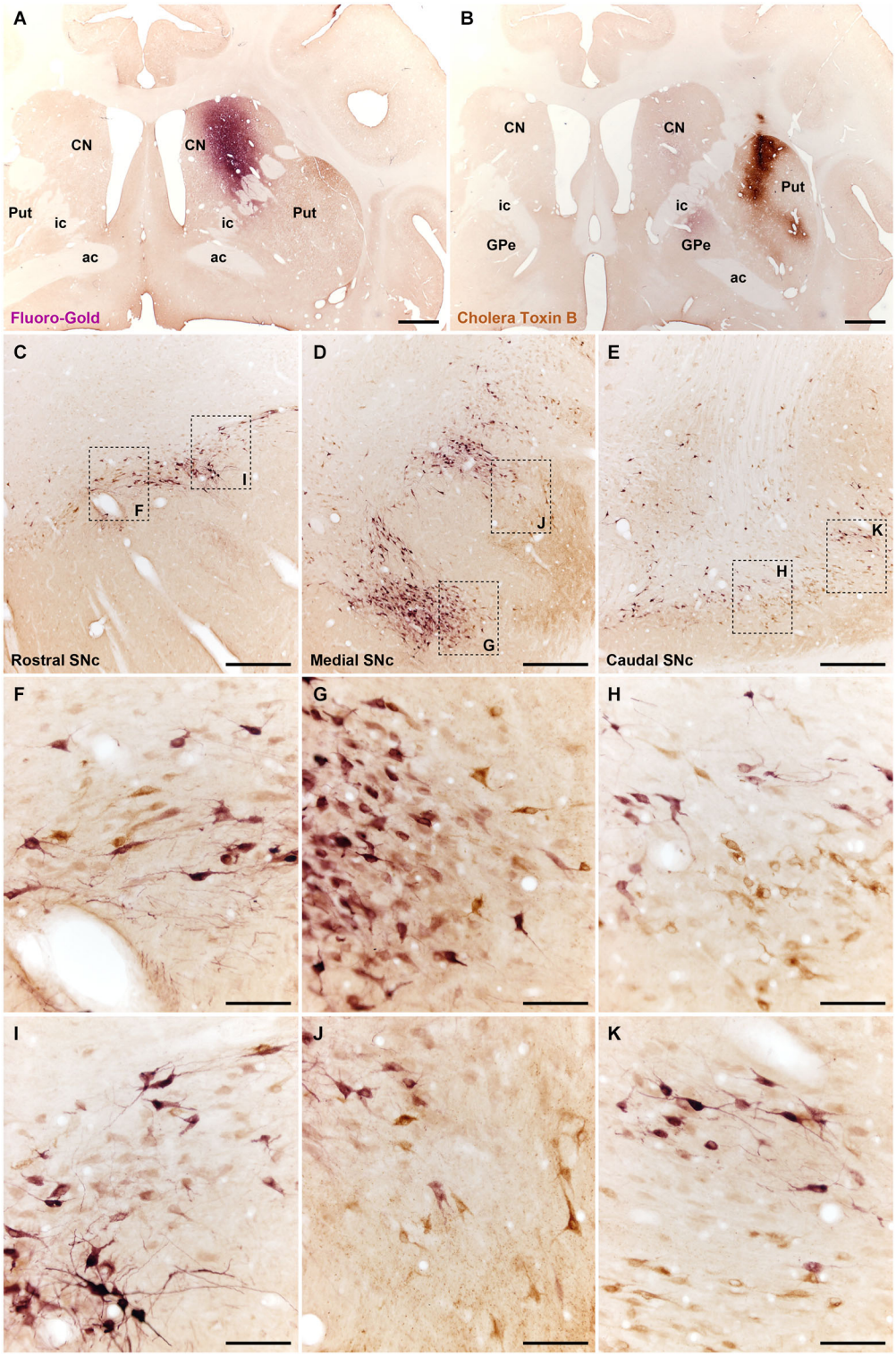

FIGURE 3 | Different types of nigrostriatal-projecting neurons identified following the delivery of retrograde tracers in the head of the caudate nucleus (A; injection of FG) and in dorsolateral territories of the postcommissural putamen (B; CTB deposit). Both injections are restricted to the targeted area, without any noticeable tracer leakage through the injection tract. **(C–E)** coronal sections through the substantia nigra taken from rostral **(C)**, medial **(D)** and caudal **(E)** levels. Neurons innervating either the caudate nucleus (purple-stained; labeled with FG) or the putamen (brown-stained; labeled with CTB) are distributed in clusters containing both subtypes of neurons intermingled with each other. **(F–K)** Insets taken from **(C–E)** at higher magnification to better appreciate the cellular composition of the clusters containing projection neurons. It is worth noting that double-labeled cells, e.g., neurons innervating both the

(*Continued*)

FIGURE 3 | Continued
caudate and the putamen were never noticed. Scale bar is 2,000 μm in panels **(A)** and **(B)**; 500 μm in panels **(C–E)**, and 100 μm in panels **(F–K)**. Abbreviations: putamen (Put), caudate nucleus (CN), external division of the globus pallidus (GPe), internal capsule (ic), anterior commissure (ac).

found in the SNcd, since SNcv neurons lacked CB expression. Those results are also comparable to the TH and CB immunoreactivities observed in the human midbrain, where CB-ir cells were only found in the VTA and SNcd (Damier et al., 1999b).

As expected, the MPTP treatment produced severe dopaminergic depletion in the macaque striatum (German et al., 1988; Varastet et al., 1994; Jackson-Lewis et al., 1995; Mazloom and Smith, 2006; Rico et al., 2010), together with a gradient of neuronal loss in midbrain dopaminergic nuclei (German et al., 1988; Lavoie and Parent, 1991; Rico et al., 2010). After MPTP administration, TH-ir neurons in the SNcv are almost completely removed, followed by neurons in the SNcd, and to a

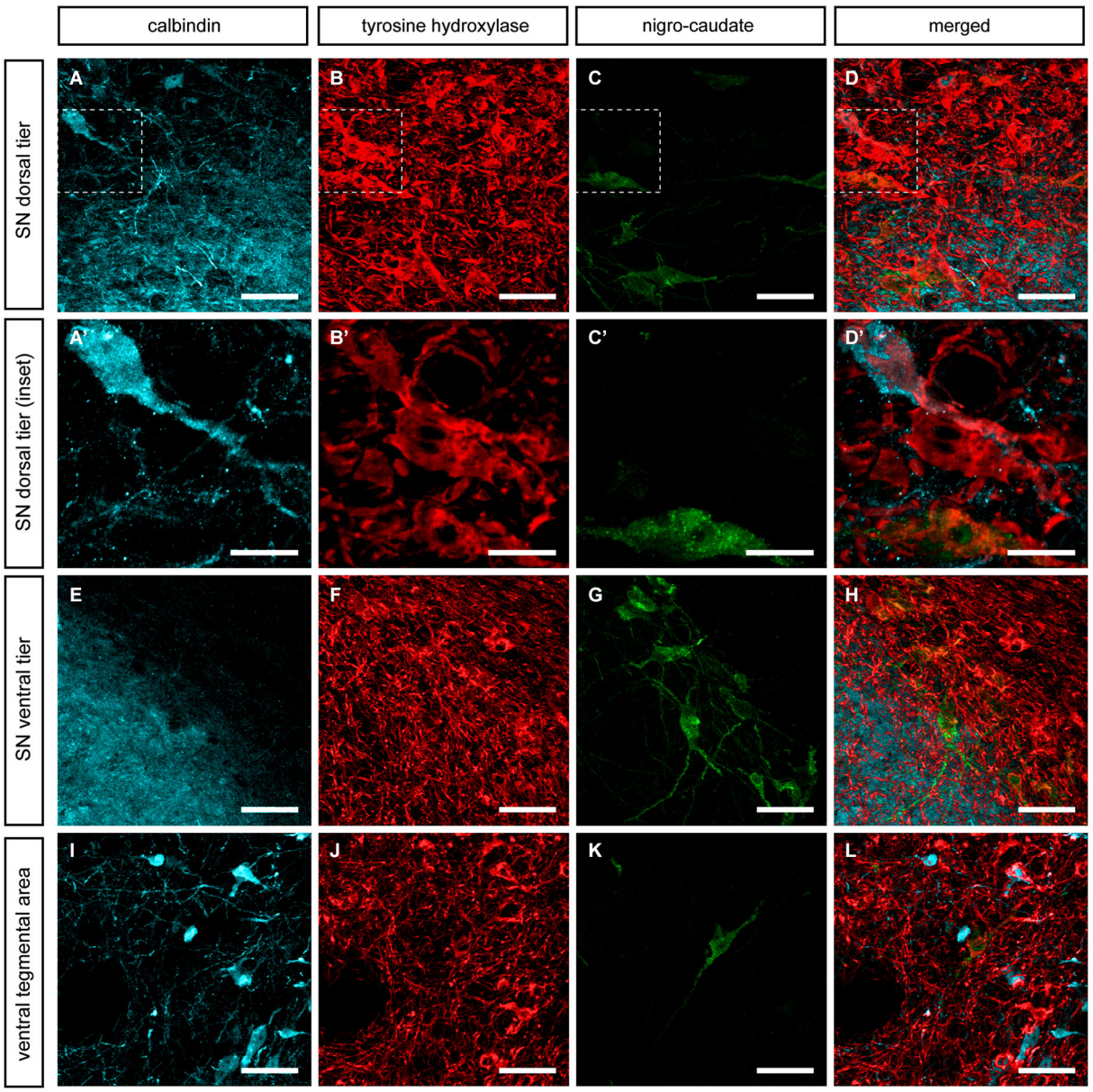

FIGURE 4 | Nigrostriatal neurons innervating the caudate nucleus. Following the delivery of FG in the head of the caudate nucleus, retrogradely-labeled neurons (green channel) were found in the SNcd **(C)**, in the SNcv **(G)** and to a lesser extent in the VTA **(K)**. All these FG-ir neurons are TH-ir and negative for CB. **(A'–C')** Insets taken from **(A–D)** showing one FG+/TH+ neuron, one CB-ir/TH-ir neuron as well as another neuron single-stained for TH only. It is worth noting that FG-ir/TH-ir/CB-ir neurons were never seen. Scale bar is 25 μm for panels **(A–D, E–H and I–L)** and 5 μm in panels **(A'–D')**.

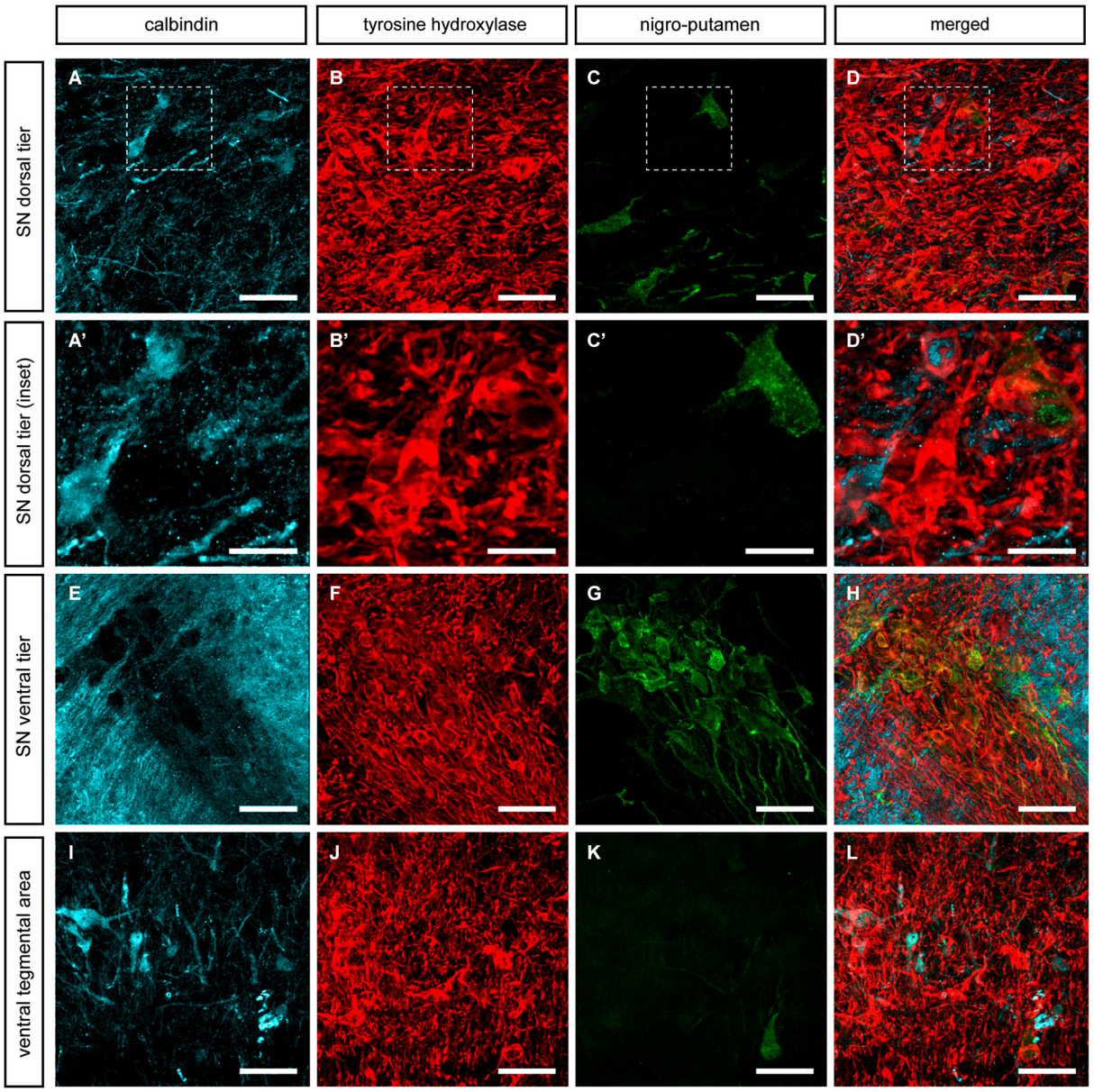

FIGURE 5 | Nigrostriatal neurons innervating the putamen nucleus. Following the delivery of CTB in dorsolateral territories of the postcommissural putamen, retrogradely-labeled neurons (green channel) were found in the SNcd **(C)**, in the SNcv **(G)** and to a lesser extent in the VTA **(K)**. All these CTB-ir neurons are TH-ir and negative for CB. **(A'–C')** Insets taken from **(A–D)** showing one CTB-ir/TH-ir neuron, two CB-ir/TH-ir neurons as well as few more neurons single-stained for TH only. It is worth noting that FG-ir/TH-ir/CB-ir neurons were never seen. Scale bar is 25 μm for panels **(A–D, E–H and I–L)** and 5 μm in panels **(A'–D')**.

lesser extent those dopaminergic neurons in the VTA. This heterogeneous pattern of cell loss properly mimicked the one observed in PD patients (German et al., 1988, 1996; Varastet et al., 1994; Liang et al., 1996; Oiwa et al., 2003; Fitzpatrick et al., 2005). Comparing control and MPTP-treated macaques, similar percentages of TH/CB co-localization were found in the VTA (58.2 vs. 62.1, respectively), whereas at the level of the SNcd, most of the surviving dopaminergic neurons expressed CB following MPTP treatment (88.6% compared to 34.7% in control animals).

Although these data supported the potential neuroprotective effect of CB against MPTP-induced dopaminergic cell degeneration, data gathered from the retrograde tracing studies conducted here envisioned a different argument by showing that nigrocaudate- and nigroputaminal-projecting TH-ir neurons did not expressed CB in the VTA and in the SNcd nuclei. In other words, TH-ir/CB-ir neurons in VTA and SNcd do not innervate the caudate/putamen. Data provided here showed that nigrostriatal-projecting neurons lacked CB, whereas those expressing CB (the most resistant ones against MPTP) were

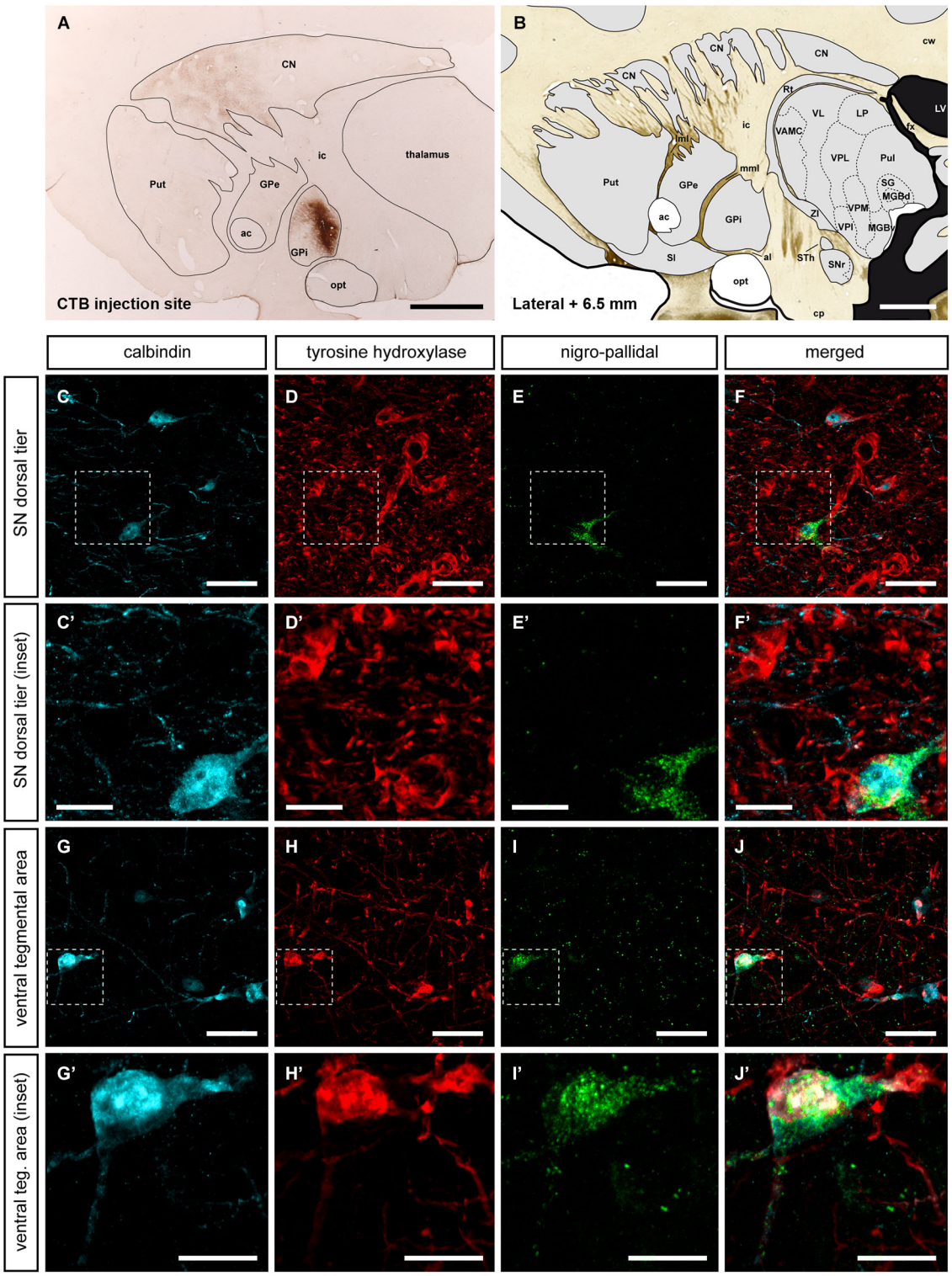

FIGURE 6 | Nigroextrastriatal neurons innervating the internal division of the globus pallidus (GPi). Following the delivery of the retrograde tracer CTB **(A, B)**, very few CTB-labeled neurons were found in the SNcd and in VTA **(E, E'; I, I')**. It is worth noting that nigropallidal-projecting neurons were never found in SNcv. Even at low magnification **(panel A)**, a substantial number of CTB-labeled neurons were easily noticed in the caudate nucleus and to a lesser extent in medial territories of the putamen. At the level of the SNcd **(panels C–F')**, all CTB-ir neurons (green channel) were also positive for both TH and CB. The same holds true when considering the VTA area, since all the VTA neurons projecting to GPi co-expressed CTB, TH and CB **(panels G–J')**. Scale bar is 25 μm in panels **(C–F and G–J)** and 5 μm in panels **(C'–F' and G'–J')**.

those neurons innervating the GPi through nigroextrastriatal projections. It is worth noting that TH-ir fibers are still observed in the GPi after chronic MPTP treatment (Parent et al., 1990; Lavoie and Parent, 1991; Varastet et al., 1994) and indeed earlier reports already suggested that nigroextrastriatal-projecting dopaminergic neurons are less prone to MPTP-induced degeneration (Schneider et al., 1987; Parent et al., 1990; Schneider and Dacko, 1991; Varastet et al., 1994). Nevertheless, it is worth noting that the CTB retrograde tracer deposits made here were all located in the dorsolateral postcommissural putamen and therefore the potential co-localization of CB and TH in VTA/SNcd neurons innervating striatal territories other than the dorsolateral postcommissural putamen cannot be ruled out. Furthermore, nigroextrastriatal targets other than the GPi nucleus such as the subthalamic nucleus and the external division of the globus pallidus were not investigated in this study.

CONCLUDING REMARKS

The potential role of the nigroextrastriatal system in PD pathophysiology has long been neglected (Rommelfanger and Wichmann, 2010) and indeed some studies have suggested that this system plays an important role in the early compensatory changes in PD (Obeso et al., 2004). Here we have found that midbrain dopaminergic neurons innervating extrastriatal targets were the only ones containing CB. Data reported here sustain the presence of a potential imbalance between the nigrostriatal and nigroextrastriatal systems in advanced diseases states. It seems clear that dopaminergic inputs reaching the GPi nucleus are better preserved that striatal dopaminergic innervation. Compared to the early degeneration of the nigrostriatal system, the nigroextrastriatal system is less prone to degenerate, a phenomenon that merits further research efforts in order to properly elucidate their role both in the normal and diseased basal ganglia.

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Striatal vessels receive phosphorylated tyrosine hydroxylase-rich innervation from midbrain dopaminergic neurons

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Nowadays it is assumed that besides its roles in neuronal processing, dopamine (DA) is also involved in the regulation of cerebral blood flow. However, studies on the hemodynamic actions of DA have been mainly focused on the cerebral cortex, but the possibility that vessels in deeper brain structures receive dopaminergic axons and the origin of these axons have not been investigated. Bearing in mind the evidence of changes in the blood flow of basal ganglia in Parkinson's disease (PD), and the pivotal role of the dopaminergic mesostriatal pathway in the pathophysiology of this disease, here we studied whether striatal vessels receive inputs from midbrain dopaminergic neurons. The injection of an anterograde neuronal tracer in combination with immunohistochemistry for dopaminergic, vascular and astroglial markers, and dopaminergic lesions, revealed that midbrain dopaminergic axons are in close apposition to striatal vessels and perivascular astrocytes. These axons form dense perivascular plexuses restricted to striatal regions in rats and monkeys. Interestingly, they are intensely immunoreactive for tyrosine hydroxylase (TH) phosphorylated at Ser19 and Ser40 residues. The presence of phosphorylated TH in vessel terminals indicates they are probably the main source of basal TH activity in the striatum, and that after activation of midbrain dopaminergic neurons, DA release onto vessels precedes that onto neurons. Furthermore, the relative weight of this "vascular component" within the mesostriatal pathway suggests that it plays a relevant role in the pathophysiology of PD.

Keywords: dopamine, midbrain, striatum, Ser 19, Ser 40, cerebral blood flow, Parkinson's disease

INTRODUCTION

DA is a neurotransmitter and neuromodulator involved in a wide range of brain functions including control of voluntary movements, reward-seeking behavior, cognitive processes and circuit formation during development (Schultz, 2007; Money and Stanwood, 2013; Morita et al., 2013). DA also exerts important actions in peripheral organs, with those being on the vascular dynamics control of particular relevance (Tayebati et al., 2011). Studies carried out in the 1970s and 1980s showed that DA and DA agonists have vasomotor effects on major extracerebral arteries and pial arterioles of the cortical surface, promoting a general increase in cerebral blood flow (Toda, 1976; Ingvar et al., 1983; Edvinsson et al., 1985). More recently, it has been shown that DA analogs also modify blood flow into discrete brain regions (Breiter et al., 1997; Marota et al., 2000), and that this effect is prevented by dopaminergic lesion (Chen et al., 1997; Nguyen et al., 2000; Jenkins et al., 2004). In sum, the involvement of DA in the regulation of regional cerebral blood flow is well-established nowadays. However, our knowledge on this issue is still fragmentary and relevant aspects have not been elucidated. For example, while some data suggest that vascular effects of DA require activation of postsynaptic D_1 DA receptors (Knutson and Gibbs, 2007), dopaminergic contacts have also been described in small vessels of the monkey frontal cortex (Krimer et al., 1998), and DA receptor expression has been found in cultured endothelial cells and astrocytes (Bacic et al., 1991; Bal et al., 1994; Zanassi et al., 1999; Choi et al., 2006), suggesting a direct DA effect on vessels and surrounding astrocytes. On the other hand, most interest in cerebral blood flow regulation has been focussed on the cerebral cortex, whereas mechanisms operating in deep brain centers, including the striatum, have been less investigated. It is noteworthy that in spite of the striatum being the main target of midbrain dopaminergic inputs, there is no evidence that striatal vessels and surrounding astrocytes

receive midbrain dopaminergic contacts. The clarification of this question could contribute to a better understanding of metabolic and hemodynamic events associated with striatal processing and their role in the pathophysiology of Parkinson's disease (PD). This possibility has been explored here by using nigral injections of an anterograde tracer in combination with immunofluorescence for dopaminergic, vascular and astroglial markers, confocal microspcopy and dopaminergic lesion.

MATERIAL AND METHODS

Experiments were carried out on 16 male Sprague-Dawley rats (250–300 g; Charles River, L'Arbresle, France) and three male rhesus monkeys (*Macaca fascicularis*, 6–7 years old, 3.5–4.8 Kg). Nine rats were used for the injection of the anterograde neuronal tracer biotinylated dextran amine (BDA) in the dopaminergic cell groups of ventral midbrain, and seven rats for 6-hydroxydopamine (6-OHDA) lesion. The three monkeys were processed for immunofluorescence. Experimental protocols were approved by the Ethical committee of the University of La Laguna (Reference # 091/010), and are in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU) regarding the care and use of animals for experimental procedures.

SURGICAL PROCEDURES

BDA injections were performed according to Lanciego and Wouterlood (2006). A 10% solution of BDA (biotin, dextran 10 kDa; Molecular Probes, Leiden, The Netherlands) in 10 mM phosphate buffer pH 7.25 was iontophoretically delivered in the midbrain dopaminergic formation (1.2–1.8 mm lateral to midline, 5.7–6.0 mm posterior to bregma and 8.0–8.5 mm below the dura, according to Paxinos and Watson, 1998) using a glass micropipette (inner tip diameter 20–30 μm) and a positive-pulsed direct current (7 s on/off) for 7 min. The micropipette was left in place for 5 min before removal. Animals were killed 1 week after injection.

A rat model based on the intracerebroventricular injection of 6-OHDA was used for dopaminergic lesion. This model causes bilateral degeneration of mesostriatal dopaminergic neurons, and a motor syndrome composed of hypokinesia, purposeless chewing and catalepsy (Rodríguez et al., 2001; Rodríguez-Díaz et al., 2001; González-Hernández et al., 2004). Rats were injected in the third ventricle (midline, 2 mm posterior to bregma and 8 mm below the dura, according to Paxinos and Watson, 1998) with vehicle (0.9% saline solution with 0.3 μg/μl ascorbic acid, sham group, $n = 3$) or a single dose (400 μg) of 6-OHDA (6-hydroxydopamine hydrochloride, Sigma, St. Luis, MO; in 8 μl of vehicle per injection; 1 μl/min, 6-OHDA groups, $n = 4$). Anesthesia, pre-surgery treatment and intraventricular injection protocols followed Rodríguez et al. (2001). Bearing in mind that the bilateral degeneration of DA-cells can cause adipsia and aphagia (Zigmond and Stricker, 1973), the intake of food and water was monitored following the 6-OHDA injection. No body weight loss was observed and rats were killed 2 weeks after injection.

TISSUE PROCESSING

Animals were deeply anesthetized with an overdose of sodium pentobarbital and transcardially perfused with heparinized ice-cold 0.9% saline (150 ml in rats, 1 l in monkeys) followed by 4% paraformaldehyde in phosphate buffer saline 0.1 M pH 7.4 PBS; 300 ml in rats and 2.5 l in monkeys). The brains were then removed, the midbrain and forebrain blocks were stored in the same fixative at 4°C (8 h in rats and 18 h in monkeys), cryoprotected in a graded series of sucrose-PBS solutions and stored at −80°C until processing. Coronal sections (25 μm in rats, 40 μm in monkeys) were obtained with a freezing microtome, collected in parallel series and processed for single and double immunohistochemical labeling.

For detecting BDA-stained fibers, floating sections were immersed for 30 min in 3% H_2O_2 to inactivate endogenous peroxidase, washed several times in PBS, and then incubated for 90 min in either ExtrAvidin-peroxidase (1:5000, Sigma) or Cy2-conjugated ExtrAvidin (1:1000; Amersham, Buckinghamshire, England) and 0.3% TX-100 in PBS. In sections incubated in ExtrAvidin-peroxidase, stained fibers were visible after immersion for 5–10 min in 0.005% 3′-3′-diamiobenzidine tetrahydrochloride (DAB, Sigma) and 0.001% H_2O_2 in cacodylate buffer 0.5 N, pH 7.6.

Sections incubated in Cy2-conjugated ExtrAvidin were washed several times in PBS, and incubated for 60 min at room temperature (RT) in 4% normal goat serum (NGS, Jackson ImmunoResearch, West Grove, PA) in PBS, and overnight in PBS containing 2% NGS and one of the primary antibodies: mouse anti-tyrosine hyroxylase (TH) monoclonal antibody (Sigma, 1:12,000), rabbit anti-TH phosphorylated at Ser19 (THp19) polyclonal antibody (PhosphoSolutions, Aurora, CO, 1:2000), rabbit anti-THp31 polyclonal antibody (PhosphoSolutions, 1:600), rabbit anti-THp40 polyclonal antibody (PhosphoSolutions, 1:600), goat anti-dopamine transporter (DAT) polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-endothelial nitric oxide synthase (eNOS) monoclonal antibody (1:1000, Sigma), mouse anti-glial fibrilary acidic protein (GFAP) monoclonal antibody (1:2000, Sigma), or mouse anti-vimentin monoclonal antibody (1:400, Abcam, Cambrige, UK). Immunofluorescent labeling was visible after incubation for 3 h in Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:150; Molecular Probes, OR) and Rhodamine (TRITC) -conjugated goat anti-guinea-pig IgG (1:100; Jackson ImmunoResearch), Lissamine Rhodamine-conjugated donkey anti-goat IgG (1:100; Jackson ImmunoResearch) or Lissamine Rhodamine-conjugated goat anti-mouse IgG (1:100; Jackson ImmunoResearch) in PBS containing 1:200 NGS.

After several rinses, the sections were mounted on gelatinized slides, air dried, coverslipped with Vectashield (Vector), and examined under a confocal laser scanning microscopy system (Olympus FV1000, Hamburg, Germany) using appropriate filters. Sections were first examined using low-magnification lenses, and the areas of interest were analyzed at high magnification (×63 oil-immersion Plan-Apochromat objective lens, NA 1.4) at a resolution of 1024 × 1024 pixels, and acquired in Z-stack mode (10 μm total thickness, 6 z-steps). Selected high-resolution confocal images were then deconvoluted by using the MetaMorph 6.1r 0 software (Molecular Devices, Downingtown, PA). Deconvolution is a post-adquisition computational process for correcting the optical blur inherent to any image adquisition system, restoring

the appearance of original image with the highest degree of confidence (Sibarita, 2005; Salin et al., 2008).

In addition, a quantitative analysis of double labeled terminals (THp19-DAT and THp40-DAT) was performed in high magnification images. Four striatal sections 100 μm apart were randomly selected in five rats. Four vessel-centered 200 μm × 200 μm square regions were also randomly selected in each section. Simple and double labeled terminals were counted within 100 μm from the putative border of the vessel. Data are expressed as the percentage of double labeled terminals per section ± standard error of the mean.

RESULTS

Striatal terminals were studied in rats whose BDA injection was restricted to the midbrain dopaminergic cell groups (substantia nigra pars compacta (SNC) and ventral tegmental area (VTA), **Figure 1A**). Cases with injection involving neighboring centers were excluded. Consistent with previous reports (Gerfen et al., 1987; Joel and Weiner, 2000), the regional distribution of striatal BDA positive fibers varied with the location of the injection site. Injections in VTA and/or the rostromedial region of the SNC provided a terminal field preferentially localized in the ventral striatum and the ventral part of the dorsal striatum, while after injections in the caudolateral region of the SNC, terminals spread out across the dorsal striatum.

Axons through the ventral and dorsal striatum showed collateral fibers emitting terminals in close apposition to the wall of blood vessels of diverse diameters (range 50–500 μm). They usually arise in the form of individual branches that emit isolated perivascular endings (**Figures 1C,E,G**), but sparse perivascular terminal plexuses were also found (**Figures 1B,D,F**). Double labeling for BDA and vimentin (a marker of intermediate filaments present in vessels and astrocytes) or GFAP (an astroglial marker) confirmed the presence of BDA-positive terminal and "en passant" buttons abutted on the wall of striatal vessels (**Figure 1H**) and astroglial cells wrapping them (**Figure 1I**).

In order to confirm the dopaminergic nature of this projection, striatal sections of BDA-injected rats were further processed for TH (the rate-limiting enzyme in DA synthesis) and DAT. For TH immunohistochemistry, antibodies against the native (non-phosphorylated) as well as the phosphorylated forms of TH at serine 19 (THp19), serine 31 (THp31) and serine 40 (THp40) were used. Immunochemistry for native TH resulted in a diffuse striatal staining (**Figure 2A**), making it difficult to identify double TH-BDA positive axons. Consistent with previous reports supporting the idea that under basal conditions only a very small proportion of TH is phosphorylated (Haycock et al., 1998; Salvatore et al., 2001; Bobrovskaya et al., 2004), immunostaining for the phosphoryated TH (THp) forms was significantly lower than for native TH, and was restricted to discrete striatal regions. Thus, immunoreactivity for THp31 was virtually undetected in our material (data not shown), and for THp19 and THp40 was confined to the olfactory tubercle (OT), the accumbens shell and small patches throughout the ventral and dorsal striatum (**Figures 2B,C**). The similarity in shape and distribution between THp patches and striosomes (see Graybiel and Ragsdale, 1978) prompted us to perform double labeling for THp19 or THp40 and mu-opioid receptor (a marker of striosomes; Desban et al., 1993). However, no colocalization between both markers was found (**Figure 2D**), indicating that THp-rich patches preferentially localize in the matriceal compartment of the striatum. Interestingly, all the patches surround vascular profiles. The combination of immunolabeling for THp (Ser19 or Ser40) and two vessel markers, the endothelial form of nitric oxide synthase (eNOS, **Figure 2E**) and vimentin (**Figure 2F**), confirmed that THp-rich patches form dense terminal plexuses around striatal vessels, even penetrating their external layers (**Figures 2J,K** arrows). The presence of THp perivascular plexuses was also explored in monkeys. As shown in **Figures 2G–I**, small and large vessels in the ventral and dorsal striatum of monkeys were also surrounded by dense plexuses of THp terminals. Furthermore, double labeling for THp (Ser19 or Ser40) and GFAP in rats showed that THp-terminals are in close apposition to perivascular astroglial processes (**Figures 2L,M** arrows).

As previously reported (González-Hernández et al., 2004), immunostaining for DAT showed a homogeneous field of well-defined striatal terminals (**Figure 3A**). A low magnification view of double labeling for DAT and THp (Ser 19 and Ser 40) in rats showed co-localization in practically all perivascular plexuses (**Figures 3A–C**). The count of double labeled terminals at higher magnification revealed that within 100 μm from striatal vessels, virtually 100% (98.4 ± 0.9%) of positive THp19 terminals were immunoreactive for DAT, and 97.7 ± 1.1% of DAT terminals were also immunoreactive for THp19 (**Figures 3D–F**). Furthermore, 97.4 ± 0.8% of THp40 terminals were immunoreactive for DAT, but THp40 immunoreactivity was not detected in 12.8 ± 1.3% of DAT terminals. These findings confirm the dopaminergic nature of THp plexuses and that both serine residues are phosphorylated in most perivascular terminals. Further supporting the midbrain dopaminergic origin of the THp-rich innervation of striatal vessels, BDA-positive axons and perivascular terminals were immunoreactive for both DAT (**Figures 3G–I**) and THp19 (**Figures 3J–L**) or THp40 (**Figures 3M–O**). Moreover, striatal THp perivascular plexuses disappeared after midbrain dopaminergic lesion in parallel with the loss of midbrain DA-cells (6-OHDA, **Figure 4**). In sum, our results indicate that striatal vessels receive midbrain dopaminergic inputs that form dense perivascular plexuses of THp-rich terminals.

DISCUSSION

The cerebral blood flow is controlled by two major neurovascular systems. One of them, known as the extrinsic system, consists of fibers from sympathetic and parasympathetic extracranial ganglia and the sensory trigeminal ganglion that release different vasoactive substances on the wall of extracerebral arteries and pial arterioles for the regulation of the global cerebral perfusion (Drake and Iadecola, 2007; Willie et al., 2014). The other one, known as the intrinsic system, consists of fibers arising from different neurons in the brain which project directly to parenchymal microvessels or astroglial end-feet adjoining them for the regulation of highly localized changes in blood flow coupled with regional synaptic activity (Iadecola, 2004; Hamel, 2006; Drake and Iadecola, 2007; Attwell et al., 2010; Lecrux and

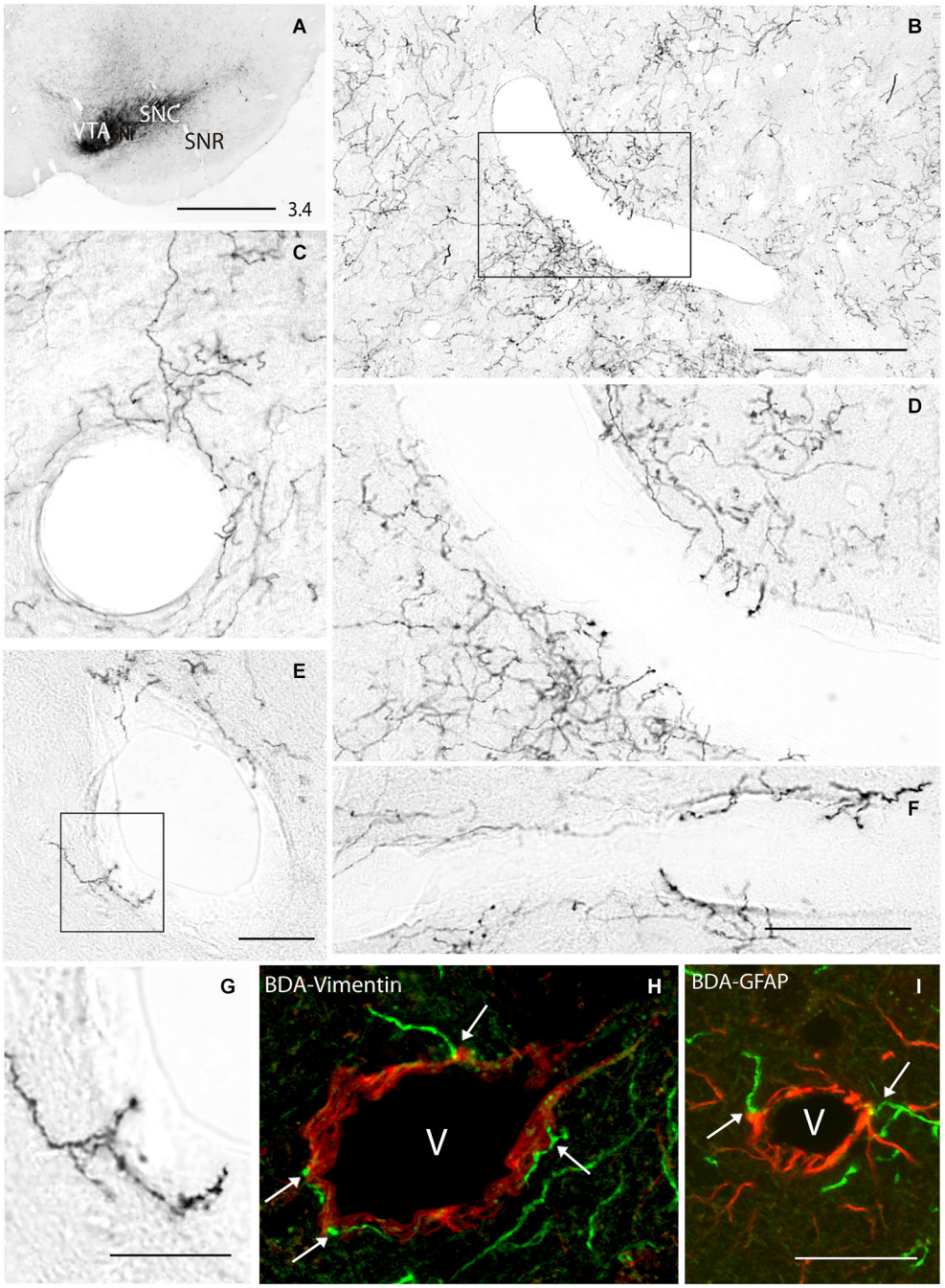

FIGURE 1 | (A) Biotinylated dextran amine (BDA) injection in the substantia nigra pars compacta (SNC) and ventral tegmental area (VTA) of the rat. **(B–G)** BDA-positive fibers emitting terminals in close apposition to striatal vessels, some of them in the form of individual axons **(C,E,G)**, and others forming sparse plexuses **(B,D,F)**. **(H,I)** Double labeling for BDA and vimentin **(H)** or GFAP **(I)** showing terminals abutted on striatal vessels and surrounding astrocytes (arrows in **H** and **I**). **(D,G)** Boxed areas in **(B)** and **(E)** respectively. The number at the bottom right in **(A)** indicates the distance from the interaural axis in millimeters. SNR, substantia nigra pars reticulata. v, vessel lumen. Bar in **(A)**, 1 mm; in B, 400 μm; in **(F)** (for **D and F**), 150 μm; in **(G)**, 100 μm; in **(I)** (for **H and I**), 100 μm.

Hamel, 2011). This system, preferentially studied in the cerebral cortex, involves local GABAergic, peptidergic and glutamatergic collaterals, as well as cholinergic, noradrenergic and serotoninergic ascending inputs (Attwell and Iadecola, 2002; Cauli et al., 2004; Iadecola, 2004; Lecrux and Hamel, 2011). The use of anterograde tracers, specific neurotoxins and electron microscopy shows that these projections come from the nucleus basalis, locus coeruleus, and raphe nuclei respectively (Reinhard et al., 1979;

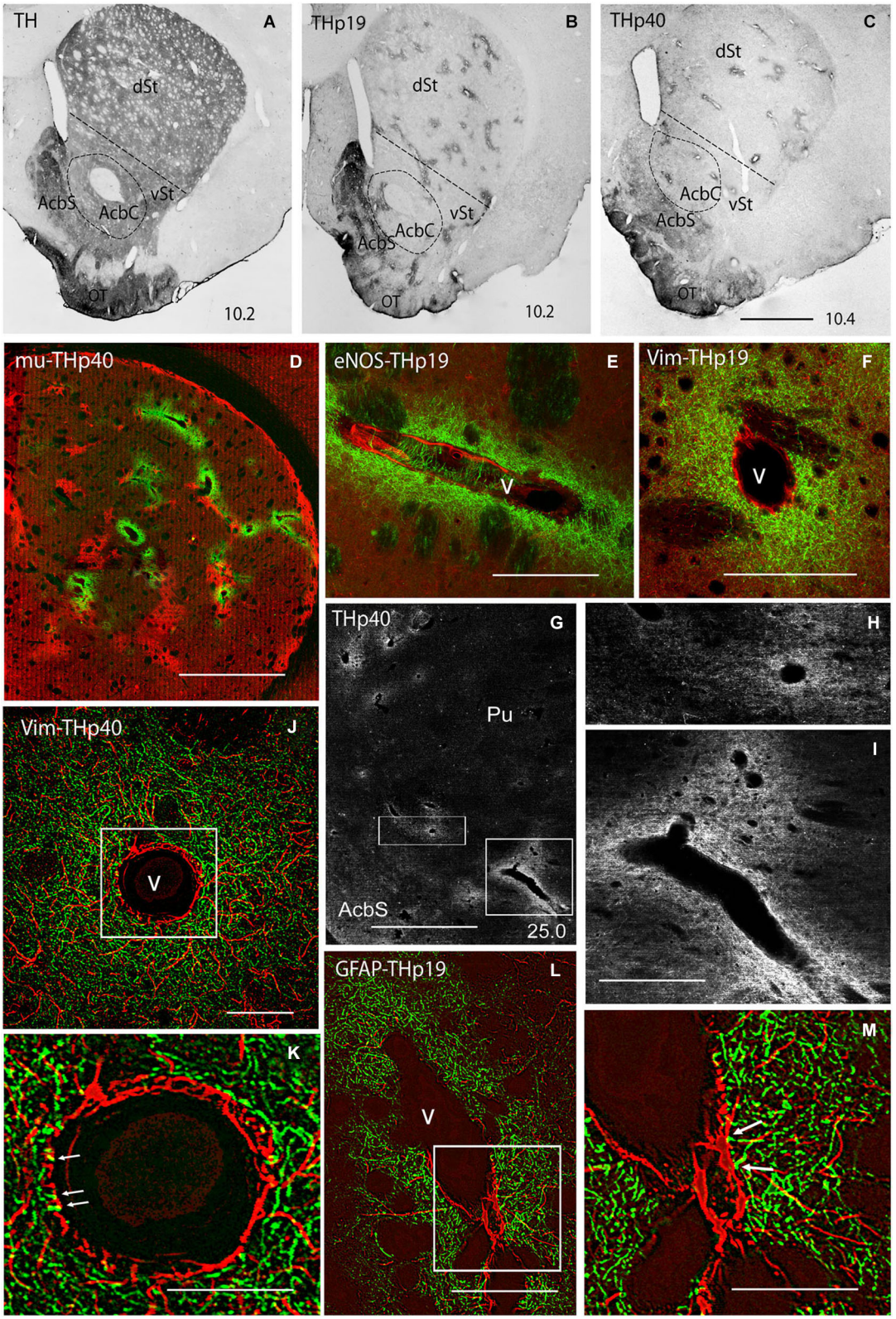

FIGURE 2 | (A–C) Immunostaining for the non-phosphorylated (TH; **A**) and phosphorylated forms of tyrosine hydroxylase at Ser19 (THp19; **B**) and Ser40 (THp40; **C**) in rat striatum. We note that in contrast to the homogeneous labeling for TH **(A)**, THp19 and THp40 immunostaining is restricted to the olfactory tubercle (OT), accumbens shell (AcbS), and discrete regions throughout the ventral (vSt) and dorsal (dSt) striatum. **(D)** Double labeling for mu-opioid receptor (mu, a marker of striosomes; red) and THp40 (green) showing that THp40 is not localized in striosomes. **(E,F)** Double labeling for endothelial nitric oxide synthase (eNOS; **E**, red) or

(*Continued*)

FIGURE 2 | Continued
vimentin (Vim; **F**, red) and THp19 (green) showing dense plexuses of THp19-positive terminals surrounding longitudinal- **(E)** and transversally **(F)** cut vessels. **(G)** Immunostaining for THp40 in the monkey striatum showing intense immunoreactivity in perivascular terminals. **(H,I)** Boxed areas in **(G)**. **(J)** Double labeling for vimentin (Vim; red) and THp40 (green) in rat striatum subjected to deconvolution processing. **(K)** Boxed area in **(J)** showing THp40 terminals in close apposition to or penetrating (arrows) the vessel wall (arrows). **(L)** Double labeling for GFAP (red) and THp19 (green) in rat striatum subjected to deconvolution processing. **(M)** Boxed area in **(L)** showing THp19 terminals touching astroglial processes (arrows). Doted line in **(A–C)** indicates the putaitve border between vSt and dSt. The number at the bottom right in **(A–C,G)** indicates the distance from the interaural axis in millimeters. AcbC, accumbens core; Pu, putamen; v, vessel lumen. Bar in **(C)** (for **A–C**), 450 μm; in **(D)**, 200 μm; in **(E)**, 75 μm; in **(F)**, 75 μm; in **(G)**, 3 mm; in **(I)** (for **H and I**), 1 mm; in **(J)**, 50 μm; in **(K)**, 20 μm; in **(L)**, 50 μm; in **(M)**, 20 μm.

Vaucher and Hamel, 1995; Cohen et al., 1997), and that their endings make contact with both the components of the neurovascular unit, the vessel wall and surrounding astrocytes (Vaucher and Hamel, 1995; Cohen et al., 1997). Dopaminergic neurotransmission has also been implicated in both neurovascular regulatory systems. Firstly, through *in vitro* and *in situ* studies showing that DA and its analogs promote vasoactive changes in convexity cerebral arteries and pial arterioles (Toda, 1976; Edvinsson et al., 1978a,b, 1985; Forster et al., 1983), and more recently, from the evidence of dopaminergic contacts in capillaries and perivascular astrocytes (Krimer et al., 1998), and the induction of hemodynamic changes in discrete brain regions by dopaminergic drugs (Marota et al., 2000; Choi et al., 2006; Sander et al., 2013) and their inhibition after dopaminergic lesion (Chen et al., 1997; Nguyen et al., 2000; Jenkins et al., 2004). However, the anatomical origin of dopaminergic inputs to intracerebral vessels has not been elucidated. The results here, based on the injection of an anterograde neuronal tracer, combination of vessel, astrocyte and dopaminergic markers, confocal microscopy and dopaminergic lesion, indicate that striatal vessels receive inputs from midbrain dopaminergic neurons. We are aware that only electron microscopy provides high enough magnification to properly identify synaptic structures. Nevertheless, high-resolution confocal laser scanning followed by adequate post-adquisition computer processing markedly reduces the resolution gap between optical and electron microscopies (Sibarita, 2005; Salin et al., 2008). Thus, one can suggest that, similar to dopaminergic and non-dopaminergic inputs to cortical vessels (Vaucher and Hamel, 1995; Cohen et al., 1997; Krimer et al., 1998), ascending dopaminergic terminals make contact with both components of the neurovascular unit in the rat striatum. In any case, we know that besides the synaptic transmission which requires interneuronal contacts, neurotransmitters and neuromodulators, including DA, can signal through extrasynaptic receptors after flowing short or long distances in the extracellular space. Interestingly, electron microscopy studies show that 50% of DA D_1 and D_2 receptors in the rat striatum are extrasynaptic (Yung et al., 1995), suggesting that most dopaminergic afferents can operate via both synaptic and extrasynaptic receptors. According to Fuxe et al. (2012), this sort of intercellular communication called volume transmission (Agnati et al., 1986), is the main communication pathway in neuroglial and neurovascular interactions. The evidence of DA D_1 and D_2 receptors in endothelial and astroglial cells (Bacic et al., 1991; Bal et al., 1994; Zanassi et al., 1999; Choi et al., 2006), together with the time (0–2 s) of vascular response to DA release in the striatum (Knutson and Gibbs, 2007) agree with the suggestion of Fuxe et al. (2012), and therefore the evidence of terminal contacts becomes less relevant.

Our results further show that dopaminergic axons innervating striatal vessels form dense perivascular plexuses immunoreactive for phosphorylated TH at Ser19 and Ser40. We know that phosphorylation is the primary mechanism responsible for short-term activation of TH, the rate-limiting enzyme of catecholamine synthesis, and that TH may be phosphorylated by different protein kinases at four serine residues (8, 19, 31 and 40) near N-terminus (Haycock and Wakade, 1992; Dunkley et al., 2004; Nakashima et al., 2009). However, under basal conditions no more than 5–7% TH is phosphorylated, and a substantial proportion of TH becomes phosphorylated only after protein kinase activating stimuli (Pocotte et al., 1986; Haycock, 1993; Haycock et al., 1998; Salvatore et al., 2001; Bobrovskaya et al., 2004). Consistent with these data, Xu et al. (1998) reported reduced immunoreactivity for phosphorylated TH in comparison with that for the native form, with phosphorylation being restricted to Ser19 and localized in the accumbens nucleus and the OT. Our results confirm these findings but enlarging them show that TH is also constitutively phosphorylated in dopaminergic terminals encircling vessels throughout the ventral and dorsal striatum. In addition, as demonstrated in rats and monkeys, phosphorylation also involves Ser40 residue.

The functional meaning and advantages of having multiple phosphorylatable positions have not been yet elucidated (for review see Daubner et al., 2011), but there is a general consensus in that Ser40 is critical for TH activity. Thus, while no activation mechanisms and functional consequences have been found for phosphorylation at Ser8 and phosphorylation at Ser19 and Ser31 promote no more than 2-fold increases in TH activity, phosphorylation at Ser40 results in a 300-fold decrease in TH affinity for catecholamines with a 20-fold increase in TH activity (Bevilaqua et al., 2001; Bobrovskaya et al., 2004; Dunkley et al., 2004; Daubner et al., 2011). In addition, although Ser19 phosphorylation "*per se*" does not significantly affect TH activity, it modifies TH structure thereby accelerating its phosphorylation at Ser40 (Bevilaqua et al., 2001). Consequently, one can assume that dopaminergic terminals with TH phosphorylated at Ser19 and Ser40 are responsible for the basal TH activity in the striatum, and that their DA content ready to be released is higher than in those without phosphorylated TH.

Studies about the striatal processing in associative learning indicate that the activity of striatal neurons in each trial is preceded by adaptive local hemodynamic changes which are mediated by DA (Knutson and Gibbs, 2007; Peterson and Seger, 2013). Midbrain dopaminergic neurons fire in response to reward-predicting cues leading to an increase in extracellular DA levels in the striatum (Roitman et al., 2004). Changes in extracellular DA levels are paralleled by an increase in the regional

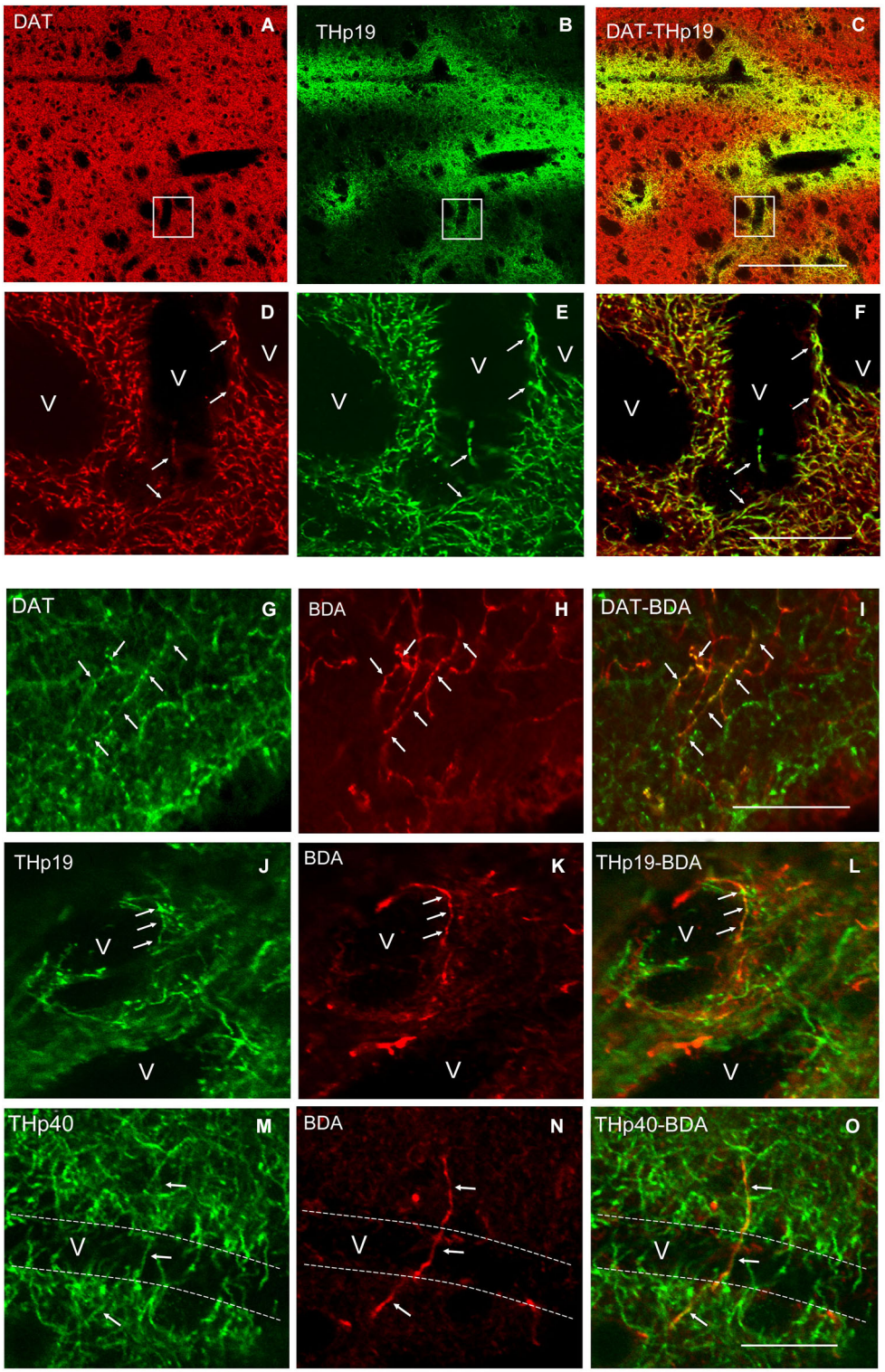

FIGURE 3 | (A–C) A low magnification view of double labeling for the dopamine transporter (DAT) and THp19 in the dorsal striatum of the rat. **(D–F)** Boxed areas in **(A)**, **(B)** and **(C)** respectively showing that all perivascular THp19 fibers and terminals are dopaminergic (DAT-immunoreactive). **(G–O)** Double labeling for DAT, THp19 or THp40 and BDA showing that BDA-positive ascending axons **(H)** and perivascular terminals **(K,N)** are immunoreactive for DAT **(G–I)**, THp19 **(J–L)** and THp40 **(M–O)**. v, vessel lumen. Dotted lines in **(M–O)** indicate the probable localization of the vessel wall. Bar in **(C)** (for **A–C**), 130 μm; in **(F)** (for **D–F**), 40 μm; in **(I)** (for **G–I**), 40 μm; in **(O)** (for **J–O**), 40 μm.

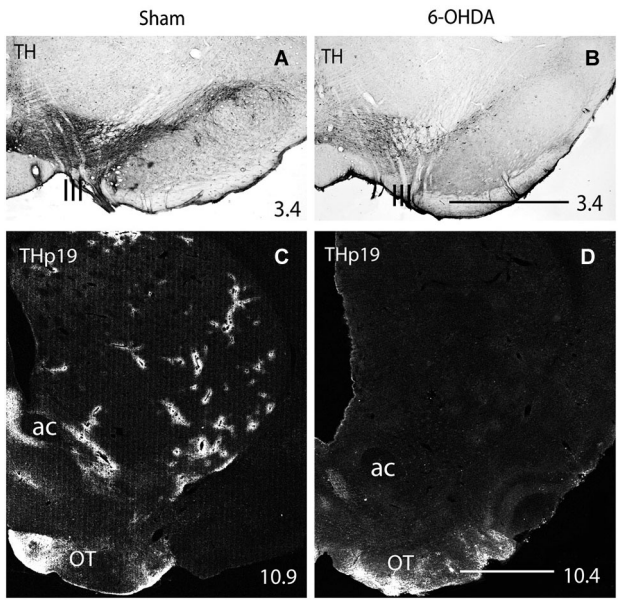

FIGURE 4 | (A,B) Immunohistochemistry for TH in the rat midbrain after sham **(A)** and 400 μg 6-OHDA **(B)** intracerebroventricular injection. **(C,D)** Immunofluorescence for THp19 in the striatum of sham **(C)** and 400 μg 6-OHDA **(D)** injected rats. One can see the decrease in the number of TH-positive midbrain cells after 6-OHDA injection (compare **A** and **B**), and that THp-19 striatal perivascular plexuses disappear after midbrain DA-cell degeneration (compare **C** and **D**). ac, anterior commissure; OT, olfactory tubercle; III, fibers of the third cranial nerve emerging in the surface of the ventral midbrain. Numbers at the bottom right indicate the distance from the interaural axis in millimeters. Bar in **(B)** (for **A** and **B**), 1 mm; in **(D)** (for **C** and **D**), 1 mm.

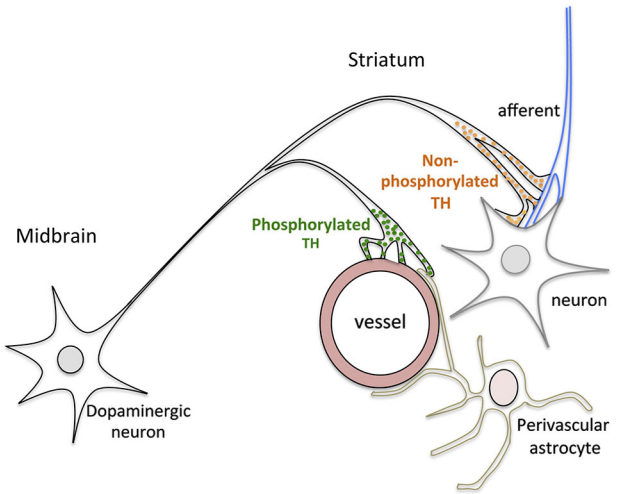

FIGURE 5 | Schematic drawing summarizing the main results of this study. Dopaminergic terminals on vessels contain phosphorylated TH while those on striatal neurons and afferents contain non-phosphorylated TH, suggesting that DA release onto vessels precedes DA release onto neurons.

cerebral blood flow that is inhibited by dopaminergic lesion (Chen et al., 1997; Nguyen et al., 2000; Mandeville et al., 2001; Jenkins et al., 2004). Consequently, the prior release of DA in discrete striatal regions is nowadays considered a key factor in the regulation of blood flow depending on local metabolic demands. It is notable that dopaminergic signaling has also been involved in anticipatory hemodynamic changes in the cerebral cortex (Tan, 2009). However, in the light of our and previous results, this should be quite different to that in the striatum. In contrast to the conspicuous perivascular plexuses involving small to large vessels from the OT to the dorsal striatum, dopaminergic terminals are sparse and restricted to small vessels in the cerebral cortex (Krimer et al., 1998). Furthermore, as shown in rats and monkeys, TH is not constitutively phosphorylated in vessel terminals in the cerebral cortex. The restricted localization of phosphorylated TH in dopaminergic terminals of striatal vessels suggests that it is related to specific aspects of striatal processing. As shown in **Figure 5**, a striatal neurovascular unit and the neighboring neuropil probably receive projections from the same midbrain neuron. Consequently, dopaminergic inputs should reach both compartments at the same time. However, the fact that dopaminergic terminals in perivascular plexuses contain phosphorylated TH facilitates that the release of DA onto vessels precedes that onto the neighboring neuropil, and then, that DA vascular actions precede DA neuronal (non phosphorylated TH) actions.

We know that the striatum receives the most intense dopaminergic input in the brain, and that DA actions are exerted through different DA-receptors in striatal neurons and cortical and subcortical afferents arriving to different striatal regions (Obeso et al., 2008). In addition, the loss of DA signaling in striatal circuits is considered to cause motor symptoms as well as less noticeable cognitive and psychiatric manifestations of PD (Rodriguez-Oroz et al., 2009). However, as our results show, an important contingent of dopaminergic axons reaches striatal vessels rather than neurons, suggesting that these axons can play a role in the pathophysiology of PD. Beyond the concept of vascular parkinsonism (Kalra et al., 2010), the relationship between genuine PD and cerebral perfusion has mostly been focused on whether vascular co-morbidities can aggravate motor symptoms as a result of additive effects of two independent disorders (Kotagal et al., 2014), or the possibility that vascular aspects can act synergistically with other factors contributing to dopaminergic cell degeneration. In support of the latter, morphological changes have been found in nigral vessels of PD patients and animal models of PD (Faucheux et al., 1999; Barcia et al., 2005), and the density of nigral microvessels has also been found to be low in aged rats (Villar-Cheda et al., 2009). In addition, recent studies by Rodriguez-Perez et al. (2013) reveal that chronic brain hypoperfusion can "*per se*" induce dopaminergic cell degeneration and exacerbate the degeneration promoted by intrastriatal injection of 6-OHDA. Our findings suggest that independently of the vascular involvement in dopaminergic degeneration, the striatal blood flow can also be substantially affected as a result of dopaminergic cell degeneration. Consequently, the supply of oxygen and nutrients coupled to hemodynamic changes preceding neuronal activity might also be altered in the striatum, contributing as an additional factor in the pathophysiology of PD from its first stages. Interestingly, although the relationship between neuronal activity and oxygen

consumption is the basis of functional imaging technologies widely used in the diagnosis of PD (Mahlknecht et al., 2010), nowadays blood flow is considered to merely serve as a metabolic support in this paradigm (Moore and Cao, 2008). Moreover, changes in striatal perfusion in the course of PD are still a matter of controversy. While some authors report an increase of the regional blood flow (Wolfson et al., 1985; Feigin et al., 2002; Hsu et al., 2007), others find no changes (Bissessur et al., 1997) or a decrease (Markus et al., 1994; Van Laere et al., 2004). These discrepancies reflect differences in data processing and the clinical profile of patients studied. In this respect, Imon et al. (1999) found a decrease in striatal blood flow at early stages of PD, and an increase at later stages, suggesting that changes can depend on the disease stage. Bearing in mind the relative weight of the "vascular component" in the mesostriatal pathway, it is possible that hemodynamic changes may be detected and monitored from the early stages of PD with the systematic use of functional imaging.

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The degeneration and replacement of dopamine cells in Parkinson's disease: the role of aging

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Available data show marked similarities for the degeneration of dopamine cells in Parkinson's disease (PD) and aging. The etio-pathogenic agents involved are very similar in both cases, and include free radicals, different mitochondrial disturbances, alterations of the mitophagy and the ubiquitin-proteasome system. Proteins involved in PD such as α-synuclein, UCH-L1, PINK1 or DJ-1, are also involved in aging. The anomalous behavior of astrocytes, microglia and stem cells of the subventricular zone (SVZ) also changes similarly in aging brains and PD. Present data suggest that PD could be the expression of aging on a cell population with high vulnerability to aging. The future knowledge of mechanisms involved in aging could be critical for both understanding the etiology of PD and developing etiologic treatments to prevent the onset of this neurodegenerative illness and to control its progression.

Keywords: Parkinson's disease, aging, dopamine, nigrostriatal cells, etiopathology

Parkinson's disease (PD) is a neurodegenerative illness whose onset and progression is clearly linked to aging (Driver et al., 2009; Buchman et al., 2012). The discovery of cell loss and eosiniphilic intracitoplasmic aggregates (Lewy bodies) in the substantia nigra (SN) of these patients during the early twentieth century (Greenfield and Bosanquet, 1953) led a number of groups to investigate the etio-pathology of PD in this center. Although recent studies have reported neurodegeneration in many other brain centers, the degeneration of SN cells is still the hallmark for a diagnosis of PD.

In the 1960s Hornykiewicz reported a decrease of striatal dopamine (DA) and an effective therapeutic response to levodopa (a DA precursor) suggesting that nigrostriatal DA-cells (nsDAc) are the SN cells which mainly degenerate in PD, a possibility also supported by the loss of neuromelanin+ cells in this center (this pigment is a by-product of DA oxidation) (Hornykiewicz, 1966, 2010; Hirsch et al., 1988). This possibility was then supported by studies showing that most degenerated cells express proteins involved in the synthesis (e.g., tyrosine hydroxylase -TH- and l-dopa decarboxylase -DD-), degradation (monoamine oxidase -MAO-), and transport (dopamine transporter, DAT) of DA (Lloyd and Hornykiewicz, 1970; Kastner et al., 1993). The aforementioned findings are frequently used to support the possibility that the nigral DA cell (DAc) loss is a specific characteristic of PD. However, a similar degeneration has been observed in the SN of aged healthy subjects who also show a decrease in the number of: (1) total SN neurons (Hirai, 1968; McGeer et al., 1977; Stark and Pakkenberg, 2004; Morterá and Herculano-Houzel, 2012); (2) pigmented SN neurons (which decrease 7–10% per decade) (Ma et al., 1999; Stark and Pakkenberg, 2004; Rudow et al., 2008); (3) TH+ and DAT+ neurons (Kastner et al., 1993; Rudow et al., 2008; Kordower et al., 2013); (4) DD+ neurons (Lloyd and Hornykiewicz, 1970); and (5) MAO+ neurons (Saura et al., 1997). Thus, the nsDAc loss cannot be considered as a discriminating characteristic of PD.

It has been suggested that the nigral DA-cell subgroups (González-Hernández and Rodriguez, 2000) which degenerate in PD are not the same subgroups which degenerate during the normal aging. PD degeneration mainly affects snDAc located in the ventral tier of the posterior-lateral regions of the SN compacta (Fearnley and Lees, 1991; Damier et al., 1999) which innervate the dorsal-lateral region of the striatum (Kish et al., 1988; Hornykiewicz, 1989). However, this snDAc subgroup also shows the highest degeneration rate during aging, a fact observed in both monkeys (Kanaan et al., 2008; Collier et al., 2011) and humans (Reeve et al., 2014). In addition, the striatal distribution of the DA denervation is also similar in PD (Kish et al., 1988; Hornykiewicz, 1989) and aging (Kish et al., 1992; Haycock et al., 2003). Therefore, the difference between the DA-cell degeneration in PD and aging may be the intensity of the degeneration process more than the type of cells which degenerate.

Both PD (Olanow and Tatton, 1999; Obeso et al., 2010) and aging (Olson, 1987; Peto and Doll, 1997) are probably the consequence of the simultaneous and persistent action of a number of damaging agents, with oxidative stress being one of the most relevant factors in both cases. *Oxidative stress* has proved to be critical for aging (Gerschman et al., 1954; Brack et al., 2000; Toussaint et al., 2000), affecting proteins, lipids, and nucleic acids in a variety of organs and animals (Sohal and Weindruch, 1996; Perez et al., 2009; Oliveira et al., 2010). The oxidative stress in mammals is mainly generated by the mitochondrial production of energy. The nsDAc has an unmielinated axon (Orimo et al., 2011) and a large number of synaptic terminals (hundreds of thousands) (Matsuda et al., 2009) which require a high amount of energy, thereby increasing oxidative stress. The metabolization and autooxidation of DA, together with the high concentration of intracellular iron, are additional sources of free radicals in these cells (Kidd, 2000; Berg and Hochstrasser, 2006). These characteristics increase the vulnerability of the snDAc to the aging process.

The DAc is protected from oxidative stress by different mechanisms including the superoxide dismutase and glutatione peroxidase activity (which prevent the oxidant action of oxygen species), and by the DAT and the vesicular monoamine transporter 2 activity (which moves DA from the extracellular medium to synaptic vesicles preventing its metabolization and self-oxidation). These protecting mechanisms are altered in PD where a disruption of the mitochondrial electron transport chain increases the generation of free radicals (Parker et al., 1989; Bender et al., 2006). This, and the down-regulation of the superoxide dismutase, glutatione peroxidase, DAT and vesicular monoamine transporter 2 activities observed in PD (Riederer et al., 1989; Zeevalk et al., 2008), suggest high oxidative stress in the SN of these patients. This possibility is also supported by the high oxidative damage of lipids (Bosco et al., 2006), proteins and DNA (Nakabeppu et al., 2007) found in the SN of these patients (Jenner, 2007). However, all these facts have also been observed in the aged brain and cannot be considered as a selective characteristic of the PD brain (Sohal and Brunk, 1992; Oliveira et al., 2010). In fact, increasing the resistance to oxidative stress via caloric restriction is often considered as the most effective way of delaying aging in animals (Yu, 1996; Bokov et al., 2004), although this neuroprotecting possibility is still to be properly tested in PD.

The most direct impact of oxidative stress is produced on the *mitochondria*. The DNA of mitochondrias (mtDNA) is highly vulnerable to mutations because it is located near the mitochondrial source of free radicals (electron transport chain) and because it is not protected by histones. mtDNA shows a high number of delections in PD patients, and epidemiological studies and cybrid models have suggested that the mtDNA damage is important in PD (Gu et al., 1998; Kraytsberg et al., 2006). Similar mtDNA damage has been observed in the healthy brain, where the mtDNA mutations normally accumulate with aging (Linnane et al., 1989; Bender et al., 2006). Sporadic mtDNA mutations in single mitochondrias are not enough to induce severe cell damage, but the aggregation of random mutations in an increasing number of mitochondrias can reduce cell viability. This fact probably enhances neurodegeneration in both aged and age-associated diseases such as PD (Cantuti-Castelvetri et al., 2005; Smigrodzki and Khan, 2005; Maruszak et al., 2006).

The mitochondrial population of cells is normally protected from damage by different repair mechanisms, including *fission/fusion processes* (which use healthy mitochondrias to recuperate the functions of damaged mitochondrias) and *mitophagy* (an autophagic process which eliminates the most damaged mitochondrias preventing their accumulation). Proteins involved in these repair mechanisms (e.g., parkin and PINK1) behave anomalously in both PD (Ethell and Fei, 2009) and aging (Palikaras and Tavernarakis, 2012), with autophagy also being altered in both cases (Cuervo et al., 2004; Ethell and Fei, 2009; Hubbard et al., 2012). The movement of mitochondrias across the axon is necessary to preserve an efficient quality control of neuronal mitochondrias. Most synaptic mitochondrias are synthesized in the neuronal somata and moved along axons (anterograde motion). Axonal transport is also necessary to move dysfunctional mitochondrias from synaptic bottoms to the cell somata (retrograde motion) where they can be destroyed by mitophagy and other mechanisms (Cheng et al., 2010). Different proteins involved in the axonal transport (e.g., α-synuclein, parkin and PINK1-Miro-Milton complex) are involved in both PD and aging as well. The axonal damage observed in DAc of the PD brain (Cheng et al., 2010) has been found in the aging brain too (Gilley et al., 2012), which shows that the anomalous behavior of axons is also a characteristic shared by the PD and the aging brain.

The anomalous conformations of α-synuclein facilitate the formation of Lewy bodies in the nsDAc of PD patients (Lansbury and Brice, 2002) as well as in healthy aged subjects (Li et al., 2004; Moore et al., 2005). Similarly, the mutation of parkin has been associated to both PD (Lücking et al., 1998; Lucking et al., 2000; Moore et al., 2005; Reeve et al., 2014) and aging (Rodríguez-Navarro et al., 2007; Vincow et al., 2013). The UCH-L1 mutation impairs the ubiquitin-proteasome system (Osaka et al., 2003; Li et al., 2004), promoting both PD (Leroy et al., 1998) and aging (Marzban et al., 2002). PINK1 facilitates axonal transport and degradation of damaged mitochondrias (Valente et al., 2004a; Liu, 2014), and this PINK1 activity is altered in both the PD (Valente et al., 2004b; Albanese et al., 2005; Gelmetti et al., 2008) and aging (Wood-Kaczmar et al., 2008; Vincow et al., 2013) brain. The DJ-1 protein protects cells against oxidative stressors (Moore et al., 2005). Its anomalous behavior has been linked to a familiar parkinsonism (Bonifati et al., 2003a,b; Ibanez et al., 2003) and to aging (Marzban et al., 2002; Meulener et al., 2006). These proteins have been associated with the different familiar early onset parkinsonisms which present mutations of their genes, but also with idiopathic (or sporadic) PD and with normal aging where their activity may change (Cookson and Bandmann, 2010).

Many of the altered cell groups in PD show similar changes in the aged brain. This is the case of *astrocytes*, cells whose physiological functions (Sofroniew and Vinters, 2010; Rodriguez et al., 2012) change in PD and aging (Raivich et al., 1999; Morales et al., 2013). Astrocytes prevent neuronal damage by releasing neuroprotecting agents (glutathione, basic fibroblast growth factor, glial cell line-derived neurotrophic factor...) (Saavedra et al., 2006; Deierborg et al., 2008), and by removing toxic molecules from the extracellular medium (e.g., α-synuclein)

(Braak et al., 2007; Lee et al., 2010). The neuroprotecting abilities of astrocytes decrease with age (Pertusa et al., 2007; Mansour et al., 2008; Chinta et al., 2013), which increases DAc vulnerability (Mirza et al., 2000; Song et al., 2009) and enhances the development of PD (Halliday and Stevens, 2011).

It has been suggested that the slow DAc decline during life is normally compensated by a slow cell repopulation provided by the subventricular zone (SVZ; Doetsch et al., 1997, 1999; Quiñones-Hinojosa et al., 2006). SVZ *stem cells* normally differentiate into astrocytes and neuroblasts which later migrate to the olfactory bulb. The differentiation and migration of these cells are modulated by the DA released from nsDAc terminals (Freundlieb et al., 2006; Borta and Höglinger, 2007). Some neurons generated by SVZ stem cells express a DAergic phenotype and migrate to the olfactory bulb where they modulate olfaction. However, neuroblasts can also migrate to other brain loci, particularly when the target areas have been damaged (ictus.) (Macas et al., 2006). It has been suggested that stem cells can migrate to the SN (Kay and Blum, 2000; Zhao et al., 2003; Zhao and Janson Lang, 2009), where they could compensate for the DAc loss induced by aging. Thus, an insufficient repopulation of the DAc loss induced by senescence may also be a cause of PD (Armstrong and Barker, 2001). This possibility is supported by the low neurogenesis observed in the SVZ (Höglinger et al., 2004) and anterior olfactory nucleus (Pearce et al., 1995; Hawkes et al., 1997) of PD patients. A similar low neurogenesis has been observed during aging. Healthy subjects present a noticeable decrease of SVZ stem cell proliferation during the last third of life which is when the incidence of PD increases (Galvan and Jin, 2007; Conover and Shook, 2011). Nevertheless, the cell repopulation hypothesis is currently a matter of debate because the DAergic repopulation of the SN has not been definitively proved (Frielingsdorf et al., 2004). The new astrocytes derived from SVZ stem cells could also prevent DAc degeneration by replacing the damaged astrocytes in PD patients (Gonzalez-Perez and Quinones-Hinojosa, 2012; Mack and Wolburg, 2013). Bearing in mind the neuroprotecting role of astrocytes, this repopulation could also be necessary to keep the DAc alive in the aged brain. In this case, aging and PD could be the final result of a deficient gliogenesis and of the consequent deterioration of the astrocyte population supporting the snDAc. Therefore, the reduced neurogenesis and gliogenesis secondary to the senescence of the SVZ could be involved in both aging and PD.

The *microglia* has been linked to the neurodegenerating process in PD. *Microglia* is activated in the presence of aggregated forms of α-synuclein (Zhang et al., 2005), expressing macrophage markers and releasing IL-1β, IL-6 and TNF-α which can damage the DAc (Croisier et al., 2005; Orr et al., 2005). This activation has been found in both PD (Hunot et al., 1996; Knott et al., 2000) and aged brains (Godbout and Johnson, 2004; Gelinas and McLaurin, 2005; Campuzano et al., 2009), suggesting that the neurotoxic action of these cells is similar in both conditions (Ouchi et al., 2005; Streit et al., 2008; Cunningham, 2013).

Recent technological advances have made it possible to obtain *pluripotent stem cells* (iPSC; Takahashi and Yamanaka, 2006) from the skin of healthy subjects and patients with different illnesses including PD (disease-specific iPSC) (Lee and Studer, 2010). The DAc derived from iPSC shows an abnormal phenotype (with respect to aged-matched controls) when produced from patients with familiar parkinsonisms (PINK1, SNCA, parkin, LRRK2...) (Sánchez-Danés et al., 2013) but not when produced from patients with sporadic PD (Soldner et al., 2009). However, cells from sporadic PD patients show the typical alterations of the nsDAc when they are kept for a long time in a culture medium which *in vitro* simulates *in vivo* aging (more than 2 months in a culture medium which induces chronic cellular stress) (Sánchez-Danés et al., 2012). In these conditions, the DAc derived from iPSC of sporadic PD patients shows morphological (reduced number of neurites and accumulation of autophagic vacuoles) and neurochemical (accumulation of α-synuclein in their cytoplasm) characteristics similar to those of the DAc in PD (Sánchez-Danés et al., 2012). Thus, aging, in this *in vitro* model, seems to be a condition for developing the DAc characteristics observed in PD, which also supports aging as a basic mechanism for PD.

In summary, the studies reviewed above show that the DAc degeneration in PD is similar to that observed in aging, suggesting that aging is not simply another agent to add to the etiology of PD. The progressive course of aging and PD could be induced by the same multi-factorial etiology, including astrocytic and microglia alterations, oxidative stress, anomalous action of different proteins, mitochondrial disturbances, and alterations of the mitophagy and the ubiquitin-proteasome system. To this effect, PD could be the expression of aging on a cell population which, due to its characteristics (number of synaptic terminals, unmielinated axon etc...), is particularly vulnerable to damage. Repeated injuries accumulated throughout a person's lifespan may go unnoticed until the DAc loss exceeds a critical value. DAc degenerated over the years could be regularly replaced by new neurons derived from brain stem cells. Since stem cells are also affected by aging, the DAc loss induced by aging could be increased by an insufficient cell replacement. The progressive imbalance between the DAc loss and DAc neurogenesis eventually leads to a large enough decrease in the number of DAc to trigger the onset of motor disturbances of PD. This DAc loss is usually considered as a sign of brain aging until it crosses the above mentioned clinical threshold and PD can be diagnosed. In our opinion, a better understanding of the mechanisms involved in aging would help to explain the etiopathology of PD.

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Oxidative stress and Parkinson's disease

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Parkinson disease (PD) is a chronic, progressive neurological disease that is associated with a loss of dopaminergic neurons in the substantia nigra pars compacta of the brain. The molecular mechanisms underlying the loss of these neurons still remain elusive. Oxidative stress is thought to play an important role in dopaminergic neurotoxicity. Complex I deficiencies of the respiratory chain account for the majority of unfavorable neuronal degeneration in PD. Environmental factors, such as neurotoxins, pesticides, insecticides, dopamine (DA) itself, and genetic mutations in PD-associated proteins contribute to mitochondrial dysfunction which precedes reactive oxygen species formation. In this mini review, we give an update of the classical pathways involving these mechanisms of neurodegeneration, the biochemical and molecular events that mediate or regulate DA neuronal vulnerability, and the role of PD-related gene products in modulating cellular responses to oxidative stress in the course of the neurodegenerative process.

Keywords: mitochondrial dysfunction, dopamine, neuroinflammation, Parkinson disease, oxidative stress

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Introduction

Parkinson's disease (PD) is associated with the selective loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) and DA levels in the corpus striatum of the nigrostriatal DA pathway in the brain. This loss of DA causes a deregulation in the basal ganglia circuitries that leads to the appearance of motor symptoms such as bradykinesia, resting tremor, rigidity, and postural instability as well as non-motor symptoms such as sleep disturbances, depression, and cognitive deficits (Rodriguez-Oroz et al., 2009). The exact etiology of PD still remains elusive and the precise mechanisms that cause this disease remain to be identified (Obeso et al., 2010). At the cellular level, PD is related to excess production of reactive oxygen species (ROS), to alterations in catecholamine metabolism, to modifications in mitochondrial electron transporter chain (METC) function or to enhancement of iron deposition in the SNpc. The failure of normal cellular processes that occur in relation to the aging process are also believed to contribute to the increased vulnerability of DA neurons (Schapira and Jenner, 2011; Rodriguez et al., 2014).

While the familial forms of PD, that have been described, involve mutations in a number of genes (Kieburtz and Wunderle, 2013; Trinh and Farrer, 2013), mitochondrial dysfunction, neuroinflammation and environmental factors are increasingly appreciated as key determinants of dopaminergic neuronal susceptibility in PD, and are a feature of both familial and sporadic forms of the disease (Ryan et al., 2015). In both cases, oxidative stress is thought to be the common underlying mechanism that leads to cellular dysfunction and, eventual cell death. ROS

are continuously produced *in vivo* by all body tissues. However, oxidative stress occurs when there is an imbalance between ROS production and cellular antioxidant activity. Oxidants and superoxide radicals are produced as products of oxidative phosphorylation, making mitochondria the main site of ROS generation within the cell. ROS can affect mitochondrial DNA which can cause modulations in the synthesis of METC components like adenosine triphosphate (ATP) production as well as the leakage of ROS into the cell's cytoplasm (Brieger et al., 2012).

Although the precise mechanism corresponding to ROS generation related to PD is still unknown, in this review, we summarize the major sources of oxidative stress generated by the DA neurons, like DA metabolism, mitochondrial dysfunction, and neuroinflammation (**Figure 1**).

Dopamine Metabolism

Selective degeneration of the DA neurons of the SNpc suggests that DA itself may be a source of oxidative stress (Segura-Aguilar et al., 2014). DA is synthesized from tyrosine by tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase. Following this, DA is stored in synaptic vesicles after uptake by the vesicular monoamine transporter 2 (VMAT2). However, when there is an excess amount of cytosolic DA outside of the synaptic vesicle in damaged neurons, i.e., after L-DOPA treatment, DA is easily metabolized via monoamine oxidase (MAO) or by auto-oxidation to cytotoxic ROS (Zucca et al., 2014). For example, mishandling of DA in mice with reduced VMAT2 expression was sufficient to cause DA-mediated toxicity and progressive loss of DA neurons (Caudle et al., 2007).

This oxidative process alters mitochondrial respiration and induces a change in the permeability transition pores in brain mitochondria (Berman and Hastings, 1999). Also, the auto-oxidation of DA produces electron-deficient DA quinones or DA semiquinones (Sulzer and Zecca, 2000). Some studies have demonstrated a regulatory role for quinone formation in DA neurons in the L-DOPA-treated PD model induced by neurotoxins and in methamphetamine neurotoxicity (Asanuma et al., 2003; Miyazaki et al., 2006; Ares-Santos et al., 2014). DA quinones can modify a number of PD-related proteins, such as α-synuclein (α-syn), parkin, DJ-1, Superoxide dismutase-2 (SOD2), and UCH-L1 (Belluzzi et al., 2012; Girotto et al., 2012; da Silva et al., 2013; Hauser et al., 2013; Toyama et al., 2014; Zhou et al., 2014) and have been shown to cause inactivation of the DA transporter (DAT) and the TH enzyme (Kuhn et al., 1999; Whitehead et al., 2001), as well as mitochondrial dysfunction (Lee et al., 2003), alterations of brain mitochondria (Gluck and Zeevalk, 2004) and dysfunction in Complex I activity (Jana et al., 2007, 2011; Van Laar et al., 2009). Additionally, DA quinones can be oxidized to aminochrome, whose redox-cycling leads to the generation of the superoxide radical and the depletion of cellular nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), which ultimately forms the neuromelanin (Sulzer et al., 2000) known to be accumulated in the SNpc of the human brain (Ohtsuka et al., 2013, 2014; Plum et al., 2013).

Significant increases in cysteinyl adducts of L-DOPA, DA, and DOPAC have been found in substantia nigra of PD patients, suggesting the cytotoxic nature of DA oxidation (Spencer et al., 1998). Also, DA terminals actively degenerated proportionally to increased levels of DA oxidation following a single injection of DA into the striatum (Rabinovic et al., 2000). Recently, it has been shown that increased uptake of DA through the DAT in mice results in oxidative damage, neuronal loss and motor deficits (Masoud et al., 2015).

Mitochondrial Dysfunction

Mitochondrial dysfunction is closely related to increased ROS formation in PD (Schapira, 2008). Oxidative phosphorylation is the main mechanism providing energy to power neural activity in which the mitochondria use their structure, enzymes, and energy released by the oxidation of nutrients to form ATP (Hall et al., 2012). Consequently, this metabolic pathway is the main source of superoxide and hydrogen peroxide, which, at the same time, lead to propagation of free radicals contributing to the disease.

Complex I deficiencies of the respiratory chain account for the majority of unfavorable neural apoptosis generation and is considered one of the primary sources of ROS in PD. Complex I inhibition results in an enhanced production of ROS, which, in turn, will inhibit complex I. Reduction in complex I activity in the SNpc of patients with sporadic PD has been well described (Schapira et al., 1990; Hattori et al., 1991; Hattingen et al., 2009). Additionally, mitochondrial complex I deficiency in different brain regions (Mizuno et al., 1989; Parker et al., 2008), fibroblasts (Mytilineou et al., 1994), blood platelets (Krige et al., 1992; Blandini et al., 1998), skeletal muscle (Blin et al., 1994), and lymphocytes (Yoshino et al., 1992; Haas et al., 1995) of PD patients has been shown before as well.

As such, complex I inhibitors like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or rotenone show preferential cytotoxicity to the DA neurons (Blesa and Przedborski, 2014).The mechanism by which MPTP crosses the blood–brain barrier and is oxidized to 1-methyl-4-phenylpyridinium (MPP+) is well known (Blesa and Przedborski, 2014). The MPP+ accumulates in the mitochondria where it inhibits complex I in the METC, therefore disrupting the flow of electrons along the METC, which results in decreased ATP production and increased generation of ROS (Mizuno et al., 1987). Like MPTP, rotenone is another mitochondrial complex I inhibitor. Interestingly, rotenone toxicity is involved in oxidative damage to proteins and Lewy body-like inclusions (Betarbet et al., 2000; Sherer et al., 2003a,b; Greenamyre et al., 2010). The events downstream to complex I inhibition that lead to neuronal cell death by these toxins are still unknown (Schapira, 2010).

Other evidence for mitochondrial dysfunction related to oxidative stress and DA cell damage comes from findings that mutations in genes of proteins like α-syn, parkin, DJ-1, or PINK are linked to familial forms of PD. The convergence of all of these proteins on mitochondrial dynamics uncovers a common function in the mitochondrial stress response that might provide a potential physiological basis for the pathology

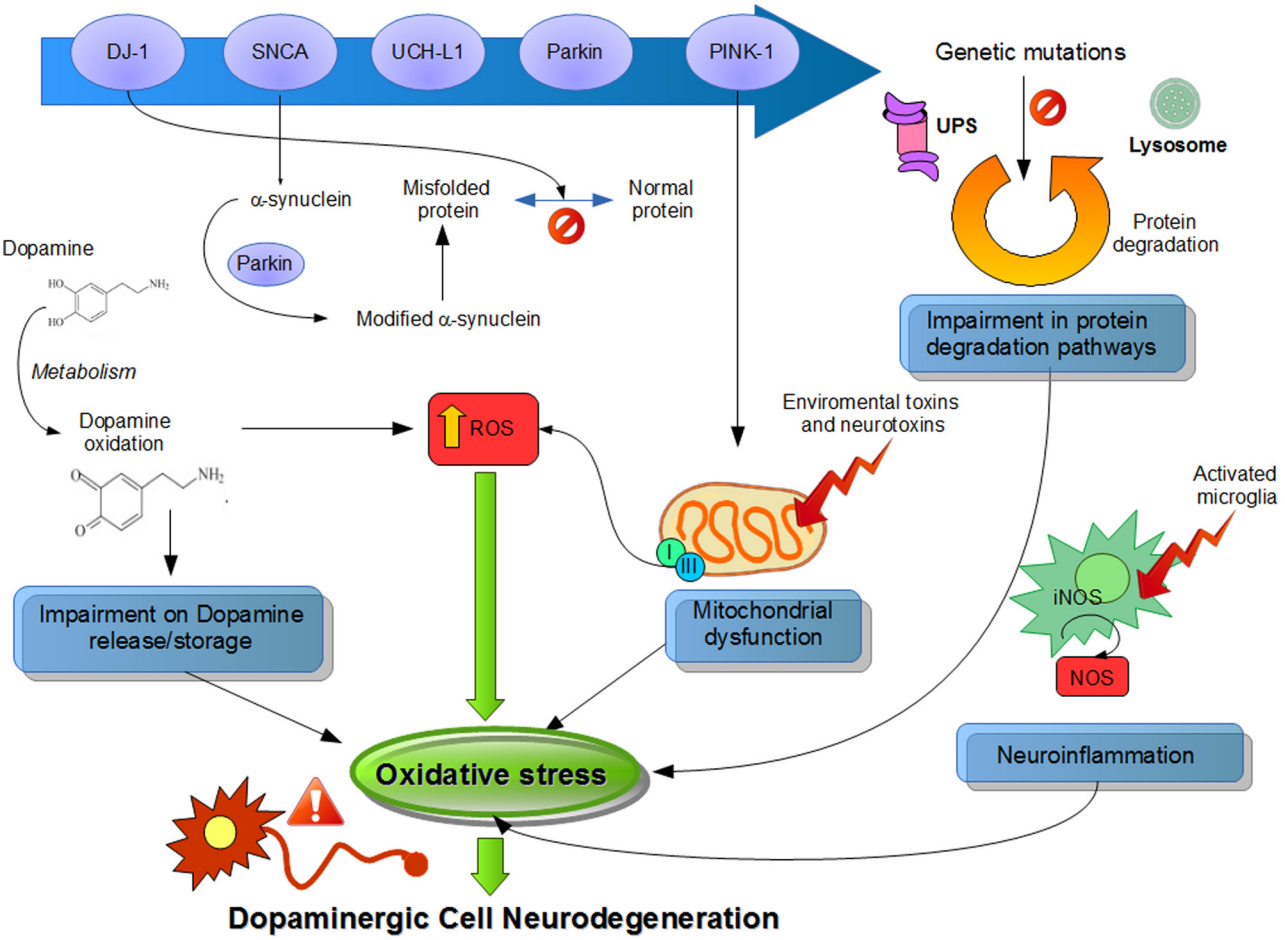

FIGURE 1 | Suggested physiological processes related to pathogenesis of Parkinson's disease (PD). Different pathways and their dysfunctions resulting from genetic modifications in PD-related genes and lead to an increased oxidative stress. Mutations or altered expression of these proteins result in mitochondrial impairment, oxidative stress, and protein misfolding. Also, dopamine metabolism may be oxidized to reactive dopamine quinones contributing to increased levels of reactive oxygen species. α-Synuclein becomes modified and accelerate its aggregation. Increased oxidative stress provokes impaired function of the UPS that degrades misfolded or damaged proteins and hereby further affecting cell survival. Environmental toxins impair mitochondrial function, increase the generation of free radicals, and lead to aggregation of proteins, including α-synuclein. Mitochondrial dysfunction by complex I inhibition affects by adding an increase in oxidative stress and a decline in ATP production, leading to damage of intracellular components and to cell death. Also, neuroinflammatory mechanisms might contribute to the cascade of consequences leading to cell death. In summary, all these several cellular mechanisms attributed to oxidative stress are implicated in the selective degeneration of dopaminergic neurons.

of PD (Norris et al., 2015; van der Merwe et al., 2015). Overall, these observations show that mutations in these genes affect mitochondrial function and integrity and, are associated with increases in oxidative stress (Zuo and Motherwell, 2013). ROS influence proteasomal, lysosomal, and mitochondrial function, which, in turn, regulate the cellular response to oxidative damage (Cook et al., 2012). The correct elimination of damaged proteins by effective proteolysis and the synthesis of new and protective proteins are vital in the preservation of brain homeostasis during periods of increased levels of ROS. Consequently, this can lead to protein misfolding (i.e., α-syn), preventing the ability of some of these proteins to be unfolded and degraded by the systems that regulate protein clearance, like the ubiquitin proteasome system or autophagy. Indeed, protein misfolding, together with the dysfunction of these protein degradation systems, may play a key role in the appearance of deleterious events implicated in the neurodegenerative process of PD (Schapira et al., 2014).

Parkin and PINK1 are localized in the mitochondria and their functions are tightly connected to the normal functioning of the mitochondria (Scarffe et al., 2014). PINK1 accumulates on the outer membrane of damaged mitochondria and recruits Parkin to the dysfunctional mitochondrion (Pickrell and Youle, 2015). In humans with parkin mutations, mitochondrial complex I activity is impaired (Müftüoglu et al., 2004). Overexpression of parkin in mice reduced DA neuronal cell loss induced by MPTP through the protection of mitochondria and the reduction of α-syn (Bian et al., 2012). On the other hand, parkin KO mice showed decreased amounts of several proteins that are involved in mitochondrial function and oxidative stress as well as increases in protein oxidation and lipid peroxidation (Palacino

et al., 2004). Also, *Drosophila*, lacking, or deficient in parkin, exhibit mitochondrial deficits and high vulnerability to oxidative stress (Saini et al., 2010). PINK1 mutations in humans lead to mitochondrial defects and respiratory chain abnormalities (Hoepken et al., 2007; Piccoli et al., 2008). PINK1 KO in human and mouse DA neurons causes decreases in membrane potential and increases in ROS generation (Wood-Kaczmar et al., 2008). The decrease in mitochondrial membrane potential is not due to a proton leak, but to respiratory chain defects like complex I and complex III deficiency (Amo et al., 2011, 2014). Therefore, PINK1 is required for maintaining normal mitochondrial morphology of SNpc DA neurons in culture and exerts its neuroprotective effect by inhibiting ROS formation (Wang et al., 2011). In animal models, studies show that the lack of PINK1 resulted in abnormal mitochondrial morphology, loss of SNpc DA neurons, reduction in complex I activity, and enhanced vulnerability to oxidative stress (Clark et al., 2006; Kitada et al., 2007; Gautier et al., 2008). These defects can be ameliorated and rescued by the enhanced expression of parkin (Yang et al., 2006; Exner et al., 2007). This last scenario seems to involve PINK1 and Parkin in a common pathway that regulates mitochondrial physiology and cell survival in which PINK1 seems to be functioning upstream of Parkin, at least as observed in *Drosophila* disease models (Clark et al., 2006).

α-syn is a soluble protein that is highly enriched in the presynaptic terminals of neurons. Accumulation of α-syn as intracellular filamentous aggregates is a pathological feature of both sporadic and familial PD (Goedert et al., 2013). Accumulation of wild-type α-syn in DA neurons reduced mitochondrial complex I activity, elevated ROS production leading to cell death (Martin et al., 2006). It has been shown that α-syn inclusions elevate dendritic mitochondrial oxidative stress in DA neurons (Dryanovski et al., 2013). This mitochondrial dysfunction occurs many months before the occurrence of striatal DA loss (Subramaniam et al., 2014). The nuclear translocation of α-syn increases susceptibility of MES23.5 cells to oxidative stress (Zhou et al., 2013). Exposure to rotenone or other stimuli that promote ROS formation and mitochondrial alterations correlate well with mutant α-syn phosphorylation at Ser129 (Perfeito et al., 2014). Oxidative stress promotes uptake, accumulation, and oligomerization of extracellular α-syn in oligodendrocytes (Pukass and Richter-Landsberg, 2014) and induces posttranslational modifications of α-syn which can increase DA toxicity (Xiang et al., 2013). It has been suggested that the NADPH oxidases, which are responsible for ROS generation, could be major players in synucleinopathies (Cristóvão et al., 2012).

DJ-1 is another gene reported to cause a familial early onset PD (Puschmann, 2013). DJ-1 binds to subunits of mitochondrial complex I and regulates its activity (Hayashi et al., 2009). Although a portion of DJ-1 is present in mitochondria matrix and inter-membrane space (Zhang et al., 2005), the degree of translocation of DJ-1 into mitochondria is stimulated by oxidative stress (Canet-Avilés et al., 2004). Mitochondrial-targeted sequence-conjugated DJ-1 has been shown to be more protective against oxidative stress-induced cell death (Junn et al., 2009). DJ-1 KO mice displayed nigrostriatal DA neuron loss (Goldberg et al., 2005). Also, these DJ-1 KO mice showed altered mitochondrial respiration and morphology, reduced membrane potential, and accumulation of defective mitochondria (Irrcher et al., 2010; Krebiehl et al., 2010; Giaime et al., 2012). These defects can be reversed by DJ-1 overexpression, which points to the specific role of DJ-1 in mitochondrial function (Heo et al., 2012). Recently, following oxidative stress, DJ-1 was shown to be involved in the oxidative stress response that leads to the upregulation of the proteasome, thus inhibiting its activity and rescuing partially unfolded proteins from degradation (Moscovitz et al., 2015).

Neuroinflammation

Neuronal loss in PD is associated with chronic neuroinflammation, which is controlled primarily by microglia, the major resident immune cells in the brain (Barcia et al., 2003) and, to a lesser extent, by astrocytes and oligodendrocytes (Perry, 2012). Microglial activation has been found with a greater density in the SNpc (Lawson et al., 1990) and in the olfactory bulb of both sporadic and familial PD patients (McGeer et al., 1988; Doorn et al., 2014a,b). Additionally, activated microglia have been found in the SNpc and in the striatum of PD animal models (Pisanu et al., 2014; Stott and Barker, 2014) and have been associated with different PD-associated gene/proteins like α-*syn* or *LRRK2* (Daher et al., 2014; Sacino et al., 2014). In response to certain environmental toxins and endogenous proteins, microglia can shift to an over-activated state and release ROS which can cause neurotoxicity (Block et al., 2007). Accumulating evidence indicates that activation of different enzymes like NADPH oxidase (NOX2) in microglia is neurotoxic not only through the production of extracellular ROS that damage neighboring neurons but also through the initiation of redox signaling in microglia that amplifies the pro-inflammatory response (Surace and Block, 2012).

Neuromelanin confers the dark pigmentation that is produced from DA oxidation and is so characteristic of the SNpc appearance. High levels of catecholamine metabolism in the midbrain are associated with increased levels of neuromelanin in the same region and, it is neuromelanin that is thought to be one of the molecules responsible for inducing chronic neuroinflammation in PD. Neuromelanin released from dying DA neurons in the SNpc activate microglia, increasing the sensitivity of DA neurons to oxidative stress-mediated cell death (Halliday et al., 2005; Li et al., 2005; Beach et al., 2007; Zhang et al., 2009). The ability of neuromelanin to interact with transition metals, especially iron, and to mediate intracellular oxidative mechanisms have received particular attention. Increased levels of iron result in increased ROS and increased oxidative stress and has been shown to be involved in aging and PD. Iron homeostasis is modulated by angiotensin in DA neurons and microglia, and glial cells play an essential role in the efficient regulation of this balance (Garrido-Gil et al., 2013).

Dopamine neurons containing neuromelanin are especially more susceptible, indicating a possible role for neuromelanin in MPTP-toxicity (Herrero et al., 1993). MPTP induces a glial response, increased levels of inflammatory cytokines and

microglial activation in mice (Członkowska et al., 1996; Jackson-Lewis and Smeyne, 2005) and monkeys (Barcia et al., 2004, 2009). Angiotensin is one of the most important inflammation and oxidative stress inducers, and produces ROS by activation of the NADPH-oxidase complex. It has been suggested that the inflammatory response in the MPTP model could be mediated by brain angiotensin and microglial NADPH-derived ROS (Joglar et al., 2009). Moreover, oral treatment with NADPH oxidase antagonists mitigates the clinical and pathological features of parkinsonism in the MPTP marmoset model (Philippens et al., 2013). Also, microglia play an important role in mediating rotenone-induced neuronal degeneration through NADPH (Gao et al., 2003, 2011; Pal et al., 2014). Rotenone increased microglial activation in both the SNpc and striatum in rats (Sherer et al., 2003a), activated microglia via the NF-κB signaling pathway (Gao et al., 2013) and induced neuronal death by the microglial phagocytosis of neurons (Emmrich et al., 2013).

Parkinson's disease-associated proteins like α-syn, parkin, LRRK2, and DJ-1 have also been reported to activate microglia (Wilhelmus et al., 2012). Extracellular α-syn released from neuronal cells is an endogenous agonist for Toll-like receptor 2 (TLR2), which activates the microglial inflammatory responses (Kim et al., 2013a). An increased number of activated microglia and increased levels of TNF-α mRNA and protein were detected in the striatum and in the SNpc of mice over-expressing WT human α-syn (Watson et al., 2012). Moreover, in α-syn KO mice, microglia secreted higher levels of proinflammatory cytokines, TNF alpha and IL-6 (interleukin-6) compared to WT mice (Austin et al., 2006). Intracerebral injection of recombinant amyloidogenic or soluble α-syn induces extensive α-syn intracellular inclusion pathology that is associated with a robust gliosis (Sacino et al., 2014). LRRK2 increases proinflammatory cytokine release from activated primary microglial cells which results in neurotoxicity (Gillardon et al., 2012). In contrast, LRRK2 inhibition attenuates microglial inflammatory responses (Moehle et al., 2012). Additionally, lipopolysaccharide induces LRRK2 up-regulation and microglial activation in mouse brains (Li et al., 2014) but they down regulated Parkin expression via NF-κB (Tran et al., 2011). Abnormal glial function is critical in parkin mutations, increasing vulnerability to inflammation-related nigral degeneration in PD (Frank-Cannon et al., 2008) and its role increases with aging (Solano et al., 2008). DJ-1 expression is up-regulated in reactive astrocytes in PD patients (Bandopadhyay et al., 2004). DJ-1 negatively regulates inflammatory responses of astrocytes and microglia by facilitating the interaction between STAT1 and its phosphatase SHP-1 (Kim et al., 2013b). Astrocyte cultures from DJ-1 KO mice treated with lipopolysaccharide have increased NO production and an up-regulation of different pro-inflammatory mediators like COX-2 and IL-6 (Waak et al., 2009).

Conclusion

The elements that potentially cause oxidative stress in PD are still unknown. DA metabolism, mitochondrial dysfunction and neuroinflammation all play critical roles in the etiology of this disease. Exposure to environmental factors or mutations in PD-associated genes of patients with either sporadic or familial PD may cause mitochondrial dysfunction that ultimately results in PD. All of these share common linkages and influence each other greatly. Limiting the early inflammatory response will reduce further both elevated oxidative stress and microglial activation that are key to slowing the death of the neurons in the SNpc. Development of potential drugs able to delay the neurodegenerative process is crucial to ameliorating the deleterious effects of oxidative stress in neurodegenerative diseases. Neuroprotective therapies will need to target multiple pathological pathways such as mitochondrial dysfunction and neuroinflammation in the next few years.

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Brain renin-angiotensin system and dopaminergic cell vulnerability

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Although the renin-angiotensin system (RAS) was classically considered as a circulating system that regulates blood pressure, many tissues are now known to have a local RAS. Angiotensin, via type 1 receptors, is a major activator of the NADPH-oxidase complex, which mediates several key events in oxidative stress (OS) and inflammatory processes involved in the pathogenesis of major aging-related diseases. Several studies have demonstrated the presence of RAS components in the basal ganglia, and particularly in the nigrostriatal system. In the nigrostriatal system, RAS hyperactivation, *via* NADPH-oxidase complex activation, exacerbates OS and the microglial inflammatory response and contributes to progression of dopaminergic degeneration, which is inhibited by angiotensin receptor blockers and angiotensin converting enzyme (ACE) inhibitors. Several factors may induce an increase in RAS activity in the dopaminergic system. A decrease in dopaminergic activity induces compensatory upregulation of local RAS function in both dopaminergic neurons and glia. In addition to its role as an essential neurotransmitter, dopamine may also modulate microglial inflammatory responses and neuronal OS *via* RAS. Important counterregulatory interactions between angiotensin and dopamine have also been observed in several peripheral tissues. Neurotoxins and proinflammatory factors may also act on astrocytes to induce an increase in RAS activity, either independently of or before the loss of dopamine. Consistent with a major role of RAS in dopaminergic vulnerability, increased RAS activity has been observed in the nigra of animal models of aging, menopause and chronic cerebral hypoperfusion, which also showed higher dopaminergic vulnerability. Manipulation of the brain RAS may constitute an effective neuroprotective strategy against dopaminergic vulnerability and progression of Parkinson's disease.

Keywords: aging, angiotensin, dopamine, NADPH-oxidase, neurodegeneration, neuroinflammation, oxidative stress, parkinson

INTRODUCTION

The renin-angiotensin system (RAS) was initially considered as a circulating humoral system, with functions in regulating blood pressure and in sodium and water homeostasis. The RAS is phylogenetically one of the oldest hormone systems. It has been suggested that the RAS played an important role in human evolution, and it is possible that our ancestors may have survived on little salt, thanks to RAS activation (Lev-Ran and Porta, 2005). Angiotensin II (AII), which is the most important effector peptide of the RAS, is formed by the sequential action of two enzymes -renin and angiotensin converting enzyme (ACE)- on the precursor glycoprotein angiotensinogen. The actions of AII are mediated by two main cell receptors: AII type 1 and 2 (AT1 and AT2) receptors (Unger et al., 1996; Oro et al., 2007; Jones et al., 2008). In addition to the afore mentioned components of the RAS, several other components that are involved in secondary mechanisms of this system have emerged (Cuadra et al., 2010; Wright and Harding, 2013). The AT1 receptor mediates most of the classical peripheral actions of AII. It is generally considered that AT2 receptors exert actions directly opposed to those mediated by AT1 receptors thus antagonizing many of the effects of the latter (Chabrashvili et al., 2003; Jones et al., 2008). However, the relationships between AT1 and AT2 are probably more complex and remain to be fully clarified.

THE LOCAL (TISSUE OR PARACRINE) RAS. ROLE IN OXIDATIVE STRESS, INFLAMMATION AND TISSUE DEGENERATION

It is now known that, in addition to the "classical" humoral RAS, many tissues have local (tissue or paracrine) RAS that contain the different components previously described for the circulating RAS (Ganong, 1994; Re, 2004). Although both circulating

RAS and local RAS act together in different tissues, it is generally accepted that circulating components are far less important than local formation of angiotensins for functioning of the system. Abnormal upregulation of local AII induces oxidative stress (OS) damage and exacerbates of inflammation. AII is a major activator of the NADPH-oxidase complex (Zalba et al., 2001; Touyz, 2004; Hoogwerf, 2010) which is the most important intracellular source of reactive oxygen species (ROS) other than mitochondria (Babior, 1999, 2004; Cai, 2005). It is known that NADPH-dependent oxidases are upregulated in major aging-related diseases such as hypertension, diabetes and atherosclerosis (Griendling et al., 2000; Münzel and Keany, 2001). It is usually considered that activation of AT2 receptors inhibits NADPH-oxidase activation and counteracts the deleterious effects of AT1 activation. In peripheral tissues, the upregulated AII acts, via AT1 receptors, on the resident cells (i.e., endothelial cells, smooth muscle cells) leading to OS, and subsequent production of chemokines, cytokines, and adhesion molecules, which contribute to the migration of inflammatory cells into the injured tissue (Ruiz-Ortega et al., 2001; Suzuki et al., 2003). Furthermore, AII acts on inflammatory cells to induce inflammatory responses and to release high levels of ROS mainly by activation of the NADPH complex (Okamura et al., 1999; Yanagitani et al., 1999; Qin et al., 2004; Touyz, 2004).

Finally, in addition to the "classical" humoral RAS and the local or tissue RAS, a number of recent studies support the existence of third level of RAS in several types of cells (Baker et al., 2004): the intracellular or intracrine RAS. The existence of functional intracellular RAS opens up new perspectives for understanding the effects of the RAS and for the management of RAS-related diseases (Kumar et al., 2007, 2009).

THE BRAIN RAS. LOCAL RAS IN THE NIGROSTRIATAL DOPAMINERGIC SYSTEM

The role of the RAS on brain function was initially associated with effects of the circulating RAS in areas involved in the central control of blood pressure and sodium and water homeostasis, which are located in circumventricular organs lacking the blood-brain barrier (von Bohlen und Halbach and Albrecht, 2006; Phillips and de Oliveira, 2008). However, over the last two decades, all components of the classical RAS have been identified in different brain areas inside the blood-brain barrier, and the brain RAS has been suggested to be involved in additional functions and disorders (Kerr et al., 2005; Maul et al., 2005; Saavedra, 2005; Saab et al., 2007). Interestingly, it has been observed that brain levels of AII are much higher than circulating levels (Hermann et al., 1984), and that the precursor protein angiotensinogen is mainly produced by astrocytes (Stornetta et al., 1988; Milsted et al., 1990), although it is also produced at low levels in neurons (Kumar et al., 1988; Thomas et al., 1992). Major components involved in the effects of AII in peripheral tissues such as NADPH-oxidases have also been located in neurons (Noh and Koh, 2000; Wang et al., 2004) and glial cells (Gao et al., 2003; Wu et al., 2003). Several studies have shown that, as previously observed in peripheral organs, AT1 receptor blockers and ACE inhibitors (ACEIs) decreased the inflammatory response in the CNS (Platten et al., 2009; Stegbauer et al., 2009; Saavedra, 2012). In accordance with their inhibitory effect on brain inflammation, beneficial effects of AT1 inhibition have been observed in a number of processes mediated by microglial activation and neuroinflammation, including animal models of Alzheimer's disease (Kehoe and Wilcock, 2007; Mogi and Horiuchi, 2009), brain ischemia (Lou et al., 2004; Iwanami et al., 2010) and multiple sclerosis (Platten et al., 2009; Stegbauer et al., 2009).

Several studies have reported the presence of RAS components in the basal ganglia, particularly in the nigrostriatal system (Quinlan and Phillips, 1981; Simonnet et al., 1981; Brownfield et al., 1982; Chai et al., 1987; Allen et al., 1992). In recent studies (Rodriguez-Pallares et al., 2008; Joglar et al., 2009; Valenzuela et al., 2010; Garrido-Gil et al., 2013b), we used laser confocal microscopy and other methods to demonstrate the presence of AT1 and AT2 receptors in nigral dopaminergic neurons and glial cells (i.e., astrocytes and microglia) in rodents and primates, including humans (Garrido-Gil et al., 2013b), as well as in primary mesencephalic cell cultures (Rodriguez-Pallares et al., 2004, 2008; Joglar et al., 2009). Furthermore, we demonstrated the presence of different cytoplasmatic and membrane subunits of the NADPH complex in mesencephalic dopaminergic neurons, astrocytes and microglia (Rodriguez-Pallares et al., 2007, 2008; Joglar et al., 2009). Recently, we have described, for the first time, prorenin receptors in nigral dopaminergic neurons and microglial cells in humans, monkeys and rats (Valenzuela et al., 2010; Garrido-Gil et al., 2013b). Interestingly, the labeling for prorenin, AT1 and AT2 receptors was not only located at the cell surface but also intracellularly in dopaminergic neurons and glial cells (Garrido-Gil et al., 2013b). Therefore, our observations support the existence of an intracellular/intracrine RAS in neurons, and particularly in dopaminergic neurons, as previously suggested for other cell types (Baker et al., 2004; Kumar et al., 2007, 2009).

INCREASED LOCAL RAS ACTIVITY ENHANCES DOPAMINERGIC CELL VULNERABILITY: MECHANISMS INVOLVED

In several recent studies, we used 6-OHDA and MPTP models of parkinsonism to study the possible role of the brain RAS in dopaminergic degeneration: the results suggest that enhanced levels of AII, via AT1 receptors, exacerbate dopaminergic cell death and may play a synergistic role in the pathogenesis and progression of PD. Recent experimental data from other laboratories also support the involvement of brain RAS in dopaminergic degeneration (Grammatopoulos et al., 2007; Zawada et al., 2011; Sonsalla et al., 2013). It was observed that AII increased the neurotoxic effect induced by low doses of dopaminergic neurotoxins, and that treatment with ACEIs (Lopez-Real et al., 2005; Muñoz et al., 2006; Sonsalla et al., 2013) or blockage of AT1 receptors (Rey et al., 2007; Rodriguez-Pallares et al., 2008; Joglar et al., 2009) led to significant reduction in the loss of dopaminergic neurons and levels of protein oxidation and lipid peroxidation induced by the neurotoxins (Sanchez-Iglesias et al., 2007). Interestingly, the neuronal loss was also reduced by inhibitors of NADPH-oxidase activation, which suggests that NADPH activation and NADPH-derived ROS are involved in the AII-enhanced dopaminergic neuron death (Rey et al., 2007; Rodriguez-Pallares et al., 2008; Joglar et al., 2009).

In peripheral tissues (see above) and brain (Benicky et al., 2009; Wright and Harding, 2013), abnormal upregulation of local AII induces OS damage and exacerbates inflammation. Oxidative stress and neuroinflammation (including microglial NADPH-oxidase activation) constitute early components of dopaminergic cell death and both factors act synergistically with others to induce progression of PD (Gao et al., 2003; Wu et al., 2003; Rodriguez-Pallares et al., 2007). In the substantia nigra, AII receptors and NADPH-oxidase were observed in dopaminergic neurons and glial cells. Therefore, AII may also enhance dopaminergic degeneration through several mechanisms, as previously observed in the vessel wall (**Figure 1**). First, AII acts on neurons (i.e., resident cells) via AT1 receptors and stimulates production of low levels of intraneuronal ROS by activation of neuronal NADPH-oxidase. ROS act as second messengers in several signaling pathways, including those involved in triggering the inflammatory response and the migration of inflammatory cells into the lesioned area; NADPH-derived ROS also modulate neuronal levels of ROS by interacting with mitochondria-derived ROS and with ROS from other sources such as dopaminergic neurotoxins or activated microglia. Feed-forward cross-talk signaling between NADPH oxidase-derived ROS and mitochondria-derived ROS has been observed in several types of cells (Doughan et al., 2008; Wosniak et al., 2009). This interaction has recently been confirmed in a dopaminergic cell line treated with the neurotoxin MPP^+ and angiotensin (Zawada et al., 2011) and in our recent studies with primary cultures of dopaminergic cells (Rodriguez-Pallares et al., 2009, 2012). Second, AII acts on microglia (i.e., inflammatory cells), in which NADPH oxidase activation produces high concentrations of ROS, which are released extracellularly and affect neurons; AII also produces low levels of microglial intracellular ROS, which act as a second messenger in several microglial signaling pathways involved in the inflammatory response (Babior, 2004; Qin et al., 2004). We have recently shown that activation of the microglial RhoA/ROCK pathway (Villar-Cheda et al., 2012a; Borrajo et al., 2014b), release of microglial TNF-α (Borrajo et al., 2014a), and altered iron homeostasis (Garrido-Gil et al., 2013a) are involved in the enhancing effect of AII/AT1 activation on the microglial response and dopaminergic degeneration. Activation of peroxisome proliferator-activated receptor gamma (PPAR-γ) also mediates the neuroprotective and anti-inflammatory effects of AT1 receptor inhibition (Garrido-Gil et al., 2012).

FACTORS THAT MAY INCREASE RAS ACTIVITY IN THE NIGROSTRIATAL DOPAMINERGIC SYSTEM

Interaction between dopamine and angiotensin was initially suggested to occur in the basal ganglia because acute administration of AII directly into the striatum (via microdialysis probes) induced an increase in extracellular levels of dopamine in normal rats, which was blocked by co-administration of AT1 receptor antagonists (Mendelsohn et al., 1993; Brown et al., 1996). This suggested that AII, via AT1 receptors, facilitates the release of dopamine. However, acute or chronic administration of AT1 receptor antagonists alone did not alter striatal dopamine levels (Dwoskin et al., 1992; Mendelsohn et al., 1993; Brown et al., 1996; Jenkins, 2008). This was attributed to possible compensatory mechanisms, which we have recently investigated in normal rats and dopaminergic denervated rats (Dominguez-Meijide et al., 2014). Therefore, it seems reasonable to assume that a decrease in dopaminergic activity may induce a compensatory increase in RAS activity to increase striatal dopamine. However, if the dopaminergic system is impaired (e.g., in the initial stages of dopaminergic lesions or aging), normal dopaminergic levels cannot be reached and the resulting overactivation of the RAS may exacerbate the microglial inflammatory response and produce OS, leading to progression of dopaminergic vulnerability and neurodegeneration. In a series of recent studies, we have confirmed that a decrease in dopaminergic activity induces compensatory upregulation of local RAS function in both dopaminergic neurons and glia (**Figure 1**). It is known that both angiotensin (Rodriguez-Pallares et al., 2004, 2008; Joglar et al., 2009; Garrido-Gil et al., 2013b) and dopamine (Miyazaki et al., 2004; Färber et al., 2005) receptors are located in neurons, microglia and astrocytes. In the nigrostriatal system, we observed that dopamine depletion induced a significant increase in AT1 and AT2 receptor expression, and NADPH-oxidase complex activity, which decreased as dopamine function was restored (Villar-Cheda et al., 2010). More recently, we investigated the possible interactions between angiotensin and dopamine receptors in D1-, D2-, and AT1-deficient mice, as well as mice over-expressing D2 receptors. A counter-regulatory mechanism between dopamine and angiotensin receptors was observed in the striatum and substantia nigra of these mice (Villar-Cheda et al., 2014). A similar interaction between dopamine and angiotensin receptors has recently been demonstrated in peripheral tissues, particularly in relation to the regulation of renal sodium excretion and cardiovascular function (Zeng et al., 2006; Khan et al., 2008; Gildea, 2009; Padia et al., 2012).

Other factors may induce an increase in RAS activity independently or before the loss of dopamine (**Figure 1**). It is known that dopaminergic neurotoxins such as MPP^+ can act directly on astrocytes to induce an increase in production of proinflammatory factors (Henze et al., 2005; Block et al., 2007); astrocytes are the main source of angiotensinogen/angiotensin (Stornetta et al., 1988; Milsted et al., 1990), which may then act on neurons and microglial cells as indicated above. A loss of estrogen and other mechanisms that inhibit the neuroinflammatory response may also induce RAS activation and lead to an increase in dopaminergic neuron vulnerability as detailed below.

DOPAMINERGIC VULNERABILITY IN AGING, MENOPAUSE AND BRAIN HYPOPERFUSION. ROLE OF RAS HYPERACTIVITY

In recent studies, we investigated whether enhanced RAS activity in the nigra may be involved in the increased vulnerability of dopaminergic neurons to degeneration observed in aging, postmenopause or chronic cerebral hypoperfusion. Several studies have shown that normal aging is associated with a proinflammatory and pro-oxidant state that may favor an exaggerated response to injury and degenerative diseases (Csiszar et al., 2003; Ungvari et al., 2004; Choi et al., 2010). We have confirmed that, in aged male rats, aging enhances levels of neuroinflammation, OS markers and dopaminergic cell death induced by dopaminergic neurotoxins. The nigral RAS is involved in these effects, and levels

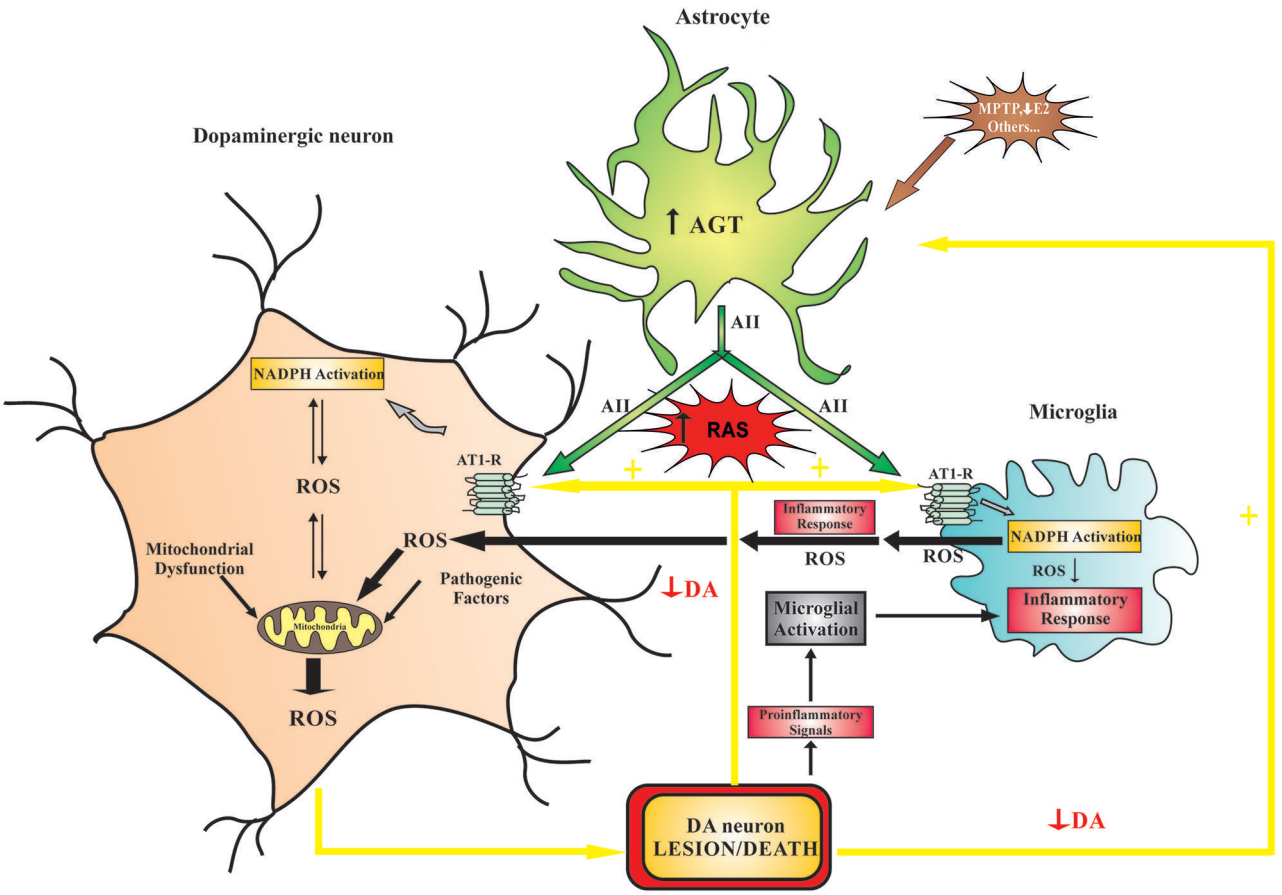

FIGURE 1 | Model of the role that brain RAS plays in dopaminergic cell vulnerability. Different pathogenic factors (e.g., mitochondrial dysfunction, aging-related changes, neurotoxins, etc.) may initiate dopaminergic lesions and diminish dopaminergic function, which leads to increased RAS activation and progression of the dopaminergic degeneration. Furthermore, neurotoxins and proinflammatory factors may act directly on astrocytes and induce an increase in ANG/AII production, which leads to an increase in RAS activity and dopaminergic vulnerability. In dopaminergic neurons, increased RAS activity (via AT1 receptors) increases NADPH-oxidase activity, which enhances intraneuronal ROS production (in an interaction with mitochondria) and pro-inflammatory signals. In microglial cells, increased RAS activity stimulates the NADPH-oxidase complex, which enhances the inflammatory response, promoting extracellular release of high levels of ROS, activation of ROCK, and the release of cytokines and different neurotoxic factors. ANG, angiotensinogen; AII, angiotensin II; AT1, angiotensin type I receptors; DA, dopamine; E2, estrogen; NADPH, NADPH-oxidase complex; RAS, renin-angiotensin system; ROCK, Rho-associated kinase; ROS, reactive oxygen species.

of neuroinflammation, OS markers and dopaminergic cell death are reduced by treatment with the AT1 antagonist candesartan (Villar-Cheda et al., 2012b, 2014). Numerous studies in animal models and humans have shown aging-related loss of striatal D2 and D1 receptors (Wang et al., 1998; Ishibashi et al., 2009; Rieckmann et al., 2011), and that the dopaminergic system is altered during normal aging (Kubis et al., 2000; Collier et al., 2007). Therefore, the RAS upregulation that we observed in aged rats may be part of the compensatory changes related to decreased levels of dopamine or dopamine receptors (Villar-Cheda et al., 2012b, 2014). However, other factors may also be involved (Cruz-Muros et al., 2007, 2009), as aging has been shown to be associated with overactivation of RAS in a number of tissues (Thompson et al., 2000; Min et al., 2009; Cassis et al., 2010). Thus, the upregulation of AT1 receptors observed in aged rats may be part of the compensatory changes related to changes in the dopaminergic system; however, the compensatory upregulation of AT2 receptors observed in young rats with similar changes in the dopaminergic system was not observed in aged rats.

Menopause has also been identified as a prominent risk factor for PD. Numerous experimental studies have shown that estrogen exerts protective effects against dopaminergic cell degeneration (Leranth et al., 2000; Callier et al., 2002). The anti-inflammatory effects of estrogen play a major role in the neuroprotective effects (Suzuki et al., 2007; Vegeto et al., 2008), although direct anti-apoptotic (Das et al., 2011; Brendel et al., 2013) and trophic (López-Martín et al., 1999; Campos et al., 2012) effects on neurons have also been suggested. A number of epidemiological studies have also reported that the incidence and prevalence of PD is higher in postmenopausal women and men than in premenopausal women of similar age (Currie et al., 2004; Ragonese et al., 2006a,b). However, some reported effects of estrogen replacement therapy are controversial (Shulman, 2002; Popat et al., 2005), and the age and duration of lack of estrogen

in women receiving the treatment appear to be major factors in the discrepancies. Interestingly, estrogen-induced regulation of the RAS mediates beneficial effects of estrogen in several tissues (Nickenig et al., 1998; Dean et al., 2005; Chen et al., 2008), and interactions between estrogen and AII receptors have also been observed (Liu et al., 2002; Tsuda et al., 2005; Xue et al., 2007; Hoshi-Fukushima et al., 2008). In several recent studies we have observed that the lack of estrogen increases RAS activity in the substantia nigra in females (Rodriguez-Perez et al., 2010, 2011, 2012). We compared young ovariectomized rats (i.e., early surgical menopause) with aged rats (i.e., natural menopause). Both groups of menopausal rats showed increased RAS activity. However, estrogen therapy significantly reduced 6-OHDA-induced dopaminergic cell loss in young rats but not in aged rats, suggesting that other factors are involved in aged females. Interestingly, treatment with the AT1 antagonist candesartan significantly reduced dopaminergic neuron loss in both groups of menopausal rats (Rodriguez-Perez et al., 2012).

Dopaminergic cell loss and signs parkinsonism have been observed in elderly people without PD (almost 40%; Buchman et al., 2012), and presynaptic dopaminergic function is reduced in most patients with vascular parkinsonism (Zijlmans et al., 2007), suggesting an interaction between aging-related cerebrovascular disease/brain hypoperfusion and dopaminergic degeneration. This was confirmed in a recent study with animal models of chronic brain hypoperfusion (Rodriguez-Perez et al., 2013), in which we have shown that chronic hypoperfusion increases dopaminergic cell death by enhancing the deleterious effects of other factors (such as low doses of dopaminergic neurotoxins). This suggests that hypoperfusion derived from aging and/or vascular disease may increase the risk of development of parkinsonism. The mechanistic links between hypoperfusion/vascular disease and neurodegeneration are unknown. However, chronic hypoperfusion led to increased expression of inflammatory markers such as IL-1β and increased levels of OS markers such as NADPH activity (Rodriguez-Perez et al., 2013), which have been shown to be involved in the progression of dopaminergic cell death in animal models of PD and in PD patients (Wu et al., 2003; Koprich et al., 2008). Interestingly, these changes were accompanied by increased RAS activity in the substantia nigra, and they were inhibited by chronic treatment with the AT1 receptor antagonist candesartan (Rodriguez-Perez et al., 2013).

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Inflammation in Parkinson's disease: role of glucocorticoids

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Chronic inflammation is a major characteristic feature of Parkinson's disease (PD). Studies in PD patients show evidence of augmented levels of potent pro-inflammatory molecules e.g., TNF-α, iNOS, IL-1β whereas in experimental Parkinsonism it has been consistently demonstrated that dopaminergic neurons are particularly vulnerable to activated glia releasing these toxic factors. Recent genetic studies point to the role of immune system in the etiology of PD, thus in combination with environmental factors, both peripheral and CNS-mediated immune responses could play important roles in onset and progression of PD. Whereas microglia, astrocytes and infiltrating T cells are known to mediate chronic inflammation, the roles of other immune-competent cells are less well understood. Inflammation is a tightly controlled process. One major effector system of regulation is HPA axis. Glucocorticoids (GCs) released from adrenal glands upon stimulation of HPA axis, in response to either cell injury or presence of pathogen, activate their receptor, GR. GR regulates inflammation both through direct transcriptional action on target genes and by indirectly inhibiting transcriptional activities of transcriptional factors such as NF-κB, AP-1 or interferon regulatory factors. In PD patients, the HPA axis is unbalanced and the cortisol levels are significantly increased, implying a deregulation of GR function in immune cells. In experimental Parkinsonism, the activation of microglial GR has a crucial effect in diminishing microglial cell activation and reducing dopaminergic degeneration. Moreover, GCs are also known to regulate human brain vasculature as well as blood brain barrier (BBB) permeability, any dysfunction in their actions may influence infiltration of cytotoxic molecules resulting in increased vulnerability of dopamine neurons in PD. Overall, deregulation of glucocorticoid receptor actions is likely important in dopamine neuron degeneration through establishment of chronic inflammation.

Keywords: glucocorticoid receptor, Parkinson's disease (PD), neuroinflammation, neurodegeneration, microglia

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Introduction

Parkinson's disease (PD) is a common age-related neurodegenerative disorder characterized by cardinal motor symptoms that include bradykinesia with resting tremor, rigidity and gait disturbance. These motor symptoms become evident when already 70–80% of nigrostriatal terminals have degenerated. At present, therapeutic treatments, for example, levodopa, mostly address motor symptoms. However, a wide spectrum of non-motor clinical

features such as REM (Rapid eye movement) sleep disturbances, autonomic dysfunction, depression, anxiety, cognitive impairment or falling are associated with PD, and are moreover debilitating and unresponsive to dopamine-related treatments. Thus, PD is a complex systemic disorder with non-motor symptoms often preceding motor symptoms and worsening with disease progression (Berg et al., 2014; Goldman and Postuma, 2014). PD has an age-adjusted incidence of 13.5–13.9 per 100.000 person-years, and a prevalence of 315 per 100,000 individuals (the second most common worldwide). As PD affects predominantly older people, its prevalence increases with age from 428 at 60–69 years to 1,903 per 100.000 in 80 years old people (Abdullah et al., 2014; Pringsheim et al., 2014).

The major neuropathological hallmarks of PD are progressive degeneration of dopaminergic neurons in the Substantia Nigra pars compacta (SNpc); presence of proteinaceous inclusions called Lewy bodies (LBs) and chronic inflammation. However, the initial causes and underlying mechanisms pertaining to these neuropathological features in the majority of patients, classified as sporadic PD, remain unknown. Recent studies indicate that combined genetic, environmental factors and aging confer risk for developing sporadic PD rather than genetic or environmental factor individually. Approximately 5–10% of PD patients present the familial form of the disease with either autosomal dominant or recessive mode of inheritance. Epidemiological analysis confirm that up to 40% of PD patients with age at onset of less than 30 years and 17% of those with age at onset of less than 50 years will probably present the familial form of the disease. At least 18 loci as well as 12 genes with Mendelian inheritance and highly penetrant mutations causing rare monogenic forms have been identified. The genetic discovery of point mutations, duplication or triplication of *SNCA* (synuclein) gene coding for α-synuclein protein (reviewed in Goedert et al., 2013) with demonstration by Spillantini et al. (1997) that α-synuclein is a major component of LBs, led Braak et al. (2003) to staging PD according to appearance of α-synuclein containing LBs and Lewy neuritis with disease severity. Accordingly to Braak's hypothesis PD progresses in neuronally-connected ascending manner to dorsal motor nucleus of the glossopharyngeal and vagus nerves likely from gut and/or olfactory mucosa (stage 1 and 2), then from lower brain stem to midbrain including nigral regions (stage 3 and 4) and lastly to the neocortical regions (stage 5 and 6). However, it has been suggested that LB pathology alone is not sufficient and that associated neuronal loss leads to Parkinsonism (Buchman et al., 2012).

In last few years, large genetic and genome-wide association (GWA) studies together with meta-analyses have led to significant and rapid advances in the genetic basis of sporadic PD, with realization both for its wide implication and complexity (Lill et al., 2012; Clarimón and Kulisevsky, 2013). There is great expectation for further insights with advent of new DNA sequencing technologies (exome and whole-genome sequencing) and the NeuroX genotyping platform (Nalls et al., 2015). These studies have identified at least 20 susceptibility loci as risk for sporadic PD (Nalls et al., 2014). As yet, the true significance of many of these loci is still unknown. Of interest, susceptibility related to *SNCA* and *LRRK2* (Leucine rich repeat kinase 2) loci consistently observed are also genes that have been identified in the monogenic, autosomal dominant familial PD patients. Occurrence of somatic mosaicism has been also hypothesized in the etiology of some cases of PD (Kim and Jeon, 2014). Cases of somatic mosaicism in many central nervous system (CNS) disorders have been reported, for example, somatic mutation in the *presenilin-1* gene associated with Alzheimer's disease (Beck et al., 2004), in *SPG4/SPAST* (spastic paraplegia4/spastin) causing spastic paraplegia (Depienne et al., 2007) or *MECP2* (methyl CpG binding protein) resulting in Rett syndrome (Topçu et al., 2002). Thus far, no cases of PD with somatic mosaicism are known, as well, in this regard results of study on *SNCA* somatic mutations by Proukakis et al. (2014) were negative.

Summing up: (a) although at present there is a rapid progress and evolution in technology to unravel genetic basis of PD, most PD risk is not understood; (b) the pathogenicity arising from several of identified gene mutations remains to be determined; (c) highly penetrant gene mutations (as *DJ-1, LRRK2, Parkin, PINK1* (PTEN-induced putative kinase 1), and *SNCA*) cause rare monogenic forms of the disease; (d) somatic mosaicism could shed light on the heterogeneity of PD; and (e) additive mechanisms is suggested in risk for PD, increasing with the number of risk alleles carried by a single subject e.g., in HLA (human leucocyte antigen) region (Hill-Burns et al., 2011).

There is strong epidemiological evidence to show that aging is a single most important risk factor for PD, with increase in incidence between fifth to eight decades. Modifications that occur in specific brain regions during aging, such as increased oxidative and nitrative stress, changes in glial functions, dysfunction of proteasomes and lysosomes and altered α-synuclein protein are also manifestations of PD (Collier et al., 2011; Kieburtz and Wunderle, 2013). Environmental toxins identified as risk for PD are herbicides (e.g., paraquat or rotenone), heavy metals such as manganese and lead, nanoparticles as air pollutants, head trauma or well water. Thus, for example, it was shown that people exposed to pesticides and harboring Cytochrome P450 2D6 (CYP2D6) genotype with poor metabolic capacity for xenobiotics are at increased risk for developing PD (Elbaz et al., 2004). Epidemiologic link also exists between rotenone and PD. Rotenone is a powerful inhibitor of mitochondrial complex 1 and interestingly complex I deficiency is found in PD. The role of viral infections as risk factor has been evoked ever since the famous and controversial von Economo's encephalitis lethargica pandemic suspected to be caused by H1N1 (Hemagglutinin1 neuraminidase1) influenza virus where patients exhibited Parkinsonism symptoms (Ravenholt and Foege, 1982). Recently, animals infected with highly pathogenic H5N1 virus showed clear motor deficits as well human cases with encephalitis have been reported (Jang et al., 2009). Epigenetic modifiers could be potential mediators of environmental factors (Portela and Esteller, 2010). Aberrant epigenetic modifications include changes in gene functions or gene expression but without changing the DNA sequence: non-coding RNA-mediated changes of gene expression, DNA methylation or post-transcriptional

modifications and acetylation of histones (Nalls et al., 2014). For instance, the methylation of the Tumor Necrosis Factor alpha (TNF-α) promoter is significantly decreased in the SNpc of PD patients compared with controls or with the methylation in the cortex (Pieper et al., 2008) suggesting increased susceptibility of dopamine neurons to TNF-α mediated inflammation (Barcia et al., 2005, 2011).

Thus, aging together with genetic susceptibility and cumulative environmental factors such as air pollutants, pesticides, infections or exposure to heavy metals likely have a role in the development of idiopathic PD.

Immune System and the Etiology of Parkinson's Disease

All of the above environmental factors together with multiple cellular changes occurring during aging can impact immune functions. There is now a growing realization, particularly from genetic studies, that immune system is most likely involved in the etiology as well as early phases of PD, thus the inflammatory component of the disease may simply not be a consequence of neuronal dysfunction and neurodegeneration. In GWA studies, a number of susceptibility loci that have been identified as strong risk factors, are related to both innate and adaptive immune functions, for example, *HLA-DQB1*, *LRRK2*, *GPNMB* (glycoprotein NMB), or *BST-1* (bone marrow stromal cell antigen) (Liu et al., 2011; Pihlstrøm et al., 2013). In this regard, *LRRK2*, *Parkin*, *PLA2G6* (phospholipase A2, group VI), *DJ-1* and *SNCA* genes mutated in both familial and idiopathic PD are also known to function in microglia and astrocytes (Russo et al., 2014). Interestingly, several studies have identified risk of PD with polymorphisms present in the promoter regions of *IL-1β* and *TNF-α* genes that augment the expression of these genes and whose protein products have potent pro-inflammatory activity (Wahner et al., 2007). Moreover, polymorphisms reported in other pro-inflammatory genes e.g., CD14, *HLA-DBQ1*, *HLA-DRA*, HLA-DRB1, *HLA-DRB5* can also increase the risk for PD (Ahmed et al., 2012). In the analysis of potential markers of motor and cognitive progression, SNPrs 6482992 of clarin3 (CLRN3) was described as the best predictor of cognitive deterioration (Chung et al., 2012) whereas SNPrs 10958605 as involved in neuroinflammatory pathways (Cappellano et al., 2013). The implication of early involvement of immune system is also reinforced by epidemiological studies showing a prolonged use of NSAIDs (Nonsteroidal anti-inflammatory) particularly ibruprofen subsequently lowers the risk of PD (Rees et al., 2011).

Neuroinflammation in PD

As a progressive neurodegenerative disorder, PD is a multifactorial complex disease most likely evolving because of the genetic and environmental risk factors, as well as cellular alterations and aging. Inflammatory component in PD not only encompasses deregulation of inflammatory pathways resulting from genetic vulnerability but also immune alterations associated with aging and with primary activation of glia in the face of neuronal injury. Aging affects the functions of immune system, resulting in so-called "immune senescence". Specifically, advancing age has been associated with chronic mild inflammation in the SNpc, thereby rendering dopaminergic neurons vulnerable to degeneration (Kanaan et al., 2010). Increasing evidence points to the role of active peripheral inflammation in PD that can contribute to the initiation and/or the progression of the disease by, for example, exacerbating and synergizing with the discordant central inflammatory response to drive dopaminergic neurodegeneration. Combination of aging, heritable risk factors and exposure to environmental agents has been suggested as potential host-pathogen specific pathophysiologic elements that can cause deregulation of both innate and adaptive immune system responses (Kanaan et al., 2008; Chao et al., 2014). Thus, in both sporadic and familial PD, immune activation occurring at multiple levels would play an important role in PD pathology.

Evidence of an on-going neuroinflammation in affected brain regions in PD stems from analyses of pro-inflammatory cytokines (Interferon gamma, IFN-γ; TNF-α; Interleukin-6, IL-6; or Interleukin-1β, IL-1β) showing their accumulation in both cerebrospinal fluid and post-mortem brain (Mogi et al., 1994; Dobbs et al., 1999; Reale et al., 2009a,b). Recently, it has been demonstrated that the serum levels of IL-6 and the chemokine ligand 5 (CCL5) also known as Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) were significantly increased in PD patients, and importantly, RANTES levels correlated with the severity and duration of the disease (Tang et al., 2014). Furthermore, the augmentation of iNOS (Inducible nitric oxide synthase) observed in SN and striatum of PD (Hunot et al., 1996) suggests that the toxicity originating both from cytokines/chemokines and inflammation-derived oxidative stress could contribute to dopaminergic neuronal degeneration and progression of the disease (Orr et al., 2005; Wilms et al., 2007). Numerous studies in experimental PD models indicate that dopamine neurons are particularly vulnerable to both oxidative stress and inflammatory attack (McGeer and McGeer, 2008; Pott Godoy et al., 2008). Interestingly, in this regard, Lipopolysaccharide (LPS)-activated microglia in the vicinity of dopamine neurons in SN induce degeneration of these neurons whilst sparing GABAergic and serotonergic neurons, suggesting a selective dopamine neuron vulnerability to inflammation (Liu and Bing, 2011).

Inflammation and immune-related responses may be viewed not only as determinant factors in disease progression but also as pathogenic processes in the onset of both familial and sporadic PD (Halliday and Stevens, 2011; Chao et al., 2014; Dzamko et al., 2014). On this point, presence of activated microglia, visualized by PET (positron emission tomography) analysis using radioligand ^{11}C-PK-11195, was recently reported in the SN and putamen of PD patients diagnosed within a year from clinical onset (Iannaccone et al., 2013). This, together with study of Ouchi et al. (Ouchi et al., 2005) suggests a microglial-mediated inflammatory process in early stage of PD. Several lines of evidence also point to relevant actions of different PD-linked gene mutations e.g., *SNCA* or *LRRK2* in stimulating inflammatory responses through activation of microglia and astrocytes thereby participating directly in chronic

PD progression (Gillardon et al., 2012; Moehle et al., 2012; Harms et al., 2013). Both central and peripheral inflammation occurs in the prodromal stage of PD, which thus sustains disease progression (Dzamko et al., 2014; Su and Federoff, 2014). Overall, accumulation of pathological α-synuclein in PD brain leads to neurodegeneration with T-cell infiltration, microglial activation and increased production of inflammatory cytokines and chemokines (Harms et al., 2013). The detection of T lymphocytes and activated microglia in the SN of Parkinsonian patients is striking because systemic immune cells have to penetrate several barriers in order to reach the brain parenchyma.

The CNS was considered as an immunologically privileged site because of the lack of lymphatic vessels, the absence of classical major histocompatibility complex (MHC) positive antigen presenting cells, and the presence of barriers as the tanycytic barrier around the circumventricular organs or the neurovascular unit of the BBB. The latter is composed of endothelial cells, pericytes and astrocytes and associated strong, tight junctions prevent the entry of immune cells into the brain parenchyma. BBB is a metabolic and physical barrier that separates the CNS from the peripheral circulation, actively allowing the transports of nutrients to the brain but limiting passive diffusion of blood-borne solutes. However, in aging and in PD, a BBB disruption has been described with loss of the barrier permeability leading to secondary leukocyte migration within the brain parenchyma, reactive gliosis and damage to neurons (Stolp and Dziegielewska, 2009; Cabezas et al., 2014). BBB dysfunction in PD favors an invasion of immune cells (and/or peripheral mediators and factors as toxins or elements of adaptive immunity) into the brain parenchyma that provokes a progressive and self-perpetuating degenerative process (Monahan et al., 2008). Additionally, it has been demonstrated that PD patients have increased permeability of the intestinal epithelial barrier (Forsyth et al., 2011) as well as chronic enteric/colonic inflammation (Devos et al., 2013). As proposed by Braak et al. (2003), an environmental pathogen can cross the monolayer of polarized epithelial cells (the intestinal epithelial barrier) (Sharkey and Savidge, 2014) and enter into the terminal axons of the submucosal plexus spreading to the medulla oblongata via the vagal preganglionic innervation of the gut (Hawkes et al., 2009). Moreover, brain injuries or systemic infections can induce systemic inflammatory responses that easily communicate with brain. Both Alzheimer's disease and PD have been associated with both the HLA region (Ahmed et al., 2012; Wissemann et al., 2013), and with the production of autoantibodies (Maetzler et al., 2014) suggesting putative genetic susceptibility to inflammation that could initiate the neuronal dysfunction.

Microglia are the resident innate immune cells in the brain. Being only 5–15% of the total cells of the brain, microglia functions include tissue repair and cellular homeostasis after neuronal injury. Activated microglia produce neurotoxic molecules, for example, pro-inflammatory cytokines, chemokines, complement proteins or nitric oxide. Additionally, activated microglia acquire phagocytic properties and develop neuro-immune interactions involving the expression of surface molecules as CD200/CD200R, CD47/CD172a, CX3C chemokine ligand 1 and its receptor (CX3CL1/CX3CR) and the complement regulatory proteins, complement components C1q and C3 in order to eliminate cellular debris and damaged neurons by gliapses (Barcia et al., 2012). However, microglial responses can have neuroprotective as well as harmful consequences mainly if there is a continuous exposure to a pro-inflammatory environment with a persistent release of inflammatory mediators (Bardou et al., 2014) as activated microglia can still persist even years after the toxic insults (Barcia et al., 2004; Jackson-Lewis and Smeyne, 2005; Block et al., 2007). In fact, if as a defense mechanism of the organism, an inflammatory response starts and continues without control, a chronic persistent inflammation environment in the brain can result in tissue destruction and progressive neurodegeneration.

Glucocorticoids, Inflammation and Parkinson's Disease

Inflammation is normally a tightly regulated process that acts to prevent pathogen invasion as well as cellular injury, whilst at the same time enabling tissue repair. Several endogenous mechanisms act to regulate the immune cell functions, which are involved in triggering an inflammatory process. Among them, the steroid hormone, glucocorticoid, is a known major regulator of immune system and inflammation. Glucocorticoids (GCs) are one of the most potent and effective anti-inflammatory agents in clinical use ever since the isolation of cortisone and its clinical application in the early 1950s by the Nobel Prize winners Hench, Kendall and Reichstein (Hench et al., 1950; Reichstein, 1951).

GCs (cortisol in humans and corticosterone in rodents) are endogenous steroid hormones synthesized in adrenal glands and secreted into systemic circulation. The GC secretion occurs in ultradian pulsatile manner (Hellman et al., 1970; Veldhuis et al., 1989) and over-riding this pattern is acute GC rise in response to a stressor (psychogenic or physical e.g., tissue injury or pathogen invasion) whereby increased levels of GCs exert important adaptive actions in multiple tissues to restore homeostasis (Young et al., 2004; McEwen, 2007). Both ultradian/circadian and stress-evoked GC secretion is tightly controlled by various negative feedback mechanisms affecting each component of HPA axis, notably synthesis and release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus and adrenocorticotropic hormone (ACTH) from anterior pituitary. Any change in negative feedback loops will affect HPA axis, resulting in altered ultradian/circadian rhythm of GC release often with abnormally high basal GC levels, which in turn could lead to GC resistance.

Measurement of plasma cortisol in idiopathic PD patients has consistently shown significantly elevated levels compared to age-matched control subjects, and as well correlating with impulsive behaviors (Bellomo et al., 1991; Stypula et al., 1996; Hartmann et al., 1997; Charlett et al., 1998; Djamshidian et al., 2011; Ros-Bernal et al., 2011). The high cortisol levels seem unrelated to L-DOPA treatment or disease duration (Müller et al., 2007). Elevated cortisol levels are observed in many other neurodegenerative diseases including Alzheimer disease (Huang

et al., 2009). In PD, however, the normally quiescent nocturnal cortisol secretory pattern is particularly affected (Hartmann et al., 1997) raising the question as to whether the circadian control of HPA axis by suprachiasmatic nucleus is altered. The underlying causes of HPA deregulation and whether or how it impacts PD pathology is presently not well understood. However, presence of LBs in both adrenal glands and hypothalamus in PD has been reported (Wakabayashi and Takahashi, 1997; Braak et al., 2006), which may imply a role of α-synuclein pathology in HPA axis deregulation. In addition to neuronal networks regulating HPA axis through feed back loops, cytokines liberated by peripheral immune cells can also stimulate HPA axis in several ways. Potent inflammatory cytokines (TNF-α, Il-1β and Il-6) can induce release of GCs by directly stimulating CRH synthesizing neurons of PVN or indirectly by stimulating production of prostaglandin E2 synthesis in perivascular cells (Ericsson et al., 1994; Kang et al., 2006; Serrats et al., 2010). In addition, IL-6 was shown to directly act on anterior pituitary cells as well as in adrenal glands, via its receptor, to stimulate the synthesis of ACTH and GC respectively (Zarković et al., 2008). Thus, deregulated immune responses with elevated levels of pro-inflammatory cytokins may lead to chronic activation of HPA axis.

Once secreted, GCs act on diverse physiological processes ranging from metabolism, immune responses to cognition and behavior. Their therapeutic potential, however, has limitations as chronic use with sustained high levels of GCs can result in serious side effects such as diabetes, obesity, dyslipidemia, hypertension, osteoporosis or behavioral anomalies. GCs clearly exert anti-inflammatory actions especially in an inflammatory setting, however, a number of recent studies indicate that they also exert pro-inflammatory responses, which are cell-type dependent. Thus, in response to acute stress resulting in increased GC levels, high levels of pro-inflammatory mediators such as IL-1β were found (Dhabhar, 2002; O'Connor et al., 2003; Sorrells et al., 2007). In a microarray study by Galon et al. (2002) on human mononuclear cells, dexamethasone treatment was found to induce the expression of several innate-immune related genes in addition to down-regulation of pro-inflammatory genes. It is believed that this opposing action of GCs "prepares" the immune system to respond rapidly to harmful stimulus and subsequently GCs act to down-regulate the immune response to restore homeostasis.

Glucocorticoid Regulation of Inflammation through GR

In brain, GC signaling is mediated by almost ubiquitously expressed GRs (GRs) as well as mineralocorticoid receptors (MR) that have restricted expression in neurons. However, it should be noted that MR is also expressed in glia (Sierra et al., 2008). GR, a prototype member of nuclear receptor superfamily (designated as NR3C1 in nomenclature of nuclear receptor family) is a ligand-activated transcription factor, it can also exert non-genomic actions (Groeneweg et al., 2011). GR is a modular protein with an N-terminal transactivation domain, a C-terminal ligand binding domain (LBD) and a central Zinc fingers-containing DNA-binding domain (DBD) that recognizes a specific DNA sequence. The LBD is the high affinity binding site for cortisol and other ligands. In humans, two major isoforms of GR, hGRα and hGRβ arising from alternative splicing have been described (Zhou and Cidlowski, 2005) and they differ in their C-terminal ligand-binding domain such that hGRβ cannot bind to endogenous or synthetic GCs. Experimental evidence indicates that hGRβ is expressed at low levels and it antagonizes the transcriptional activity of hGRα thus acting as dominant negative inhibitor of hGRα. However, recent genome-wide microarray studies indicate that hGRβ also regulates gene transcription (Kino et al., 2009). Interestingly, reduction in hGRα:hGRβ ratio has been associated with behavioral and mood disorders such as depression and schizophrenia (Perlman et al., 2004; Matsubara et al., 2006). In addition, alternative translational initiation sites generating 8 different GR proteins both in mouse and humans have been described (Oakley and Cidlowski, 2013).

Recent evidence shows that pulsatile pattern of GC secretion is crucial to proper GR transcriptional activity, thus loss of GC oscillatory pattern can result in continuous transcription with abnormal protein accumulation or GR targeting inappropriate genes leading to undesirable outcomes. GR is normally inert in the cytoplasm, in association with complex of proteins including heat-shock chaperones (HSP90, HSP70, HSP40, HSP23) and immunophilins such as FKBP51 (FK506 binding protein51), FKBP52, CP44 and PP5 (Grad and Picard, 2007). GC binding to this complex results in conformational change in GR exposing a nuclear localization signal resulting in importin-mediated translocation through the nuclear pore to the nucleoplasm.

In the nucleus, GR regulates transcription of its target genes in multiple, complex ways as well in highly cell- and context-specific manner. The transcriptional activity of GR has been especially studied with respect to its actions on metabolism and regulation of immune responses in the periphery. Multitude of studies indicates that GCs through GR influence each stage of inflammatory response i.e., from initiation, effector to resolution phases of an inflammatory reaction. Inflammatory response is triggered by specific receptors in immune cells and in this regard toll-like receptors (TLRs) activation and intracellular signaling cascade is thus far best characterized (Kawai and Akira, 2010) resulting in activation of transcriptional factors such as Nuclear factor kappa B (NF-κB), Activator Protein 1 (AP-1) or interferon response factors (IRFs). Each TLR family member (from 1–13 in mouse; all expressed in microglia) recognizes specific molecular signature present in either pathogens (PAMPs-Pathogen Associated Molecular Patterns) or molecules released by injured cells called DAMPs (Damage-associated Molecular Patterns). GR is reported to regulate key components of TLR signaling e.g., transforming growth factor beta-activated kinase 1 (TAK1; Bhattacharyya et al., 2010).

The GR regulation of inflammation is a result of both its transcriptional stimulatory and repressive activity. Classically, GR stimulates transcription of genes that act to inhibit inflammation and conversely it inhibits transcription of pro-inflammatory genes. GR stimulates transcription as homodimer binding to specific cognate DNA sequence,

GAGAACAnnnTGTTCT, called Glucocorticoid Responsive Elements (GREs) present in the promoter regions of its target genes. This transcriptional activity of GR requires the presence of chromatin modifiers (e.g., Nuclear receptor coactivator NCoA1), basal transcriptional machinery and co-factors (CREB binding protein CBP, p300) (Rosenfeld and Glass, 2001). This mode of transcriptional activity has been notably described for genes coding for proteins of metabolic pathways such as glucose-6-phosphotase, fatty acid synthase or tyrosine aminotransferase as well as anti-inflammatory genes e.g., (Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB-α), MAPK phosphatase (MPK-1), IL-4, IL-10 or annexin-1 (De Bosscher et al., 2003). GR can also bind to negative GREs (nGREs) to repress transcription, thus among the genes identified containing nGREs are CRH as well as ACTH receptor in adrenal glands (Dostert and Heinzel, 2004; Surjit et al., 2011). Importantly, with regards to inflammation, GR can also inhibit transcription by tethering (i.e., through protein-protein interactions) or modulating the activity of other transcriptional factors, for example NF-κB, AP1 or IRF (Chinenov et al., 2013). This action mediated by GR monomers has been particularly studied in peripheral immune cells involving inhibition of expression of powerful pro-inflammatory genes as well as resolution of inflammation. The cross talk between AP1, NF-κB and GR is well documented (De Bosscher et al., 2003). As it has pertinence in the effects of GR observed in microglia it will be briefly reiterated here.

AP1 is comprised of heterodimers of c-Fos (C-Fos, FosB, Fra1 and 2), Jun (c-N, B-Jun, D-Jun) as well as ATF (Activating transcription factor) families of transcription factors, which controls expression of many cytokines. AP1 activity is stimulated by MAPK cascade resulting in activation of c-Jun-N terminal kinase (JNK), which phosphorylates c-Jun. GR regulates AP1 activity by associating with Jun-Fos complex at AP1 DNA elements in promoter regions of genes, inducing a conformational change in the complex that is not functional (Diamond et al., 1990). Additionally, GR also stimulates transcription of MAPK-phosphatase 1 (MKP-1) by binding to GRE elements present in its promoter region (as mentioned above) resulting in MKP-1 termination of JNK phosphorylation activity on c-Jun.

NF-κB signaling in positive immune regulation has been thoroughly characterized especially in the periphery (Karin and Greten, 2005). NF-κB comprises of RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105 subunits) and NF-κB2 (p50/p100) proteins and the transcriptionally active dimers identified are: p65/p50 (classic NF-κB), p65/p65, p65/c-Rel, RelB/p50, RelB/p52 and, of note, Rel domains of these proteins bind to DNA. The p65/p50 NF-κB protein is normally sequestered in the cytoplasm by IκB family of proteins. NF-κB translocates to nucleus following phosphorylation of IκB by IKK kinases followed by rapid degradation of IκB. Importantly, phosphorylation of the p65 subunit is important for NF-κB activation. This involves phosphorylation of Serine 27Kuro6 of p65 by protein kinase A (PKA) catalytic subunit in complex with NF-κB and IκB as well as by nuclear localized MAPK-activated mitogen- and stress-activated protein kinase 1 (MSK1) in the nucleus. Interestingly GR was shown to decrease the nuclear pool of MSK1 thus down regulating NF-κB activity (Beck et al., 2008). With regards to its interaction with NF-κB, it was shown that upon GR activation by GC, GR is acetylated. In the nucleus, GR is deacetylated by histone deacetylase (HDAC2) (Ito et al., 2006) before it can physically bind to p65 subunit of NF-κB, functioning as transcriptional antagonist. Another manner by which GR can terminate NF-κB activation is by directly stimulating transcription of IκB-α as mentioned above.

Innate Immune Regulation by GRs in Microglia during Dopamine Neurodegeneration

In the CNS the role of endogenous GCs in regulating expression of pro-inflammatory cytokines such as IL-1β, TNF-α or IL-6 was shown originally following peripheral administration of LPS in adrenalectomized mice (Goujon et al., 1996). The finding that HPA axis is reactive to CNS inflammation triggered by an intrastriatal LPS injection was revealed through prior challenge with systemic LPS that resulted in rise in systemic corticosterone levels with concomitant and significant reductions in proinflammatory TNF-α, Monocyte chemoattractant protein MCP-1, IκBα transcripts in lesioned striatal/cortical region (Nadeau and Rivest, 2002). Interestingly in this paradigm, LPS does not trigger neuronal degeneration. However, neuronal death was observed by prior treatment with GR antagonist RU486 suggesting that GCs acting through GR prevent neuronal degeneration (Nadeau and Rivest, 2003). Recently, the role played by microglial GR in regulating neuronal survival in this intrastriatal model of LPS was shown conclusively in mice with selective inactivation of GR in microglia/macrophages, $GR^{LysMCre}$ mice (Carrillo-de Sauvage et al., 2013). Inflammation triggered by low dose of LPS (1–2 μg) injection has negligible effect on striatal or cortical neurons (Carrillo-de Sauvage et al., 2013), however the same dose of LPS injection in substantia nigra causes specific loss of dopamine neurons (Castaño et al., 2002) indicating a selective vulnerability of dopamine neurons to microglial inflammatory response mediated by LPS-activated TLR4. However, the fact that endogenous GCs activating GRs in microglia are neuroprotective during LPS-induced inflammation in cortex/striatum but not in midbrain substantia nigra implies that their actions in microglia during TLR4 activation may be region-specific. In this regard, recently the concept of microglial heterogeneity with respect to their functional capabilities, for example LPS/TLR4 signaling, has been evoked (Noh et al., 2014).

Nigral dopamine neurodegeneration triggered by MPTP is significantly reduced by pharmacological treatments with GC agonists e.g., corticosterone that artificially increase GCs above endogenous levels, conversely adrenalectomy augments dopamine neuronal loss (Kurkowska-Jastrzebska et al., 2004; Sugama et al., 2009; Ros-Bernal et al., 2011) indicating that high levels of GCs present during MPTP intoxication protect dopamine neurons. Immuno-labeling of GR revealed its localization mainly in the nucleus of microglia and its quantification was carried out in substantia nigra and striatum

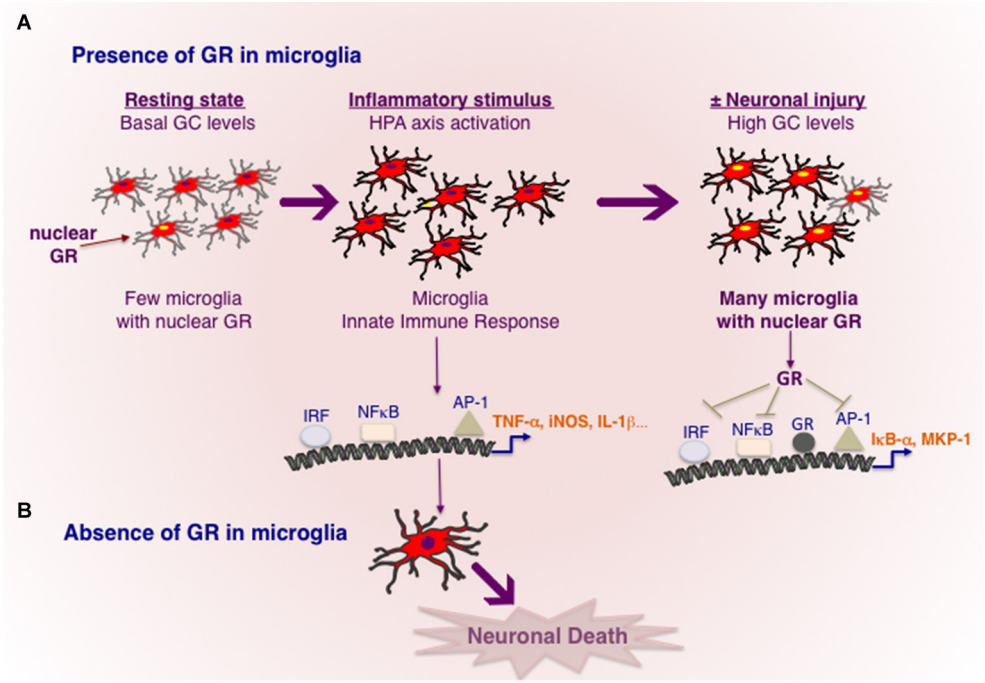

FIGURE 1 | Transcriptional regulation of inflammation by GR in microglia. **(A)** In resting, healthy state without any cellular injury or pathogen invasion, GR is inactive in most microglia. Toxins e.g., MPTP or powerful inflammogen LPS would rapidly trigger innate immune response in microglia. In the case of LPS, TLR4 present in microglia results in activation of major transcription factors NF-κB, IRF and AP-1, known to orchestrate an inflammatory response. Cellular injury or pro-inflammatory cytokines would activate HPA axis and increased GC secretion. High circulating GC levels results in GR activation in microglia, which act to repress the transcriptional activity of NF-κB, IRF and AP-1 and also stimulating the expression of genes such as IκB-α and or MKP-1 known to inhibit NF-κB and AP-1 respectively. Neuronal injury and death are prevented. **(B)** In the absence of GR activity in microglia, microglia remain in activated state causing neuronal death.

in saline and MPTP injected mice. The results showed that number of microglia with nuclear GR augmented from 35% in resting state to 70–80% 3 days after MPTP injections, which then declined to almost normal levels after 3 weeks. Measurement of endogenous corticosterone levels showed a three-fold rise 1 day after MPTP (Ros-Bernal et al., 2011). Importantly, these results indicate that GR activation during endogenous rise in corticosterone levels is progressive concurring loss of dopamine neurons (**Figure 1**). However increasing GC levels by corticosterone treatment results in significant neuroprotection likely because GR activation in microglia is rapid enough to counteract the inflammatory response mounted by activated glia.

The precise actions of GR in microglia during dopamine neurodegeneration were studied using $GR^{LysMCre}$ mice (Ros-Bernal et al., 2011). Functionally, absence of GR in microglia/macrophages resulted in significant dopamine neuronal loss in two paradigms of MPTP intoxication: (a) acute toxicity (4 injections/day) which is accompanied by intense microglial and astroglial activation of short duration; and (b) sub-chronic treatment (1 injection for 5 days) the loss of dopamine neurons is less, and morphologically, microglial activation is less apparent. In microglial GR mutant mice, both MPTP paradigms augmented microglial activation i.e., number hypertrophied microglia, compared to controls. Additionally, in sub-chronic paradigm, GR was found to prolong the duration of activation. Several molecules released by degenerating dopamine neurons can potentially trigger morphological and functional changes in microglia i.e., its activation status or its mobility (e.g., Matrix metalloprotease MMP-9, α-synuclein, Annese et al., 2015), however how the primary signals emitted from degenerating dopamine neurons trigger microglial activation is not well elucidated. With regards to regulation of inflammation, microglial GR was found to modulate 3 classes of inflammatory genes: (a) increasing expression of pro-inflammatory molecules in particular, TNF-α, iNOS, Intercellular adhesion molecule (ICAM); (b) anti-inflammatory genes e.g., MKP-1 (as described above for inhibiting AP1 transcriptional activity) and IL-1R2 which is a decoy receptor for IL-1 receptor1; (c) inflammatory caspases, i.e., caspases 1 and 4 as well as TLR3, TLR4, TLR9 and MyD88. The inflammatory caspases and TLRs are core components of innate immunity important for stimulating the transcriptional activity of AP1, NF-κB and IRF and thereby expression of plethora of inflammatory mediators. These findings indicate that GR not only inhibits the molecules like TNF-α known to execute the inflammatory reaction but also prevent excessive expression of upstream activators that initiate an inflammatory reaction.

Nuclear expression of p65 subunit of NF-κB, indicative of transcriptional activity NF-κB, was observed in microglia of SNpc in PD patients as well as in mice treated with MPTP.

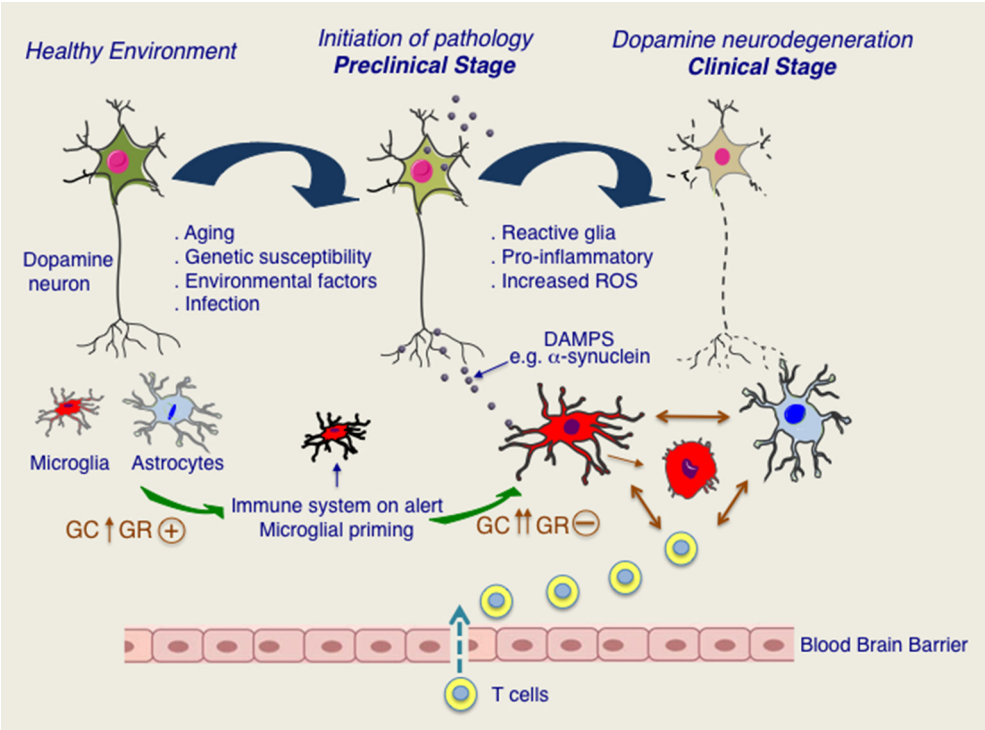

FIGURE 2 | Putative roles of glucocorticoids (GC) and glucocorticoid receptor (GR) in progression to chronic inflammation and dopamine neurodegeneration. In healthy state, microglia and astroglia surrounding are quiescent. Aging as well as other stressors such as infections or PD-related genetic and environmental factors would put immune system on alert and possibly also stimulating HPA axis. Activation of HPA axis results in increase in GC levels and activation of GR. In pre-clinical stage, secretion of DAMPS, such as pathological form of α-synuclein would activate immune system as well as HPA axis. Persistent activation of HPA axis results in loss of its regulation and chronically high GC levels. Chronic GCs are known to result in GR dysfunction in immune cells. Microglia and astroglia remain activated creating a pro-inflammatory environment and augmenting oxidative stress. Disruption in blood brain barrier resulting in T cell infiltration further promotes glial activation. Dopamine degeneration is progressively increased leading to clinical manifestation of PD.

Moreover inhibiting NF-κB in mice significantly protected dopamine neurons against MPTP toxicity (Ghosh et al., 2007). Thus sustained transcriptional activity of NF-κB is likely involved in chronic activation of microglia in PD. Interestingly, GR was found to associate with p65 subunit of NF-κB in microglial cultures, as well in luciferase reporter assays GR inhibited its transcriptional activity (Ros-Bernal et al., 2011; Carrillo-de Sauvage et al., 2013). *In vivo*, Serine 276 phosphorylation of P65 subunit of NF-κB, indicative of its activation, was sustained in MPTP-lesioned SNpc and striatum of mutant GR microglial mice (Ros-Bernal et al., 2011).

The halting of inflammation is central to immune response. A failure to limit the amplitude and duration of this process as well as initiate a resolution phase can lead to chronic inflammatory state. In addition to GR, other members of nuclear receptor family e.g., Peroxisome proliferator activated receptor gamma (PPAR-γ), Liver X receptor (LXR), Estrogen receptor ER-β, Nuclear receptor NR4A family (Nurr 77, Nurr1) are expressed in microglia, thus they can also control microglial activation. In this regard, Nurr1 inhibition was found to increase NF-κB activity in glia resulting in exaggerated expression of pro-inflammatory mediators and increased loss of dopamine neurons following LPS injection in substantia nigra (Saijo et al., 2009).

Regarding GCs, it is possible that in PD, GR functions in immune cells are compromised because of chronically elevated levels of cortisol. The putative scenario of dysfunction of GR signaling in PD is illustrated in **Figure 2**. Different stressors such as aging, infections, environmental and genetic susceptibility factors would activate HPA axis resulting in augmentation of circulating GC levels and activation of GR. In parallel, activation of peripheral immune system would result in increased circulating levels of pro-inflammatory molecules e.g., Il-1β known to activate HPA axis and also to induce microglial priming such that any subsequent insult exacerbates microglial inflammatory phenotype. Persistent activation of HPA axis with chronically high cortisol levels would compromise GR functions (Dejager et al., 2014). Further studies are needed to understand how GR activity is affected in microglia during chronically active HPA axis, as is the case for PD patients and whether GR inflammatory function is affected in PD. In addition, it would be important to understand the redundant and non-redundant functions of GR with closely related nuclear receptor members such as Nurr1 for envisaging therapeutic potentials in PD.

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Abbreviations

ACTH, Adrenocorticotropic hormone; AP1, Activator protein 1; ATF, Activating transcription factor; BBB, Blood brain barrier; BST-1, Bone marrow stromal cell antigen; C1Q, Complement component 1q; C3, Complement component 3; CBP, CREB Binding protein; CCL5, Chemokine ligand 5; CD14, cluster of differentiation 14; CD200, Cluster of Differentiation 200; CD47/CD172A, Cluster of Differentiation 47; CLRN3, Clarin3; CNS, Central nervous system; CRH, Corticotropin-releasing hormone; CX3CL1, Chemokine (C-X3-C motif) ligand 1; CX3CR1, CX3C chemokine receptor 1; CYP2D6, Cytochrome P450 2D6; DAMP, Damage-associated molecular pattern; DBD, DNA-binding domain; ER-β, Estrogen Receptor β; FKBP51, FK506 binding protein; GC, Glucocorticoids; GPNMB, Glycoprotein NMB gene; GR, Glucocorticoid receptor; GRE, Glucocorticoid response element; H1N1, Hemagglutinin neuraminidase; HDAC2, Histone deacetylase 2; HLA, Human leucocyte antigen; HPA, Hypothalamic-pituitary-adrenal; HSP90, Heat shock protein 90; ICAM, Intercellular adhesion molecule; IFN-γ, Interferon γ; IκBa, Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor; IKK, IκB kinase; IL-1β, Interleukin-1β; IL-6, Interleukin-6; iNOS, Inducible nitric oxide synthase; IRF, Interferon response factor; JNK, c-Jun N-terminal kinase; LB, Lewy bodies; LBD, Ligand-binding domain; LPS, Lipopolysaccharide; LRRK2, Leucine rich repeat kinase 2; LXR, Liver X receptor; MAPK, Mitogen-activated protein kinase; MCP1, Monocyte chemoattractant protein-1; MECP2, Methyl CpG binding protein 2; MHC, Major histocompatibility complex; MKP1, MAPK phosphatase 1; MMP9, Matrix metalloprotease 9; MPTP, 1-methyl, 4-phenyl, 1, 2, 3, 6-tetrahydropyridine; MR, Mineralocorticoid receptor; MSK1, Mitogen- and stress-activated protein kinase-1; MYD88, Myeloid differentiation primary response gene 88; NCOA1, Nuclear receptor coactivator 1; NF-κβ, Nuclear factor κ-light-chain-enhancer of activated β cells; NR3C1, Nuclear Receptor Subfamily 3, Group C, Member 1; NR4A, Nuclear receptor 4A; NSAID, Nonsteroidal anti-inflammatory drugs; P300, E1A binding protein p300; PAMP, Pathogen-associated molecular patterns; PD, Parkinson's disease; PET, Positron emission tomography; PINK1, PTEN-induced putative kinase 1; PKA, Protein kinase A; PLA2G6, Phospholipase A2, group VI; PPAR-γ, Peroxisome proliferator-activated receptor γ; PVN, Paraventricular nucleus of hypothalamus; RANTES, Regulated on activation, normal T cell expressed and secreted- CCL5; REM, Rapid eye movement (REM) sleep; SN, Substantia nigra; SNCA, Synuclein, α; SNP, Single Nucleotide Polymorphism; SPG4/SPAST, Spastic paraplegia-4/spastin; TAK1, Transforming growth factor activated kinase-1; TLR, Toll-like receptor; TNF-α, Tumor necrosis factor-α.

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Neuronal MHC-I expression and its implications in synaptic function, axonal regeneration and Parkinson's and other brain diseases

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Neuronal expression of major histocompatibility complex I (MHC-I) has been implicated in developmental synaptic plasticity and axonal regeneration in the central nervous system (CNS), but recent findings demonstrate that constitutive neuronal MHC-I can also be involved in neurodegenerative diseases by playing a neuroinflammtory role. Recent reports demonstrate its expression *in vitro* and in human postmortem samples and support a role in neurodegeneration involving proinflammatory cytokines, activated microglia and increased cytosolic oxidative stress. Major histocompatibility complex I may be important for both normal development and pathogenesis of some CNS diseases including Parkinson's.

Keywords: major histocompatibility complex class I, neurons, neuroinflammation, neurodegeneration, plasticity

INTRODUCTION

The major histocompatibility complex (MHC) gene family encodes molecules on the surface of cells that enable the immune system to recognize presented self- and foreign-derived peptides (Chemali et al., 2011). The MHC genes (in human, HLA-A, -B, -C and HLA-DP, -DM, -DOA, -DOB, -DQ, -DR; in mouse, H-2-K, -D, -L, and 2-I-A and I-E allomorphs) are generally divided into three categories: class I, II or III. Class I MHC (MHC-I) molecules are expressed by nearly every mammalian cell class, while MHC class II (MHC-II) molecules are restricted to cells of the immune system, such as macrophages and lymphocytes. In human, genes encoding for MHC-I and MHC-II have a large number of alleles, leading to a great diversity of sets of MHC molecules in our species. MHC class III genes code for other immune system proteins, including components of the complement system and proinflammatory cytokines, as well as proteins not involved in immune function (Janeway et al., 2001).

MHC-I consists of two non-covalently linked polypeptide chains, known as alpha (α) and beta 2 microglobulin (β2m) chains (Cresswell et al., 2005). The complex can bind a large set of antigenic peptide fragments derived from degradation of intracellular proteins by the proteasome, which requires the "transporter associated with antigen processing" (TAP; Van Kaer et al., 1992). The MHC-I/peptide complex is then transferred to a vesicle that fuses with the plasma membrane to present the peptide fragment extracellularly. These antigens can then be identified by cytotoxic T lymphocytes (CTLs) or natural killer cells as "self" or "non-self" peptides, which leads to various responses depending on their receptors (Fleischer et al., 1986; Pawelec et al., 1986). If CTLs recognize the peptides as non-self antigens, they kill the presenting cells through the Fas or perforin pathways and/or indirectly by the release of cytokines (Andersen et al., 2006; **Figure 1**).

It has generally been presumed that the central nervous system (CNS) is immune-privileged and that MHC-I is not expressed by neurons (Lampson, 1995). However, accumulating data have demonstrated MHC-I expression by subsets of neurons in both adult and developing mammalian brain (for review, see Cullheim and Thams, 2010). Many of these reports describe a role for neuronal MHC-I in synaptic plasticity, brain development and axonal regeneration. Recent studies suggest that neuronal expression of this molecule is involved in neuroinflammatory processes and participates in immune-mediated neurodegeneration. In particular, there have been many reports linking neuroinflammation and Parkinson's disease (PD; Tansey and Goldberg, 2010), and new data from our group suggests that expression of MHC-I by substantia nigra (SN) and locus coeruleus (LC) neurons may be involved in these inflammatory processes (Cebrián et al., 2014).

This review summarizes the pattern of expression and the different implications of neuronal MHC-I in the brain, and focuses in particular in the potential role of constitutive MHC-I expression by specific subsets of neurons in neurodegenerative diseases such as PD.

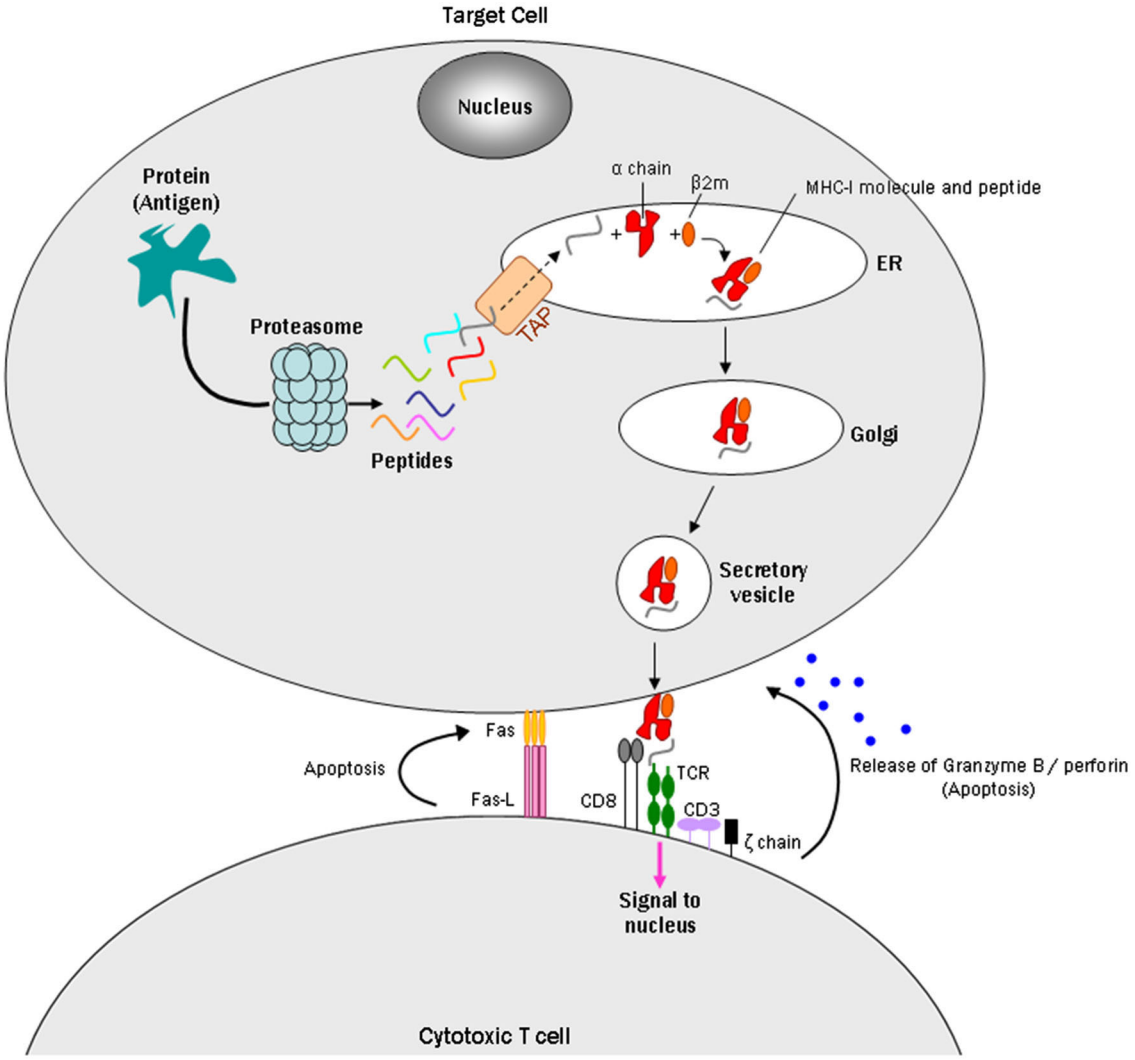

FIGURE 1 | Model of a CD8+ cytotoxic T cell targeting an MHC-I expressing cell. Intracellular antigens are digested by the proteasome into small peptides. A specialized carrier, the *transporter associated with antigen processing* (TAP) complex, translocates the peptide into the endoplasmic reticulum (ER), allowing the antigen to bind MHC class I, which consists of an alpha and beta 2 microglobulin (β2m) chain. Endoplasmic reticulum-derived vesicles that contain the complex fuse with the Golgi. The complex is then packaged in secretory vesicles that fuse with the plasma membrane to insert the complex on the cell surface. Major histocompatibility complex I molecules present antigens to $CD8^+$ cytotoxic T cells. The T cell $CD8^+$ molecule recognizes MHC-I, while TCRs recognize specific antigenic peptides. The TCR complex contains CD3 and ζ(zeta) chains. Once the $CD8^+$ T cell recognizes the target cell, apoptosis can occur in two ways: one uses the Fas ligand protein, which is expressed on the surface of the $CD8^+$ T cells and binds to the Fas receptor on the target cell, which triggers apoptosis through the classical caspase cascade. The other uses secretion of granzyme B and perforin from T cell granules into the intercellular space between the cells.

PATTERN OF MHC-I EXPRESSION IN CNS NEURONS

Although the presence of MHC-I in the mature rodent CNS was for many years thought to be confined to glial cells (Wong et al., 1984), ensuing reports demonstrate MHC-I expression by some neuronal populations, both *in vitro*, usually triggered by exposure to interferon gamma (IFN-γ), and *in vivo*. The initial such study showed that MHC-I genes expression were induced by IFN-γ in cultured rat hippocampal neurons (Neumann et al., 1995).

Subsequently, mRNA for MHC-I was identified in nuclei of neonatal and adult rodent brain including the SN, brainstem motor neurons (Lindå et al., 1999), lateral geniculate nucleus (LGN), cortex, hippocampus (Huh et al., 2000) and cerebellum (Letellier et al., 2008). In aged rat motoneurons, mRNA for MHC-I and β2m increased with age (Edström et al., 2004).

Multiple groups reported expression of MHC-I subunits by immunolabel in CNS regions including cingulate cortex and hippocampus (Needleman et al., 2010; Liu et al., 2013), with expression gradually decreasing as neonatal mice reached adulthood (Liu et al., 2013). A recent study shows that MHC-I proteins are widely expressed in the developing mouse CNS at mid-gestation

(E9.5–10.5), including the neuroepithelium and olfactory placode (Chacon and Boulanger, 2013).

In human brain, MHC-I expression was initially reported in microglia and endothelial cells of the hippocampus in control individuals and Alzheimer's disease patients (Tooyama et al., 1990), but not in neurons. In contrast, MHC-II immunolabeling microglia, but not neurons, was shown in the SN of patients with Alzheimer's disease and PD (McGeer et al., 1988), and in the hippocampus of patients with dementia with Lewy bodies (Imamura et al., 2005).

The neuronal expression of MHC-I in human brain has to date only been reported in a few studies. The first was a study of a childhood viral infection, Rasmussen's encephalitis, in which immunolabel for the MHC-I component, β2m, was present in cortical and hippocampal neurons (Bien et al., 2002); more recently, MHC-I immunolabel was observed in dysmorphic/dysphasic cortical neurons of focal cortical dysplasia, tuberous sclerosis complex and gangliolioma cases (Prabowo et al., 2013).

Most of the reports on MHC-I expression in human CNS neurons have been restricted to early development. In the embryo, β2m immunolabel was observed at 29–31 gestational weeks in the LGN of the dorsal thalamus, but was nearly absent by postnatal day 55, and was completely absent in the adult (Zhang et al., 2013b). In the human visual cortex, MHC-I was not observed at any gestational or postnatal stage (Zhang et al., 2013b), while the expression of MHC-I was very low in the hippocampus at 20 gestational weeks and slowly increased during weeks 27–33. A rapid increase in MHC-I molecule expression was found in the subiculum that reached high levels at 31–33 gestational weeks, but no expression of MHC-I was found in the adult hippocampus (Zhang et al., 2013a).

Several neurodegenerative disorders including PD are well established to display neuroinflammatory components and neuronal death (Tansey and Goldberg, 2010). As MHC-I is involved in antigen presentation and cell death (Chemali et al., 2011), we investigated its role in the degeneration of catecholamine neurons that are targeted in PD. Using immunolabel, mass spectroscopy, and mRNA analysis from laser captured neurons of adult control individuals and PD patients (Cebrián et al., 2014), we found that MHC-I is expressed by SN dopaminergic (DA) and LC norepinephrinergic (NE) neurons. Further analysis of isolated neuromelanin (NM) from these neurons by mass spectroscopy identified specific HLA alleles of MHC-I recovered from SN neurons, providing means to genotype the HLA type from neurons in postmortem human brain. Most of the immunolabel appeared to be present in NM, which are contained in modified autophagic lysosomes, but membrane preservation in postmortem human tissue is too poor to clearly ascertain whether the MHC-I is actually present on the plasma membrane at the time of death.

Our data from human tissue were supported by *in vitro* experiments that show DA human neurons derived from human embryonic stem cells normally do not express MHC-I but will do so following exposure to IFN-γ. Cultured primary catecholamine murine neurons also normally do not express MHC-I, but do so upon exposure to IFN-γ, activated microglia or exposure to high levels of L-dihydroxyphenylalanine (L-DOPA), and are far more susceptible to MHC-I induction than other neuronal populations tested, including cortical, striatal and thalamic neurons (Cebrián et al., 2014). These findings suggest that neuronal MHC-I expression and antigen display in catecholamine neurons may be triggered by microglial activation or high cytosolic DA, which in the presence of the appropriate antigen and CTLs could play a role in neuronal death during diseases in which CNS inflammation is robust. Thus, these results suggest reason to further explore roles for activated microglia, antigen presentation, neuronal MHC-I expression and recruitment of CTLs in neurodegenerative diseases, including PD, that feature the presence of T cells, activated microglia, intracellular oxidative stress and aggregates of alpha-synuclein (α-syn) in the SN and LC. These data may set a stage for understanding selective CTLs/MHC-I mediated neurodegeneration and set the basis for redefining the immunological component of PD, as well as provide evidence for a novel mechanism of neuronal death due to T-cell activity on which new therapies and treatments could be based.

ROLES FOR MHC-I IN BRAIN DEVELOPMENT AND SYNAPTIC PLASTICITY

It is well established that developing neurons express MHC-I (Shatz, 2009). A phenomenon implicated in development and maintenance of neuronal circuitry in the visual system (for review see Higenell and Ruthazer, 2010), the hippocampus, the cerebellum and the cortex (Ribic, 2012). Major histocompatibility complex I expression by developing neurons may be involved in retrograde signaling that regulates synaptic structure (Goddard et al., 2007). Neuronal MHC-I signaling may in some cases require regulators of cellular differentiation (Potthoff and Olson, 2007) known as myocyte enhancer factor 2 transcription factors (Elmer et al., 2013) to eliminate synapses during brain development.

In the visual system, mice deficient for MHC-I or the T cell receptor (TCR) subunit, CD3zeta (also known as CD247), exhibit reduced retinal synaptic activity, incomplete developmental refinement of connections between retina and its central targets, and reduced retinal ganglion cell dendritic motility with increased dendritic density (Xu et al., 2010). Ocular dominance plasticity during development was enhanced in mice lacking PirB, an innate immune receptor that binds MHC-I, or mice lacking H2-Kb and H2-Db, the two classical MHC-I αchains of C57BL/6 mice (Datwani et al., 2009). Mice that lacked H2-Db and H2-Kb also showed defects in synapse elimination and formation of eye-specific layers in visual processing areas of the brain, which was rescued by restoring H2-Db expression selectively in CNS neurons (Lee et al., 2014). Interestingly, in retina-thalamic cocultures, a soluble form of MHC-I inhibited retinal outgrowth to thalami that expressed high neuronal MHC-I (Washburn et al., 2011).

There is also evidence that neuronal MHC-I acts to regulate synapses in the hippocampus. An initial report (Corriveau et al., 1998) showed that MHC-I expression was increased by seizure. Later reports showed a variety of effects on synaptic plasticity. In mice deficient for the TCR subunit CD3zeta, hippocampal long-term potentiation was enhanced and long-term depression was absent (Huh et al., 2000). Other reports indicate

that neuronal MHC-I inhibits NMDAR function (Fourgeaud et al., 2010) and is critical for hippocampus-dependent memory (Nelson et al., 2013). These effects may in part be due to altered synaptic morphology, as hippocampal neurons with high levels of MHC-I maintain accelerated neurite outgrowth and polarization with more primary neurites (Bilousova et al., 2012). There thus appears to be a number of interacting pathways that can be influenced by MHC-I expression in hippocampal synapses.

Neuronal MHC-I expression has also been shown to regulate long-term depression and limit motor learning in cerebellum (McConnell et al., 2009) and the density and function of cortical synapses *in vitro* and *in vivo* (Glynn et al., 2011).

ROLES FOR MHC-I IN AXONAL REGENERATION

Neuronal MHC-I expression is further implicated in models of axonal regeneration. Peripheral nerve transection in MHC-I knockout mice resulted in more extensive detachments from presynaptic terminals from perikarya and dendrites of axotomized neurons than wild-type animals (Oliveira et al., 2004). These results suggest that MHC-I molecules regulate the ability of neurons to regenerate axons.

A subsequent study found that in mice with strong axonal regrowth potential, axotomy produced a pronounced upregulation of MHC-I in spinal cord and a rapid loss of afferents, but that C57BL/6J mice, which exhibit poor axonal regenerative potential, displayed less MHC-I increase and a slower stripping of the synapses. These results suggest that neuronal expression of MHC-I during the first week after lesion enhances axonal regeneration (Sabha et al., 2008), and support the observation that elevated neuronal MHC-I expression promotes the recovery of locomotor abilities after spinal cord injury (Joseph et al., 2011). It has thus been suggested that MHC-I and MHC-I receptors may provide new targets to promote neurorepair following injury (Wu et al., 2011).

Previous data suggest that enhanced levels of neuronal MHC-I facilitate axonal regeneration, although it is still unclear how this molecule might help axons to recover after a lesion. Shatz (2009) have shown that PirB, an immune receptor that binds MHC-I, is highly expressed in neurons of particular brain regions, including the cerebral cortex, the olfactory bulb and the cerebellum. PirB is also located in growth cones and axons of cerebral cortical neurons *in vitro* (Syken et al., 2006). It has been proposed that MHC-I is located postsynaptically near glutamate receptors, whereas PirB is present presynaptically in axonal growth cones of cortical neurons *in vitro*. In that model, PirB would signal when bound to MHC-I located across the synapse. Since neural activity regulates MHC-I expression levels, PirB could also regulate downstream signaling cascades in an activity-dependent manner (Shatz, 2009). This sequence of events may explain why an increase of MHC-I/PirB molecules facilitates axonal regeneration.

An alternate explanation is based on a recent report on Cx3cr1, a chemokine receptor highly expressed in microglia (Wolf et al., 2013). Cx3cr1 deficiency causes a transient reduction of microglia during the early postnatal period and a consequent deficit in synaptic pruning, which is associated with weak synaptic transmission and decreased functional brain connectivity (Zhan et al., 2014). A lack of neuronal MHC-I could be related to a decrease of microglia, while increased neuronal MHC-I could promote a higher number of microglial cells that according to Zhang et al. (2014) could improve synaptic pruning and recovery of axons after a lesion.

IS NEURONAL MHC-I IMMUNOLOGICALLY FUNCTIONAL?

During a period when neurons were generally regarded as MHC-I deficient, Medana et al. (2000) identified immunological functions of MHC-I in cultured neurons. They induced MHC-I and Fas receptor in murine hippocampal neurons with IFN-γ: the Fas receptor promotes apoptosis when it interacts with Fas ligand, a type-II transmembrane protein on T cells (Wajant, 2002; **Figure 1**). The MHC-I positive neurons were then challenged with the peptide GP33, an epitope of the lymphocytic choriomeningitis virus envelope glycoprotein, and with alloreactive CTLs to GP33. The MHC-I-expressing neurons pulsed with GP33, but not a control peptide, were killed by GP33-specific CTLs in a manner that did not require perforin, a pore-forming cytolytic protein released from CTLs granules (Tschopp et al., 1986), but did require Fas/FasL (Medana et al., 2000). A subsequent study reported that perforin, however, can play a role in CTL/neuron interactions by silencing neuronal activity prior to cell death (Meuth et al., 2009). Together, the Medana and Meuth studies introduced the hypothesis that MHC-I expressing neurons could be selectively targeted and destroyed by T cells.

ROLES FOR NEURONAL MHC-I IN VIRAL MEDIATED NEUROINFLAMMATION, BRAIN DISEASE AND NEURODEGENERATION

Pereira and Simmons (1999) showed that the H2 heavy chain and β2m components of the MHC-I molecule were both present on the surface of primary sensory neurons within 1–2 weeks after herpes simplex virus infection; some of these neurons were in close proximal association with T cells *in vivo*, suggesting a possible immunological interaction between neuronal MHC-I and T cells. Such an interaction was recently demonstrated as brain-isolated CTLs were found to destroy neurons infected with the neurotropic Borna disease virus in an antigen- and MHC-I dependent manner. Neuronal apoptosis were detected only hours after initial contact (Chevalier et al., 2011). Together, these studies indicate that the virus-specific CTLs can act as immune effectors in CNS viral infections.

The identification of neuronal MHC-I expression in human is relatively recent, starting with a report on a childhood viral infection, Rasmussen's encephalitis (Bien et al., 2002). In human autopsy, immunolabel for the MHC-I component, β2m, was identified in cortical and hippocampal neurons, and CTLs were found in close apposition to these neurons. Analysis of the T cells demonstrated that there were clonal expansions of a subset of $CD8^+$ T cells, but that the distribution $CD4^+$ cells was normal. Granzyme B, a T cell cytotoxic molecule, was observed in CTLs in close appositions to neurons and astrocytes (Schwab et al., 2009). Together, these

data strongly support antigen-driven MHC-I restricted, CTL-mediated attack against neurons and astrocytes in Rasmussen's encephalitis.

Recently, a strong upregulation of neuronal MHC-I was reported in focal glioneuronal lesions associated with intractable epilepsy. This induction of MHC-I in neuronal cells may also be a feature of type II focal cortical dysplasia, tuberous sclerosis complex and ganglioglioma (Prabowo et al., 2013).

Another recent study suggests that human LilrB2, an immune cell receptor that binds MHC class I molecules and inhibits immune response (Barrow and Trowsdale, 2008), is a β-amyloid receptor, and that its murine homolog PirB regulates synaptic plasticity in an Alzheimer's disease rodent model (Kim et al., 2013).

Multiple sclerosis (MS) has been linked to MHC-I and CTLs in the CNS (Friese and Fugger, 2005). Axonal injury and loss is a central determinant of irreversible neurological deficit and disease progression in patients with MS, and axon injury is most prominent within active, inflammatory demyelinated MS lesions enriched in CTLs (Bjartmar and Trapp, 2001). A recent study shows that axons are injured by antigen-specific CTLs through a MHC-I and granzyme B-dependent mechanism (Sauer et al., 2013), and suggests that CTLs may provide therapeutic targets in MS.

Our findings in human postmortem samples of adult control individuals and PD patients show that MHC-I is expressed by SN DA and LC NE neurons (Cebrián et al., 2014) which may have further implications for neurodegeneration. The proportion of catecholamine LC neurons that expressed MHC-I in humans was higher in controls than in PD subjects, which suggests that neurons with MHC-I expression may be more prone to cell death during the disease. The preference for MHC-I expression by catecholamine neurons was replicated in cultured SN DA murine neurons in which MHC-I was induced by IFN-γ and microglia activated by NM or α-syn, substances found extracellularly in postmortem PD brain, or by chronic exposure to the DA precursor, L-DOPA, which may be related to intracellular oxidative stress due to high cytosolic levels of its metabolite, DA. We found that cultured SN murine neurons can process and present foreign protein antigens by MHC-I, and that in the presence of the appropriate antigen and CTLs, the neurons are killed by CTLs (Cebrián et al., 2014). These findings suggest that neuronal MHC-I expression and antigen display by catecholamine neurons can be triggered by microglial activation or high cytosolic DA, features thought to be typical of PD, and that in the presence of the appropriate antigen and CTLs, MHC-I could play a role in neuronal death in diseases with robust CNS inflammation.

CONCLUSIONS

Neuronal MHC-I expression plays multiple roles. First, it regulates synaptic plasticity during brain development. Second, it regulates axonal regeneration and the appropriate specification of synaptic inputs following injury. Third, in neuronal diseases including neurotropic viral infections, neuronal MHC-I expression is upregulated and may initiate T cell mediated responses. While current research in each of these areas is ongoing, the suggestion of a role in neurodegenerative disease is the most recent and the least understood: while there is a consensus that many neurodegenerative diseases feature a robust inflammatory response, it remains unclear how this is related to chronic disease processes.

Our recent study demonstrates neuronal MHC-I expression in both normal and PD adult brain; such expression to date appears to be particular for catecholaminergic/monoaminergic neurons. *In vitro* experiments indicate that DA primary human neurons derived from human embryonic stem cells and primary catecholamine murine neurons are more susceptible to MHC-I induction by IFN-γ than other neuronal populations (Cebrián et al., 2014), which may be related to unusually high oxidative stress in these neurons. The findings suggest that an immunologically-based mechanism may link activated microglia, increased cytosolic oxidative stress and neuronal death of catecholamine neurons in PD and other diseases of this system. For PD, microglia activated by NM, native α-syn, modified α-syn, or mutant α-syn release IFN-γ that in turn can induce MHC-I expression in these neurons. The capacity of catecholamine neurons to process and display antigens may thus render them selective targets for T cell mediated cell death.

These possibilities are consistent with recent demonstrations that microglia can be activated by substances released from degenerating neurons in PD, such as α-syn (Zhang et al., 2007; Béraud et al., 2013) or NM (Zhang et al., 2011, 2013c), and that activated microglia can elicit neurotoxicity (Block et al., 2007; Lull and Block, 2010; Zhao et al., 2013). Both NM and α-syn are found extracellularly in the postmortem brain of PD patients (Double, 2012), a disorder that features high levels of activated microglia in the SN (Foix and Nicolesco, 1925) and high levels of intracellular oxidative stress (Fahn and Sulzer, 2004). Parkinson's disease patient brain features increased IFN-γ (Mogi et al., 2007) and chemokines (Harris et al., 2012), as well as a compromised blood brain barrier (Farkas et al., 2000; German et al., 2012) that may explain why CTLs are substantially higher in PD patients than age-matched controls (Hisanaga et al., 2001; Brochard et al., 2009). CD4+ T helper cells have also been shown to infiltrate the brain in human PD postmortem samples and exert a cytotoxic effect in mouse brain following nigrostriatal injury (Brochard et al., 2009); thus, T cells and antigen presentation, as well as activated microglia could play a role in PD pathogenesis.

Neuronal display of antigenic MHC-I could participate in a range of additional neurological disorders. For example, Japanese encephalitis virus can induce MHC-I expression in non-neuronal cells by interferon type 1 (Abraham et al., 2010), while in mice, IFN-γ plays a role in paraquat-induced neurodegeneration (Mangano et al., 2012). Central nervous system-directed expression of IFN-γ produces basal ganglia calcification and nigrostriatal degeneration (Chakrabarty et al., 2011). In human case studies, a link is described between chronic hepatitis C patients who were treated with type 1 interferon and developed PD-like symptoms that reversed when the treatment was halted (Almeida et al., 2009).

Together, these data indicate that in human brain, neuronal MHC-I expression, antigen presentation and the presence of T cells could occur simultaneously under certain circumstances, leading to the death of targeted neurons. In some

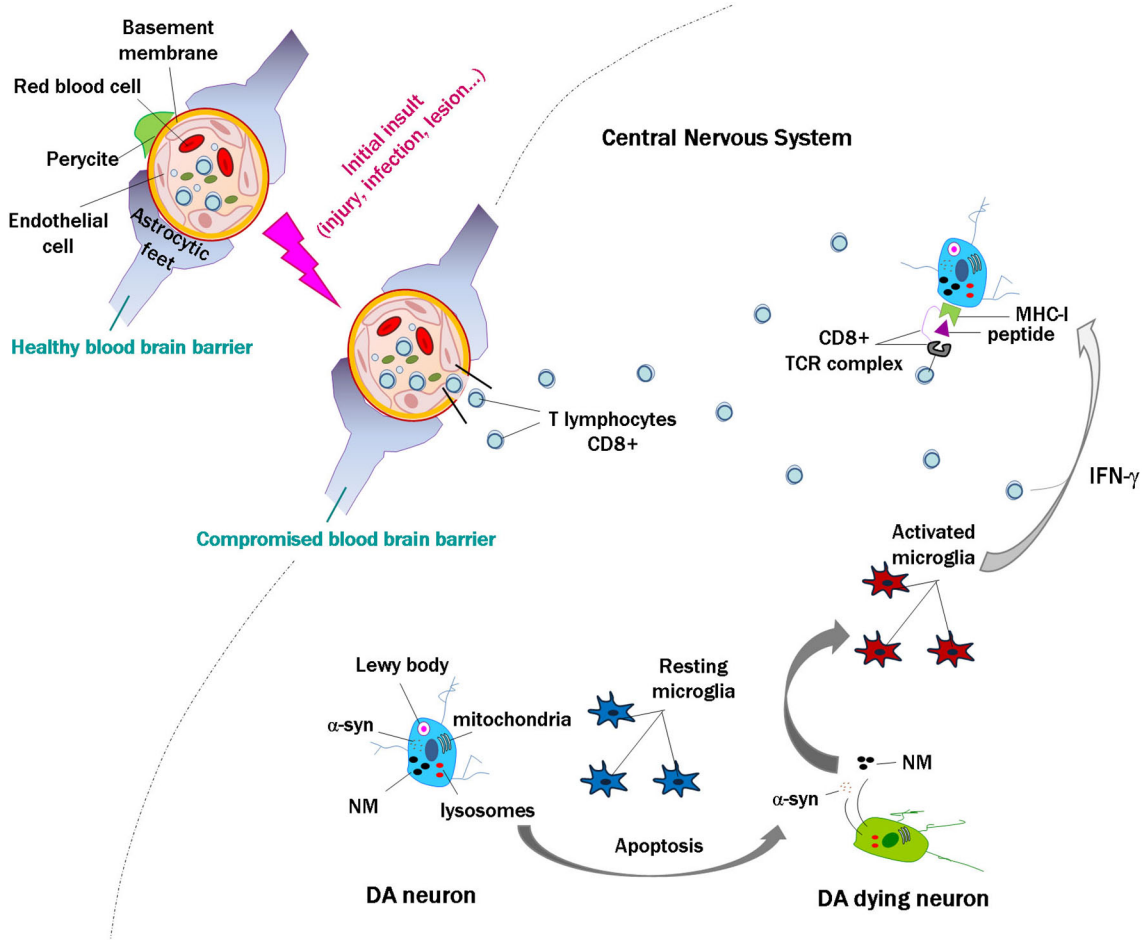

FIGURE 2 | Scheme modeling an initial insult and disruption of the blood brain barrier, with penetration of lymphocytes into the brain. In the event of death of a few catecholamine neurons, alpha-synuclein (α-syn) and neuromelanin (NM) are released to the extracellular space, activating microglial cells that in turn release proinflammatory substances such as interferon gamma (IFN-γ). This would induce major histocompatibility class I expression (MHC-I) in the membrane of catecholamine neurons, and if they contain proteins that are misfolded or cannot be normally degraded, these might be presented as antigens by MHC-I. Cytotoxic lymphocytes that enter the brain may recognize these presented antigens and kill the neurons, which would again release α-syn and NM. This could continue a vicious cycle that may contribute to the death of catecholamine neurons in Parkinson's disease.

neurodegenerative diseases, and in particular for PD, we propose that activation of lymphocytes in the periphery may occur in response to self or non-self proteins, or as an initial insult and disruption of the blood brain barrier, with a subsequent penetration of lymphocytes in the brain. When catecholamine neurons die with subsequent release of α-syn and NM to the extracellular space, the activation of microglial cells will release proinflammatory substances such as IFN-γ, leading to an upregulation of MHC-I in the membrane of catecholamine neurons that could present neuronally derived antigens. If lymphocytes are close, they could recognize these antigens and target and kill the cell, which would again release α-syn and NM (**Figure 2**), leading to a vicious cycle that would enhance with time the neuronal death and pathogenesis of PD.

Future studies are necessary to identify which antigens are presented by MHC-I expressing catecholamine neurons and how T cells might interact with them. If these interactions occur, immune therapies used in other diseases including classical autoimmune disorders such as Type 1 diabetes or MS may be adapted to provide future treatments for PD.

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Morphological changes of glutamatergic synapses in animal models of Parkinson's disease

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The striatum and the subthalamic nucleus (STN) are the main entry doors for extrinsic inputs to reach the basal ganglia (BG) circuitry. The cerebral cortex, thalamus and brainstem are the key sources of glutamatergic inputs to these nuclei. There is anatomical, functional and neurochemical evidence that glutamatergic neurotransmission is altered in the striatum and STN of animal models of Parkinson's disease (PD) and that these changes may contribute to aberrant network neuronal activity in the BG-thalamocortical circuitry. Postmortem studies of animal models and PD patients have revealed significant pathology of glutamatergic synapses, dendritic spines and microcircuits in the striatum of parkinsonians. More recent findings have also demonstrated a significant breakdown of the glutamatergic corticosubthalamic system in parkinsonian monkeys. In this review, we will discuss evidence for synaptic glutamatergic dysfunction and pathology of cortical and thalamic inputs to the striatum and STN in models of PD. The potential functional implication of these alterations on synaptic integration, processing and transmission of extrinsic information through the BG circuits will be considered. Finally, the significance of these pathological changes in the pathophysiology of motor and non-motor symptoms in PD will be examined.

Keywords: Parkinson's disease, striatum, subthalamic nucleus, synaptic plasticity, glutamatergic synapses, vGluT, astrocytes plasticity

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Basal Ganglia Nuclei and Connectivity

The basal ganglia (BG) are a collection of interconnected subcortical nuclei, including the striatum, globus pallidus (GP), substantia nigra, and subthalamic nucleus (STN), which closely interact with the cerebral cortex and thalamus. While historically considered as key components of the motor system, the BG receive cortical projections from all functional areas of the cerebral cortex and contribute to both motor and non-motor functions (Alexander et al., 1986; Mink, 1996). The information flow through the BG circuitry is segregated into motor, associative, and limbic/emotional domains based on their relationships with specific cortical projection areas and the engagement of these regions in various behaviors (Alexander et al., 1986; Lanciego et al., 2012). A large number of findings discussed in this review were gathered from the motor-related nuclei of the primate BG. The striatum, the major input structure of the BG, receives projections from the cerebral cortex, brainstem, and thalamus. The GP consists of two anatomically and functionally separate nuclei, the external and internal pallidal segments [GPe and GPi, respectively in primates; GP and entopeduncular nucleus (EPN) in rodents]. The substantia nigra also comprises two separate nuclei, the

GABAergic pars reticulata (SNr) and the pars compacta (SNc), which contains pigmented dopamine (DA)-containing neurons. The dopaminergic neurons of the SNc project primarily to the striatum, but also provide significant innervation of other BG nuclei and the thalamus (particularly in primates; Smith and Kieval, 2000; García-Cabezas et al., 2009; Rommelfanger and Wichmann, 2010). The glutamatergic STN is a small nucleus which is intercalated between GPe and GPi. In addition to the striatum, the STN is also considered as a major entry for cortical information to the BG network (Nambu et al., 2000; DeLong and Wichmann, 2010), while the GPi (or EPN in non-primates) and SNr are the two main output nuclei of the BG.

The striatum and the STN receive topographically organized projections from functionally diverse regions of the cerebral cortex (Parent and Hazrati, 1995; Nambu et al., 1996). Because information flows more rapidly to the BG output nuclei via the corticosubthalamic projection than via the direct and indirect trans-striatal pathways, the trans-subthalamic route is commonly referred to as the "hyperdirect" pathway of the BG (Nambu et al., 2000, 2002; Sano et al., 2013; Smith and Wichmann, 2015). While the corticosubthalamic projection is less extensive than the corticostriatal system, it originates from motor, associative and limbic cortical regions and is a powerful source of excitation to STN neurons through which cortical inputs can rapidly regulate the activity of downstream BG output nuclei, the GPi and SNr (Monakow et al., 1978; Nambu et al., 2000; Haynes and Haber, 2013). Corticosubthalamic axons target dendritic spines and distal dendritic shafts of STN neurons in rats (Bevan et al., 1995; Mathai et al., 2015).

The GPe is another key structure of the BG which receives its main inputs from the striatum (the so-called indirect pathway) and the STN. In turn, two different populations of GPe neurons provide GABAergic innervation to all other BG nuclei. The so-called "arkypallidal" cells are the main sources of the pallidostriatal system, while the "prototypic" cells project massively to the STN, with collateral to the GPi and SNr (Shink et al., 1996; Smith et al., 1998a,b; Mallet et al., 2012; Dodson et al., 2015). There is recent evidence for a direct GABAergic/cholinergic pallidocortical projection in mouse (Saunders et al., 2015).

The BG outflow is directed at specific thalamic and brainstem nuclei via the GPi and SNr, largely through collateralized axonal projections (Parent and De Bellefeuille, 1982; Parent et al., 1983). The BG-receiving ventral motor thalamic nuclei project to widespread areas of the frontal lobe and send projections back to the striatum, while descending projections from the BG to the brainstem terminate massively in the pedunculopontine nucleus (PPN) which, in turn, provide significant ascending and descending projections to the thalamus, BG, reticular formation and spinal cord (Rye et al., 1988; Lavoie and Parent, 1994; Parent and Hazrati, 1995; Mena-Segovia et al., 2004). Recent evidence indicates that the descending trans-PPN projections may play an important role in regulating brainstem and spinal motor mechanisms related to gait and balance (Pahapill and Lozano, 2000; Garcia-Rill et al., 2011). The PPN is also part of several feedback circuits with projections to the BG and the thalamus (Rye et al., 1988; Lavoie and Parent, 1994; Mena-Segovia et al., 2004). Other projections from the SNr reach the superior colliculus, which is involved in coordinating head and eye movements, while a specific subset of peripallidal GPi neurons project massively to the lateral habenula, and play a role in the modulation of reward and limbic mechanisms (Wurtz and Hikosaka, 1986; Wickens, 2008; Hikosaka, 2010).

Recent studies suggest the existence of a direct glutamatergic cortico-pallidal projection in mammals, including humans (Mathai et al., 2012; Smith et al., 2014c; Milardi et al., 2015; Smith and Wichmann, 2015). This "cortico-pallidal" system is separate from the descending cortico-spinal and cortico-pontine axons that travel through the internal capsule (Naito and Kita, 1994; Milardi et al., 2015; Smith and Wichmann, 2015), and bypasses the traditional direct, indirect, and hyperdirect corticofugal pathways. The existence of this direct glutamatergic cortico-pallidal projection could have a significant impact on our present understanding of transmission and processing of information through the BG circuits in normal and diseased states (Smith and Wichmann, 2015).

The Striatum: Main Entry to the BG Circuitry

The dorsal striatum, made up of the putamen and caudate nucleus in primates, is mainly innervated by sensorimotor (post-commissural putamen) and associative (caudate nucleus and pre-commissural putamen) cortices, respectively, while the ventral striatum (nucleus accumbens and olfactory tubercle) is the main target of limbic-related inputs from the hippocampus, amygdala and medial prefrontal cortices (Russchen et al., 1985; Alexander et al., 1986; McGeorge and Faull, 1987; Haber et al., 1995; Parent and Hazrati, 1995; Fudge et al., 2002). In the human literature the term "lenticular" or "lentiform" nucleus is commonly used to refer to the putamen and the GP (Carpenter and Sutin, 1983).

Each striatal region also receives prominent functionally-related thalamic inputs from intralaminar, relay, associative and midline nuclei. Among those, the caudal intralaminar nuclear group, the centre median (CM) and parafascicular complex (Pf), which innervates preferentially the putamen or caudate nucleus, respectively (Smith et al., 2004, 2009a; Galvan and Smith, 2011) is the predominant source of thalamostriatal projections. Massive dopaminergic innervation from either the SNc (to the dorsal striatum) or the ventral tegmental area (VTA; to the ventral striatum) provides key modulatory influences upon striatal processing of extrinsic cortical and thalamic information (Smith and Bolam, 1990; Nicola et al., 2000; Gerfen and Surmeier, 2011). Additional extrinsic inputs from the hypothalamus, GP, STN, raphe, locus coeruleus and PPN have also been described (Smith and Parent, 1986; Parent and Hazrati, 1995; Smith et al., 1998a,b; Ellender et al., 2011).

Striatal Projection Neurons and Interneurons

The main targets of extrinsic inputs to the striatum are the GABAergic medium spiny neurons (MSNs), which represent

90–97% of all striatal neurons (Kemp and Powell, 1971a,b,c; Oorschot, 1996; Wickens et al., 2007a). These GABAergic neurons can be categorized into two main populations based on their hodological and chemical phenotypes. The "direct" pathway neurons send their main axonal projections directly to the output nuclei of the BG (i.e., GPi and SNr), and express preferentially the D1 DA receptors (D1R) and the neuropeptides substance P (SP) and dynorphin (DYN). On the other hand, the "indirect" pathway neurons project preferentially to the GPe, and express D2 receptors (D2R) and the neuropeptide enkephalin (ENK; Gerfen et al., 1990; Sidibé and Smith, 1999; Lanciego et al., 2004; Lei et al., 2004, 2013; Smith et al., 2009a, 2014a,b,c; Galvan and Smith, 2011; Gerfen and Surmeier, 2011; Huerta-Ocampo et al., 2014). Albeit less frequent, it is noteworthy that some striatal MSNs project to both GPe and GPi/SNr and co-express D1 and D2 DA receptor subtypes (Kawaguchi et al., 1990; Surmeier and Kitai, 1993; Surmeier et al., 1996; Wu et al., 2000).

The dendritic trees of both populations of striatal MSNs are covered with spines, which are the main targets of glutamatergic inputs from the cerebral cortex and thalamus. In rodents, the dendrites of individual MSNs harbor as many as 5000 spines (Wickens et al., 2007a). In addition to their glutamatergic innervation, striatal spines also receive synaptic inputs from midbrain dopaminergic neurons which frequently terminate onto the neck of the spine or a nearby segment of the dendritic shaft, thereby providing an anatomical substrate for close synaptic interactions between glutamatergic and dopaminergic inputs at the level of spines (Freund et al., 1984; Smith and Bolam, 1990; Smith et al., 1994, 2009a, 2014a; Nicola et al., 2000; Wickens et al., 2007a; Moss and Bolam, 2008). These functional interactions are critical for the development and maintenance of long-term synaptic plasticity of glutamatergic corticostriatal synapses (Nicola et al., 2000; Calabresi et al., 2007; Surmeier et al., 2007, 2010; Gerfen and Surmeier, 2011; Picconi et al., 2012). Although D1R and D2R MSNs display very similar morphological characteristics, the D2R MSNs exhibit increased excitability and harbor a less extensive dendritic tree than D1R cells in mice (Gertler et al., 2008; Kreitzer and Malenka, 2008; Fieblinger et al., 2014b), and each type of MSNs is differentially modulated by DA in normal and diseased states (Surmeier et al., 2007; Day et al., 2008; Kreitzer and Malenka, 2008; Shen et al., 2008; Kreitzer, 2009; Fieblinger et al., 2014b).

The aspiny interneurons are far fewer in number, accounting for about 3–10% of the total striatal population (Tepper and Bolam, 2004; Bernácer et al., 2005, 2007, 2012). Anatomically, they can be categorized into medium-sized GABAergic cells and large cholinergic neurons (Kawaguchi et al., 1995; Bernácer et al., 2007, 2012; Gonzales and Smith, 2015). Medium-sized GABAergic interneurons can be further classified histochemically into different subtypes: (a) parvalbumin-positive; (b) somatostatin-, neuropeptide Y-, and nitric oxide synthase-positive; (c) calretinin-positive (Tepper and Bolam, 2004; Bernácer et al., 2005, 2007, 2012); and (d) tyrosine hydroxylase (TH)-positive (Tepper et al., 2010). It is noteworthy that the latter subtype is rare in the normal primate striatum, but undergoes an upregulation after striatal DA denervation (Betarbet et al., 1997; Mazloom and Smith, 2006; Bernácer et al., 2012). It remains unclear if the various subtypes of TH-positive GABAergic cells described in TH-Cre mice (Tepper et al., 2010) represent the same neuronal phenotype as those seen in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys and Parkinson's disease (PD) patients.

DA Mesostriatal System

DA plays a fundamental role in normal BG function. The mesostriatal dopaminergic system, which comprises the mesolimbic and the nigrostriatal pathways, enables BG control of motor planning and action selection (Wurtz and Hikosaka, 1986; Berns and Sejnowski, 1998; Gurney et al., 2001). Because of its involvement in a wide array of physiologic and pathologic processes, the anatomical and functional organization of the DA mesostriatal systems has been the topic of extensive studies for many years (for reviews, see Wickens et al., 2007b,c; Kreitzer, 2009; Gerfen and Surmeier, 2011). Despite such interest, the exact role of DA in normal BG function is complex and remains poorly understood. The whole striatum is densely innervated by dopaminergic axons and terminals (Lavoie et al., 1989; Prensa and Parent, 2001; Matsuda et al., 2009; Bolam and Pissadaki, 2012; Pissadaki and Bolam, 2013) that originates from the ventral midbrain including the SNc (A9), VTA (A10) and retrorubral Area (RRA; A8). The A9 group is the most densely packed group of midbrain dopaminergic cells located in the SNc. Projections from SNc and RRA neurons terminate in the dorsal striatum, while VTA neurons are the main source of DA innervation to the ventral striatum (Gerfen et al., 1987; Lynd-Balta and Haber, 1994a,b). Dopaminergic terminal boutons represent nearly 10% of all striatal terminals (Groves et al., 1994). Like other monoamines, there is evidence that DA can mediate its effects in striatal and extrastriatal brain regions through neurotransmitter diffusion (Arbuthnott et al., 2000; Cragg and Rice, 2004; Arbuthnott and Wickens, 2007; Wickens et al., 2007b; Descarries et al., 2008; Moss and Bolam, 2008; Rice and Cragg, 2008; Rice et al., 2011). Consistent with this hypothesis, most DA receptors in the striatum are located extrasynaptically in spines and dendrites of striatal neurons (Hersch et al., 1995; Yung et al., 1995; Delle Donne et al., 1996, 1997; Nicola et al., 2000; Wang and Pickel, 2002; Gerfen and Surmeier, 2011).

GABA and Glutamate: Co-transmitters of the Nigrostriatal System

Recent evidence indicates that DA neurons in the SNc and VTA are capable of co-releasing GABA with DA, and inhibit striatal projection neurons (Tritsch et al., 2012, 2014). It is estimated that 5–10% of SNc DA neurons express GAD65 and fewer than 1% contain the vesicular glutamate transporter 2 (vGluT2) in rodents (González-Hernández et al., 2001; Bérubé-Carrière et al., 2009; Hnasko et al., 2010). Therefore, distinct subpopulations of DA neurons may release GABA or glutamate,

and reliable detection of IPSCs and EPSCs may result from innervation of SPNs by several DA neurons (Matsuda et al., 2009). Future studies are needed to better understand the physiological (or pathological) conditions under which GABA, glutamate and DA are released or co-released from nigrostriatal axons.

The release of GABA from DA terminals is independent of vesicular GABA transporter (vGAT), but requires activity of the vesicular monoamine transporter 2 (vMAT2) for vesicular loading. The inhibitory GABAergic synaptic transmission from DA neurons does not depend on synthesis of GABA by either GADs (GAD65, GAD67) or GABA transaminase, suggesting that DA neurons inhibit MSNs by releasing GABA they acquire from the extracellular space using membrane uptake of GABA (Tritsch et al., 2014). Although the actions of DA are not believed to be spatially localized (Arbuthnott and Wickens, 2007), this co-release of GABA may confer dopaminergic neurons an additional point-to-point mode of action, and the flexibility to differentially control GABAergic transmission in a target-dependent manner across their extensive axonal arbors (Tritsch et al., 2014). It is noteworthy that evidence for GABA expression and release from other populations of monoaminergic neurons has been reported in other brain regions (Iijima, 1993; Trottier et al., 2002; Maher and Westbrook, 2008; Hirasawa et al., 2009; Broadbelt et al., 2010). Together, these findings expand the repertoire of synaptic mechanisms available to monoaminergic cells, and suggest that perturbations of GABA co-transmission might contribute to the etiology of monoaminergic pathologies or to the therapeutic efficacy of vMAT2 antagonists in specific brain disorders.

There is also evidence that a certain contingent of SNc and VTA DA neurons can store and release glutamate via the vGluT2, providing an additional level of chemical heterogeneity to the nigrostriatal system (Sulzer et al., 1998; Chuhma et al., 2004; Bérubé-Carrière et al., 2009; Yamaguchi et al., 2013; Antal et al., 2014; Morales and Root, 2014; Trudeau et al., 2014). The localization of metabotropic glutamate receptor 5 at the edges of striatal dopaminergic synapses in the monkey striatum (Paquet and Smith, 2003) is consistent with these observations.

Striatal DA Receptor Subtypes

In addition to the strong and segregated expression of D1R and D2R in direct and indirect pathway MSNs, both GABAergic and cholinergic interneurons also express different subtypes of DA receptors, and their activity is tightly regulated by DA, most particularly that of cholinergic interneurons, which express both D2R and D5R (Yan and Surmeier, 1997; Yan et al., 1997; Day et al., 2006; Wang et al., 2006; Surmeier et al., 2007; Kreitzer, 2009; Gerfen and Surmeier, 2011). D3R and D4R are also expressed in both the dorsal and ventral striata (Landwehrmeyer et al., 1993; Rivera et al., 2002; Centonze et al., 2003). DA receptors are expressed to variable degree in other BG nuclei, providing a substrate for extrastriatal DA functions (Smith and Kieval, 2000; Rommelfanger and Wichmann, 2010). In addition to their post-synaptic localization, DA receptors are localized pre-synaptically in glutamatergic and GABAergic terminals throughout the BG circuitry, providing multiple targets through which DA regulatory influences can impact neurotransmission in normal and diseased states. The readers are referred to comprehensive reviews of the topic for additional information (Arbuthnott et al., 2000; Reynolds and Wickens, 2002; Costa, 2007; Rice and Cragg, 2008; Surmeier et al., 2010; Gerfen and Surmeier, 2011; Rice et al., 2011).

Glutamatergic Synaptic Plasticity in PD and its Models

Striatal Spine Loss in PD

Striatal spine loss has been reported in the striatum of various animal models of PD and in parkinsonian patients. In both MPTP-treated monkeys and PD patients, the extent of spine pruning is tightly correlated with the extent of striatal dopaminergic denervation (Ingham et al., 1989; Stephens et al., 2005; Zaja-Milatovic et al., 2005; Smith and Villalba, 2008; Smith et al., 2009b; Villalba et al., 2009; Toy et al., 2014; **Figures 1A,B**, **4**).

Striatal Spine Loss on Direct vs. Indirect Striatofugal Neurons

Although there has been some controversy as to whether the striatal spine loss targets preferentially direct (D1R-positive) vs. indirect (D2R-positive) striatal MSNs, recent evidence indicates that both neuronal subtypes are affected, but through different mechanisms. Some authors reported that D2R striatopallidal neurons, but not D1R striatonigral neurons, selectively lose spines in reserpine-(systemic administration) and 6-hydroxydopamine (OHDA)-treated (injection in the medial forebrain bundle) mice with striatal DA depletion (Day et al., 2006). However, other reports described spine loss on both direct and indirect pathway neurons in intrastriatal 6-OHDA-treated or systemically MPTP-treated mice (Suárez et al., 2014; Toy et al., 2014). Similarly, both populations of striatal projection neurons undergo significant spine loss in monkeys chronically treated with low doses of MPTP (Villalba et al., 2009; Villalba and Smith, 2010, 2013). These findings are consistent with the homogeneous loss of spines across large populations of striatal MSNs described in Golgi studies of human parkinsonians and animal models of parkinsonism (Ingham et al., 1989; Stephens et al., 2005; Zaja-Milatovic et al., 2005; Smith and Villalba, 2008; Smith et al., 2009b; Villalba et al., 2009; Villalba and Smith, 2010, 2013). However, other monkey studies, using an acute regimen of MPTP toxicity, suggested a decrease in D2R spines accompanied with an increase in the density of D1R spines in the caudate nucleus of MPTP-treated cynomolgus monkeys (Scholz et al., 2008). The use of different animal models, different regimens and locations of neurotoxin administration, variable quantitative methods and observations of different striatal regions may contribute to these discrepancies.

In addition to spine pruning, recent evidence showed that the length and complexity of the dendritic tree of both direct and indirect pathway MSNs are significantly reduced in

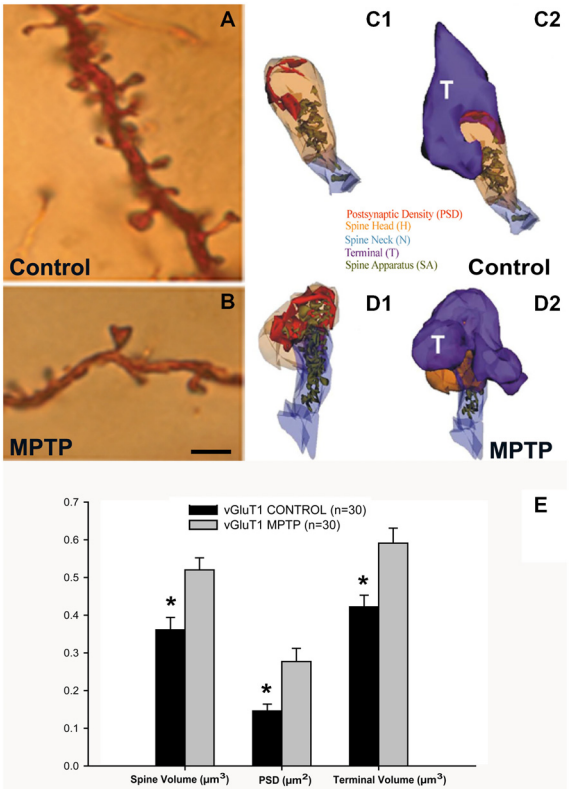

FIGURE 1 | Dendritic spines in the monkey striatum. (A,B) Light micrographs of dendrites from Golgi-impregnated medium spiny neurons (MSNs) in the caudate nucleus of a control **(A)** and a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated **(B)** monkey. Note the dramatic spine loss of the dendrite of the MSN from the MPTP-treated monkey compared with control. **(C1–D2)** Three-dimension (3D)-reconstructed images of glutamatergic axo-spinous synapses from control **(C1,C2)** and MPTP-treated **(D1,D2)** monkeys. **(E)** Histogram comparing the morphometric measurements (mean ± SEM) for spine volume (μm^3), post-synaptic density (PSD) area (μm^2) and terminal volume (μm^3) of structural elements at corticostriatal (vGluT1-positive) glutamatergic synapses using 3D reconstruction method of serial ultrathin sections collected from 30 axo-spinous synapses in each group from three control and three MPTP-treated animals. The spine volumes, the PSD areas, and the volume of vGluT1-containing terminals are significantly larger in MPTP-treated parkinsonian monkeys than in controls (*t-test; $p < 0.001$). Scale bar in **(B)** (applied to **A**) = 5 μm (See Villalba et al., 2009; Villalba and Smith, 2010, 2011a, 2013).

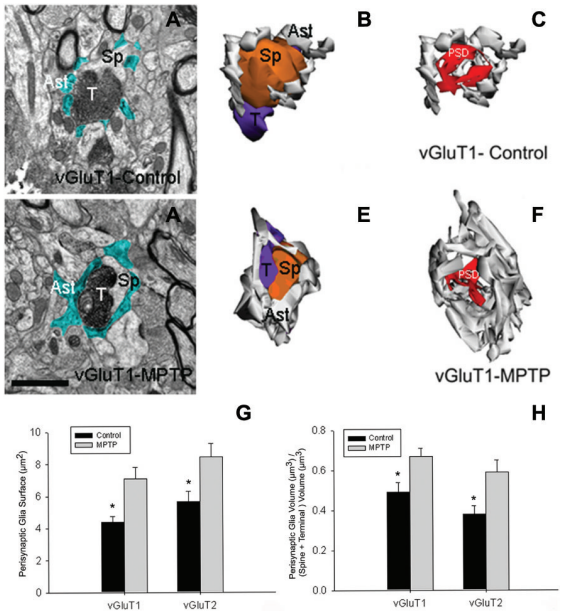

FIGURE 2 | Tripartite synapses (TS) in the monkey striatum. (A,D) Electron micrographs of perisynaptic astrocytic processes (Ast) wrapping a vGluT1 axo-spinous synapse in control **(A)** and MPTP-treated **(D)** animal. **(B,C,E,F)** The three-dimensional (3D) reconstruction of a vGluT1-immunoreactive TS highlight the differences in the extensions of the astrocytic processes between control **(B,C)** and MPTP **(E,F)**. In the TS of control animals, the perimeters of the axon-spinous interfaces were only partially surrounded by astroglial processes **(B,C)**. In MPTP-treated animals, TS vGluT1-containing synapses displayed a large increase in astroglial processes ensheatment **(E,F)**. **(G)** Histograms comparing the surface area of perisynaptic glia associated with vGluT1- and vGluT2-immunopositive axo-spinous synapses in control ($N = 3$) and MPTP-treated ($N = 3$) monkeys (mean ± SEM). The surface of the perisynaptic glia was significant larger (*, t-test, $p = 0.017$ for vGluT1 and $p = 0.006$ for vGluT2) in MPTP-parkinsonian monkeys than in control. **(H)** Histograms comparing the ratio of the volume of the perisynaptic glia over the total volume of spine and the axon terminals in TS formed by vGluT1- or vGluT2-immunoreactive terminals. This ratio was significantly larger in MPTP than in control condition (*, t-test, $p = 0.049$ for vGluT1 and $p = 0.028$ for vGluT2). No significant difference was found between TS formed by vGluT1- or vGluT2-immunoreactive terminals. Total number of reconstructed spines = 32 (8 per group). Statistics were performed by using SigmaPlot (version 11.0). Abbreviations: Ast, astrocyte; PSD, post-synaptic density; Sp, dendritic spine; T, axon terminal (see Villalba and Smith, 2011b for details).

6-OHDA-treated mice (Fieblinger et al., 2014a). In contrast to spine loss, that responds to L-DOPA therapy in this animal model, dendritic arbor atrophy is unresponsive to DA replacement therapy (Fieblinger et al., 2014a).

Does Striatal Spine Loss Affect Corticostriatal and Thalamostriatal Synapses?

Using unbiased stereological synaptic counts, Ingham et al. (1998) reported ∼20% decrease in the total number of axo-spinous asymmetric synapses in the striatum of 6-OHDA-treated rats. Recent findings from our laboratory also show a significant decrease in the total number of putative glutamatergic terminals (as revealed by asymmetric synaptic specializations) in the putamen of MPTP-treated parkinsonian monkeys (Villalba et al., 2013). To determine if this terminal loss is accounted for by a reduction in the number of cortical vs. thalamic boutons, antibodies raised against the vesicular glutamate transporter 1 (vGluT1) or vGluT2 were used as specific markers of corticostriatal or thalamostriatal terminals, respectively. Findings obtained in these studies remain controversial. On one hand, data from chronically MPTP-treated parkinsonian monkeys revealed that the relative density of vGluT1- or vGluT2-positive terminals in the putamen and the caudate nucleus is either unchanged or significantly increased compared with controls (Raju et al., 2008). These findings are consistent with human data showing a slight increase in the amount

of vGluT1 protein expression in the putamen of PD patients compared with controls (Kashani et al., 2007). However, data from unilateral 6-OHDA-treated rats or mice indicate a profound reduction in the number of vGluT1-positive terminals, without any significant alteration in vGluT2-postive thalamic boutons, in these animals (Zhang et al., 2013; Fieblinger et al., 2014a). Whether these discrepancies are due to differences in the toxin being used (6-OHDA vs. MPTP), or the chronic nature of the MPTP regimen administered in monkeys compared with the acute 6-OHDA-induced lesion of the nigrostriatal projection in rodents remains to be determined.

Anatomical and functional data indicate that the loss of spines induces various forms of structural and functional synaptic homeostatic adaptations in MSNs of parkinsonian animals. For instance, the various components of vGluT1- and vGluT2-positive corticostriatal and thalamostriatal synapses undergo structural changes consistent with an increased synaptic strength, ie increase in the volume of the spines, increase in the size of the pre-synaptic terminals, increase in the area and complexity of the post-synaptic densities (PSD) and massive growth of the spine apparatus (**Figures 1C–E**), in the putamen of chronically MPTP-treated monkeys (Villalba and Smith, 2010, 2011a, 2013). Similar changes have been associated with intraspinous increase in protein synthesis and calcium buffering in other brain regions (Fifková et al., 1983; Bourne and Harris, 2008; Plotkin et al., 2013), thereby providing further evidence for increased corticostriatal glutamatergic transmission at these remaining synapses. However, this remains to be demonstrated using adequate electrophysiological approaches.

In a recent study, Fieblinger et al. (2014a) used glutamate uncaging approach at specific axo-spinous corticostriatal synapses, and found that the intrinsic excitability of direct pathway MSNs was increased, while that of indirect pathway neurons was decreased, in 6-OHDA-treated mice. On the other hand, the excitatory corticostriatal synaptic connectivity on indirect, but not direct, striatofugal neurons was lower in 6-OHDA-treated mice than controls. Finally, they also reported that in neither case was the strength of corticostriatal connections globally scaled (Fieblinger et al., 2014a). Together, these observations indicate that striatal MSNs undergo complex homeostatic (or pathologic) changes of glutamatergic synapses in response to striatal DA depletion that could affect differentially the direct and indirect striatofugal pathways in PD.

Is the 6-OHDA-treated Rodent Model of PD Suitable to Study Striatal Spine Plasticity in PD?

Together, these recent findings (Zhang et al., 2013; Fieblinger et al., 2014a; Suárez et al., 2014) and previous studies (Ingham et al., 1989, 1998; Meshul et al., 2000; Day et al., 2006; Deutch et al., 2007; Neely et al., 2007) highlight the complex nature of the plastic changes striatal MSNs undergo in the 6-OHDA-treated rodent model of PD. However, the translation of these findings to the parkinsonsian state in humans must be achieved with caution because of the differential pathology of striatal glutamatergic afferents between the models under study and PD patients. Most importantly, PD is characterized by a massive degeneration of CM/Pf neurons (Henderson et al., 2000a,b; Smith et al., 2014a; Villalba et al., 2014), the main sources of the glutamatergic thalamostriatal system. The loss of these neurons and their corresponding axonal projections to the striatum is likely to further contribute to the synaptic homeostasis and scaling properties of remaining glutamatergic synapses in the PD striatum. Thus, the translation of morphological and functional studies of glutamatergic synapses in the striatum of PD models to the human parkinsonian condition must take into consideration the extent of CM/Pf degeneration (Villalba et al., 2013, 2014).

Although chronically MPTP-treated rhesus monkeys display 40–50% neuronal loss in CM/Pf (Villalba et al., 2013, 2014), the extent of Pf neuronal loss reported in various rodent models of PD is variable. While some authors did not find evidence for Pf degeneration 3 months after unilateral 6-OHDA nigrostriatal dopaminergic lesion in rats (Henderson et al., 2005; Kusnoor et al., 2012), other studies reported significant Pf cell loss in the same animal model (Aymerich et al., 2006; Sedaghat et al., 2009), or after systemic MPTP administration in mice (Freyaldenhoven et al., 1997). Some authors also showed that intrastriatal administration of 1-methyl-4-phenylpyridinium ion (MPP+) induces significant Pf cells damage in rats (Ghorayeb et al., 2002a). It remains to be determined whether these discrepancies were the result of differences in the neurotoxin exposure protocols, animal strains or other technical differences between these studies.

The need of animal models that include degeneration of the thalamostriatal system from CM/Pf is warranted for future studies of the plastic reorganization of striatal glutamatergic afferents in PD (Smith et al., 2014b; Villalba et al., 2014). Based on recent studies and others, it appears that MPTP toxicity might be a more reliable tool to induce CM/Pf neuronal loss and degeneration of the thalamostriatal system in mice and monkeys (Ghorayeb et al., 2002b; Smith et al., 2014a,b; Toy et al., 2014; Villalba et al., 2014).

L-DOPA-induced Dyskinesias (LID) and Striatal Spine Plasticity

Although striatal spine loss has long been recognized in the striatum of DA-depleted animals and PD patients, the effects of DA replacement therapy on spine pruning, reorganization of synapic connectivity and homeostatic plasticity remains poorly understood. However, recent studies showed that L-DOPA therapy partly restores some structural and functional aspects of corticostriatal connection in rodent models of PD (Zhang et al., 2013; Nishijima et al., 2014; Suárez et al., 2014; Fieblinger and Cenci, 2015). Some authors, indeed, reported that the loss of spines and vGluT1-positive terminals in the striatum of 6-OHDA-treated rats and mice could be reversed by chronic treatment with L-DOPA (Zhang et al., 2013; Suárez et al., 2014). However, in animals that developed L-DOPA-induced dyskinesia (LID), the spines displayed abnormal synaptic relationships with vGluT1-positive terminals such

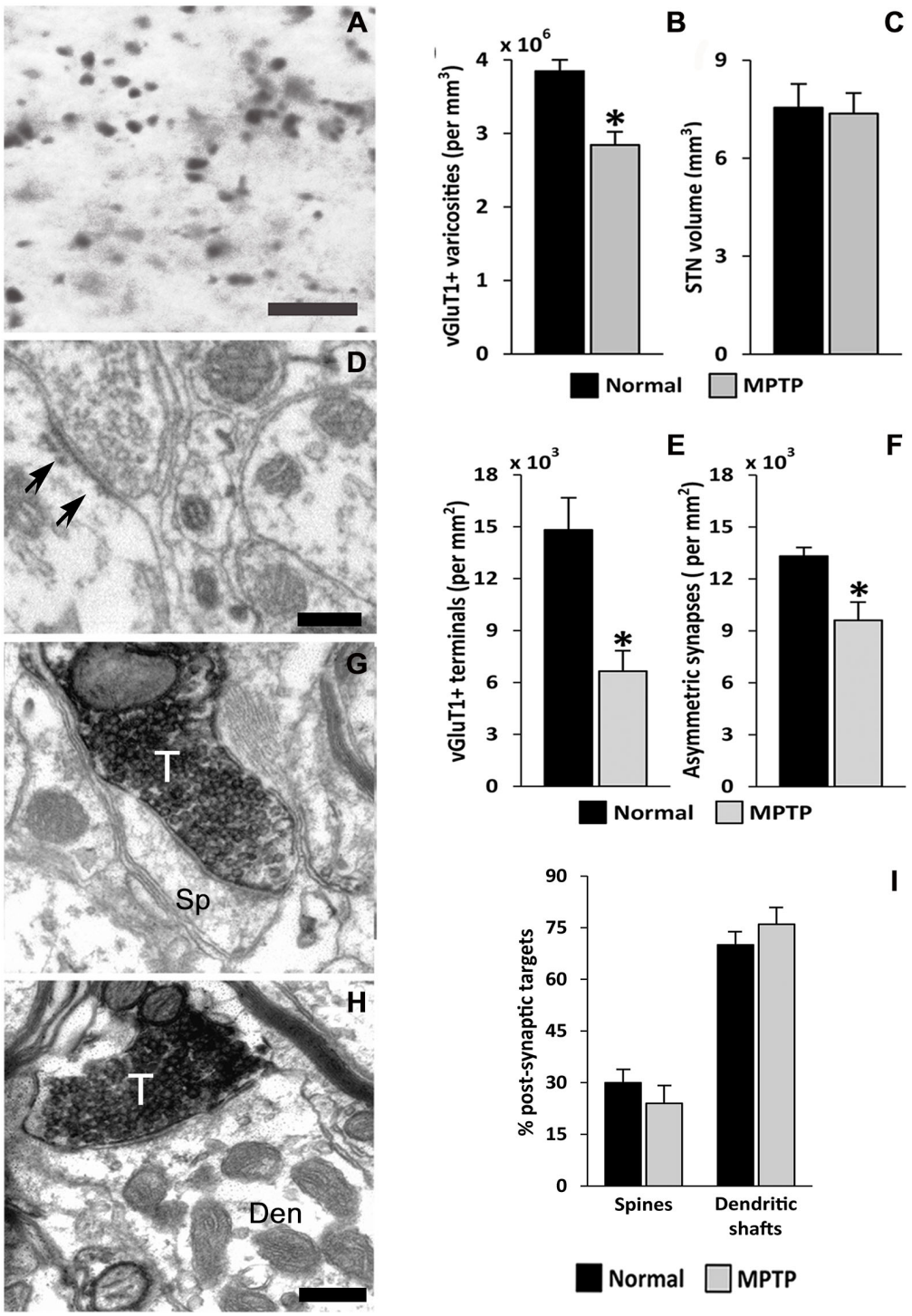

FIGURE 3 | vGluT1-positive innervation in the monkey subthalamic nucleus (STN). (A) Light micrograph showing vGluT1-positive varicose processes. **(B)** Average density (mean ± SEM; *N* = 3) of vGluT1-immunoreactive varicosities in the dorsolateral STN of normal and parkinsonian monkeys (*, *t*-test, $p = 0.012$). **(C)** Comparison of the average STN volume (mean ± SEM; *N* = 3) between normal and parkinsonian monkeys. **(D)** Electron micrograph showing an asymmetric synapse (arrows) in the dorsolateral monkey STN. **(E)** Average density (mean ± SEM; *N* = 3) of vGluT1-immunopositive terminals in the dorsolateral STN of normal and parkinsonian monkeys (*, *t*-test, $p = 0.02$). **(F)** Average density (mean ± SEM; *N* = 3) of asymmetric synapses in the dorsolateral STN of normal and pakinsonian monkeys (*, *t*-test, $p = 0.029$). **(G,H)** Electron micrographs showing vGluT1-containing terminals forming asymmetric synapses with a spine **(G)** and a dendritic shaft **(H)**. **(I)** Post-synaptic targets of vGluT1-immunopositive terminals in the dorsolateral STN. No differences were found in the proportion of vGluT1-immunoreactive terminals forming asymmetric synapses with dendritic shafts and spines in normal and parkinsonian animals. Scale bar **A** = 10 μm and in (**D**; applies also to **G**) and **H** = 0.2 μm. Abbreviations: Den, dendrite; Sp, dendritic spine; T, axon terminal (See Mathai et al., 2015).

that single spines often received synaptic inputs from 2 or more vGluT1-positive terminals (Zhang et al., 2013). Because this pathology was not found in non-dyskinetic L-DOPA-treated animals, the authors concluded that aberrant cortical innervation of striatal MSNs may be an important substrate of dysfunctional neuronal communication associated with LID (Zhang et al., 2013). Another main conclusion of this study was that neither the 6-OHDA lesion nor the L-DOPA treatment affected the prevalence and synaptic connections of vGluT2-positive thalamostriatal terminals in this animal model (Zhang et al., 2013). These observations were recently confirmed and extended in a recent study, which showed that both direct and indirect pathway MSNs manifest complex, and opposite, changes in homeostatic plasticity that affect their average firing rate in PD and LID states (Fieblinger et al., 2014a). Results of this study further demonstrated that the only adaptation found to be exclusively associated with LID was the restoration of excitatory axo-spinous synapses on the surface of indirect pathway neurons (Fieblinger et al., 2014a; see also Suárez et al., 2014).

As discussed above, an important shortcoming of these studies is the lack of evidence for thalamostriatal degeneration in the animal models used in these studies. The use of animal models of PD with CM/Pf pathology is essential to relate the neuroplastic properties of striatal MSNs and their glutamatergic responses to the human PD state (Smith et al., 2014b).

Cellular, Molecular and Genetic Mechanisms for Striatal Spine Loss in PD

Although the mechanisms underlying striatal spine loss in PD remain unclear, there is converging evidence that intraspinous calcium (Ca^{2+}) dysregulation likely contributes to this pathology (Segal et al., 2000; Sabatini et al., 2001; Oertner and Matus, 2005; Day et al., 2006; Deutch et al., 2007; Surmeier et al., 2007, 2011; Chen et al., 2008; Soderstrom et al., 2010; Surmeier and Schumacker, 2013). The Cav1.3α1 channels on D2R-containing neurons appear to be particularly important in mediating the spine pruning on indirect striatofugal neurons in mice (Day et al., 2006; Deutch et al., 2007; Surmeier et al., 2007; Soderstrom et al., 2010; Fieblinger et al., 2014b). In line with evidence that abnormal Ca^{2+} homeostasis may participate in this pathology, striatal MSNs devoid of the Ca^{2+} buffering protein, calbindin D-28k (CaB; Francois et al., 1994), such as those in the postcommissural putamen (sensorimotor striatal territory), display the most severe striatal spine pruning in parkinsonian monkeys (Smith and Villalba, 2008; Smith et al., 2009b; Villalba et al., 2009).

In vitro data suggest that the activation of the Ca^{2+}-dependent protein phosphatase, calcineurin, and the up-regulation of the transcriptional activity of the myocyte enhancer factor 2 (MEF2) and related regulatory genes (Nurr77, Arc) participate in the loss of glutamatergic synapses and spines in the striatum (Pulipparacharuvil et al., 2008; Tian et al., 2010; Villalba and Smith, 2013). Evidence that cholinergic signaling through M1 muscarinic receptors and Kir2 potassium channels may trigger the loss of glutamatergic synapses in rodent models of parkinsonism has also been suggested (Shen et al., 2008).

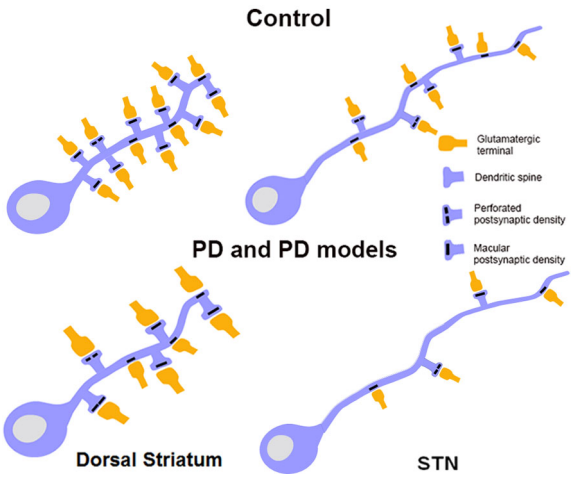

FIGURE 4 | Schematic showing morphological changes in dendritic spines and glutamatergic afferents in striatal MSNs and projection neurons in the STN in MPTP-treated parkinsonian monkeys. In the striatum of parkinsonian monkeys, there is a significant reduction in the density of Sp on MSNs, but the remaining spines and terminals display an increase in volume. The size of the PSD at corticostriatal and thalamostriatal synapses is also increased and more commonly perforated in parkinsonian animals than controls. In the STN, there is an overall decrease in the prevalence of vGluT1-positive cortical terminals in contact with dendrites and spines of STN neurons in parkinsonian animals. Potential changes in the ultrastructure of spines and afferent glutamatergic terminals, as shown in the striatum, remain to be determined in the STN.

Because of its role in the regulation of neurite length and branching, LRRK2 mutation in PD may contribute to striatal spine pathology (MacLeod et al., 2006; Parisiadou et al., 2009; Lee et al., 2010).

Changes in the Morphology of Astrocytes Associated with Glutamatergic Synapses in the Striatum of MPTP-Treated Monkeys

Data from our laboratory showed that in addition to the structural remodeling of the pre-synaptic terminals and post-synaptic spines at cortical and thalamic glutamatergic synapses (Villalba and Smith, 2010, 2011a, 2013), there is a significant growth in the extent of glial coverage of striatal glutamatergic synapses in parkinsonian monkeys (Villalba and Smith, 2011b; **Figure 2**). Perisynaptic astrocytes exhibit an interdigitated finger-like morphology in control animals (**Figure 2A**), while there is an expansion of astrocytic processes to cover a larger extent of the perimeter of axo-spinous complexes after MPTP-treatment (**Figure 2**). In MPTP-treated monkeys, the appositions between the axo-spinous complex and the astroglial processes are much tighter and continuous than in controls (**Figures 2E,F**). These differences between the normal and MPTP conditions were seen for both vGluT1- and vGluT2-positive glutamatergic synapses (Villalba and Smith, 2011b; **Figures 2G,H**).

A recent comparative study using 3D reconstruction in four animal models of PD, as well as in human PD, have shown

that in response to DA denervation, astrocytes in both the striatum and GP occupy a larger striatal volume (Charron et al., 2014). This increase in striatal volume occupied by astrocytes in parkinsonism is due to an enlargement of astrocyte cell body and processes reorganization at the level of asymmetric synapses (Charron et al., 2014), but also to an increase in the number of astrocytes, a change known as reactive gliosis (Dervan et al., 2004; Henning et al., 2008; Charron et al., 2014). These morphological and ultrastructural changes in the perisynaptic astrocytes might underlie an active participation of glial processes in structural plasticity in the striatum, as previously shown in the hypothalamus (Theodosis et al., 2008) and hippocampus (Ventura and Harris, 1999; Witcher et al., 2007, 2010), suggesting that both glial and neuronal elements of axo-spinous glutamatergic synapses in the primate striatum are endowed with a high level of structural and functional plasticity. It is likely that such a synaptic arrangement is not homogeneous across all excitatory synapses (Ventura and Harris, 1999; Witcher et al., 2007, 2010), suggesting that some glutamatergic synapses may be more leaky and prone to spill over glutamate in the extracellular medium to activate extrasynaptic glutamate receptors than others.

These modifications in astrocytes morphology and in their spatial relationships with glutamatergic synapses in PD models, together with the different molecular mechanisms by which astrocytes respond to changes in neuronal activity, suggest that pathological changes in striatal astrocytes might play a key role in triggering and/or contributing to the morphological and functional changes in striatal network plasticity in parkinsonism (Villalba and Smith, 2011b). A better understanding of glia-neuronal communication in normal and pathological conditions might help to develop new PD neurotherapeutic strategies.

Breakdown of the Corticosubthalamic Projection in Parkinsonism

The striatum and the STN are the main entry points for cortical information to the BG. Glutamatergic inputs to the STN originate from the cerebral cortex (Monakow et al., 1978; Nambu et al., 1996; Haynes and Haber, 2013), the thalamus (Sadikot et al., 1992), the brainstem PPN (Lavoie and Parent, 1994) and local axon collaterals of STN neurons (Kita et al., 1983; Kita and Kita, 2012). The parkinsonian state is associated with ultrastructural remodeling of synaptic connections which may contribute to activity changes in the BG. So far, such changes have been documented for the corticostriatal, thalamostriatal and pallido-subthalamic projections (Ingham et al., 1989; Meshul et al., 2000; Villalba et al., 2009; Villalba and Smith, 2011a, 2013; Fan et al., 2012). In line with evidence that the activity of the hyperdirect corticosubthalamic projection is altered in PD (Mathai and Smith, 2011; Yamawaki et al., 2012; Shimamoto et al., 2013; de Hemptinne et al., 2013; Delaville et al., 2015), we found a significant breakdown of the corticosubthalamic projection, characterized by a profound loss of vGluT1-positive terminals in the STN of parkinsonian monkeys (Mathai et al., 2015; **Figures 3**, **4**).

However, the functional impact of this pathology on the corticosubthalamic transmission and the downstream BG-thalamocortical circuitry remains to be clarified (Mathai et al., 2015). As shown in the striatum, possible homeostatic (or pathologic) changes in the strength and connectivity of remaining glutamatergic and GABAergic terminals in the STN might be induced (Ingham et al., 1989; Meshul et al., 2000; Smith et al., 2009b; Villalba and Smith, 2011a,b, 2013; Fieblinger et al., 2014a; Mathai et al., 2015). The known increase in the baseline (Bergman et al., 1994), and the greater degree of synchrony of STN neurons with cortical activity in PD are, indeed, in line with aberrant changes in corticosubthalamic transmission in the PD state (Williams et al., 2002, 2003, 2005; Moran et al., 2008; Gatev and Wichmann, 2009; Moshel et al., 2013; Shimamoto et al., 2013; Devergnas et al., 2014).

Thus, together with evidence for significant synaptic remodeling and altered glutamatergic transmission of the corticostriatal system in PD (Raju et al., 2008; Villalba and Smith, 2013; Fieblinger et al., 2014a), these findings suggest significant changes in the integration, processing and transmission of extrinsic cortical information to the BG in PD.

Concluding Remarks

For the past 25 years, it has been well recognized that degeneration of the nigrostriatal DA system induces loss of spines and complex plastic changes in the anatomical and functional organization of glutamatergic synapses in the mammalian striatum (**Figure 4**; for a review, see Villalba and Smith, 2013). The loss of spines has been demonstrated in various animal models and confirmed in PD patients. It has also been shown that the extent of spine loss in the striatum is tightly correlated with the degree of striatal DA denervation, but not with the severity of parkinsonian motor features, at least in MPTP-treated monkeys (Zaja-Milatovic et al., 2005; Smith and Villalba, 2008; Smith et al., 2009b; Villalba et al., 2009). Controversies remain as to whether direct or indirect pathway neurons are preferentially affected by this spine pathology. The animal species and the toxin being used, the chronic vs. acute regimen of intoxication and the time points at which observations are being made post-lesion likely contribute to the variability of results obtained in recent years (Ingham et al., 1989, 1998; Stephens et al., 2005; Zaja-Milatovic et al., 2005; Day et al., 2006; Scholz et al., 2008; Villalba et al., 2009; Suárez et al., 2014; Toy et al., 2014). Although indirect pathway neurons appear to be more sensitive than direct pathway neurons at early time points after DA depletion induced by 6-OHDA or reversible DA depleting agent like reserpine (Day et al., 2006; Fieblinger et al., 2014a), chronic MPTP toxicity in non-human primates and mice models of PD induces more widespread pathological effects upon both populations of striatofugal neurons (Villalba et al., 2009; Toy et al., 2014). In addition to spine loss, it has become clear that striatal MSNs also undergo a significant reduction in the length and number of dendritic branches in rodent models of PD, and that such changes affect invariably both populations of striatofugal cells (Fieblinger et al., 2014a).

The impact of striatal spine loss on the anatomical and functional connectivity of cortical and thalamic glutamatergic afferents has also generated significant interest in recent years, but significant issues remain to be addressed. Although authors agree that striatal spine loss is associated with a decrease in the number of total striatal glutamatergic synapses in the striatum, controversy remains as to whether these are accounted for by the loss of cortical over thalamic synapses (Raju et al., 2008; Villalba et al., 2013; Zhang et al., 2013; Fieblinger et al., 2014a). In acute, 6-OHDA-treated animals, vGluT1-positive corticostriatal terminals are selectively affected, without any impact on thalamostriatal vGluT2-positive boutons (Zhang et al., 2013; Fieblinger et al., 2014a), while the total number of vGluT1-immunoreactive boutons and amount of vGluT1 protein expression in the striatum is not significantly affected in chronically MPTP-treated parkinsonian monkeys and PD patients (Kashani et al., 2007; Raju et al., 2008; Villalba et al., 2013). In regards to the impact of spine loss on the prevalence of thalamic terminals and synaptic organization of the thalamostriatal system, the situation remains unclear, and also appears to be affected by the animal model being used (Kashani et al., 2007; Raju et al., 2008; Villalba et al., 2013; Zhang et al., 2013; Fieblinger et al., 2014a).

The concerns raised in this review about the animal model being used to address issues related to glutamatergic plasticity in PD is particularly important in the case of the thalamostriatal system because of the differential extent of CM/Pf (or Pf in rodents) cell loss in various models of PD (Freyaldenhoven et al., 1997; Ghorayeb et al., 2002a; Henderson et al., 2005; Aymerich et al., 2006; Sedaghat et al., 2009; Kusnoor et al., 2012; Smith et al., 2014a,b; Villalba et al., 2014). The lack of information about CM/Pf cell loss in some rodent models used in previous studies of striatal synaptic plasticity is a major limiting factor that complicates the use of this model to assess neuroplastic properties of striatal neurons and glutamatergic afferents in relation to PD. Because CM/Pf neuronal loss is a key pathological feature of PD (Henderson et al., 2000a,b; Smith et al., 2014a; Villalba et al., 2014), combined with the fact that the CM/Pf is the main source of thalamic inputs to the striatum, we believe that studies of striatal glutamatergic systems plasticity must be achieved in animal models that display thalamic pathology (Toy et al., 2014; Villalba et al., 2014).

Another interesting issue that has been put forward in recent years in regards to striatal spine loss in PD is the fact that L-DOPA can restore the loss of spines on subsets (mainly D2R indirect pathway neurons) of striatal neurons in 6-OHDA-treated rats and mice. However, the chronic use of L-DOPA and the subsequent development of LID in this model is linked with the development of aberrant and excessive corticostriatal axo-dendritic and axo-spinous synapses (Zhang et al., 2013). It is unclear as to whether this pathological plasticity of the corticostriatal projection is also seen in other animal models of LID or in dyskinetic patients.

Although the striatum remains the BG structure that received most attention in studies of synaptic plasticity, recent evidence indicates that afferents to the STN are also morphologically and functionally disrupted in PD models. In MPTP-treated monkeys, a significant loss of vGluT1-containing cortical terminals has been reported, suggesting a partial degeneration of the hyperdirect corticosubthalamic pathway (Mathai et al., 2015). On the other hand, GABAergic GPe terminals also undergo major plastic changes that result in an increased strength of the pallidosubthalamic system in rodent models of PD (Fan et al., 2012). Ongoing studies are in progress to better understand the underlying mechanisms and the functional consequences of these plastic changes on the transmission, integration and processing of extrinsic information by STN neurons in PD.

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Alterations in neuronal activity in basal ganglia-thalamocortical circuits in the parkinsonian state

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In patients with Parkinson's disease and in animal models of this disorder, neurons in the basal ganglia and related regions in thalamus and cortex show changes that can be recorded by using electrophysiologic single-cell recording techniques, including altered firing rates and patterns, pathologic oscillatory activity and increased inter-neuronal synchronization. In addition, changes in synaptic potentials or in the joint spiking activities of populations of neurons can be monitored as alterations in local field potentials (LFPs), electroencephalograms (EEGs) or electrocorticograms (ECoGs). Most of the mentioned electrophysiologic changes are probably related to the degeneration of diencephalic dopaminergic neurons, leading to dopamine loss in the striatum and other basal ganglia nuclei, although degeneration of non-dopaminergic cell groups may also have a role. The altered electrical activity of the basal ganglia and associated nuclei may contribute to some of the motor signs of the disease. We here review the current knowledge of the electrophysiologic changes at the single cell level, the level of local populations of neural elements, and the level of the entire basal ganglia-thalamocortical network in parkinsonism, and discuss the possible use of this information to optimize treatment approaches to Parkinson's disease, such as deep brain stimulation (DBS) therapy.

Keywords: Parkinson's disease, parkinsonism, basal ganglia, electrophysiology, extracellular recording, LFP, animal models

INTRODUCTION

Parkinson's disease (PD) is primarily characterized by movement deficits, but encompasses also many non-motor problems. The term "parkinsonism" refers to a characteristic constellation of motor impairments that are associated with PD, including decreased and slow movement (akinesia and bradykinesia), muscular rigidity, gait instability, and tremor at rest. The presence of bradykinesia and at least one other signs is required for the formal diagnosis of PD (Hughes et al., 1992).

Parkinsonism results, in large part, from the degeneration of dopaminergic neurons in the pars compacta of the substantia nigra (SNc; Bernheimer et al., 1973), and the resulting reduction in the levels of dopamine in the striatum, the main synaptic target of SNc axons (Hornykiewicz and Kish, 1987). The loss of dopamine is correlated with profound changes in the activity of neurons in the basal ganglia-thalamocortical circuits. In this review, we summarize the current knowledge of some of the electrophysiological changes that occur in the basal ganglia and related thalamic and cortical areas in parkinsonism. We focus on results obtained with extracellular recordings in parkinsonian animals or in human PD patients. Studies of functional abnormalities can also be conducted using non-electrophysiological methods (for instance, by using markers of metabolism or imaging techniques). The results of these studies are not covered here in any detail, but have been discussed in other publications (e.g., Galvan and Wichmann, 2008; Lindenbach and Bishop, 2013).

CIRCUIT ANATOMY AND PATHOLOGY OF PARKINSON'S DISEASE

FUNCTIONAL ANATOMY OF THE BASAL GANGLIA-THALAMOCORTICAL CIRCUITS

The basal ganglia (**Figure 1**, "Normal") consist of the neostriatum (caudate nucleus and putamen), the external and internal pallidal segments (GPe, GPi), the subthalamic nucleus (STN), the substantia nigra pars reticulata (SNr), and the SNc. These structures are part of larger functional and anatomical parallel circuits that also include areas of the frontal cortex and of the ventral thalamus. Depending on the function of the frontal cortical area of origin, these basal ganglia-thalamo-cortical circuits are designated as "motor", "associative/cognitive" and "limbic" (**Figure 2**, Alexander et al., 1986, 1990; Middleton and Strick, 2000).

The general anatomical arrangement of the basal ganglia is similar across these circuits. Glutamatergic excitatory cortical input reaches the basal ganglia via the striatum and STN, and, to a lesser extent, via the thalamus. The information is then transferred to the output nuclei of the basal ganglia, the GPi

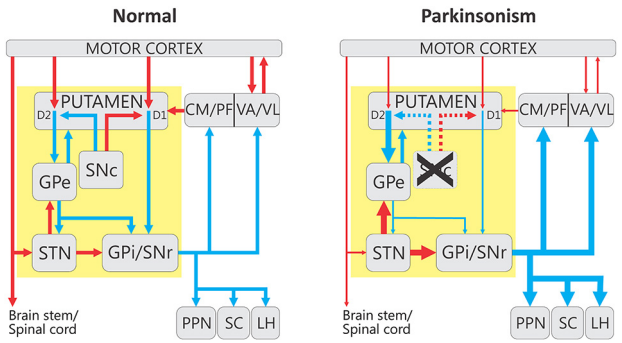

FIGURE 1 | Firing rate changes in the basal ganglia-thalamo-cortical motor circuit in parkinsonism. The yellow box indicates the interconnected basal ganglia nuclei that receive extrinsic input from the cortical and thalamic regions. For simplicity some connections have been omitted from this diagram. The left panel indicates the circuits in the "Normal" state, and the right shows the overall changes in activity that have been associated with parkinsonism. Blue and red arrows indicate inhibitory and excitatory connections, respectively. The thickness of the arrows corresponds to their presumed activity. Abbreviations: CM, centromedian nucleus of thalamus; D1 and D2, dopamine receptor subtypes; GPe, external segment of the globus pallidus; GPi, internal segment of the globus pallidus; LH, lateral habenula; PF, parafascicular nucleus of the thalamus; PPN, pedunculopontine nucleus; SC, superior colliculus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; VA, ventral anterior nucleus of thalamus; VL, ventrolateral nucleus of thalamus.

and SNr. The projections from the striatum to the output nuclei are divided into the monosynaptic "direct" pathway, and the "indirect" pathway, a polysynaptic projection that traverses GPe and STN before reaching the output nuclei. The STN is another major entry station for extrinsic cortical information into the basal ganglia. The cortico-subthalamic route, together with its continuation to GPi/SNr has been termed the "hyperdirect" pathway because information flowing along this projection reaches the basal ganglia output structures with a shorter delay than information transmitted along the direct and indirect corticostriatofugal systems (Nambu et al., 2002).

GPi and SNr neurons project to the thalamus, the lateral habenula, and brainstem structures, including the superior colliculus, and the pedunculopontine nucleus. There are also thalamic projections to the basal ganglia which reach primarily the striatum, with lesser projections to GPe and STN (Sadikot and Rymar, 2009; Smith et al., 2014a).

The obvious topographic separation of motor and non-motor functions at the cortical level is maintained throughout the subcortical course of the cortico-basal ganglia-thalamocortical circuits(Alexander et al., 1986). As mentioned below, abnormal activity patterns in the *motor* circuit are correlated with the appearance of parkinsonism. This circuit originates in somatosensory, motor, and premotor cortices, which innervate the post-commissural putamen, preserving a somatotopic organization. This area of the striatum projects, in turn, to motor regions of GPe, STN, GPi and SNr. The output nuclei send their axons to motor regions of thalamus, specifically to the ventral anterior and ventrolateral nuclei of the thalamus (VA/VL), which project back to motor regions of cortex. The GPi and SNr also send collaterals to the intralaminar centromedian and parafascicular thalamic nuclei (CM/PF)

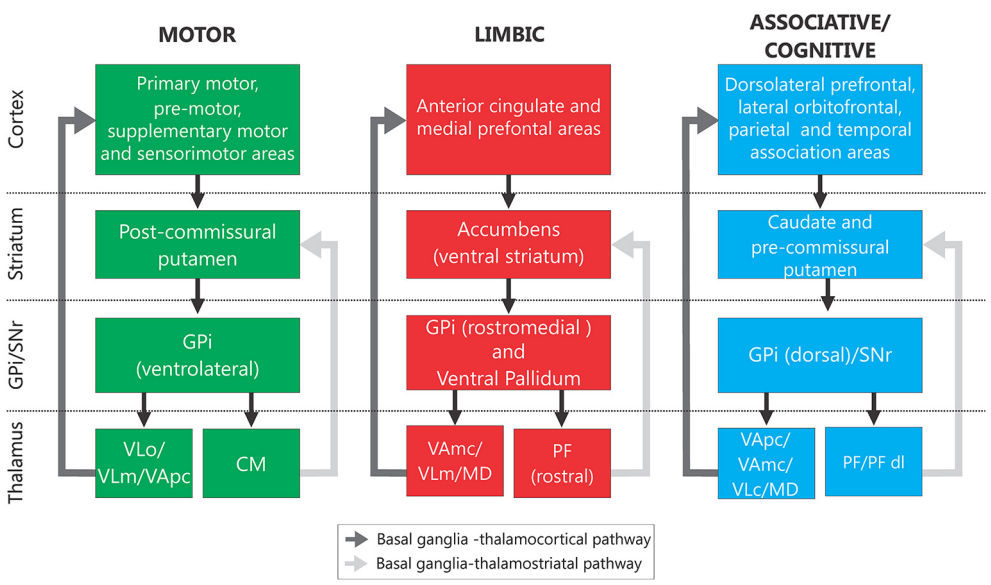

FIGURE 2 | Segregated basal ganglia—thalamocortical and basal ganglia—thalamostriatal functional loops. Information related to each functional modality is processed through segregated regions of the cerebral cortex, the basal ganglia and thalamic nuclei. Abbreviations: See **Figure 1** and MD, mediodorsal nucleus; PFdl, dorsolateral extension of the PF; VAmc, part magnocellular of VA; VApc, part parvocellular of VA; VLc, caudal part of VL; VLd, dorsal VL; VLo, pars oralis of VL; VLm, medial part of VL (based on Alexander et al., 1986, 1990; Middleton and Strick, 2000).

which, in turn, send glutamatergic efferents to the striatum (Sadikot and Rymar, 2009; Smith et al., 2014a). Of these, the CM nucleus receives movement-related output from GPi, and projects to the movement-related area of the striatum (the putamen), while PF receives largely non-motor-related inputs from the basal ganglia, and projects to the non-motor caudate nucleus.

While most basal ganglia structures are composed of either GABAergic (GPe, GPi and SNr) (Oertel and Mugnaini, 1984; Smith et al., 1987; Ilinsky et al., 1997) or glutamatergic (STN) projection neurons (Smith and Parent, 1988), the striatum contains a large population of GABAergic medium-spiny projection neurons (MSNs), and a smaller, but functionally important, proportion of interneurons, including cholinergic and various types of GABAergic interneurons (Kawaguchi, 1993; Tepper and Bolam, 2004). Besides these intrinsic sources of GABA, the striatum also receives GABAergic input from a subset of GPe neurons (Bevan et al., 1998; Sato et al., 2000; Mallet et al., 2012; Mastro et al., 2014).

In addition, the striatum receives a dense dopaminergic innervation from neurons in the SNc. Dopamine is a critical neuromodulator of striatal activity, acting both presynaptically and postsynaptically. We will describe its (proposed) actions in some detail here, because knowledge of the effects of the physiologic dopamine will facilitate an understanding of the effects of dopamine loss in PD.

At the presynaptic level, dopamine acts to decrease the release of glutamate from the terminals of cortical or thalamic projections, as well as the release of GABA from interneurons (reviewed in Tritsch and Sabatini, 2012). Postsynaptically, dopamine modulates the excitability and responsiveness of MSNs and striatal interneurons (Tritsch and Sabatini, 2012).

Dopamine D1-like and D2-like receptors are differentially expressed on the direct and indirect pathway MSNs, respectively (**Figure 1**, Gerfen et al., 1990). Because these receptor families are coupled to different second messenger system (through Ga_s or Ga_{olf} for D1-like receptors, and Ga_i or Ga_o for D2-like receptors, Tritsch and Sabatini, 2012), activation of D1 receptors increase the excitability of direct pathway MSNs, and facilitates long-term potentiation at glutamatergic synapses terminating on them, while activation of D2 receptors decreases the excitability of indirect pathway MSNs, and promotes long-term depression at synapses of the cortical projections terminating on their dendrites (Surmeier et al., 2011, 2014; Tritsch and Sabatini, 2012).

By increasing activity along the direct pathway, dopamine is thought to lead to an inhibition of the output nuclei of the basal ganglia. In contrast, by suppressing the activity of indirect pathway MSNs, dopamine facilitates GPe-STN transmission, thus increasing the inhibition of STN neurons (downstream from GPe) and their projections to GPi and SNr. The combination of these effects is that GPi and SNr activity is reduced by dopamine release in the striatum, leading to a reduction of the inhibition of thalamocortical projection neurons that receive the input from these nuclei. The differential dopaminergic modulation of the strength of synapses on direct and indirect pathway MSNs has been proposed to be relevant for the formation of reward-based procedural memory. The general functional model of the interactions between direct and indirect pathway in the regulation of motor activity was proposed decades ago (**Figure 1**, Albin et al., 1989; DeLong, 1990), and has received strong experimental support in the last few years (Kravitz et al., 2010, 2012; Cui et al., 2013; Freeze et al., 2013; Sano et al., 2013).

LOSS OF DOPAMINE AND PLASTIC CHANGES IN THE BASAL GANGLIA IN PD

In PD, the dopaminergic nigrostriatal pathway progressively degenerates. The dopaminergic projections to the sensorimotor striatum are affected more strongly than those to the associative and limbic striatal regions, contributing to the preponderance of movement problems in PD (Kish et al., 1988; Brooks et al., 1990). The loss of striatal dopamine is associated with morphological (non-dopaminergic) changes throughout the basal ganglia, including a reduction in the density of dendritic spines of MSNs in the striatum and alterations in intrastriatal and pallido-subthalamic connectivity, as we discuss below.

Although the loss of dopaminergic SNc cells remains the best known pathological feature of PD, it is clear that other types of neurons also degenerate, including neurons in the serotonergic raphe nuclei, the noradrenergic locus coeruleus, the olfactory tubercle, the intralaminar nuclei of the thalamus, and regions of the cerebral cortex and the peripheral nervous system (Braak and Braak, 2000; Braak et al., 2006; Sulzer and Surmeier, 2013). It is still not well understood how these changes in the brain stem, which occur relatively early in the disease, contribute to the motor and non-motor manifestations of parkinsonism.

TECHNIQUES USED TO STUDY PATHOPHYSIOLOGIC CHANGES IN PARKINSONISM

ANALYTICAL APPROACHES TO EXTRACELLULAR RECORDINGS AND DEFINITION OF TERMS

Most electrophysiologic *in vivo* recordings utilize extracellular recording methods, thus reflecting electrical potentials at the sites of electrodes that are positioned in the extracellular space, at some distance from the sources of the electrical activity, i.e., neuronal cell bodies or axons. The principal contributors to the recorded potentials are neuronal events in which ions are moving, i.e., action potentials and synaptic currents. The former can be detected as spikes in extracellular recordings, while the latter are recordable (at least in principle) as low-frequency fluctuations of recorded potentials.

ANALYSIS OF SINGLE CELL DISCHARGE (ACTION POTENTIALS)

The shape of individual action potentials is heavily dependent on the distance of the recording site from the current-generating source, and the geometric arrangement of the two. **Figure 3** shows examples of traces obtained during extracellular recordings of single neurons in the basal ganglia of monkeys. The analysis of neuronal action potential activity focuses therefore mostly on the timing of these potentials, and is

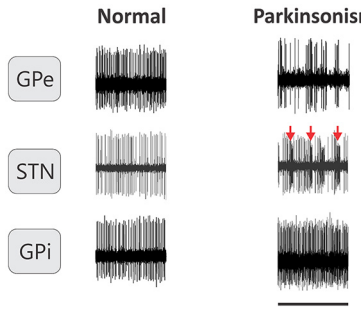

FIGURE 3 | Examples of extracellular recordings of individual neurons in the GPe, GPi and STN of monkeys in normal conditions and after MPTP treatment. Arrows indicate examples of bursts in an STN neuron. Horizontal line represents 1 s.

conventionally expressed in the form of inter-spike intervals (ISIs).

The most basic parameters that can be provided by an analysis of ISIs from an individual neuron are the static firing rate, along with measurements of the variability of discharge, such as a calculation of the coefficient of variation of ISIs, and assessments of the distribution of the ISIs (e.g., Kaneoke and Vitek, 1996).

Other analyses examine the preponderance of specific firing patterns, specifically bursts of action potentials. Such bursts are intuitively defined as sudden episodic unexpected accelerations in discharge rates, and analyzed by a variety of formal burst detection algorithms (Grace and Bunney, 1984; Legendy and Salcman, 1985; Aldridge and Gilman, 1991; Wichmann and Soares, 2006; Shimo and Wichmann, 2009).

Another set of features that can be conveniently studied is oscillatory activity. When applied to recordings of spiking activity of single neurons, the term "oscillation" is used to describe periodic fluctuations in the instantaneous firing rate of the neuron. Oscillatory activity can be most easily analyzed by frequency-domain methods, including global or time-resolved power spectral analyses of a neuron's discharge. Oscillatory activity is often grouped into larger frequency bands, based on conventions from the EEG literature, where rhythmic activity within specific frequency ranges is found in certain distribution patterns in scalp recordings, or is thought to have biological significance. Such frequency bands include the delta band (below 4 Hz), the theta band (4–8 Hz), the alpha band (8–13 Hz), the beta band (13–30 Hz) and the gamma band (above 30 Hz). The use of these band designations has led to much confusion in the field. One problem is that splitting the frequency spectrum in the EEG-based way may not be useful for other types of brain recordings. Many researchers therefore break the larger frequency ranges into smaller ones (such as the "low-beta" and "high-beta" band), or use the terminology loosely (for instance, using the term "beta band" for oscillations that encompass parts of the classical alpha- and beta bands). Another reason that argues against the unquestioning use of the traditional band descriptions is that bands of specific biological or pathological significance may differ between species, so that beta band oscillations in one species may be equivalent to oscillations in other bands in another (discussed in ref. Stein and Bar-Gad, 2013).

Oscillations of firing can occur with or without bursting (Wichmann and Soares, 2006; Wichmann and Dostrovsky, 2011). When slow oscillations occur in the form of periodically recurring bursts, this may represent the coupling of two different oscillatory phenomena (the amplitude of high frequency oscillations within the bursts is modulated by the phase of the low oscillation represented by the grouping of bursts), as is discussed to also be a prominent feature of cortical field potential recordings in parkinsonian patients (de Hemptinne et al., 2013).

Time domain measures (such as firing rates) and frequency domain measures (such as power spectra) are not sensitive to "nonlinear" features of discharge. The analysis of such non-linear features remains currently in its infancy (e.g., ref. Rodríguez et al., 2003; Darbin et al., 2006; Dorval et al., 2008), and is an interesting area of exploration.

Another level of analysis of single cell discharge is that of examination of the relatedness of activity patterns of multiple neurons. A commonly used time-domain technique is to compute the cross-correlation, a measure that examines the likelihood of firing in one neuron at pre-determined time intervals before or after another cell's firing. If oscillatory activity is the focus of the analysis, frequency-domain techniques (such as cross-spectra or coherence measurements) can also be applied.

ANALYSIS OF ELECTRICAL ACTIVITY OF NEURONAL POPULATIONS

Field potential recordings (including recordings of local field potentials (LFPs), electrocorticograms (ECoGs), and electroencephalograms (EEGs)) offer a rich source of information. It is generally accepted that LFPs are generated by transmembrane flow of current, and thus reflect synaptic activity within a population of neurons in an area several hundred micrometers in diameter around the electrode (Buzsáki et al., 2012) (but see Kajikawa and Schroeder, 2011). While relatively easy to record, the interpretation of these data is not straightforward. Field potential recordings are most often analyzed using frequency domain analysis methods, comparing the spectral content of the signals in different behavioral states. Another use of these signals is to analyze the presence of stimulation- or event related potentials, i.e., synaptic potentials related to electrical stimulation of the tissue, or potentials that precede or follow a behavioral event. Individual evoked potentials are often not visible, so that averaging techniques have to be used to extract stereotypically recurring waveforms.

Field potential signals recorded simultaneously at different locations within the brain can also be used for cross-spectral or coherence analyses which can provide information on the coupling of activity of brain regions. Phase shifts in such studies have been used to identify causality relationships, or to determine the direction of information flow within the network under study.

EXTRACELLULAR RECORDINGS IN HUMANS

In recent years, functional neurosurgical interventions, such as lesioning or DBS procedures, have been increasingly used to treat patients with PD. The placement of lesions or DBS

electrodes in the brain is often done guided by intraoperative electrophysiologic recordings. Microelectrode recordings from these surgeries have been extensively used to analyze the activity of the basal ganglia and related cortical and thalamic structures in PD patients. In addition, researchers have also used the implanted DBS electrodes as (macro-) electrodes to record LFPs intraoperatively, or in the immediate postoperative period.

Recordings in humans suffer from the unavoidable limitation that there are no available recordings in healthy subjects to use as controls. In some cases, recordings from patients with other basal ganglia-related neurological conditions (e.g., essential tremor, dystonia) are used for comparison instead.

ANIMAL MODELS OF PARKINSON'S DISEASE

Animal models of PD can be divided into several categories, including models that seek to replicate some of the genetic abnormalities that can lead to parkinsonism (Dawson et al., 2010; Blesa et al., 2012); models that attempt to mimic the spread of pathology (such as the intraparenchymal infusion of alpha-synuclein oligomers, e.g., Bezard et al., 2013), models that use pharmacological compounds to acutely deplete dopamine or to antagonize dopaminergic transmission (Bezard and Przedborski, 2011), and models that utilize neurotoxins that damage dopaminergic brain neurons (Beal, 2001; Dauer and Przedborski, 2003; Blesa et al., 2012). Electrophysiologic studies have heavily relied on the toxin models, so, to a large extent, our knowledge of the "pathophysiology of the parkinsonian state" is a description of the consequences of dopamine cell loss.

The two most common toxins used to create animal models of PD are 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Other toxins that have been described include pesticides and herbicides such as rotenone and paraquat (Betarbet et al., 2000; Hirsch et al., 2003; Thiruchelvam et al., 2003; Bové et al., 2005; Blesa et al., 2012). 6-OHDA was the first compound used to selectively lesion dopaminergic pathways (Ungerstedt, 1968). This toxin is injected directly in the brain parenchyma, targeting either the SNc itself, at the level of the dopaminergic terminals in the striatum or in the median forebrain bundle, which carries ascending dopaminergic projections to the forebrain (Schwarting and Huston, 1996b). Most frequently, 6-OHDA injections are done unilaterally, while the contralateral side serves as an (imperfect) control (Ungerstedt, 1968; Schwarting and Huston, 1996a,b). The toxin is effective in rats, mice, monkeys and other species, but has been most frequently used in rats. Dopamine depletion after unilateral injection of 6-OHDA results in motor asymmetry which can be easily quantified with behavioral methods (Ungerstedt and Arbuthnott, 1970; Ungerstedt, 1971; Schwarting and Huston, 1996a).

MPTP was discovered to induce dopamine loss in the striatum in the early 1980's, when a group of drug users developed parkinsonism after accidentally self-administering the toxin (Langston et al., 1983). Shortly after this discover, MPTP was shown to produce irreversible phenotypically convincing parkinsonism in monkeys as well (Burns et al., 1983; Langston et al., 1984; Langston and Irwin, 1986), which can be alleviated by dopaminergic agonists (e.g., Nomoto et al., 1985; Nomoto and Fukuda, 1993), deep brain stimulation (DBS, e.g., Benazzouz et al., 1993; Johnson et al., 2009; Rosin et al., 2011; Vitek et al., 2012) and other antiparkinsonian treatments. In monkeys, MPTP is most commonly administered by systemic injection, resulting in bilateral parkinsonism (Przedborski et al., 2001). Daily recurring treatment with high-dose MPTP results in rapidly evolving severe parkinsonism, while less frequent chronic administration of small doses of MPTP for a prolonged period of time (for instance, weekly injections for several months) results in slowly developing parkinsonism (Albanese et al., 1993). The latter model has been useful in studying the progression of electrophysiological changes in the basal ganglia in relation to the appearance of motor deficits (Devergnas et al., 2014). Another method of applying MPTP is administration of the toxin via intracarotid infusion, resulting in hemiparkinsonism (Bankiewicz et al., 1986).

MPTP can also induce degeneration of dopaminergic neurons in mice and rats, although, much higher doses of the toxin are needed than those required for monkeys (Chiueh et al., 1984; Heikkila et al., 1984; Kopin and Markey, 1988; Giovanni et al., 1994a,b). MPTP treatment in rodents tends to have more variable and unstable behavioral outcomes, and is therefore not commonly used for pathophysiologic studies.

CHANGES IN NEURONAL ACTIVITY IN PARKINSONISM

Early studies of the electrophysiologic alterations in MPTP-treated monkeys described mostly changes in the firing rates of individual neurons after dopamine depletion (Filion, 1979; Miller and DeLong, 1987, 1988), including decreases in firing rate in GPe neurons, and increased firing in STN, GPi and SNr cells. These modifications in firing rates are accompanied by changes in firing patterns, such as an increased tendency of neurons to fire in bursts of action potentials, enhanced oscillatory (rhythmic) activity within each nucleus and among structures, and increased synchrony among neighboring neurons.

In the following sections we will describe in more detail the changes in neuronal firing at the single cell and population levels for each basal ganglia structure, as well as for the associated cortical and thalamic regions. Although each nucleus will be presented separately, it is important to keep in mind that parkinsonian-related changes are the result of network alterations, thus affecting simultaneously all elements of the basal ganglia thalamocortical circuitry. **Table 1** shows a summary of the changes reported for each structure.

STRIATUM

The original "rate" model of the basal ganglia dysfunction in PD (**Figure 1**, "Parkinsonism", Albin et al., 1989; DeLong, 1990), proposed that striatal MSNs that give rise to the direct or indirect pathway are differentially affected by the striatal dopamine loss in PD. While direct pathway neurons reduce their firing rate, those in the indirect pathway increase their activity. As a result, the firing in GPe neurons is reduced, which leads to disinhibition of STN

Table 1 | Electrophysiological changes reported in parkinsonism in the basal ganglia and related thalamic and cortical regions.

	Firing rate	Bursting	Oscillations (single cell studies)	Oscillations (LFP studies)	Interneuronal synchrony
Striatum					
NS	INC. (R, P) (Chen et al., 2001; Liang et al., 2008)			INC. (M, beta) (Costa et al., 2006).	
MSNd	DEC. (R) (Mallet et al., 2006; Kita and Kita, 2011)				
MSNi	INC. (R) (Mallet et al., 2006; Kita and Kita, 2011)				
TAN			INC. (P, beta) (Raz et al., 1996)		INC. (P) (Raz et al., 1996)
GPe	DEC. (P, R, H) (Miller and DeLong, 1987; Pan and Walters, 1988; Sterio et al., 1994)	INC. (P, R, H) (Hutchison et al., 1994; Vila et al., 2000; Wichmann and Soares, 2006).	INC. (P, R, theta, alpha) (Raz et al., 2000; Magill et al., 2001)		INC. (P, R) (Nini et al., 1995; Mallet et al., 2008a)
STN	INC. (R, P, H) (Bergman et al., 1994; Hassani et al., 1996)	INC. (P, H) (Bergman et al., 1994; Steigerwald et al., 2008)	INC. (P, theta, alpha; H, beta) (Bergman et al., 1994; Levy et al., 2002b)	INC. (H, R, beta) (Priori et al., 2004; Sharott et al., 2005a) DEC. (H, gamma) (Brown et al., 2001)	Prominent (H, patients with limb tremor) Levy et al. (2002b)
GPi/SNr	INC. (P, H) (Miller and DeLong, 1987; Hutchison et al., 1994; Wichmann et al., 1999)	INC. (P, H) (Raz et al., 2000; Chan et al., 2011)	INC. (P, theta, alpha) (Raz et al., 2000; Soares et al., 2004)	INC. (P, alpha; H, beta) (Brown et al., 2001; Devergnas et al., 2014)	INC. (P, H) (Nini et al., 1995)
Thalamus (VA/VL)	Not consistent (P, H, R) (Vitek et al., 1994; Molnar et al., 2005; Pessiglione et al., 2005)	INC. (P, H) (Zirh et al., 1998; Magnin et al., 2000; Guehl et al., 2003)	INC. (P, theta, alpha) (Kammermeier et al., 2014)	INC. (H, theta) (Sarnthein and Jeanmonod, 2007)	
Motor cortex					
NS	DEC. (P, movement- related) (Doudet et al., 1990)	INC. (P) (Goldberg et al., 2002)		INC. (H, delta; H, P, theta; P, alpha; R, beta) (Neufeld et al., 1994; Sharott et al., 2005a; Devergnas et al., 2014) DEC. (P, gamma) (Devergnas et al., 2014)	INC. (P) (Goldberg et al., 2002)
Cortico-striatal	DEC. (R, to MSNd) (Mallet et al., 2006) UNCH. (P) (Pasquereau and Turner, 2011)	UNCH. (P) (Pasquereau and Turner, 2011)			
Cortico-spinal	DEC. (P) (Pasquereau and Turner, 2011)	INC. (P) (Pasquereau and Turner, 2011)			

Limits used to define frequency bands in the columns referring to oscillations: delta, <4 Hz; theta, 4–8 Hz; alpha, 8–13 Hz; beta, 13–30 Hz; gamma >30 Hz (see Section 1.4.1). Abbreviations: NS, not specified; MSNd, medium spiny neurons of the direct pathway; MSNi medium spiny neurons of the indirect pathway; TAN, tonically active neurons; Inc, increased; Dec, decreased; Unch, unchanged; P, MPTP-treated primates); R, 6-OHDA treated rats; H, PD patients; M, DAT-KO mice (see ref. Costa et al., 2006). A few representative examples of the studies in which these changes are demonstrated are cited in the table. Other references can be found in the main text.

neurons and, subsequently, excessive excitation of STN targets (GPi and SNr). The increased activity of GPi and SNr neurons is further reinforced by the lack of inhibition from direct pathway neurons. The final outcome of these changes is that the basal ganglia exert a greater than normal inhibition on their thalamic and brainstem targets.

Evidence in favor of increased activity of some neurons in the striatum was found in parkinsonian rats and monkeys (Chen et al., 2001; Liang et al., 2008). By using antidromic stimulation or juxtacellular labeling, studies in 6-OHDA treated rats have identified MSNs from the direct or the indirect pathway. These results indicate that, after dopaminergic depletion, direct pathway MSNs are less active, while the spontaneous firing of indirect pathway MSNs is increased (Mallet et al., 2006; Kita and Kita, 2011). The changes in firing rate in MSNs could be, at least in part, a consequence of changes in corticostriatal projections rather than an intrinsic change in activity, since cortical input to direct pathway MSNs (identified by showing antidromically mediated responses to stimulation of the SNr) show decreased spontaneous activity in 6-OHDA treated rats (Mallet et al., 2006), while inputs to neurons that did not respond antidromically to the SNr stimulation (presumed to be indirect pathway MSNs) were unchanged. It is not clear whether this finding also applies to primates, as studies in monkeys have suggested that corticostriatal projection neurons show few (if any) changes in activity in the parkinsonian state (Pasquereau and Turner, 2011). The rate model has recently received additional support from the application of optogenetic techniques in parkinsonian mice, in which selective activation of opsin-expressing direct pathway MSNs alleviated the motor deficits resulting from dopamine depletion (Kravitz et al., 2010). This study, however, does not provide direct evidence that low activity of direct pathway MSNs caused the motor deficits.

Due to the low number of interneurons in the striatum, changes in the electrical activity of these neurons in parkinsonism have been studied to a very limited extent. A specific group of cells, however, the tonically active neurons (TANs presumed to be cholinergic interneurons, Goldberg and Reynolds, 2011), has received considerable attention. These cells have a regular tonic firing at rest, a wide action potential, and a stereotypic electrophysiological response to rewarding events (Apicella, 2002). After MPTP treatment, the number of TANs showing this characteristic response to reward was drastically reduced, and a large proportion of TANs develop abnormal oscillatory activity around 16 Hz, while the already high degree of correlation between these neurons was slightly increased (Raz et al., 1996).

Few studies have analyzed parkinsonism-related changes in oscillatory activities in LFP signals in the striatum. In mice, spectral power in the delta and beta ranges was increased in LFPs recorded in the dorsal striatum during pharmacologically-induced acute akinesia, and such increases were reversed by administration of L-DOPA (Costa et al., 2006). While intrastriatal blockade of D2- and D1-receptors induce severe akinesia, only blockade of D2-receptors increases delta and beta oscillations and decreases gamma activity in the striatum (Burkhardt et al., 2009). Other studies have suggested that the effect of dopamine depletion on LFP oscillations in the striatum may be task- and learning-dependent (Lemaire et al., 2012), and that it is strongly influenced by oscillatory activity in the cortex, as shown by simultaneous recordings of striatal LFPs and EEGs (Courtemanche et al., 2003; Berke et al., 2004).

The parkinsonian state has frequently been associated with alterations in the somatotopic organization of the basal ganglia-thalamo-cortical circuits. In the striatum of parkinsonian rodents, there is a disruption of the normal organization of striatal neurons that response to sensory inputs (Cho et al., 2002). This finding may indicate that cortical-striatal inputs are abnormal (perhaps related to the morphological changes discussed below), or could reflect pathological processing of information within the striatum itself.

Several morphological changes occur in the striatum after dopaminergic depletion and may contribute to the appearance of abnormal activity patterns in this nucleus. A major alteration, first described in 6-OHDA rats and later in PD patients and MPTP-treated monkeys, is the loss of dendritic spines of MSNs (Ingham et al., 1989; Stephens et al., 2005; Zaja-Milatovic et al., 2005; Day et al., 2006; Villalba et al., 2009). Since the dendritic spines on MSNs are the main recipients of cortico-striatal (and a proportion of thalamo-striatal) afferents, it is likely that the spine loss affects glutamatergic transmission in the striatum (Smith and Villalba, 2013). As studied in MPTP-treated monkeys, the remaining spines, along with the corticostriatal and thalamostriatal terminals that contact them, have an increased volume compared to that found in normal animals, which may signify increased activity at these synapses (Villalba and Smith, 2011). Studies in 6-OHDA treated mice have also demonstrated that the dendritic arborization is reduced in both direct and indirect pathway MSNs (Fieblinger et al., 2014) (These morphological alterations are accompanied by functional changes, as demonstrated recently in *in vitro* studies, which show that after dopaminergic lesions with 6-OHDA the intrinsic and dendritic excitability of indirect pathway MSNs was lower, while the intrinsic excitability of direct pathway MSNs was increased (Fieblinger et al., 2014), compared to the normal state. These changes were interpreted as homeostatic adaptations that could contribute, in early stages of parkinsoninsm, to keep a balance activity of both striatal patwhays, but that are insufficient in later stages (Mallet et al., 2006). Such plastic changes remain to be fully explored in *in vivo* animal models of PD (Ma et al., 2012).

Other plastic changes in striatal connectivity have been described in parkinsonian rodents. Studies in transgenic mice showed that collateral connections across MSNs of direct and indirect pathway are reduced by dopamine depletion (Taverna et al., 2008), while fast-spiking GABAergic striatal interneurons selectively increase the number of connections established with indirect-pathway MSNs (Gittis et al., 2011). These changes could enhance the degree of synchronization among striato-pallidal MSNs, and could induce alterations in the activity of the target regions. These plastic changes, however, have not yet been described in primates.

GPe

The GPe strongly influences the activity of almost all of the other basal ganglia structures. Early studies in MPTP-treated monkeys

reported that the average firing rate of GPe neurons was lower in MPTP-treated monkeys than in normal animals (Miller and DeLong, 1987, 1988). This observation has been corroborated in primate and rodent models of PD (**Figure 3**, Pan and Walters, 1988; Filion and Tremblay, 1991; Heimer et al., 2002; Soares et al., 2004; Mallet et al., 2008a). Recordings in PD patients found that the firing rates of GPe neurons were similar to that found in MPTP-treated monkeys (Hutchison et al., 1994; Sterio et al., 1994).

The reduced activity of GPe neurons after dopaminergic depletion could result from an increased activity of indirect pathway MSNs, as the "rate" model would predict (**Figure 1**, "Parkinsonism"). This view has also been incorporated into some of the models of basal ganglia dysfunction in the parkinsonian state (Terman et al., 2002). An alternative possibility is that reduced pallidal activity could result from a loss of autonomous pace making activity of these neurons after dopamine depletion. This idea is supported by studies in rodent models of parkinsonism in which a proportion of GPe neurons was found to show reduced spontaneous activity (Chan et al., 2010). In these studies, the number of "silent" GPe neurons correlated with the severity of the motor deficits.

In addition to the decreased firing rate, the incidence of burst discharges is increased in GPe neurons, as reported in MPTP-treated monkeys (Soares et al., 2004; Wichmann and Soares, 2006) and 6-OHDA treated rodents (Vila et al., 2000; Ni et al., 2001; Breit et al., 2007). In PD patients, there was a high degree of bursting in GPe neurons, reminiscent of that seen in MPTP-treated monkeys (Hutchison et al., 1994; Magnin et al., 2000). Also, individual neurons with oscillations in the (approximately) 3–8 and 8–15 Hz frequency bands are more frequently found in GPe of MPTP-treated than in normal monkeys (Raz et al., 2000; Soares et al., 2004). A similar observation has been made in the GP of 6-OHDA treated rats (Magill et al., 2001).

Furthermore, the normally uncorrelated activity of neighboring GPe neurons (Nini et al., 1995; Bar-Gad et al., 2003) becomes more synchronized after MPTP treatment (Nini et al., 1995; Raz et al., 2000; Heimer et al., 2002; Morris et al., 2005). The parkinsonian state is similarly associated with increased synchrony of neuronal activity in the pallidum in rodents (see below), as well as in most of the other basal ganglia structures. While the cause of the increased synchrony remains under investigation, it appears to be a pervasive feature of basal ganglia activity in parkinsonism that may contribute (or even underlie) other firing abnormalities, such as the appearance of oscillatory activity patterns or the finding of less specific sensory responses of neurons in the motor circuit (Rothblat and Schneider, 1995; Schneider and Rothblat, 1996; Boraud et al., 2000; Cho et al., 2002; Prokopenko et al., 2004).

Several recent rodent studies have demonstrated considerable heterogeneity among GPe neurons, suggesting distinctive patterns of projections, protein expression and electrophysiological activity (Sato et al., 2000; Kita, 2007; Mallet et al., 2008a, 2012; Mastro et al., 2014). Studies in rats have defined two major GPe neuronal populations with opposing phase relationships with cortical oscillatory activity, so-called prototypic and arkypallidal cells (Zold et al., 2007a,b; Mallet et al., 2008a, 2012). Compared with controls, the level of synchrony of firing between prototypic or arkypallidal cells, and between each of these groups and cortical activity is increased after 6-OHDA treatment (Mallet et al., 2008a; Zold et al., 2012). It has recently been proposed that these two types of GPe neurons, and the connections they establish among themselves and with the rest of the basal ganglia, may have an important role in the development of the pathological oscillations in parkinsonism (Nevado-Holgado et al., 2014). It is not (yet) clear whether these rodent findings can be generalized to primates. In the early reports of primate GPe electrophysiological activities, DeLong described neurons with high-frequency firing rates interspersed by pauses, and a second, less frequently encountered, type of neurons with low frequency discharge and bursts (DeLong, 1971). It has been suggested that these neuronal types might correspond, respectively to the prototypic and arkypallidal types described in rodents (Mallet et al., 2012), but the parallel remains unclear.

As in the striatum, dopamine depletion appears to be associated with morphologic and functional changes in connectivity in GPe. Thus, intrapallidal collaterals, which seem to exert only weak inhibition among neighboring neurons under normal conditions (Bar-Gad et al., 2003) appear to be functionally strengthened in 6-OHDA treated rats (Miguelez et al., 2012; Nevado-Holgado et al., 2014), and there is an increase in the abundance and strength of synaptic contacts between GPe and STN after dopaminergic depletion (Fan et al., 2012). It is not clear how these plastic changes are triggered. They may be an adaptive response, acting to reduce excessive STN activity (see below), at the price of reinforcing aberrant interneuronal synchronization.

STN

Based on the initial finding that the mean firing rate of STN neurons is increased after MPTP-treatment in monkeys (Miller and DeLong, 1987; Bergman et al., 1994), later corroborated in several primate and rodent studies (Hassani et al., 1996; Bezard et al., 1999; Vila et al., 2000; Magill et al., 2001; Soares et al., 2004), most authors agree that PD is associated with excessive activity in the STN. This concept has strongly influenced the development of models of changes in the basal ganglia thalamocortical circuit in parkinsonism, and the development of surgical strategies for PD (DeLong and Wichmann, 2012).

While increased firing of STN neurons is considered a hallmark of the pathological electrical activities in parkinsonism, other changes in firing activities in the STN may be similarly important. For instance, similar to pallidal neurons, STN neurons show increased bursting, in parkinsonian animals, and probably also in parkinsonian patients (**Figure 3**, Bergman et al., 1994; Levy et al., 2000; Soares et al., 2004; Wichmann and Soares, 2006; Steigerwald et al., 2008; Tachibana et al., 2011). Bursting-related measures, such as the intra-burst firing rate of neurons, are among the most discriminative features of parkinsonism in parkinsonian monkeys (Sanders et al., 2013) and correlate strongly with the

severity of parkinsonism in patients with PD (Sharott et al., 2014).

The proportion of neurons in STN with oscillatory firing is also markedly increased in the parkinsonian state, particularly in the 3–8 and 8–15 Hz frequency ranges in MPTP-treated monkeys (Bergman et al., 1994; Soares et al., 2004; Rivlin-Etzion et al., 2006; Moran et al., 2012; Galvan et al., 2014). These oscillations are coherent with those of neurons that are simultaneously recorded in the STN targets, GPe and GPi (Moran et al., 2012). In PD patients, many STN neurons also show oscillations, but at higher (15–30 Hz) frequencies (Levy et al., 2002b; Weinberger et al., 2006). Neurons with oscillatory activity are more likely to exhibit, in addition, an increase in tonic firing (Bergman et al., 1994; Deffains et al., 2014). Studies in humans have also shown that STN neurons in PD patients with limb tremor tend to show synchronized firing (Levy et al., 2000).

Numerous studies have analyzed the characteristics of LFP signals recorded from the STN area in patients with advanced parkinsonism. Such recordings can conveniently be made, using perioperative recordings in patients who undergo therapeutic DBS lead placement procedures that target the STN, using the implanted DBS electrodes as recording electrodes. It is worth noting that the (usually bipolar) "LFP" recordings in these cases refer to potentials between two contacts of the DBS electrode which are at least 1.5 mm apart, thus reflecting potential differences between separate groups of neurons. It is not clear how such potentials relate to the membrane potential fluctuations in small groups of neurons that are usually recorded as LFPs in animal experimentation. These studies have shown that the disease is associated with prominent oscillations in the STN in the beta band (for review, see Stein and Bar-Gad, 2013) which can be reduced by dopaminergic medications and high-frequency stimulation of the STN (Brown et al., 2001; Levy et al., 2002a; Priori et al., 2004; Wingeier et al., 2006; Kühn et al., 2008b; Bronte-Stewart et al., 2009). Some of the studies in 6-OHDA treated rats have also shown increased beta band power in STN LFPs, as compared with normal animals (Sharott et al., 2005a).

However, beta-band oscillations in LFP recordings from MPTP-treated monkeys are not as prominent as they are in humans (despite the presence of a clearly recognizable parkinsonism, Devergnas et al., 2014). Explanations for the discrepancy between these studies and those in human patients include the possibility that the degree of synchrony in the parkinsonian monkeys may not be sufficient to lead to recordable LFP oscillations or that the recording conditions are substantially different. Another important consideration is that the main frequency of normal and abnormal oscillations in these animals may differ from that in humans. It has been suggested that the 8–15 Hz frequency range in monkeys may be equivalent to the beta band in humans (Stein and Bar-Gad, 2013).

The interactions between the reciprocally connected GPe and STN (Smith et al., 1998) may be particularly important in the development of bursts and oscillations in these two structures (Plenz and Kitai, 1999; Ni et al., 2000a; Cruz et al., 2011). Modeling studies have proposed that the STN-GPe connections may generate rhythmic or irregular patterns of activity. Increased input from indirect pathway MSNs onto GPe neurons tends to promote rhythmicity and abolish irregular firing (Terman et al., 2002). After dopamine depletion, the pallidosubthalamic circuits are strengthened by an increase in the number of synaptic connections between GPe terminals and STN, as has been demonstrated in 6-OHDA treated rodents (Fan et al., 2012). Such increased inhibition from the GPe may promote hyperpolarization-induced rebound bursting of STN cells (Beurrier et al., 1999; Bevan et al., 2007), and account for changes in the temporal structures of burst discharges in pallidum and STN in MPTP-treated monkeys (Wichmann and Soares, 2006). Furthermore, studies on parkinsonian rats have suggested that oscillations that originate in striatum, cortex or thalamus may be amplified in the GPe-STN network which would act as a non-linear oscillator with self-adjusting resonance frequencies (Nevado-Holgado et al., 2014).

Rhythmic activity in the STN is closely related to that in cortex (Magill et al., 2000), and this correlation is exacerbated after dopamine depletion in 6-OHDA treated rats (Magill et al., 2001), with a peak coherent activity between cortex and STN in the beta range (Sharott et al., 2005b). Similarly correlated cortex-STN activity has been described to be dopamine-dependent in PD patients (Williams et al., 2002; Shimamoto et al., 2013).

GPi AND SNr

As could be predicted by the increased firing of STN neurons and the reduced firing of GPe neurons, the firing rates of GPi and SNr neurons are increased after dopamine depletion in most studies of parkinsonian primates (**Figure 3**, Miller and DeLong, 1987, 1988; Filion and Tremblay, 1991; Wichmann et al., 1999; Heimer et al., 2002; Soares et al., 2004). In PD patients, the average firing rate of GPi neurons was similar to that seen in MPTP-treated monkeys (Hutchison et al., 1994; Sterio et al., 1994), and it was higher than the average discharge rate in dystonia patients (Starr et al., 2005).

Compared to normal animals, in the GPi of MPTP-treated monkeys, there was a smaller proportion of neurons that decreased activity during movement onset (Leblois et al., 2006). Instead, the authors found that most GPi neurons showed increased activity around the time of movement. (Leblois et al., 2006). This abnormal movement-related excitation of GPi neurons could result in excessive inhibition of thalamic neurons (and thus, help explain bradykinesia during voluntary movements in parkinsonism).

GPi neurons of MPTP-treated monkeys also show increased oscillations and bursting activities (Raz et al., 2000; Soares et al., 2004; Leblois et al., 2007). Similarly, in PD patients burst firing is found in both oscillatory and non-oscillatory GPi cells (Chan et al., 2011). As found in GPe, synchronized activity among GPi neurons is more prominent after MPTP treatment in monkeys (Nini et al., 1995; Bergman et al., 1998; Raz et al., 2000; Morris et al., 2005; Leblois et al., 2007) and in PD patients (Hurtado et al., 1999; Levy et al., 2000). In studies of LFP signals, recorded from DBS electrodes in GPi patients (Silberstein et al., 2003; Weinberger et al., 2006), the relative power in the beta

band (11–30 Hz) was higher in parkinsonian patients than in those with dystonia, while the power in the 4–10 Hz range was higher in the dystonic group (Silberstein et al., 2003). In MPTP-treated monkeys, however, the oscillatory activities are predominant at lower frequencies (7.8–15.5 Hz, Devergnas et al., 2014).

Oscillatory activities can also be identified in the SNr in 6-OHDA treated rats. Recordings using chronically placed electrodes in the SNr of unilaterally 6-OHDA lesioned animals showed increased oscillatory LFP activity and increased synchronization (entrainment) of single cell activity to LFP oscillations in the 12–40 Hz range in the dopamine-depleted hemisphere compared to the non-lesioned side (Avila et al., 2010; Brazhnik et al., 2012, 2014). Brazhnik et al. found that "active" states, such as grooming and walking, were related to relatively higher frequencies, while rest and REM sleep were associated with lower frequencies (Brazhnik et al., 2014).

While it is not clear how such oscillations are generated or modulated, the finding of high coherence of oscillations among STN, GPe and GPi in parkinsonian individuals (Moran et al., 2012) suggests that the oscillations in the basal ganglia output nuclei may be related to oscillations in the other nuclei. For instance, in PD patients, low frequency stimulation of the STN enhances the synchronization at similar frequencies in the GPi, while higher frequency stimulation of STN suppresses the beta-range oscillations in GPi (Brown et al., 2004). It is conceivable that the altered balance between direct and indirect pathways in the parkinsonian state (Mallet et al., 2006) may allow (oscillatory) STN output to strongly "drive" GPi neurons.

There is evidence that, after dopaminergic depletion, there is a disruption of the somatory-sensory representation in the GPi, as indicated by a reduction in the specificity of responses to sensory stimuli in the entopeduncular nucleus of MPTP-treated cats (Rothblat and Schneider, 1995) and an increased number of GPi neurons that respond to movement in monkeys (Leblois et al., 2006).

THALAMUS

Studies in MPTP-treated (parkinsonian) monkeys suggest that metabolic activity is increased in VA and VL (Mitchell et al., 1989; Rolland et al., 2007), perhaps reflecting increased basal ganglia input. The downstream effects of abnormal basal ganglia output on firing rates in thalamus have been studied to a limited extent and comparative studies of neuronal activity in the basal ganglia-receiving regions of the thalamus have been inconsistent. While some studies show decreased neuronal firing (Vitek et al., 1994; Schneider and Rothblat, 1996; Ni et al., 2000b; Kammermeier et al., 2014), others found no firing rate change (Pessiglione et al., 2005), and others show increase in firing rates (Bosch-Bouju et al., 2014). In PD patients, neurons in the basal ganglia-receiving areas of thalamus have a reduced mean firing rate, compared to similar recordings from non-PD patients (Molnar et al., 2005; Chen et al., 2010).

Recent studies in rats have shown that, under normal conditions, the firing rate of neurons in the motor thalamus is modulated during reaching movements, and that the responsiveness of thalamic cells during such movements is greatly reduced in 6-OHDA lesioned (Bosch-Bouju et al., 2014). Such task-related changes in thalamic activity could contribute to some of the deficits in motor performance in parkinsonism.

Studies in MPTP-treated monkeys have suggested that burst discharges are increased in the motor thalamus (Guehl et al., 2003; Pessiglione et al., 2005) and a similarly high incidence of burst firing has been found in corresponding areas in PD patients (Zirh et al., 1998; Magnin et al., 2000; Molnar et al., 2005). The thalamic bursts were shown to fulfill the criteria of "rebound" bursts (Magnin et al., 2000), suggesting that the bursts could result from hyperpolarization of thalamic neurons driven by increased inhibitory basal ganglia input via a T-type calcium channel dependent mechanism. These results may, however, not only relate to the changed motoric state of the subject, but also to parkinsonism-related changes in the state of arousal. Indeed, patients and animals tend to be less awake in the parkinsonian state, which, independent of other factors, may contribute to the finding of a higher incidence of burst discharges in the thalamus.

Reports of the effects of parkinsonism on oscillatory activity in the motor thalamus have been inconsistent. LFP recordings from the pallidal receiving area of PD patients with drug-resistant tremor showed prominent oscillations in the tremor frequency range (4–9 Hz), strongly correlated with oscillations recorded in frontal cortex (Sarnthein and Jeanmonod, 2007). Our recent findings on oscillatory activity patterns in parkinsonian monkeys suggest that single-cell oscillatory activities increase in the 3–13 Hz range of frequencies, and are mildly reduced in the gamma-range of frequencies in the basal ganglia-receiving area of the thalamus (Kammermeier et al., 2014).

The disruption in somatosensory representation is also apparent in the motor thalamus. The sensory responses of VA/VL neurons were found to be less specific in MPTP-treated monkeys than under normal conditions (Pessiglione et al., 2005). In the thalamus of these animals, the spiking activity of neighboring VA/VL neurons was more frequently correlated than under normal conditions (Pessiglione et al., 2005).

Several of the alterations in firing described in the basal ganglia-receiving portion of the thalamus in parkinsonism, such as increased bursting and abnormal sensory processing, have also been described for the thalamic regions that receive cerebellar inputs (Guehl et al., 2003; Molnar et al., 2005; Pessiglione et al., 2005; Chen et al., 2010). In fact, a recent study of single unit activity in the cerebellar and basal ganglia-receiving regions of the thalamus in PD patients, found that oscillations of single neurons in the beta range were prominent in the cerebellar regions of the thalamus, but not in the basal ganglia area (Basha et al., 2014). The recently described interconnections between the cerebellum and the basal ganglia (Bostan et al., 2010, 2013; Bostan and Strick, 2010) could have a role in the development of electrophysiological alterations and the parkinsonian motor deficits (Lewis et al., 2013), but it is also possible that such oscillations reflect changes in cortical oscillatory patterns which may then be fed back to the thalamus via corticothalamic projections that modulate the excitability of thalamocortical

neurons. It is important to note that interventions aimed at the cerebellar receiving thalamus (lesioning or stimulation) are an established treatment for parkinsonian tremor, suggesting that the cerebello-thalamo-cortical pathway may be involved in the generation of parkinsonian tremor (Benabid et al., 1991; Schuurman et al., 2000).

Besides the VA/VL, the CM/PF nuclei also receive inputs from the basal ganglia, and then send a massive projection back to the striatum (Smith et al., 2014a). The impact of CM/PF activity on basal ganglia functions under normal conditions is just starting to emerge (Smith et al., 2011), and remains almost unexplored in the parkinsonian state. In 6-OHDA treated rats, recordings in the PF (the rodent equivalent of the primate CM, Smith et al., 2004) there is a reduction in the number of neurons with spontaneous activity, a large proportion of cells develop oscillations at 0.3–2.5 Hz, and there is a reduction in low threshold spike (LTS) bursts (Parr-Brownlie et al., 2009). The changes in PF neurons, however, do not seem to be driven by changes in the basal ganglia (Parr-Brownlie et al., 2009). LFP recordings in the CM in PD patients described an increase in gamma band activity after levodopa treatment and a decrease in beta-band activity, but the latter was seen in only one of three patients (Kempf et al., 2009). CM/PF activity changes may not only be shaped by altered inputs from the basal ganglia, but also by the fact that CM and PF neurons degenerate early in parkinsonism, as reported for PD patients (Henderson et al., 2000a,b), and MPTP-treated monkeys (Villalba et al., 2014). It is unclear how the loss of CM/PF neurons affects the activity of the remaining cells in the CM/PF complex or in other basal ganglia structures that receive afferents from it.

CORTEX

Early studies of neuronal activity of cortical neurons in primary motor (M1) or supplementary cortices in monkeys reported that the spontaneous activity of these neurons did not change with MPTP treatment, but that movement-related discharges were reduced in parkinsonian animals (Doudet et al., 1990; Watts and Mandir, 1992; Goldberg et al., 2002; Escola et al., 2003). A later study, in which M1 projection neurons were identified in primates on the basis of responses to antidromic stimulation, demonstrated that the observed reductions in firing rates were restricted to neurons projecting to the pyramidal tract, but did not affect those projecting to the striatum (Pasquereau and Turner, 2011). This suggests that the pyramidal tract projecting subpopulation of cortical neurons might be particularly involved in the expression of motor problems associated with parkinsonism.

In M1 of MPTP-treated monkeys, the number of bursting neurons is increased compared to normal animals (Goldberg et al., 2002). As reported for the changes in firing rates, corticospinal, but not corticostriatal, M1 neurons showed increased bursting after MPTP-treatment (Pasquereau and Turner, 2011). Increased neuronal synchrony has also been found among M1 neurons in MPTP treated monkeys (Goldberg et al., 2002).

In addition to the aforementioned single neuron recording studies, there is a large number of studies of cortical field potential oscillations, mostly using EEG and ECoG recordings. It is noteworthy that EEG signals are the only electrophysiologic signals from human patients for which true control data (from normal individuals) are available. The amplitude of these signals tends to be larger and more robust than similarly recorded LFP signals recorded from the basal ganglia because of the laminar organization of cortex that contrasts with the non-laminar architecture of the basal ganglia. Studies of EEG recordings have found abnormally large delta (1–3 Hz) and theta band (4–7 Hz) activities in parkinsonian patients at rest, both in M1 (Soikkeli et al., 1991; Neufeld et al., 1994; Serizawa et al., 2008) and in other cortical regions (Silberstein et al., 2005; Babiloni et al., 2011; Morita et al., 2011). Animal studies have shown an increase of beta band oscillations in ECoG signals recorded in the frontal cortex of 6-OHDA treated rats (Sharott et al., 2005b; Brazhnik et al., 2012), and an increase of low frequency oscillations (<15.5 Hz) in M1 of MPTP treated monkeys (Devergnas et al., 2014).

These cortical activity changes could be secondary to the aberrant activity in the basal ganglia and thalamus, but could also originate in the cerebral cortex itself, perhaps reflecting the effects of cortical dopamine depletion (Lindenbach and Bishop, 2013). In addition, the effects of the loss of other neurotransmitters (such as noradrenaline, acetylcholine and serotonin) (Gaspar et al., 1991; Braak et al., 2003) and the neuronal degeneration in the motor cortex in PD (MacDonald and Halliday, 2002) remain to be further studied.

ROLE OF DOPAMINE IN DEVELOPMENT OF ELECTROPHYSIOLOGICAL CHANGES IN PARKINSONISM

Although it is often mentioned that the changes in electrophysiologic activity that were discussed above are consequent to the loss of dopamine in the striatum, the link between the disrupted striatal dopaminergic transmission and altered activity patterns in basal ganglia, thalamus and cortex is not entirely clear. Several studies have attempted to investigate this issues using locally or systemically administered dopamine receptor agonists or antagonists.

In PD patients, systemic infusions of therapeutic effective doses of the non-selective dopamine agonist apomorphine was shown to decrease the firing rates of GPi cells, and of STN cells with activity related to limb tremor; it also decreased the proportion of STN and GPi cells with responses to passive movements (Levy et al., 2001). However, in other studies, dopamine receptor agonist treatment did not fully reduce burst firing in basal ganglia neurons of parkinsonian animals or patients (Tseng et al., 2000; Lee et al., 2001; Levy et al., 2001), and was shown to even promote neuronal bursting when locally applied in the STN (Baufreton et al., 2003; Galvan et al., 2014) or in GPi or SNr (Kliem et al., 2007). Furthermore, acute blockade of dopaminergic transmission failed to increase beta band activity in subthalamic LFPs of rats, despite the presence of motor symptoms (Mallet et al., 2008b).

While increased bursting in the parkinsonian state may not fully respond to dopamine replacement, the increased synchrony found among striatal MSNs, or between MSNs and GP neurons appears to be more tightly controlled by the presence or absence of dopamine (Heimer et al., 2002; Burkhardt et al., 2007),

suggesting that the asynchronous firing of basal ganglia neurons in the normal state is an actively maintained state. Details of the mechanism and extent of this identified decorrelating function of dopamine are lacking.

The effects of dopamine on oscillatory activity patterns have also been examined. Increased oscillations in the SNr in the 25–40 Hz become prominent after complete, but not partial, 6-OHDA lesions, suggesting that an extensive lesion of the dopaminergic system is a prerequisite for the appearance of increased oscillatory activity (Quiroga-Varela et al., 2013). Importantly, motor impairments are evident at early stages of the dopaminergic degeneration processes. Thus, the increased oscillatory activity may not be an indicator (or cause) of early parkinsonism.

Systemic administration of dopaminergic agents reduces the degree of beta band oscillations in LFP recordings from the STN and GP in parkinsonian patients (Brown et al., 2001; Cassidy et al., 2002; Levy et al., 2002a; Priori et al., 2004; Kühn et al., 2008a; Bronte-Stewart et al., 2009), and from the SNr in 6-OHDA treated rats (Brazhnik et al., 2014). These agents also restore, to some extent, EEG abnormalities in M1 in MPTP-treated monkeys (Devergnas et al., 2014). Interestingly, in the latter study, it was observed that beta-band activity in the STN was reduced even in the absence of primary pathologic oscillations, suggesting that levodopa may not specifically normalize pathologic oscillations in the parkinsonian state, but may more generally suppress beta-band oscillations.

As already mentioned, the lack of dopamine is (directly or indirectly) accompanied by long-term morphological (and potentially functional) changes at different levels of the basal ganglia-thalamo-cortical circuits that may contribute to the abnormal electrophysiologic activities. These morphological changes are not necessarily a direct consequence of the dopaminergic loss, and may therefore escape the regulation of acute dopaminergic interventions.

RELATIONSHIP BETWEEN THE DIFFERENT PATTERN ABNORMALITIES AND THE BEHAVIORAL MANIFESTATIONS OF PARKINSONISM

There is little question that neuronal activities in the basal ganglia-thalamo-cortical circuits are altered in PD, but it is less clear to what extent these alterations account for the motor (or non-motor) symptoms of the disease.

One experimental approach to this problem has been to examine the effects of inactivation of the dysfunctional brain region(s). Experimental and clinical evidence indicate that lesions or inactivation of the "overactive" STN or GPi ameliorate parkinsonian symptoms in parkinsonian animals and in PD patients (Bergman et al., 1990; Aziz et al., 1991; Guridi et al., 1994; Wichmann et al., 1994; Parkin et al., 2001; Alvarez et al., 2005; Coban et al., 2009; Yoon et al., 2014). The knowledge gained from such lesioning experiments is limited, however. One problem is that lesions disrupt all activity in the lesioned nucleus, thus providing little insight into the role of specific activity changes. A more general consideration is that improvement of a behavioral abnormality after a lesion does not necessarily confirm that activity in the lesioned brain region was a primary cause for the abnormal behavioral patterns. For instance, the lesion could have induced plastic changes in other brain regions that could be responsible for the behavioral improvement.

An alternative way to study the possible link(s) between the basal ganglia abnormalities and parkinsonism is to examine the temporal relationship between the electrophysiologic changes and the behavioral state. An early study in monkeys in which repeated injections of small doses of MPTP resulted in the gradual development of parkinsonian signs suggested that changes in single cell firing in STN and GPi may precede the onset of motor signs (Bezard et al., 1999). This report contrasts, however with a more recent report which found activity changes in GPi only after the development of parkinsonian motor signs (Leblois et al., 2007). A related study by Sharott et al. (2014) found that the beta-band power of single- or multiunit activities recorded in the STN of parkinsonian patients was correlated with the severity of bradykinesia, and that sub-beta (<2–13 Hz) oscillations had a positive correlation with bradykinesia and axial symptoms. Measures of burst intensity (e.g., the intra-burst firing rate) were also correlated with parkinsonian signs (Sharott et al., 2014). Interestingly, the severity of tremor did not correlated with any of the measured abnormalities. In our own recent study of parkinsonism-related changes in LFP activities in the basal ganglia of chronically MPTP-treated monkeys, we did not find a correlation between oscillatory activity in the STN and motor impairments, but found instead that the severity of the motor signs was correlated with an increase in LFP power in low frequency bands (7.8–15.5 Hz) and a decrease in higher-frequency bands (23.4 Hz) in M1 and GPi. We also found an increase in coherence between M1 and basal ganglia oscillations in the 7.8–23.3 Hz frequency range to be associated with the severity of parkinsonism (**Figure 4**, Devergnas et al., 2014). Thus, it remains unclear if the early motor parkinsonian signs appear before the electrophysiological alterations can be detected (Quiroga-Varela et al., 2013).

Another approach to the possible link between neuronal activity changes and the motor signs is to analyze whether effective antiparkinsonian treatments are accompanied by "normalization" of activity patterns in the basal ganglia-thalamocortical circuit. Many studies have provided evidence that antiparkinsonian treatments reduce burst discharges (Filion et al., 1991; Shi et al., 2006; Xu et al., 2008), beta band oscillations (Brown et al., 2001; Levy et al., 2002a; Priori et al., 2004; Wingeier et al., 2006; Kühn et al., 2008b; Bronte-Stewart et al., 2009; Brazhnik et al., 2014) and abnormal synchronization within and among basal ganglia nuclei (Levy et al., 2000; Heimer et al., 2002; Hammond et al., 2007). Similarly, cortical coherence in the 10–35 Hz range, associated with the severity of parkinsonism, is reduced with STN-DBS (Silberstein et al., 2005). DBS in the STN also reduces neuronal entropy (a measure of the "disorder" of neuronal firing) in the GPe, GPi and motor thalamus (Dorval et al., 2008). These correlations obviously do not prove causality, however. Indeed, other studies have reported that these treatments do not always completely restore normal electrical activity in basal

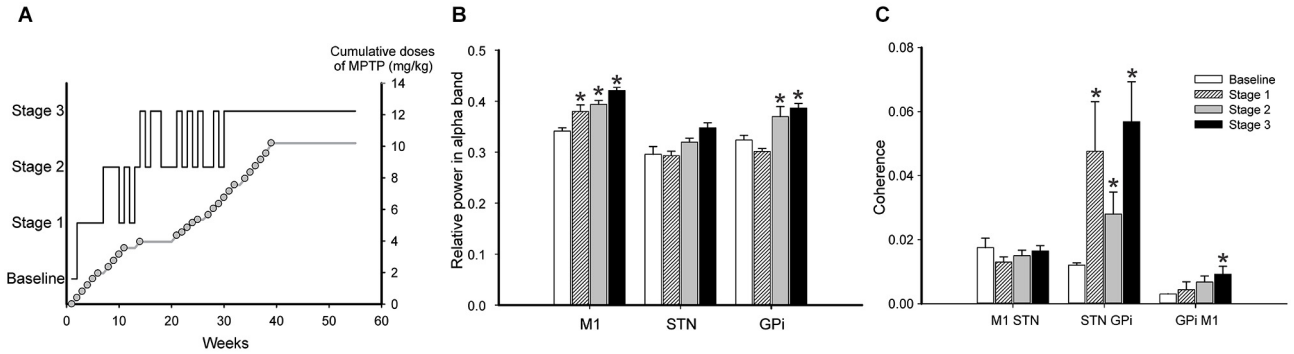

FIGURE 4 | Relationship between oscillatory activity changes in the cortico-basal ganglia network and progression of parkinsonism, in an MPTP-treated monkey. The animal was chronically instrumented for recording of EcoG signals from the primary motor cortex (M1). In addition, local field potential signals were recorded from STN and GPi through macroelectrodes that were repeatedly inserted into the same basal ganglia locations throughout the course of the experiment. **(A)** Temporal progression of the development of parkinsonism induced by MPTP injections, based on weekly evaluations. The solid black line shows the stage of parkinsonism. The cumulative dose of MPTP is shown as a gray line with open circles, where each circle corresponds to a single MPTP injection. Stages 1–3 represent mild, moderate, and moderately-severe parkinsonism, as judged by a parkinsonian rating scale. **(B)** Relative spectral power (mean ± SEM) of M1, STN and GPi signals (EEG, LFPs) across the different stages. **(C)** Coherence between M1 and STN, STN and GPi, and M1 and GPi signals in the 7.8–15.5 Hz frequency range across different stages of parkinsonism during periods of wakefulness. $*p < 0.05$ vs. baseline (2-way ANOVA with *post hoc* Holm-Sidak test). Modified from Devergnas et al. (2014), with permission.

ganglia neurons (Lee et al., 2001; Levy et al., 2001; Heimer et al., 2006; Hahn et al., 2008; McCairn and Turner, 2009), or, as already mentioned, that the anti-oscillatory properties of dopaminergic treatments may be non-specific (Devergnas et al., 2014).

Finally, it has been studied whether parkinsonian signs can be induced or worsened by experimentally imposing "pathological" frequencies or patterns on the basal ganglia circuitry. As in other studies described above, these studies have not been fully conclusive. Thus, while low-frequency stimulation of the STN, presumably inducing beta-band oscillations, was described to worsen akinesia (Timmermann et al., 2004), other human and animal studies have found subtle or no effects of this intervention (Chen et al., 2007, 2011; Eusebio et al., 2008; Syed et al., 2012). As an alternatively experimental approach to induce parkinsonian symptoms, Soares et al. (2004) used ibotenic acid to lesion the GPe in normal monkeys, with the rationale to mimic the proposed lack of GPe activity in PD (**Figure 1**, "Parkinsonism"). Such GPe inactivation increased the firing of STN and GPi neurons, but did not result in development of parkinsonian motor signs (Soares et al., 2004).

Overall, despite significant experimental study, it remains unclear whether the changes found in the neuronal signals in the parkinsonian state can be clearly linked with the emergence of parkinsonism. It is important to remember that the electrophysiological alterations could be more prominent during specific phases or types of movements (Brazhnik et al., 2012, 2014; Lemaire et al., 2012; Quiroga-Varela et al., 2013; Bosch-Bouju et al., 2014). It also remains to be further clarified which electrophysiological activity changes are most specific for PD or its specific signs and symptoms (Sanders et al., 2013; but see Sharott et al., 2014). Studies of this topic in advanced human patients are obviously complicated by the fact that long-term exposure to antiparkinsonian treatments and non-dopaminergic changes may play significant (and poorly defined) roles.

USE OF THE INSIGHTS FROM PATHOPHYSIOLOGY STUDIES TO IMPROVE SURGICAL PD THERAPIES

While a causal link between the electrophysiologic abnormalities and parkinsonian motor signs remains elusive, our knowledge of the electrophysiological activities in the basal ganglia and related structures in PD can, nevertheless, be used to improve surgical therapies, such as DBS or lesioning approaches. For example, it may be possible to use the power distribution in LFP signals as a substitute for the currently used time-consuming microelectrode recording methods to guide the placement of DBS electrodes. In studies of intra-operatively recorded LFPs from DBS electrodes that were advanced towards the STN (Chen et al., 2006; Miyagi et al., 2009), the beta band power in LFP signals was found to be maximal at the surgical target in the dorsal STN (Chen et al., 2006; Miyagi et al., 2009). However, this technique is at present clearly limited by the low spatial resolution of LFP signal recordings (Zaidel et al., 2010). A related use of recordings from DBS electrodes is to choose the best stimulation contact of already implanted electrodes based on the amount of beta-band power that can be recorded from them (Ince et al., 2010).

Another application of our electrophysiologic knowledge is the use of disease-related electrophysiological characteristics to regulate DBS parameters in "closed-loop" or "adaptive" feedback regimes that dynamically adapt the therapy to fluctuations of disease severity, and may help to prolong the battery life of the implanted devices by cutting the duty-time of the implanted pulse generator (for review, see Priori et al., 2013). The potential success of adaptive DBS for PD has been computationally modeled (Santaniello et al., 2011), and experimentally demonstrated in studies that used cortical single neuron activity patterns

to trigger STN stimulation in parkinsonian monkeys (Rosin et al., 2011). Of course, in clinical use, LFP-based methods of adaptive DBS would be more practical. The first attempt to use LFP characteristics to control stimulation parameters was recently published, using short-term changes in the beta-band power of STN LFP signals to trigger STN-DBS in PD patients (Little et al., 2012, 2013). In these experiments, the adaptive DBS was at least as effective as continuous DBS. Interestingly, the duty cycle of closed loop-controlled DBS progressively shortened in these studies, even over relatively short stimulation periods, suggesting that plastic changes could be triggered by this type of intermittent STN stimulation that may eventually make STN DBS less and less necessary to control the parkinsonian signs in a given patients. The signal analysis and control of the DBS device still required an external computer and the miniaturization and overall power consumption of an implantable signal sampling, processing and stimulation system remain important limitations (Starr and Ostrem, 2013). Other approaches have also been proposed, including specifically the use of measures of cortical phase-amplitude coupling between beta- and gamma-band ECoGs signals to trigger DBS (de Hemptinne et al., 2013), or the use of "coordinated reset" stimulation, a stimulation regime based on computer modeling, designed to minimize pathologic synchronization of activity patterns in the STN and related nuclei (Tass et al., 2012; Adamchic et al., 2014).

CONCLUSIONS

The literature review presented above shows that similar changes in neuronal activities (changes in firing rates, increases in bursting, synchrony and oscillatory activities in the beta range) have been seen in almost all basal ganglia nuclei, both in PD patients and in animal models of the disease (**Table 1**). In fact, PD remains the only neurologic disease for which this level of detailed information regarding specific network alterations is available. At the most general level, the described changes jointly paint the picture of an altered network state that is disruptive for motoric and non-motoric functions.

Despite all of the progress that has been made in this field, there is certainly much need for further investigations of the role of specific circuit nodes or interactions between them in producing or relaying the described abnormalities, and of the link(s) between these changes and the behavioral signs of the disease. There is clearly also a need for further studies of the cellular and molecular underpinnings of these changes. In addition, experimental studies need to pay close attention to the species differences in the basal ganglia circuits among rodents (and other smaller mammals) and non-human primates and patients (Parent, 1986; Smith et al., 2014b).

The potential translational payoff of the accumulated knowledge of activity changes in the parkinsonian state is very large. Insights gained from these studies may help us to develop some of these changes (such as EEG changes) into early biomarkers for treatment trials in patients, and there are already many trials underway to use them to guide neuromodulation therapies such as DBS. Furthermore, knowing how specifically neuronal activity patterns changes in the parkinsonian state may help us to develop treatments that specifically address these activity pattern changes rather than simply replace dopamine as is currently done. Combined, these efforts promise to translate into more specific therapies for patients with PD that are both, more effective and less encumbered by adverse effects than the currently available treatments.

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GDNF-based therapies, GDNF-producing interneurons, and trophic support of the dopaminergic nigrostriatal pathway. Implications for Parkinson's disease

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The glial cell line-derived neurotrophic factor (GDNF) is a well-established trophic agent for dopaminergic (DA) neurons *in vitro* and *in vivo*. GDNF is necessary for maintenance of neuronal morphological and neurochemical phenotype and protects DA neurons from toxic damage. Numerous studies on animal models of Parkinson's disease (PD) have reported beneficial effects of GDNF on nigrostriatal DA neuron survival. However, translation of these observations to the clinical setting has been hampered so far by side effects associated with the chronic continuous intra-striatal infusion of recombinant GDNF. In addition, double blind and placebo-controlled clinical trials have not reported any clinically relevant effect of GDNF on PD patients. In the past few years, experiments with conditional *Gdnf* knockout mice have suggested that GDNF is necessary for maintenance of DA neurons in adulthood. In parallel, new methodologies for exogenous GDNF delivery have been developed. Recently, it has been shown that a small population of scattered, electrically interconnected, parvalbumin positive (PV+) GABAergic interneurons is responsible for most of the GDNF produced in the rodent striatum. In addition, cholinergic striatal interneurons appear to be also involved in the modulation of striatal GDNF. In this review, we summarize current knowledge on brain GDNF delivery, homeostasis, and its effects on nigrostriatal DA neurons. Special attention is paid to the therapeutic potential of endogenous GDNF stimulation in PD.

Keywords: GDNF, Parkinson disease, parvalbumin interneurons, neurotrophic factors, mouse models, dopaminergic system, nigrostriatal pathway, striatum

INTRODUCTION

Parkinson's disease (PD) is a progressive, mainly idiopathic and age-related, neuronal disorder that affects as much as 1% of the population over 60 years (de Lau and Breteler, 2006). PD causes severe postural, motor, and physiological impairments that can reduce life expectancy. Although PD is a systemic disease, affecting central and peripheral neurons, the most disabling motor symptoms are due to the progressive death of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), a mesencephalic nucleus that sends projections to the striatum (caudate nucleus (Cd) and putamen) and is involved in motor control. Although pharmacological (pro-DA drugs) and surgical (deep brain stimulation) therapies exist to alleviate PD symptoms (see Tarazi et al., 2014), to date there is no cure for PD despite intense efforts made to develop new protocols, particularly cell replacement therapy, to substitute or protect nigrostriatal cells affected by the disease. The discovery by Lin et al. (1993) of a specific DA neurotrophic factor secreted by rat glial cells -the glial cell line-derived neurotrophic factor (GDNF)- opened a new perspective for PD pathogenesis and therapy. This review will discuss the pros and cons of using GDNF as a treatment for PD, highlighting the potential therapeutic applicability of endogenous brain GDNF activation.

GDNF ADMINISTRATION FOR TREATMENT OF PARKINSON'S DISEASE: EARLY OBSERVATIONS AND CLINICAL TRIALS

GDNF and its structurally related trophic proteins artemin, neurturin and persephin, are distant member of the transforming growth factor-β superfamily (Airaksinen and Saarma, 2002). A wealth of papers based on rodent and non-human primate models have described the benefits of GDNF treatment on nigrostriatal neurons. In early studies, GDNF showed a specific action on survival of rat E16 midbrain DA neurons in culture and proved to be a potent and selective stimulator of dopamine uptake and neurite outgrowth in tyrosine hydroxylase positive (TH+) neurons (Lin et al., 1993). These initial *in vitro* observations led to immediate testing of GDNF effects on PD animal models based on toxin-induced destruction of midbrain DA neurons. Hoffer et al. (1994) used rats unilaterally injected with 6-hydroxydopamine (6-OHDA) in the nigrostriatal pathway. This procedure elicits a rapid and permanent ipsilateral destruction

of DA neurons that is manifested by a contralateral rotation pattern in response to low doses of amphetamines, thus accurately reflecting the degree of DA neuronal loss. In 6-OHDA-treated animals, intranigral injection of 100 μg of recombinant human GDNF reduced the rotations by ∼4-fold (Hoffer et al., 1994). Similar rescue effects of GDNF were reported in an independent study on the same rat model (Winkler et al., 1996). In 1995, four articles described the potent neurotrophic effects of GDNF on mesencephalic DA (Beck et al., 1995; Tomac et al., 1995a) as well as motor (Oppenheim et al., 1995; Yan et al., 1995) neurons *in vivo*; a year later the first non-human primate data in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonian monkey model was published (Gash et al., 1996). GDNF-treated monkeys showed functional improvement of parkinsonian features along with increased levels of striatal dopamine. The benefits claimed by GDNF use were unanimous, although when it came to human patients the initial elation dissipated.

Several human studies have been performed to test the effect of striatal delivery of GDNF through a permanently implanted cannula. The degree of symptomatic relief in these clinical trials has varied from major improvement (Gill et al., 2003; Love et al., 2005; Patel et al., 2005; Slevin et al., 2005) to no benefit at all (Lang et al., 2006). Some patients enrolled in these studies developed neutralizing antibodies as part of an immune response to the recombinant human GDNF treatment (Lang et al., 2006; Tatarewicz et al., 2007), whereas others simply reacted to the placebo in a randomized trial (Lang et al., 2006). In another study, intraventricular GDNF delivery resulted in strong adverse effects (Nutt et al., 2003). A phase II clinical trial, based on improved bilateral intra-putaminal GDNF injection, has recently been launched at the Frenchay Hospital in Bristol (UK) to overcome the inconsistent results previously obtained. Progress to a treatment is hampered by the problem of delivering GDNF to brain cells across the blood-brain barrier (Boado and Pardridge, 2009). Thus, it seems that the simple administration of the GDNF protein does not represent a sustainable treatment for PD and alternative options have to be tested to exploit the benefit of the potent trophic action of GDNF on DA neurons.

ALTERNATIVE GDNF-BASED THERAPIES

Overcoming the blood-brain-barrier (BBB) obstacle for GDNF delivery to the brain using a systemic route has became a major technological objective (see **Figure 1**). Trojan horse approaches were tested by the mean of systemic administration of nanoliposomes engulfing a GDNF plasmid and engineered to cross the BBB via trancytosis after coupling to the transferrin receptor. This resulted to a near complete rescue of the nigrostriatal system from 6-OHDA neurotoxicity in the rat brain (Xia et al., 2008; Zhang and Pardridge, 2009). An attempt to fuse GDNF to a monoclonal immunoglobulin (GDNF-IgG) directed against the BBB cellular component proved to be potent in mice (Fu et al., 2010), but this method failed when it was tested on monkeys as no behavioral improvements were observed (Ohshima-Hosoyama et al., 2012). Biodegradable GDNF-loaded microspheres implanted in the striatum are an interesting alternative to overcome the BBB problem since they sustainably release recombinant GDNF for at least 8 weeks (Jollivet et al., 2004a; Garbayo et al., 2009; Herrán et al., 2013), with long protective effects lasting up to 24 weeks (Jollivet et al., 2004b). Finally, the administration of GDNF by nasal route, using cationic liposomes to increase their residence time through electrostatic interactions at the olfactory epithelium, has recently been tested. Intranasal GDNF given to rats, immediately prior to 6-OHDA lesion, provided significant protection of striatal DA neurons (Migliore et al., 2014).

In parallel with the studies based on the delivery of GDNF peptide, considerable efforts have been made towards the development of *in vivo* gene transfer by recombinant viral vectors expressing the *Gdnf* gene (**Figure 1**). Bilateral intranigral delivery of adenoviral vector constructs carrying the GDNF sequence (Ad-*Gdnf*) to adult rats prior to 6-OHDA lesion protected DA neurons from toxin-induced cell death (Choi-Lundberg et al., 1997). Although the experimental design was criticized (Pallini et al., 1997), this landmark attempt was encouraging and thus followed by several other studies based on viral vector-driven GDNF strategy in rodent and monkey models (reviewed by Kordower and Bjorklund, 2013). A key study reported that adeno-associated virus (AAV)-*Gdnf* promoted motor recovery of parkinsonian rats when injected in the striatum rather than in the SN region (Kirik et al., 2000). Furthermore, intranigral AAV-*Gdnf* exhibited histological neuroprotection on DA neuronal bodies but DA fibers sprouting and functional recovery occurred only when AAV-*Gdnf* was transduced in the striatum (Kirik et al., 2000, 2004). Several viral vector based strategies have been developed to optimize GDNF production, in particular inducible vectors in order to control the timely expression of GDNF. For instance, injection of a synthetic steroid mifepristone lead to highly increased levels of GDNF expression from the inducible AAV-*Gdnf*. This allowed the recovery of motor function in 6-OHDA lesioned rats, and was associated to DA neuron protection in the SN (Tereshchenko et al., 2014). Another newly reported approach used lentivirus (LV) vectors transgenes fused with a destabilizing domain (DD). The resulting fusion protein is unstable and rapidly cleared by the proteasome unless it is stabilized by trimethoprim (TMP). Thus, peripheral injection of TMP allows DD-GDNF stabilization in the striatum (Tai et al., 2012). When applied to 6-OHDA lesioned rats, the TMP-stabilized DD-GDNF protects the DA nigrostriatal pathway and associated functional behavior (Quintino et al., 2013). Pharmacological modulation of GDNF-expressing viral vectors, still in initial stage of development, is particularly attractive when considering new therapeutic approaches in early disease stages to protect nigrostriatal degeneration and concomitantly prevent adverse effects from sustained high GDNF delivery. Biodegradable nanoparticles encompassing a plasmid DNA coding for GDNF can get through the plasma membrane of neurotensin receptor-expressing cells, such as DA neurons. This non-viral targeted transfection has proved to be efficient when used in rat PD models (Gonzalez-Barrios et al., 2006). A set of experiments combining non-viral gene delivery with systemic route of administration gave promising results. Multiple intravenous injections of a lactoferrin (Lf)-modified vector, expressing human GDNF, protected DA neurons and highly

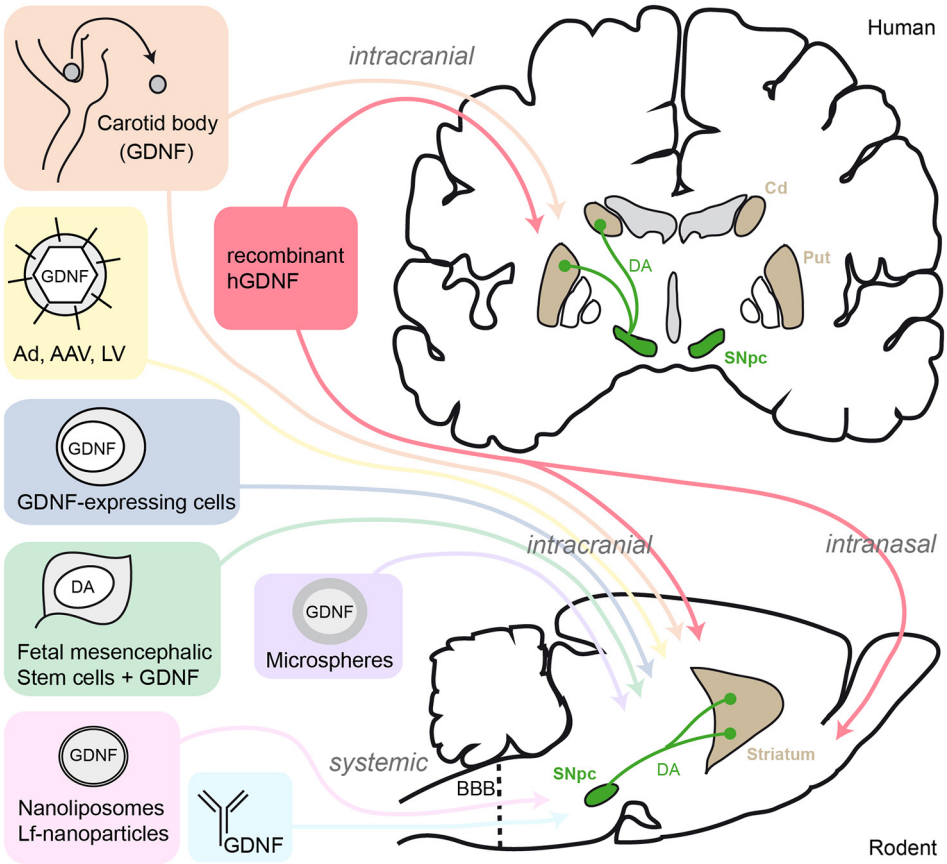

FIGURE 1 | Schematic summary of GDNF-delivery strategies tested in human PD patients and rodent models. AAV, adeno-associated virus; Ad, adenovirus; LV, lentivirus; Lf, lactoferrin; SNpc, substantia nigra pars compacta; DA, dopamine; Cd, caudate nucleus; Put, putamen; BBB, blood-brain-barrier.

reduced the amphetamine-induced rotational behavior that normally occurs after lesion by 6-OHDA intrastriatal injection (Huang et al., 2009).

Evidently, not all studies have systematically reported positive effect of viral GDNF vectors. Indeed, an herpes simplex virus (HSV)-derived vector overexpressing GDNF presented toxic effects while masking the potential benefits of GDNF (Monville et al., 2004). Intranigral lentiviral injection of a vector expressing the A30P mutant human α-synuclein provoked a selective and progressive degeneration of the nigrostriatal DA neurons in the treated rats (Lo Bianco et al., 2002). Preventive treatment by LV-GDNF vector, successfully used in a monkey PD model (Palfi et al., 2002), failed to modulate nigrostriatal degeneration induced by the α-synuclein toxicity (Lo Bianco et al., 2004). Surprisingly, the use of a tetracyclin-dependent LV-GDNF expression in the striatum in normal rats provoked a dramatic down-regulation of TH protein expression (Georgievska et al., 2004).

Cell-based GDNF therapy, i.e., transplantation of GDNF-expressing cells, has also been extensively tested. Two main strategies have been used so far: (i) introduction of GDNF-secreting cells in the lesioned nigrostriatal system; and (ii) transplantation of DA-producing cells in association with GDNF treatment to protect and to increase survival of grafted cells. Successful intrastriatal transplantation of primary astrocytes engineered to express GDNF prevented 6-OHDA-induced DA neuronal death (Cunningham and Su, 2002). Interestingly, low levels of GDNF released by these astrocytes (∼5 pg/g of striatum) provided a remarkable robust neuroprotection. Neural stem cells engineered to synthesize GDNF were also successfully used to limit DA neuron degeneration in a 6-OHDA lesion mouse model (Åkerud et al., 2001). Encapsulated GDNF-producing cells may represent a valuable option since they will not migrate out of the targeted region, the caudate-putamen, and can still be removed in the event that some adverse effects may occur (reviewed by Lindvall and Wahlberg, 2008). Pioneer work from Tseng et al. (1997) used polymer-encapsulated fibroblasts engineered to overexpress GDNF prior to be transplanted next to the SN. Nanogram levels of continuous GDNF release completely prevented degeneration of DA neurons induced by medial forebrain bundle axotomy. Alternatively, trophic factors-producing tissues, such as the carotid body (CB), have been used as a source of GDNF. The CB is highly DA, bilateral, O_2-sensing organ that contains cells which produce unusual

high levels of GDNF (López-Barneo et al., 1999; Villadiego et al., 2005). Intrastriatal transplantation of CB cells produces clear neuroprotective effects on DA neurons in rodent parkinsonian models (Espejo et al., 1998; Muñoz-Manchado et al., 2013), and amelioration, with indications of biological effects, in PD patients (Mínguez-Castellanos et al., 2007). However, the therapeutic action of CB is limited by the small amount of tissue available. To overcome this limitation, new stem cell-based procedures are being assayed to expand CB tissue before transplatation (see Pardal et al., 2007; Platero-Luengo et al., 2014). The combination of GDNF delivery and fetal DA grafts, to improve survival of transplanted cells, has been largely tested in animal models (Rodriguez-Pallares et al., 2012; Kauhausen et al., 2013), as well as in PD patients (Mendez et al., 2000). GDNF promoted survival of fetal mesencephalic cell transplants in the striatum of 6-OHDA-lesioned rats, which was associated with functional improvement (Yurek et al., 2009). However, this beneficial effect was limited in time, as 6 months later the association of grafted cells/LV-GDNF failed to support DA neuron survival. Moreover, LV-GDNF induced some down regulation of TH in the grafted cells. In similar experimental conditions, GDNF had no effect on fetal mesencephalic graft outgrowth when compared to other growth factors such as bFGF (Törnqvist et al., 2000). GDNF has also been used to increase DA differentiation and survival of embryonic (Buytaert-Hoefen et al., 2004) or bone marrow stromal (Dezawa et al., 2004) stem cell-derived DA neurons prior to transplantation. This procedure, that represents an indirect use of GDNF, substantially alleviated the rotation behavior induced by amphetamines in 6-OH dopamine-lesioned rats. However, the use of GDNF to drive stem cell-derived neuronal cells to produce DA is a procedure that calls for caution, as safety of progenitor cell transplants is always a key concern. Optimization of DA neuron maintenance and GDNF delivery protocols has permitted recent preclinical advances in the field. DA cells from ventral mesencephalon of young donors (embryonic day 10) transplanted homotopically in the nigral region, combined with the intrastriatal injection of a AAV-GDNF, allowed graft survival, integration into the medial forebrain bundle circuitry to innervate the striatum, and functional motor recovery (Kauhausen et al., 2013). Together, the data summarized in this section support a beneficial neuroprotective action of exogenous GDNF on DA nigrostriatal neurons.

GDNF SIGNALING ON DOPAMINERGIC NEURONS

GDNF shares the receptor tyrosine kinase rearranged during transcription (Ret) with artemin, neurturin and persephin. Ret activation requires association to a second glycosylphosphatidyl inositol-anchored protein named GDNF family receptor α (GFRα), of which four subtypes have been identified with different affinities for ligands of the GDNF-family. The GDNF homodimer specifically binds to two GFRα1 to form a high affinity complex with the recruitment of Ret proteins (Bespalov and Saarma, 2007). GDNF displays lower affinity for GFRα2 and GFRα3. The Ret-GFRα1 complex formation induces transphosphorylation of Ret tyrosine kinase residues which, in turn, activates downstream signaling molecules (**Figure 2**) such as the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)/Akt (Airaksinen and Saarma, 2002). *In vitro* studies suggest that the protective effect of GDNF on DA neurons involves the activation of the MAPK and PI3K intracellular pathways (Ugarte et al., 2003; Onyango et al., 2005). Aging mice (26 months) carrying a partial deletion of *Gfrα1* (heterozygous), show a decrease in TH fiber density in the striatum accompanied by a lower number of TH+ neurons in the SN. Additionally, these mice exhibit increased sensitivity of nigrostriatal DA neurons to MPTP toxicity (Boger et al., 2008). These observations suggest a pivotal role of GFRα1 in the trophic protection by GDNF signaling. Specific ablation of Ret in DA neurons (using a dopamine transporter-Cre/Ret-flox mice) results in progressive loss of nigrostriatal DA neurons. Spontaneous decrease of TH+ cells in the SNpc and striatal innervation occurred in these mice and this was associated with increased number of activated glial cells, a sign of CNS injury (Kramer et al., 2007). GDNF signaling also utilizes c-Src kinase to promote neurites outgrowth (Encinas et al., 2001). Although the GFRα1/Ret complex is the most studied GDNF receptor, it is known that this trophic factor can also bind to alternative signaling system, e.g., NCAM (Paratcha et al., 2003). This would explain why ablation of *Ret* does not produce a phenotype similar to GDNF-deficiency (see Pascual et al., 2011).

The data summarized in the previous paragraph strongly suggest the requirement of direct GDNF trophic signaling to the DA neurons for their survival. Ret and GFRα1 mRNA expressions are up-regulated in the SNpc shortly after 6-OHDA lesion, a trophic response to drug toxicity. After 3 to 6 days, the level of expression of both Ret and GFRα1 mRNA decreased dramatically, which could be explained by the loss of DA neurons observed after 6 days in this rat PD model (Marco et al., 2002). Ret is not specific to GDNF but its activation is also enhanced by other ligands such as GM1 ganglioside (Newburn et al., 2014). This observation denotes a possible pharmacological induction of the GDNF signaling cascade to promote a trophic response. The canonical neurotrophic factor action requires retrograde communication from the axon terminals to neuron cell bodies, partly explained by the "signaling endosome hypothesis" where the activated receptor is internalized and transported via the microtubules machinery for cytosolic and nuclear signaling (Howe and Mobley, 2005; Ibáñez, 2007). GDNF is no exception to this rule as it has been demonstrated that ^{125}I-GDNF injected into the rat striatum is retrogradely transported to the cell body of SNpc neurons (Tomac et al., 1995b).

The use of *Gdnf*-null mice has provided valuable data regarding the role of endogenous GDNF on DA neuron survival. Mice carrying GDNF deletion do not survive after birth due to lack of the entire enteric nervous system and kidney agenesis (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996). However, embryonic development of the midbrain DA nigrostriatal pathway is not affected by the lack of GDNF (Sánchez et al., 1996). Mice with partial deletion of *Gdnf* (*Gdnf*$^{+/-}$) suffer from higher neuro-inflammation and loss of TH-positive neurons with aging (Boger et al., 2006) or following lipopolysaccharide (LPS) treatment (Granholm et al., 2011). However, whether

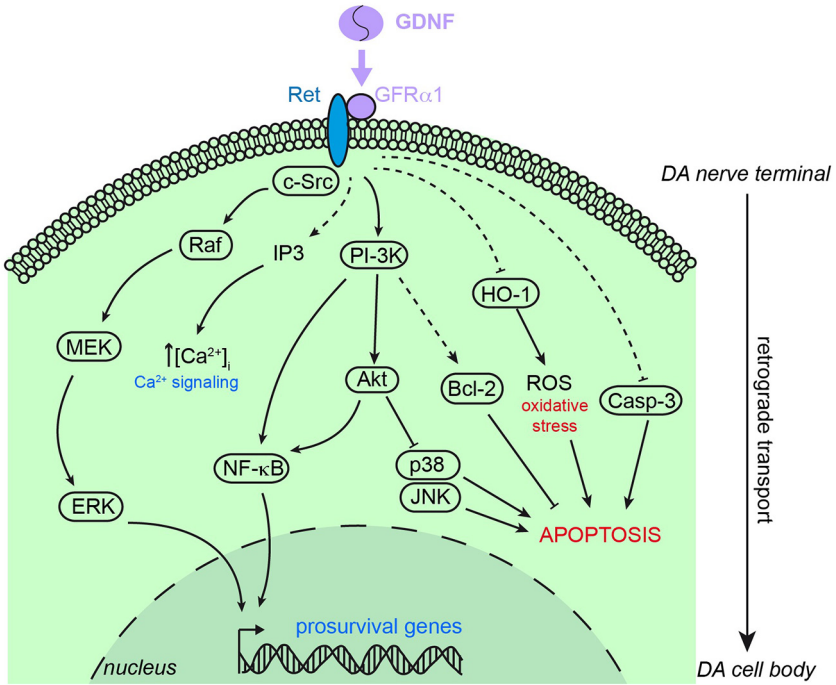

FIGURE 2 | Schematic representation of the main signaling pathways involved in the neuroprotective action of GDNF on dopaminergic neurons. GDNF principally stimulates the binding of GFRα1 and Ret to trigger intracellular signaling cascades leading to pro-survival genes expression, calcium signaling and pro-apoptosis factors inhibition. Akt, protein kinase B; Bcl-2, B cell lymphoma 2; Casp-3, caspase 3; c-Src, proto-oncogene tyrosine-protein kinase Src; ERK, extracellular signal-regulated kinase; HO1, heme oxygenase 1; IP3, inositol tris-phosphate; JNK, c-Jun N-terminal kinase; MEK, mitogen extracellular signal-regulated kinase; NF-κB, nuclear factor kappa B; PI-3K, phosphatidylinositol 3 kinase; Raf, Raf kinase; ROS, reactive oxygen species. Dashed arrows indicate indirect stimulation or inhibition.

GDNF might serve as an important target-derived neurotrophic factor for adult nigral DA neurons has remained unknown until conditional GDNF-KO mice were generated. Inducible CRE-LoxP *Gdnf*-null mice were engineered to bypass the developmental lethality caused by GDNF loss. In this model, a *floxed-Gdnf* allele was deleted in adulthood by tamoxifen-induced Cre recombinase activation, leading to a marked decrease of GDNF expression in the striatum (Pascual et al., 2008). Strikingly, these mice showed a progressive and selective death of the catecholaminergic neuronal population in the substantia nigra (SN), ventral tegmental area, and locus coeruleus with associated locomotor dysfunction (Pascual et al., 2008). These data further support the notion that adult mammalian mesencephalic catecholaminergic neurons rely on the continuous input of endogenous GDNF, an observation that remains to be demonstrated with other animals models and in the human brain.

MECHANISMS INVOLVED IN THE PROTECTIVE EFFECT OF GDNF

It is postulated that GDNF protects the DA nigrostriatal system by interacting with several cellular pathways involved in apoptosis, metabolism, and redox homeostasis (see **Figure 2**). GDNF may prevent apoptosis in the DA neuron population by directly up-regulating the anti-apoptotic proteins B cell lymphoma 2 (Bcl-2) and Bcl-X via PI3K signaling (Sawada et al., 2000). The neuroprotective action by GDNF on the nigrostriatal system might also involve the activation of protein kinase CK2 as demonstrated in parkinsonian rats (Chao et al., 2006). Moreover, GDNF induces nuclear factor κB (NF-κB) pathways to promote neuronal survival from toxic insults (Cao et al., 2008). Other targets of GDNF are caspase-3 and the endoplasmic reticulum stress-related genes. Treatment of primary mesencephalic rat cultures with lactacystin inhibits the ubiquitin-proteasome system and leads to apoptosis of DA neurons. However, pretreatment with GDNF prevents DA neuronal death by suppressing caspase-3 activation and endoplasmic reticulum stress (Li et al., 2007). Intrastriatal infusion of GDNF prevents lactacystin-induced DA neuron loss by inhibiting the pro-apoptotic molecules Jun N-terminal kinase (JNK) and p38 and activating the pro-survival Akt and MAPK pathways (Du et al., 2008).

As it occurs in the classical neurotrophic models, GDNF promotes the DA phenotype in DA neurons, and in this way exerts some of its neuroprotective actions. GDNF seems to increase cellular levels of transcription factors, such as Nurr1 and Pitx3, involved in the expression of a set of genes—TH, vesicular monoamine transporter (*Vmat2*), dopamine transporter (*Dat*) and aromatic L-amino acid decarboxylase (*Aadc*)—involved in dopamine metabolism, (Lei et al., 2011). When added to the culture medium of midbrain-derived neural stem cells (mdNSCs), GDNF induced a DA phenotype associated with Nurr1 and Pitx3 up-regulation. Transplantation of these cells

into the striatum of 6-OHDA-injected rats greatly prevented the amphetamine-induced contralateral rotation in the lesioned animals (Lei et al., 2011).

Although the causes of DA neuron degeneration in PD remain unclear, mitochondrial dysfunction and oxidative stress induced by reactive oxygen species (ROS) are known to have a pathogenic role early in the disease process (Subramaniam and Chesselet, 2013). Interestingly, striatal GDNF administration moderately enhances the activity of certain enzymes involved in the enzymatic detoxification of ROS: superoxide dismutase, catalase and glutathione peroxidase (Chao and Lee, 1999). Moreover, GDNF administration in the rat striatum prevents 6-OHDA-induced ROS formation, evidenced by protein carbonyls and 4-hydroxynonenal, and thus protects DA neurons from oxidative stress (Smith and Cass, 2007). GDNF seems to negatively regulate the expression of heme oxygenase-1 (HO-1) to reduce oxidative stress (Saavedra et al., 2005).

A proteomic analysis revealed 46 specifically regulated proteins in the striatum of MPTP mice 4 and 72 h after striatal GDNF injection. These proteins are related to cell differentiation, system development, cell structure and motility, energy pathways, transport, apoptosis, cell proliferation and response to stress-regulating genes (Hong et al., 2009). However, none of them are involved in GFRα1-Ret downstream-activated pathways. Taking into account that post-transcriptional modification, such as phosphorylation, were not detected with the aforementioned method, a thorough proteomic examination of posttranslational modifications elicited by GDNF on DA neurons would probably provide relevant information for understanding the neuroprotective action of GDNF. It has been reported that striatal GDNF inhibits Shh production by DA neurons and, in turn, Shh released at the striatal DA terminals down-regulates *Gdnf* gene expression (Gonzalez-Reyes et al., 2012). This concept is attractive, as the mutual repression of Shh and GDNF would allow DA neurons to dynamically control neurotrophic factor production in the striatum. The level of striatal *Gdnf* mRNA, and the number of GDNF-expressing parvalbumin-positive (PV+) interneurons (see below) do not seem to be affected by MPTP-derived lesions of nigrostriatal neurons (Hidalgo-Figueroa et al., 2012). However, to what extent the integrity of the DA nigrostriatal pathway modulates the survival and activity of GDNF-producing striatal interneurons is under debate. Although much progress has been done regarding the molecular mechanism of GDNF neurotrophic/neuroprotective action, whether the intracellular pathways involved are the same in normal and lesioned cells and to what extent GDNF production is cell autonomous or depend on the activity of the relevant neuronal networks are fundamental questions yet to be resolved.

In addition to its well-established neurotrophic role, GDNF may also modulate the activity of DA nerve terminals at the basal ganglia. Amperometric recordings from midbrain DA neurons showed that exposure to GDNF increases quantal release of catecholamines as well as the density of axonal varicosities (Pothos et al., 1998). GDNF enhances basal levels and release of DA and DA metabolites evoked by potassium or amphetamine in primary cultured ventral midbrain (VM) DA neurons (Wang et al., 2001), striatal slices (Gomes et al., 2006), and striatal synaptosomes (Gomes et al., 2009). Similar effects of GDNF have also been observed *in vivo* by microdialysis measurements (Hebert et al., 1996; Xu and Dluzen, 2000; Cass and Peters, 2010). Therefore, GDNF may not only prevent DA neurons from degeneration but also potentiate DA release and turnover by some as yet unknown mechanism.

ENDOGENOUS GDNF EXPRESSION: STRIATUM

Knowledge of where and when GDNF is expressed in the adult brain is fundamental to understand the physiological role of this trophic factor and the mechanisms that regulate its synthesis. Eventually, this could make it possible to pharmacologically stimulate endogenous GDNF production as a way to increase the level of GDNF available at the striatal DA nerve terminals. Unfortunately, studies on GDNF expression performed with antibodies are challenged by specificity considerations. However, there are several studies in which either *Gdnf* mRNA expression was analyzed by *in situ* hybridization (ISH), or mouse models with reporter genes were used to estimate *Gdnf* promoter activity. In rodents, *Gdnf* mRNA is broadly expressed in the developing embryo (Golden et al., 1999), although in adult mice its expression is rather limited to few organs, with the highest content found in the ovary and testis. In the adult rodent brain, *Gdnf* mRNA expression is consistently observed in restricted discrete cells of the striatum, thalamic structures, nucleus accumbens, cerebellum and hippocampus (Schaar et al., 1993; Nosrat et al., 1996; Trupp et al., 1997). Using a β-gal reporting mouse model (Sánchez et al., 1996), GDNF expression in adult mice brain was restricted to the dorsal and ventral striatum, the anteroventral nucleus of the thalamus, the septum and, interestingly, the subcommissural organ (Pascual et al., 2008). Curiously, GFRα1 and Ret do not share the same expression pattern than GDNF and are broadly expressed in the adult CNS. Noteworthy, GDNF receptor mRNAs are not detected in the striatum, but highly expressed in the SNpc (Trupp et al., 1997), which again supports that GDNF may specifically act on SNpc DA neurons that project to the striatum. This also indicates that no other striatal cells could benefit from its trophic action. GDNF protein levels have been measured by enzyme-linked immunosorbant assay (ELISA) in lysates of caudate/putamen, SN, cerebellum, frontal cortex, and the cerebrospinal fluid (CSF) of PD and non-PD postmortem human brains. GDNF concentration in the range of 40–70 pg/mg total protein was relatively constant between control and PD patients in the SN and Cd and putamen, with lower concentrations reported in the cerebellum and the frontal cortex (10–15 pg/mg). However, GDNF was no detected in the CSF (Mogi et al., 2001). Additionally, polymorphisms in the GDNF gene have been found in PD and non PD patients with no apparent correlation between mutation and disease (Wartiovaara et al., 1998). In another study, depletion of GDNF, but no other neurotrophic factors, was detected in the SN of parkinsonian patients (Chauhan et al., 2001). Although these results must be taken with caution as they are based on immunohistochemical analyses, they suggest that down regulation of GDNF might participate in the onset of PD pathophysiology. However, whether alterations in GDNF

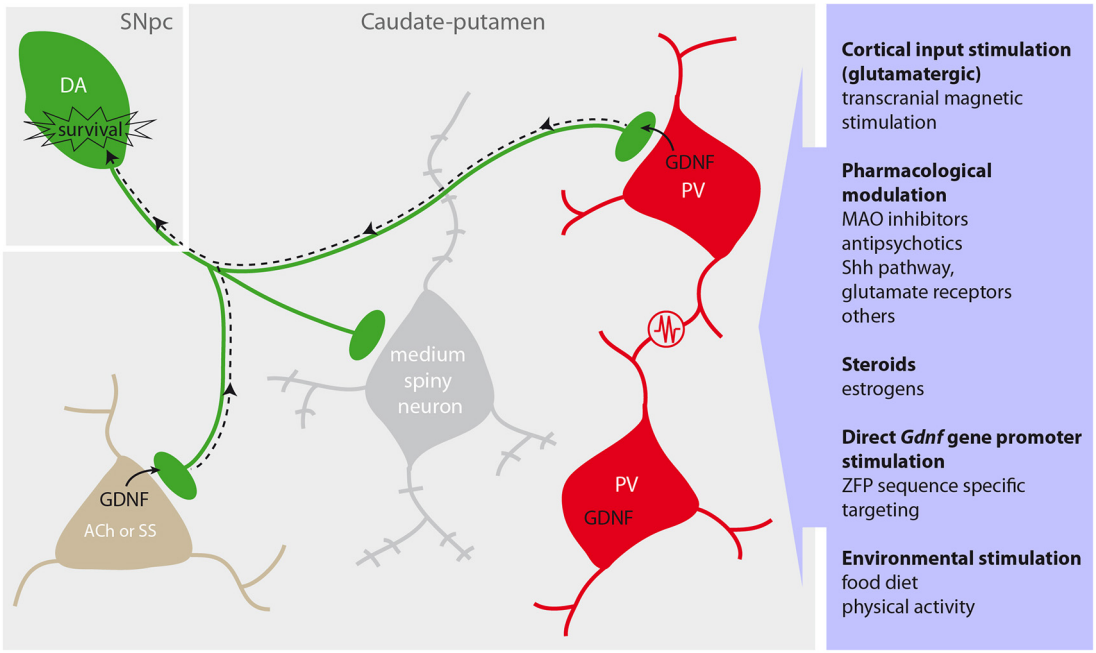

FIGURE 3 | Protection of the dopaminergic nigrostriatal pathway by striatal GDNF and activation of endogenous GDNF production. Dopaminergic (DA) neurons (*green*) located in the substantia nigra pars compacta (SNpc) innervate the caudate-putamen to modulate the activity of GABAergic medium spiny neurons (*gray*), parvalbumin (PV)-positive interneurons (red) and other cholinergic (ACh) or somatostatin (SS) interneurons (brown). PV neurons form an ensemble of synchronized cells through multiple dendrodendritic electrical synapses (*resistance* in the scheme), and release GDNF at the nerve terminals to provide trophic support to DA neurons via retrograde signaling (dotted arrow). Proposed strategies to enhance the endogenous GDNF production are summarized in the purple box (right).

synthesis and release have any causative pathogenic role in PD is for the moment unknown.

There are few studies focused on the cell distribution of striatal GDNF. In an ISH-based study over 60% of the choline acetyltransferase (ChAT) positive interneurons were reported to express *Gdnf* mRNA (Bizon et al., 1999). In the same study, a significant fraction (17–42%) of GABAergic neurons expressed *Gdnf* mRNA, however it did not discriminate between the medial spiny neurons (the most abundant cells in the striatum) and the GABAergic interneurons. As PV+ interneurons represent only a small fraction of GABA-positive cells, it could be concluded from these data that PV+ neurons account for a small proportion of striatal cells expressing GDNF (Bizon et al., 1999). However, in this study a majority of PV+ cells expressed NGF and acidic fibroblast growth factor (FGF1), which are thought to provide trophic protection to excitotoxic insult. Interestingly, some cells were found highly co-expressing GDNF and FGF1 (Bizon et al., 1999). In contrast with these observations, the use of a *Gdnf-LacZ* mouse model (Sánchez et al., 1996) unveiled a different population of GDNF-expressing cells in the striatum. *Gdnf* promoter-driven *LacZ* expression, revealed by β-galactosidase activity (XGal staining), demonstrates that *Gdnf* is expressed in more than 80% of striatal PV+ GABAergic interneurons. Moreover, ∼95% of the GDNF-positive striatal neurons are PV+, while the remaining GDNF+ cells are either cholinergic (ACh) or somatostatinergic (SS) interneurons (Hidalgo-Figueroa et al., 2012; see **Figure 3**). As yet there is no explanation for the discrepancy between these two studies performed in different models of rat (Bizon et al., 1999) and mouse (Hidalgo-Figueroa et al., 2012). However the particularly scattered distribution of PV+ cells throughout the mouse striatum, their electrical coupling by dendro-dendritic gap junctions (Fukuda, 2009) and their high resistance to excitotoxicity, make them a target of choice for pharmacological modulation. On the other hand, although the number of ACh+ and GDNF+ cells does not seem to be too high, they may have a significant contribution to striatal GDNF homeostasis, as degeneration of cholinergic interneurons following the injection of the cholinotoxin AF64α results in a 30% reduction in striatal GDNF protein content (Gonzalez-Reyes et al., 2012). This decrease of GDNF production might be directly inferred to the loss of ACh+ interneurons, or a consequence of a drop of cholinergic input to the PV+ interneurons (Chang and Kita, 1992). Despite these recent advances in the identification of GDNF-producing interneurons in the rodent striatum, the nature of the cells that produce GDNF in the human striatum remains as yet unidentified.

In the lesioned striatum, reactive astrocytosis occurs in parallel to an increase in GDNF expression (Nakajima et al., 2001). Similarly, in the DA-depleted striatum, reactive astrocytes results in expression of *Gdnf* mRNA, as shown by both quantitative RT-PCR and ISH (Nakagawa and Schwartz, 2004). However, in the *Gdnf-LacZ* mice, none of the GDNF expressing cells are of astrocyte or microglia origin 7 and 21 days post-MPTP, despite a significant increase of the astrocytic population

occurred (Hidalgo-Figueroa et al., 2012). Unilateral nigrostriatal lesions with 6-OHDA produce a 50% decrease in the number of PV+ neurons in the ipsilateral side in comparison with the contralateral side (Proschel et al., 2014). This brings an interesting contradiction with the MPTP-treated *Gdnf-LacZ* mice that displayed no difference in PV/GDNF expression in the injured striatum (Hidalgo-Figueroa et al., 2012). These differences may be due to the use of rats vs. mice and distinct parkinsonian models (neurotoxic drugs and route of administration).

STIMULATION OF STRIATAL ENDOGENOUS GDNF PRODUCTION

Since GDNF has a potent neurotrophic effect on DA neurons and it is highly expressed in the striatum, pharmacological or physical interventions aiming at up-regulating endogenous GDNF production are of major potential medical relevance. Several drugs have been tested to boost striatal GDNF expression thus far (see **Table 1**). For instance, a weeklong systemic injection of 1,25-dihydroxyvitamin D3 (calcitriol) induced *Gdnf* mRNA and protein expression in the rat striatum, presumably via the activation of vitamin D receptors. Longer treatment with calcitriol prevented DA neuron loss in 6-OHDA-lesionned rats (Smith et al., 2006). Monoamine oxidase (MAO) inhibitors rasagiline and selegiline, broadly used to treat PD patients, up-regulate *in vitro* GDNF expression via NF-κB internalization (Mizuta et al., 2000; Maruyama et al., 2004; Bar-Am et al., 2005). It would be interesting to test these MAO inhibitors *in vivo*. Valproic acid, an anti-epileptic drug, induces GDNF secretion in the culture medium of rat astrocytes, which partially prevents DA cell loss after LPS or MPTP treatment (Chen et al., 2006). Valproate is a powerful histone deacetylase inhibitor, therefore facilitating chromatin relaxation and transcriptional activation, which is suggested to facilitate transcription of neurotrophic factors (Harrison and Dexter, 2013). Indeed, treatment with histone deacetylase inhibitors increased *Gdnf* and *Bdnf* expression and preserved DA neuronal function from MPTP injury. Moreover, valproate induced a marked increase in *Gdnf* promoter activity and promoter-associated histone H3 acetylation (Wu et al., 2008). Other mood stabilizer drugs have been reported to trigger GDNF release by rat glioblastoma cell line (see **Table 1** for details). In any case, these data must be interpreted cautiously, as rat cortical primary astrocyte and cell line cultures used in these studies are experimental models very different from the striatum *in situ*.

Noribogaine, a metabolite of the naturally occurring alkaloid ibogaine, bears anti-addictive effects on alcohol and other drugs consumption. In rats, the effect of ibogaine on the reduction of ethanol intake is located in the ventral tegmental area a DA mesencephalic region medial to the SN. Systemic injection of ibogaine stimulates *Gdnf* mRNA expression in the midbrain of both rats and mice, and when added to the SH-SY5Y adrenergic cell line (He et al., 2005; Carnicella et al., 2010). Although ibogaine/noribogaine is known to act as an agonist to 5-HT2A and κ-opioid receptors and as an antagonist to NMDA receptors, the mechanism by which it induces *Gdnf* mRNA expression remains to be deciphered. Another potential stimulant of *Gdnf* mRNA and protein expression in mouse striatal neurons is the metabotropic glutamate receptor 3 agonist LY379268 (Battaglia et al., 2009). The organotellurium compound AS101 exerts diverse biologic activities and holds great potential in PD. Systemic application of this immunomodulator prevents neurotoxicity and behavioral deficits induced by 6-OHDA striatal injections in rats. Besides activation of the Ras-Raf-MEK-Erk cascade leading to cell growth and survival, AS101 up-regulates GDNF levels by inhibiting interleukin-10 in primary astrocyte cultures as well as in the rat SN (Sredni et al., 2007). It is surprising, however, that this compound has not been further studied in regard to its potential effect on GDNF expression.

Chinese medicinal plants also bring interesting molecules such as echinacoside, a polyphenol natural product that when injected peripherally alleviates MPTP-induced DA neuronal loss. Echinacoside stimulates GDNF and BDNF and prevents MPTP-induced apoptosis (Zhao et al., 2010). Puerarin, from the roots of a kudzu plant *Pueraria lobata*, partially prevents the chemically-induced DA neurodegeneration in mice and rats, and stimulates striatal GDNF (Zhu et al., 2010, 2014). Naringin is another recent example of a plant pigment (flavonoid) present in grapefruits that seems to stimulate GDNF in the SN of MPTP-treated mice (Jung et al., 2014; Leem et al., 2014).

An elegant strategy used to activate endogenous GDNF is based on an engineered zinc-finger protein (ZFP) that specifically activates the GDNF promoter (Laganiere et al., 2010). In this work, a six ZFPs sequence carried by an AAV vector was designed to target rat, human and monkey *Gdnf* promoters (hGDNF-ZFP). Microarray data from *in vitro* assays showed a very specific increase of *Gdnf* mRNA expression while the rest of the genomic activity remained unchanged. hGDNF-ZFP infused into the striatum of normal adult rats 4 weeks before triggering neurotoxicity by a 6-OHDA striatal injection, increased GDNF production in the striatum and improved motor activity in lesioned rats (Laganiere et al., 2010). This methodology could be potentially applicable to prevent DA neuron degeneration in genetic cases in which the disease can be diagnosed before appearance of the clinical symptoms. Whether hGDNF-ZFP induces GDNF expression in the striatal cells that normally synthetize the trophic factor, or if other cell types are also put to contribution, is a point that needs to be clarified. Recently, stimulation of the intracellular Sigma-1 receptor (Sig-1R) by the agonist PRE-084 (Su et al., 1991) showed neurorestorative properties in 6-OHDA-treated mice (Francardo et al., 2014). PRE-084 also induced a moderate, but significant, increase of GDNF protein in the striatum (∼6% over vehicle treatment) and in the SN (∼14%) whereas no difference was observed in the Sig-1R-*null* mice (Francardo et al., 2014). Quantification with inadequately characterized anti-GDNF antibodies remains a weak point in several of these studies (Battaglia et al., 2009; Di Liberto et al., 2011; Campos et al., 2012; Lee et al., 2013; Francardo et al., 2014). Such antibodies need to be tested on GDNF-KO tissue extracts as they may give false positive bands of the expected molecular size (authors' unpublished observation), and this may contribute to overstatement on the efficiency of certain drugs in stimulating GDNF expression.

Table 1 | *In vivo* and *in vitro* pharmacological tests employed to modulate the endogenous GDNF production.

Drug or stimulus	Administration	Model	Areas (or origin)	Duration	GDNF detection	GDNF levels	Reference
MAO inhibitors							
Rasagiline	Culture medium	SH-SY5Y cells		3–24 h	ELISA, WB, Q RT-PCR	> 10 fold ↑	Maruyama et al. (2004), Bar-Am et al. (2005)
Selegiline	Culture medium	Mouse astrocytes		24 h	ELISA	≈ 10 fold ↑	Mizuta et al. (2000)
(-)-deprenyl	Intrastriatal	MPTP mouse	St	30 min	RT-PCR	≈ 2 fold ↑	Tang et al. (1998)
Antidepressants, antipsychotics							
Valproate	Culture medium	Rat astrocytes	(VM)	24–48 h	ELISA, Q RT-PCR	265% ↑	Chen et al. (2006)
Amitriptyline, fluoxetine	Culture medium	Rat C6 glioblastoma cells		48 h	ELISA, RT-PCR	> 10 fold ↑	Hisaoka et al. (2001)
Serotonine	Culture medium	Rat C6 glioblastoma cells		48 h	ELISA	≈ 5 fold ↑	Hisaoka et al. (2004)
Quetiapine, clozapine, haloperidol	Culture medium	Rat C6 glioblastoma cells		24–48 h	ELISA	up to 4 fold ↑	Shao et al. (2006)
Chinese medicinal plants-derived molecules							
Echinacoside	i.g.	MPTP mouse	VM	14 days	WB	≈ 2 fold ↑	Zhao et al. (2010)
Puerarin	i.p.	6-OHDA rat, MPTP mouse	St	10 days	IHC, ELISA	≈ 1.5 fold ↑	Zhu et al. (2010, 2014)
Naringin	i.p.	MPTP rat	SN	7 days	WB, IHC	≈ 1.5 fold ↑	Leem et al. (2014)
Miscellaneous							
Ibogaine, Noribogaine	i.p. i.c.	Rat, mouse	VM	1–24 h	ELISA	≈ 3 fold ↑	He et al. (2005)
	Culture medium	SH-SY5Y cells		1–12 h	ELISA, RT-PCR	> 10 fold ↑	He et al. (2005), Carnicella et al. (2010)
Glutamate receptor 3 agonist (LY379268)	i.p.	Mouse	St, Ctx	6 h	ISH, Q RT-PCR, WB	up to 3 fold ↑	Battaglia et al. (2009)
	Culture medium	Mouse astrocytes	(St), (Ctx)	24 h	WB	≈ 1.7 fold ↑	Battaglia et al. (2009)
AS101	i.c.	6-OHDA Rat	SN	72 h	RT-PCR	≈ 2 fold ↑	Sredni et al. (2007)
1,25-dihydroxyvitamin D3 (Calcitriol)	i.p. s.c.	6-OHDA Rat	St, SN	8 days	ELISA	37%↑ (SN)—(St)	Smith et al. (2006)
hGDNF-ZFP	intrastriatal	6-OHDA Rat	St	4 weeks	Affymetrix, ELISA	up to 4 fold ↑	Laganiere et al. (2010)
PRE-084	s.c.	6-OHDA Mouse	St, SN	7–35 days	WB	37%↑ (SN) 14%↑ (St)	Francardo et al. (2014)
17-β-estradiol	s.c. osmotic pump	6-OHDA Rat	St, SN	8–10 days	WB	≈ 1.5 fold ↑	Campos et al. (2012)

VM, ventral midbrain; SN, substantia nigra; St, striatum; i.p., intraperitoneal; i.c., intracranial; i.g., intragastric; s.c. subcutaneous; Ctx, cortex; ELISA, enzyme-linked immunosorbant assay; WB, western blot; Q RT-PCR, quantitative reverse transcription polymerase chain reaction; ISH, in situ hybridization; IHC, immunohistochemistry.

In parallel to the pharmacological agents, noninvasive approaches are also being considered to stimulate endogenous brain GDNF production. *In vitro* analysis has revealed that GDNF is secreted both tonically and after depolarization of cells with high K^+, suggesting that *in vivo* GDNF could be released in an activity dependent manner (Lonka-Nevalaita et al., 2010). Transcranial magnetic stimulation (TMS) has been used for some time with little insights regarding its actual effect on neurons. A recent study made an attempt to use TMS on rats to assess the effect on GDNF production. Repeated TMS (rTMS), at 10 Hz, during 20 min for 4 weeks proved to be beneficial to unilaterally 6-OHDA-lesioned rats with improvement of behavioral test scores, increase of SNpc TH+ neuron number and fiber density as well as GDNF, NGF and PDGF levels in the striatum (Lee et al., 2013). However, the mechanisms leading to the positive action of rTMS on striatal neurotrophin expression and the associated neurorestorative effect are unknown. Electroconvulsive shock (ECS), a standard psychiatric therapy provoking seizures to provide relief from psychiatric illnesses, is known to improve motor function in PD animal models. ECS prevents neurodegeneration of the DA nigrostriatal pathway observed after 6-OHDA injections. Daily ECS treatment to healthy rats for 7 days stimulates GDNF protein expression in the SN but not in the striatum (Anastasia et al., 2007). Moreover, anti-GDNF IgG inhibits the neuroprotective effect of chronic ECS treatment (Anastasía et al., 2011). It is however not clear how GDNF is up-regulated in the SN since its expression is located in the striatum where no change in protein expression is observed after ECS. A far-fetched explanation would involve the participation of a large ECS-induced glutamate release, which may stimulate GDNF expression and release by the surrounding astrocytes (Yamagata et al., 2002).

Finally, physical exercise (Zigmond et al., 2009), and food restriction diets (Maswood et al., 2004), have both been suggested to have a neuroprotective effect. For example in rats, placing a cast to immobilize the limb ipsilateral to the 6-OHDA injection, thus forcing the use of the contralateral limb, reduces behavioral deficits and DA neuron loss in the lesioned striatum. This also increases GDNF protein content in the striatum (Cohen et al., 2003). Protective effect of exercise on the nigrostriatal DA system associated to an increase of GDNF protein in the 6-OHDA lesioned striatum has been reported in other studies (Tajiri et al., 2010; Lau et al., 2011). Yet, it remains unexplained how exercise can positively modulate GDNF expression, as well as other growth factors, in the striatum and SN. Altogether, the data summarized in this section demonstrate that activation of endogenous GDNF is feasible and therefore further research should be done to determine what methodology, or combination of techniques, can produce more consistent protection for DA neurons and terminals (**Figure 1**).

CONCLUDING REMARKS

Two decades have passed since the discovery of GDNF and much advance has been produced regarding its cellular effects and neuroprotective action on DA neurons. However, it still remains unclear which are the main factors determining GDNF production by brain cells and whether GDNF can effectively be used as a therapeutic agent for PD. Despite intense preclinical research and some clinical studies have been performed, intrastriatal delivery or systemic administration of GDNF have failed so far to provide robust and reproducible methodologies applicable to a large number of PD patients. Intrastriatal transplantation of GDNF-producing cells has worked well in animal models but is still confronted with several limitations (e.g., graft stability, cell survival, and sufficient cell number) for its translation to the clinical setting. The discovery of a specific set of striatal PV+ neurons, organized as a functional ensemble, responsible for production of most of the striatal GDNF, offers a well-identified target to stimulate endogenous production of GDNF. This electrically (gap-junction) interconnected PV+ neuronal pool is particularly attractive, as stimulation of a few of these cells could induce a synchronized activation of the whole population. However, the actual role of PV+ cells in nigrostriatal protection and the functional relations between the different subclasses of interneurons (GABAergic and cholinergic) need to be evaluated by selective deletion of the *Gdnf* gene in each one of these cell types. In addition to the striatum, PV+ neurons are also present in other parts of the brain, in particular in the cerebral cortex. As cortical PV+ neurons do not significantly produce GDNF, it would be interesting to investigate molecular differences between cortical and striatal PV+ neurons that make the latter capable of producing GDNF. Most of the research on GDNF has been done on non-human samples and models. The actual role of human striatal GDNF and the identification of human striatal cells producing this trophic factor are questions that should be urgently addressed by experimental work. GDNF therapy holds much hope and still remains an important field of investigation in PD. Combined with early diagnosis, neuroprotection by endogenous GDNF stimulation may be a potential preventive therapy to PD patients.

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Novel subcellular localization for α-synuclein: possible functional consequences

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α-synuclein (α-syn) is one of the genes that when mutated or overexpressed causes Parkinson's Disease (PD). Initially, it was described as a synaptic terminal protein and later was found to be localized at mitochondria. Mitochondria-associated membranes (MAM) have emerged as a central endoplasmic reticulum (ER) subcellular compartments where key functions of the cell occur. These domains, enriched in cholesterol and anionic phospholipids, are where calcium homeostasis, lipid transfer, and cholesterol metabolism are regulated. Some proteins, related to mitochondrial dynamics and function, are also localized to this area. Several neurodegenerative diseases have shown alterations in MAM functions and resident proteins, including Charcot Marie-Tooth and Alzheimer's disease (AD). We have recently reported that MAM function is downregulated in cell and mouse models of PD expressing pathogenic mutations of α-syn. This review focuses on the possible role of α-syn in these cellular domains and the early pathogenic features of PD that could be explained by α-syn-MAM disturbances.

Keywords: alpha-synuclein, Parkinson's disease, mitochondria-associated membranes, endoplasmic reticulum, phospholipid

PARKINSON DISEASE BACKGROUND

Parkinson disease (PD) is the second most prevalent neurodegenerative disease after Alzheimer disease (AD). Its main symptoms are resting tremors, rigidity, slowness of voluntary movements, freezing, and postural instability. Histopathologically, this disease is characterized by (a) a significant loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc; Braak et al., 2003) and (b) the accumulation of intracytoplasmic aggregates called Lewy bodies, composed mainly of alpha-synuclein protein (α-syn; Spillantini et al., 1997, 1998). This aggregation occurs at the SNpc and other cerebral areas such as locus ceruleus, nucleus basalis, hypothalamus, cerebral cortex, and autonomic nervous system (Maroteaux et al., 1988; Parkinson, 2002).

The majority of the PD cases are sporadic with only less than 10% of the cases related to mutations in genes such as *PARK2*, *PARK7*, *PINK1*, *LRRK2* or *SNCA* (Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004). Among these, mutations or duplication in *SNCA*, which codifies for α-syn, have been shown to cause autosomal dominant forms of familial PD (Krüger et al., 1998; Singleton et al., 2003; Zarranz et al., 2004; Schon and Przedborski, 2011).

SUBCELLULAR LOCALIZATION α-SYNUCLEIN

α-syn is a 140 aa protein, highly expressed in nervous tissues, that was identified as the precursor protein for the non–beta amyloid component of AD plaques (Uéda et al., 1993). Despite numerous research efforts, its main function remains unknown.

The majority of α-syn is soluble and resides in the cytoplasm. However, many researchers have demonstrated that α-syn, upon a yet unknown stimulus, is capable of binding to membranes and changes its N-terminal domain conformation upon this interaction (Eliezer et al., 2001; Jao et al., 2004, 2008). *In vitro*, α-syn binds preferentially to anionic phospholipids and liposomes of high curvature (Davidson et al., 1998; Fortin et al., 2004; Auluck et al., 2010). In the cell, these membrane regions are called lipid raft domains, which are detergent resistant membranes (DRM) with unique molecular characteristics (Simons and Toomre, 2000). Initially, lipid rafts were believed to form only at the plasma membrane; however, many authors have shown that these domains can also be localized intracellularly (Hayashi and Fujimoto, 2010).

In an effort to understand the function of this protein, many groups have reported several subcellular localizations for α-syn. In the last decades, multiple research data have shown α-syn located at pre-synaptic terminals (Kahle et al., 2000), participating in the regulation of the synaptic pool size and neurotransmitter release (Iwai et al., 1995; Masliah et al., 1996; Abeliovich et al., 2000; Murphy et al., 2000; Cabin et al., 2002; Gitler et al., 2008).

More recently, α-syn has been reported to bind to mitochondria (Li et al., 2007; Cole et al., 2008; Devi et al., 2008; Parihar et al., 2008; Zhang et al., 2008). This binding is especially significant in the striatum, substantia nigra (SNpc), and cortex of PD brains (Devi et al., 2008). Supporting these results, a recent study describes the existence of an N-terminal sequence

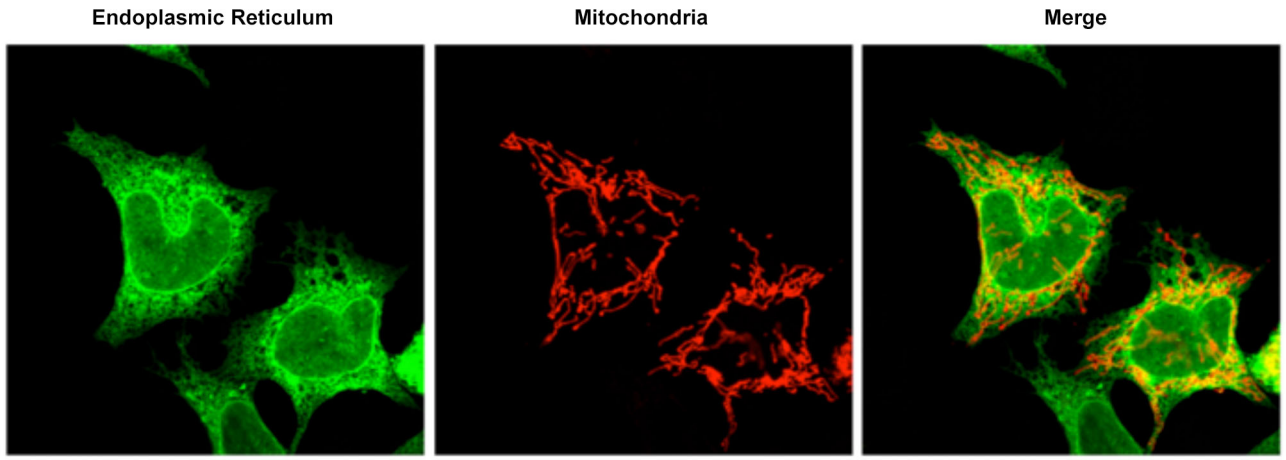

FIGURE 1 | Confocal microscopy image representative of the ER mitochondrial connections in M17 dopaminergic cell line. ER is labeled with GFP-Sec61-β (green) and mitochondria labeled with pDsRed2-mito (red). Merge image indicates the colocalization of both organelles.

in α-syn that could work as a mitochondrial targeting sequence (Devi et al., 2008). Moreover, α-syn binding to membranes is favored by the presence of cardiolipin (Zigoneanu et al., 2012), a lipid specific of the mitochondria membrane.

MITOCHONDRIA AND α-SYNUCLEIN IN THE PATHOGENESIS OF PD

Supporting α-syn localization to the mitochondria, PD patients, and cellular models containing pathogenic mutations of this protein show a deficit in mitochondrial functionality (Hsu et al., 2000; Schon and Przedborski, 2011), and, in particular, a significant decrease in complex I activity (Devi et al., 2008). In fact, the decrease in complex I activity is also present in PD brains and cellular models containing mutations in other genes related to the disease.

Moreover, exposure to a contaminant called 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), which is an inhibitor of the mitochondrial complex I, provokes parkinsonism symptoms and loss of dopaminergic neurons (Langston et al., 1983; Dauer and Przedborski, 2003). Additionally, injection of another complex I inhibitor, rotenone, caused a similar phenotype (Betarbet et al., 2000). All these data supports a role for mitochondrial dysfunction in the pathogenesis of PD.

In addition to complex I dysfunction, pathogenic mutations of α-syn have also been shown to interact with and reduce the activity of complex IV (Elkon et al., 2002). Also, there is data that relates the age-related accumulation of non-aggregated α-syn to mitochondria with a reduced dopamine phenotype in the SNpc (Chu and Kordower, 2007). Studies in one of the transgenic models of PD bearing the α-syn A53T mutation, show not only complex I inhibition, but also damaged mitochondrial DNA and aberrant mitochondrial dynamics (Martin et al., 2006; Chinta et al., 2010; Choubey et al., 2011).

It is widely known that mitochondria are dynamic organelles that undergo fusion and fission continuously (Chan, 2006). Mitochondrial movement is especially dramatic in neurons, where mitochondria travel along the axons to provide the terminals with ATP and other metabolites. Perturbations of this flux of mitochondria throughout the cell body cause defects in cell viability. Curiously, alterations in mitochondrial dynamics have been extensively reported in numerous neurodegenerative diseases, i.e., AD (Wang et al., 2008, 2009), PD (Yu et al., 2011; Cooper et al., 2012) and Charcot Marie-Tooth (Baloh et al., 2007; Chen and Chan, 2009). For instance, in the case of PD, the mitochondrial protein PINK1, whose mutation causes PD, is known to interact with the proteins Miro and Milton, both microtubule-associated proteins (Weihofen et al., 2009). Moreover, research data showed that aggregates of wild-type α-syn disrupt the mitochondrial trafficking of cargoes (Galvin et al., 1999; Lee et al., 2006).

An alternative consequence of the deregulation of mitochondrial trafficking is the alteration of the mitochondrial quality control. Spare or damaged mitochondria are degraded by mitophagy, a process in which the cell has to be able to differentiate healthy from damaged mitochondria. An indication of healthy mitochondria is a high membrane potential and low reactive oxygen species (Twig and Shirihai, 2011). The primary mechanism for degrading or minimizing damaged mitochondria is fusion and fission (Schon and Przedborski, 2011). During these processes, the cell "neutralize" unhealthy mitochondrial content, mixing it with other healthy organelles. In the case of neurodegenerative diseases, such as PD, where the fusion/fission machinery is altered, the accumulation of "bad" mitochondria can lead to the disease. More specifically, some authors link those dysfunctional mitochondria that cannot reach axonal extremes in PD with an increased expression of α-syn and aggregation (Lee et al., 2002). This could be the cause of the accumulation of mitochondrial mutations observed in the SNpc of PD patients that leads to a loss of dopaminergic neurons (Bender et al., 2006; Kraytsberg et al., 2006).

α-SYNUCLEIN IS LOCALIZED AT MITOCHONDRIA-ASSOCIATED MEMBRANES

Trying to answer this question, we revisited the exact cellular localization of α-syn. We have recently described a more accurate localization of α-syn (Guardia-Laguarta et al., 2014). Our data shows the existence of a subpopulation of α-syn that resides at the mitochondria-associated membranes or MAM. This region interconnects the endoplasmic reticulum (ER) and the mitochondria and is responsible for specific cellular functions. These membranes are composed of intracellular lipid rafts. This result is compelling, as previous works show that α-syn has an affinity for lipid rafts (Fortin et al., 2004) and negatively-charged membranes (Davidson et al., 1998) and could possibly explain the studies describing α-syn as a mitochondrial protein (Li et al., 2007; Cole et al., 2008; Devi et al., 2008; Parihar et al., 2008; Shavali et al., 2008). The lack of appropriate markers for MAM and the technical difficulty in fractionating this kind of membrane because of its association with the ER could explain previous results (Area-Gomez et al., 2012).

MAM is a subcompartment of the ER that is connected to the mitochondria (**Figure 1**; Rusiñol et al., 1994; Csordás et al., 2006; Hayashi et al., 2009). It is involved in a number of core cellular functions; i.e., calcium homeostasis (Csordás et al., 2010), cholesterol metabolism (Rusiñol et al., 1994), and phospholipid transfer from the ER to the mitochondria (Vance, 1990). Specifically, MAM has been described as the residence of several proteins related to phospholipid regulation (phosphatidylserine synthase 2: PTDSS2), cholesterol metabolism (acyl-CoA:cholesterol acyltransferase) (Rusiñol et al., 1994), and calcium transport from the ER to the mitochondria (the type 3 inositol 1,4,5-triphosphate receptor, IP3R3) (Hayashi and Fujimoto, 2010). Notably, mitochondrial distribution and dynamics are influenced by the physical connections formed by MAM (Rizzuto et al., 1998; Levine and Rabouille, 2005; Csordás et al., 2006; Hayashi et al., 2009; Friedman et al., 2011; Rowland and Voeltz, 2012). During mitochondrial fission, ER tubules appear to "embrace" mitochondria and mark sites of mitochondrial division (Friedman et al., 2011). In addition, isolated MAM from different tissues have been shown to be enriched in proteins related to the control of mitochondrial dynamics (e.g., FIS1, MFN2, and DRP1). Finally, MAM also contain some proteins involved in apoptosis (e.g., VDAC1 [voltage-dependent anion channel 1], BAX and BID (Garofalo et al., 2005; Ciarlo et al., 2010)). Indeed, calcium release at ER-mitochondrial contacts, which is important for ATP production, could be responsible for sensitizing mitochondria to apoptosis (Iwasawa et al., 2011; Tabas and Ron, 2011). The alteration of mitochondrial-ER contacts can cause deregulation of the calcium signal which results in inappropriate protein folding, metabolic alterations, and apoptosis (Csordás and Hajnóczky, 2009; Bui et al., 2010).

COULD THE PATHOGENESIS OF PD BE EXPLAINED BY EARLY ALTERATION IN MAM FUNCTION?

Scorrano's group made the first correlation between MAM disturbance and disease when they described Mfn2 as a MAM resident protein that participates as a scaffold between ER and mitochondria. Mutations of Mfn2 cause Charcot Marie Tooth type 2a (de Brito and Scorrano, 2008). More recently it has been shown that presenilin-1, presenilin-2, and γ-secretase activity—all key factors associated with the pathogenicity of AD—are highly enriched in the MAM (Area-Gomez et al., 2009). Moreover, mutation or ablation of these γ-secretase components provokes a significant upregulation of several activities located at the MAM (Area-Gomez et al., 2012). Similarly, we have also shown that mutations in α-syn cause an alteration in the regulation of MAM function (Guardia-Laguarta et al., 2014). Supporting this observation, several groups have reported alterations in the lipidic composition of membranes from PD brains (Fabelo et al., 2011). These data suggest that these molecular alterations would change thermodynamic properties, organization, and signal transduction in the PD brain.

MAM regulates the homeostasis of cholesterol through the acyl-coA cholesterol acyltransferase (ACAT) activity. ACAT is the enzyme responsible of the conversion of free cholesterol to cholesteryl esters that eventually will be stored as lipid droplets. Therefore, ACAT activity regulates the amount of free cholesterol in cellular membranes. Cholesterol regulation alterations in PD have been extensively reported in the literature (de Lau et al., 2006; Huang et al., 2007; Hu et al., 2008). Interestingly, α-syn contains two cholesterol binding domains that play a role in the regulation of its binding to membranes and perhaps aggregation (Fantini and Yahi, 2013). In fact, α-syn transgenic mice treated with statins (cholesterol-lowering drug) showed a significant reduction in α-syn aggregation (Bar-On et al., 2008). Finally, numerous reports have described the interaction of α-syn and lipid droplets (Cole et al., 2002; De Franceschi et al., 2009). Taking all of this into account, it may well be that the cholesterol alterations in PD are a consequence of a MAM dysfunction caused by mutations in α-syn, a MAM protein.

The transfer of calcium between ER and mitochondria via MAM is a highly regulated process that controls the whole calcium homeostasis in the cell (Rizzuto et al., 2009; Csordás et al., 2010). As in many other neurodegenerative diseases, calcium homeostasis is altered in PD patients and animal models. In neurons, these alterations result in excitotoxic events that may eventually cause cell death (Rizzuto et al., 2009). Brini's group was the first to show that α-syn is involved in the regulation of calcium homeostasis by altering the ER-mitochondria communication (Calì et al., 2012). Hodge and Colombini (1997) show that VDAC, a voltage-dependent calcium channel that controls mitochondrial calcium levels and mitochondrial function (and is localized in MAM), is decreased in nigral neurons positive for α-syn. Finally, recent evidence shows that increased Parkin expression improves calcium transfer through MAM; implying that Parkin mutations that cause PD could be detrimental for maintaining healthy levels of calcium (Calì et al., 2013).

Oxidative stress has been considered one of the main factors in the pathogenesis of PD (Kidd, 2000; Jenner, 2003). Increased levels of lipid hydroperoxydes have been found in SNpc and midbrain from PD patients (Yoritaka et al., 1996). Indeed, oxidative damage, lipoxidation of proteins like α-syn and oxidative DNA damage have been found in early-stages of

PD (Dalfó et al., 2005), indicating a role for oxidative stress in the disease. As a response to this insult, it has been suggested that the SNpc suffers an increase in the turnover of membrane phospholipid synthesis that may be behind the specificity of neuronal death in the SNpc in PD (Ross et al., 2001). Again, the results point to an early imbalance in a very basic function of the cell, as it is the phospholipid transfer, controlled by MAM membranes that, over time, cause a disabling neurodegenerative process.

It is also well known that ER-mitochondria connections regulate mitochondrial dynamic processes (Csordás et al., 2006; Hayashi et al., 2009; Friedman et al., 2011; Rowland and Voeltz, 2012). It has been previously described that mutations in α-syn increase mitochondrial fragmentation (Kamp et al., 2010; Nakamura et al., 2011). Correlating this to MAM dysfunction, we have confirmed this fragmented phenotype in our mutant cells (Guardia-Laguarta et al., 2014). Nevertheless, it is possible that the fragmentation observed when α-syn is mutated is not due to defects on the fusion/fission mitochondrial machinery but rather to MAM alteration (Guardia-Laguarta et al., 2014).

Next, autophagy, a strictly regulated mechanism, is altered in PD (Chinta et al., 2010). Actually, PINK1 and Parkin, are known to be part of the mitochondrial autophagy cascade, or mitophagy. There is also evidence of the relation of α-syn with mitophagy: first it was described that transgenic animals expressing A53T α-syn present alterations in mitophagy (Chinta et al., 2010). The same result was found in yeast expressing wild-type α-syn (Sampaio-Marques et al., 2012). Interestingly, it has been reported that the autophagosomes, a key step during autophagy pathway, are formed at the MAM boundaries (Hamasaki et al., 2013).

Finally, consistent with other authors (Calì et al., 2012), our results suggest that pathogenic mutations result in a lower binding of α-syn to MAM. Therefore, it is possible that a certain amount of wild-type α-syn is necessary to maintain normal function, and so, mutation in α-syn does not cause a toxic gain-of-function, but rather a loss of "relevant" function in mitochondrial morphology maintenance and in some of the main MAM functions.

FUTURE QUESTIONS

While we believe that our data help create a new way of thinking about PD pathogenesis, many questions need further research to properly address this new "MAM hypothesis".

First, α-syn was initially described as a protein with a perinuclear and pre-synaptic dual localization, hence the name. The perinuclear localization is in agreement with our data because MAM, as part of the ER, is known to be enriched around the nucleus (de Brito and Scorrano, 2010). However, in order to satisfy both the pre-synaptic and this new MAM localization, the ER-mitochondria domains should also be present at these synaptic terminals. Notably, some authors have shown that ER-mitochondrial connections (McNulty, 1980) and known MAM markers (Mavlyutov et al., 2012) can be found closely juxtaposed to synaptic membranes. Moreover, similar connections between ER and mitochondria were also observed in ganglion cell membranes close to nerve endings (Watanabe and Burnstock, 1976; Taxi and Eugène, 1995).

Second, our data does not address the question of whether the toxic effect of α-syn is due to its aggregation tendency or to its soluble state, or caused by the overexpression of the protein (Narhi et al., 1999; Goldberg and Lansbury, 2000; Ostrerova-Golts et al., 2000). Furthermore, none of the experiments carried out reveal any aggregation of α-syn in dopaminergic cells lines or tissues. It may well be that only monomeric α-syn in MAM initiate the cascade of events that ultimately leads to mitochondrial dysfunction and dopaminergic cell loss. Equally possible is that α-syn binding to MAM triggers the aggregation of this protein into oligomers.

Finally, our work focuses only on the relationship between α-syn and MAM. Whether MAM dysfunction is an event also related to mutations in other PD genes requires additional investigation. Nonetheless, cellular symptoms caused by pathogenic mutations in these other genes are practically identical to those provoked by mutations in α-syn.

CONCLUSIONS

Our data suggest that MAM alteration may play an important role during the progression of PD pathology as an early event that may cause an imbalance in basic functions of the cell. Our "MAM hypothesis" helps reconcile many of the cellular symptoms seen in PD over time, such the accumulation of unhealthy mitochondria, altered autophagy, dysfunctional calcium levels, increased lipid droplets, and altered phospholipid species that lead to neurodegeneration. In addition, the localization of α-syn in MAM may help reconcile questions regarding the role of both the ER and mitochondria in the pathogenesis of PD, and may explain some of the features of DA neuron degeneration, i.e.: the deregulation of calcium homeostasis and mitochondrial dysfunction.

We hypothesize that a more detailed study of other functions that are located in MAM will reveal other alterations related to PD progression and that MAM alteration could be a good pre-symptomatic predictor of future PD pathology.

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Lysosomes and α-synuclein form a dangerous duet leading to neuronal cell death

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Neurodegenerative diseases are (i) characterized by a selective neuronal vulnerability to degeneration in specific brain regions; and (ii) likely to be caused by disease-specific protein misfolding. Parkinson's disease (PD) is characterized by the presence of intraneuronal proteinacious cytoplasmic inclusions, called Lewy Bodies (LB). α-Synuclein, an aggregation prone protein, has been identified as a major protein component of LB and the causative for autosomal dominant PD. Lysosomes are responsible for the clearance of long-lived proteins, such as α-synuclein, and for the removal of old or damaged organelles, such as mitochondria. Interestingly, PD-linked α-synuclein mutants and dopamine-modified wild-type α-synuclein block its own degradation, which result in insufficient clearance, leading to its aggregation and cell toxicity. Moreover, both lysosomes and lysosomal proteases have been found to be involved in the activation of certain cell death pathways. Interestingly, lysosomal alterations are observed in the brains of patients suffering from sporadic PD and also in toxic and genetic rodent models of PD-related neurodegeneration. All these events have unraveled a causal link between lysosomal impairment, α-synuclein accumulation, and neurotoxicity. In this review, we emphasize the pathophysiological mechanisms connecting α-synuclein and lysosomal dysfunction in neuronal cell death.

Keywords: α-synuclein, lysosome, Parkinson's disease, cell death

INTRODUCTION

Neurodegenerative diseases are (i) characterized by a selective neuronal vulnerability to degeneration in specific brain regions; and (ii) likely caused by disease-specific protein misfolding. Parkinson's disease (PD), the second most common neurodegenerative disorder after Alzheimer's disease, is notably characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Loss of dopamine perturbates the cortico-basal ganglia-cortical control of voluntary movements. Current treatments have no proven protective or restorative effect and are only symptomatic. Moreover, their long-term use is associated with the onset of dramatic side effects i.e., fluctuated responses and L-Dopa induced dyskinesia. The important of understanding the mechanisms of neuronal death underlying neurodegenerative diseases is crucial for identifying targets for disease-modifying/curative strategies. In addition to dopaminergic neuronal cell loss, the main pathological hallmark of PD is the presence of intraneuronal proteinaceous cytoplasmic inclusions, named Lewy bodies (LB). α-Synuclein (α-syn), a major protein component of LB, has been identified as autosomal dominant cause of PD, which is found increased in expression in patients (Goedert et al., 2013; Lashuel et al., 2013). The presence of LB in PD suggests that defective protein handling contributes to the pathogenesis of the disease. Proteasomal and autophagic proteolysis are the two major pathways for degradation of cellular constituents in eukaryotic cells. Mounting evidence indicates that alterations in autophagy-lysosomal pathways (ALP) may be preferentially involved in PD. In this article, we review the close relationship between α-syn and the lysosome, two players involved in neuronal cell death in PD.

THE HARMFUL α-SYNUCLEIN

α-Syn has a central role in the pathogenesis of PD and other synucleinopathies, as dementia with Lewy bodies (DLB) and Multiple System Atrophy (MSA; Spillantini and Goedert, 2000). In 1997, the first link between PD and α-syn was described with the identification of point mutations -A53T- in the *SNCA* gene in autosomal-dominant forms of PD (Polymeropoulos et al., 1997; Athanassiadou et al., 1999; Spira et al., 2001; Ki et al., 2007; Choi et al., 2008; Puschmann et al., 2009). To date, the list of missense mutations continues to grow with A30P, E46K, H50Q, G51D, A53E (all classified as *PARK1* locus) (Krüger et al., 1998; Zarranz et al., 2004; Appel-Cresswell et al., 2013; Lesage et al., 2013; Proukakis et al., 2013; Pasanen et al., 2014). The subsequent identification of families with multiplication (duplication or triplication) of its allele (*PARK4* locus) strengthen the link between α-syn and PD (Singleton et al., 2003; Chartier-Harlin et al., 2004), suggesting that increased expression levels of the normal α-syn can be causal for PD and others synucleinopathies. Furthermore, genome-wide association studies (GWAS) have linked single nucleotide polymorphisms

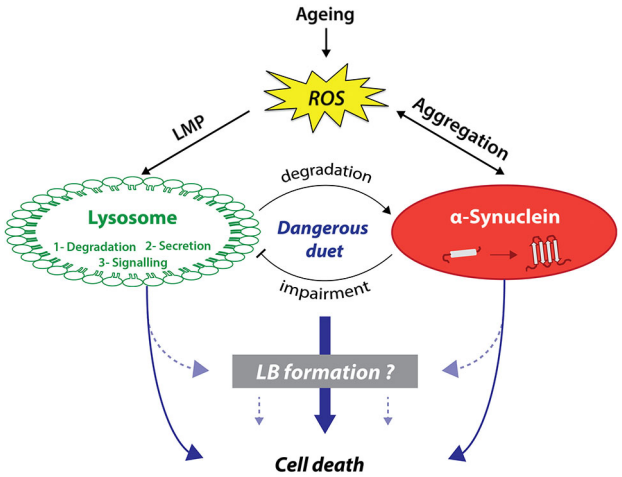

FIGURE 1 | Lysosomes and α-synuclein are involved in a vicious pathogenic loop eventually leading to cell death and LB formation. On the one hand, lysosomes have been shown to be involved in cell death activation through canonical or non-canonical pathways. On the other hand, α-synuclein (α-syn) can also trigger cell death through several distinct pathways including membranes permeabilization. Lysosomes and α-syn display a bidirectional relationship. While lysosomes predominantly degrade α-syn, α-syn aggregation can lead to lysosomal dysfunction in return. α-Syn-mediated lysosome impairment can lead to alterations of one of lysosomal main function: protein degradation, lysosome-to-nucleus signaling, and secretion. This pathogenic loop can be worsened with age and in particular ROS production, which can induce both LMP and α-syn aggregation. Interestingly, α-syn aggregation, especially specific oligomeric species, can increase ROS production. We previously suggested that this loop might be the template for the formation of LB, which remains currently unknown.

(SNPs) in the *SNCA* gene with increased susceptibility of developing PD (Simón-Sánchez et al., 2009; Edwards et al., 2010; International Parkinson Disease Genomics et al., 2011). α-Syn is a 14 kDa neuronal protein consisting of 140 amino acids mainly localized to presynaptic terminals. While the exact physiological function of α-syn remains to be fully understood, several studies have implicated its capacity to interact directly with cellular membranes, such as vesicles (Auluck et al., 2010) or mitochondria-associated membrane, which is an endoplasmic reticulum subdomain involved in lipid and calcium homeostasis (Guardia-Laguarta et al., 2014). Nevertheless, substantial evidence suggests that α-syn function is related to vesicle dynamics, neurotransmission and synaptic plasticity, the mechanisms of which have been reviewed elsewhere (Bellani et al., 2010). In its native state, the previous paradigm was that α-syn behaves as an unfolded monomer. However, a recent report now hints at a more complex picture as the predominant physiological species of α-syn is a helically folded tetramer (Bartels et al., 2011). α-Syn is, however, intrinsically defined as an aggregation-prone protein. In PD brains, α-syn antibodies strongly react in LB (Spillantini et al., 1997) and Lewy neurites (Takeda et al., 1998). Biochemical analyses have shown that α-syn is a major protein component of LB and may be part of the β-sheet enriched fibrillar structure of these inclusions (Crowther et al., 2000). α-Syn can undergo several post-translational modification such as truncation, nitration, oxidation, sumoylation, ubiquitinylation and phosphorylation (Giasson et al., 2000; Fujiwara et al., 2002; Tofaris et al., 2003; Anderson et al., 2006; Dorval and Fraser, 2006; Krumova et al., 2011). Interestingly, post-translationally modified α-syn has been found in LB and some post-translational modifications, such as oxidation or nitration, have been shown to impact its aggregation process in favor to oligomeric species (Fujiwara et al., 2002; Norris et al., 2003; Yamin et al., 2003). In the past few years, substantial progress has been made not only at elucidating how α-syn undergoes spontaneous self-aggregation, but also in its highly heterogenous aggregation process that turns its monomers into multiple oligomeric forms, then protofibrils, fibrils and aggregates. The identification of pathological species of α-syn involved in the perturbation of cellular function is an expanding area of research. Recent studies support the concept of soluble oligomers as the prominent toxic α-syn species in *in vitro* and *in vivo* settings, although the precise size and type of the toxic oligomeric species remains to be determined (Auluck et al., 2010; Winner et al., 2011; Cremades et al., 2012). Recent evidence piles up for prion-like propagation mechanisms in synucleinopathies, including PD. Indeed, α-syn might behave as a prion, responsible for initiating and spreading the pathological process in PD. Supporting this concept, α-syn can be transmitted to neighboring neurons and neuronal precursor cells (Puschmann et al., 2009; Hansen et al., 2011). *In vivo* studies have added a further piece to the puzzle with the observation that intracerebral inoculation of synthetic recombinant α-syn fibrils (Pffs) can mimic α-syn pathology in mice (Luk et al., 2012). More recently, through an innovative strategy based on the purification of aggregated α-syn from the SNpc of PD patients, intranigral or intrastriatal inoculations of PD-derived LB extracts resulted in progressive nigrostriatal neurodegeneration in both mice and monkeys (Recasens et al., 2014), which were found to originate at striatal dopaminergic terminals, Overall, these results demonstrated that human α-syn species contained in PD-derived LB are pathogenic and have the capacity to initiate a PD-like pathological process, not only in rodents but also in non-human primates (Recasens et al., 2014). Taken together, α-syn has multiple ways to cause cellular perturbations and lead to cell death. The presence of undegraded proteinaceous inclusions led the research community to wonder how is handled α-syn degradation? It is now understood that this involves both the ubiquitin-proteasome system (UPS) and the ALP. α-Syn is, however, predominantly degraded inside lysosomes, through chaperone-mediated autophagy (CMA) or endocytosis (Webb et al., 2003; Cuervo et al., 2004; Martinez-Vicente and Vila, 2013). The signals responsible for targeting α-syn (although it contains a KFERQ-like sequence, i.e., a motif recognized by heat shock cognate70 (hsc70) allowing direct lysosomal import) to a given degradation pathway are not yet fully understood, but may heavily depend on its folding state. Aggregated proteins will be preferentially routed for degradation to the lysosome through macroautophagy, whereas soluble forms would be both targeted to the proteasome or to the CMA. Overall, defective α-syn protein degradation can be recognized as an important pathogenic factor.

LYSOSOME: WHITE KNIGHT OR TWO-FACE

Lysosomes are dynamic acidic organelles that contain hydrolytic enzyme capable of degrading intracellular components, which were discovered by Christian de Duve more than 50 years ago (De Duve et al., 1955; Luzio et al., 2007). Acidic pH (around 4.6) is maintained in the lumen by proton-pumping vacuolar ATPases. Around 200 proteins have been reported as lysosomal membrane proteins such as proton pumps, secretory, plasma membrane, signaling or transport proteins (Schröder et al., 2007). The most abundant proteins are the lysosomal-associated membrane protein (LAMP)-1 and LAMP-2 as well as the lysosomal integral membrane protein (LIMP)-2 and CD63 (Saftig et al., 2010). Interestingly, lysosomes have a high intravesicular Ca^{2+} concentration (around 500-600 μM). Defective lysosomal Ca^{2+} uptake has been associated with human diseases, such as Niemann-Pick type C (Lloyd-Evans and Platt, 2011). Several lysosomal storage disorders are caused by lysosomal membrane dysfunctions (Ruivo et al., 2009). These defects are mostly due to non-enzymatic transport defects, highlighting the importance of transport and channel proteins in lysosome physiology (Ruivo et al., 2009). As mitochondrial outer membrane permeabilization (MOMP) is a major checkpoint of apoptosis pathway, lysosomal membrane permeabilization (LMP) has also been shown to induce cell death (Boya and Kroemer, 2008). Following LMP, cell death can occur through several pathway including canonical MOMP/caspase pathway but also MOMP- and caspase-independent pathways (Boya and Kroemer, 2008). The two main effects of LMP are the release of lysosomal proteases, such as cathepsins B or D (CSTB/CTSD), and cytosolic acidification. CTSD or CTSB could then directly or indirectly promote cytochrome C release from mitochondria (Boya and Kroemer, 2008). Currently, the principal inducer of LMP remains to be reactive oxygen species (ROS), although Bcl-2-associated X protein (Bax) has also shown to initiate this process (Kågedal et al., 2005).

Several pathways converge to the lysosome: phagocytosis, endocytosis, and autophagy through three different means respectively named microautophagy, CMA and macroautophagy. Autophagy (which comes from the Greek: "self-eating") is an evolutionary conserved mechanism that allows cells to degrade their own components and recycle important molecules (Wong and Cuervo, 2010; Cuervo, 2011; Boya et al., 2013). Briefly, CMA involves selective recognition by a chaperone and import through LAMP-2a, while microautophagy and macroautophagy involve direct sequestration of a portion of the cytosol (including proteins and organelles). While microautophagy requires the direct invagination of lysosomal membrane, macroautphagy involves the formation of a vesicle named autophagosome that will then fused with lysosomes to allow degradation of the sequestered material. In regards to protein aggregation, macroautophagy has been suggested to be the mammalian counterpart of the cytosolic-to-vacuole (Cvt)-pathway in yeast responsible for cargo-selective degradation (Yamamoto and Simonsen, 2011). Selective degradation of protein aggregates, named aggrephagy, has been characterized based on the observation of autophagosomes specifically containing aggregates (Filimonenko et al., 2010). Moreover, a phosphatidylinositol 3-phosphate-binding protein, Alfy, has been shown to specifically recognize and promote degradation of huntingtin aggregates (Filimonenko et al., 2010). For several decades, lysosomes have been only considered as terminal degradative compartments. However, recent studies suggest that lysosomes are involved in a vast number of cellular functions including lysosome-to-nucleus signaling, secretion, energy metabolism and cell death pathways (Rodriguez et al., 1997; Settembre et al., 2012, 2013).

Impairment of ALP, which is essential to maintain proper protein and organelle quantity and quality within cells, is increasingly regarded as a major pathogenic event in neurodegenerative diseases, including PD. The presence of LB in brains of PD patients made the first connection with ALP and led to the hypothesis that defective protein handling system might contribute to the pathogenesis of the disease. Several studies from independent groups reported ALP impairment associated with lysosomal depletion in brain tissue from idiopathic PD patients (Chu et al., 2009; Alvarez-Erviti et al., 2010; Dehay et al., 2010). More precisely, accumulation of undegraded microtubule-associated protein light chain 3 (LC3)-positive vesicles, decreased cytosolic hsc70, LAMP-1 and LAMP-2a have been reported (Chu et al., 2009; Alvarez-Erviti et al., 2010; Dehay et al., 2010). Genetic studies further strengthen the connection between PD and ALP dysfunction, which have indicated that lysosomal impairments may play a primary pathogenic role in the disease process. Interestingly, both CMA and proteasome can degrade the two proteins associated with autosomal dominant inheritance of PD, i.e., α-syn (*PARK1/PARK4* locus) and Leucine-rich repeat kinase 2 (LRRK2–*PARK8* locus) (Webb et al., 2003; Cuervo et al., 2004; Orenstein et al., 2013). However, PD-linked α-syn mutants (as well as post-translationally dopamine-modified wild-type α-syn) and mutant forms of LRRK2 block CMA activity, resulting in insufficient clearance and subsequent accumulation and aggregation of α-syn (Cuervo et al., 2004; Martinez-Vicente et al., 2008; Mak et al., 2010; Orenstein et al., 2013). Notably, two other genes encoding for lysosomal proteins have been linked to PD: the lysosomal type 5 P-type ATPase (ATP13A2—*PARK9* locus) (Ramirez et al., 2006) and the enzyme glucocerebrosidase (GBA; Aharon-Peretz et al., 2004; Di Fonzo et al., 2007; Sidransky et al., 2009). While the former has been characterized in rare families with prominent parkinsonism (Ramirez et al., 2006; Di Fonzo et al., 2007), the latter has been identified as risk factor in multicenter genetic analysis of patients (Sidransky et al., 2009). Recently, genetic analysis suggested that lysosomal dysfunction may play an important role in the etiology of DLB (Bras et al., 2014). Relevant to PD, these two proteins have been reported to be components of LB (Goker-Alpan et al., 2010; Dehay et al., 2012). Defects in one of these two proteins may result in insufficient clearance of α-syn through lysosomes, hence leading to the accumulation of this protein in both cytosol and lysosome lumen (Dehay et al., 2012). Such vicious pathogenic loop has been reported between GBA and α-syn (Mazzulli et al., 2011). One can thus imagine a similar scenario in which toxic species of α-syn "damage" lysosomes, hence leading to an impairment of α-syn clearance that subsequently favor α-syn-aggregation. Such aggregates then cause, in return, other damages, while concomitantly accumulating in lysosomes/autolysosomes to form LB (Dehay et al., 2012).

In addition to the aforementioned genes, hereditary parkinsonism has been identified in families carrying mutations for ALP-related pathways. For instance, mutations in parkin (*PARK2* locus), in the phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) (*PARK6* locus) or in DJ-1 (*PARK7* locus), which are all involved in mitophagy, lead to autosomal recessive forms of PD (Corti et al., 2011). While PINK1 and parkin belong to the same pathway, DJ-1 has been shown to be involved in an independent parallel pathway, which can rescue a loss of function of PINK1 (Hao et al., 2010; Thomas et al., 2011). A defective degradation of dysfunctional mitochondria leads to maintaining those in the neuron and hence promotes the mitochondrial dysfunctions that have been characterized in PD patients (i.e., decrease in complex I activity and accumulation of large-scale mitochondrial DNA mutations) (Schapira et al., 1989; Bender et al., 2006). Mutations in the PD-associated gene *UCH-L1* (*PARK5*) abnormally interact with LAMP-2A, also causing an increase amount of α-syn (Kabuta et al., 2008). From a genetic point of view, all genes that have been positively associated with PD (Corti et al., 2011) are also connected to ALP, which shed light on the lysosome as an important player in PD-induced cell death.

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Lysosomal function impairment and α-syn aggregation can both induce cell death either on their own or through a dramatic additive effect. Of importance, α-syn seems to induce cell toxicity through its different pathological α-syn species, which include post-translationally modified, mutant, oligomeric and aggregated forms. These can (i) disrupt its typical function in neurotransmission release (Abeliovich et al., 2000; Jenco et al., 1998); (ii) impair mitochondrial dynamics, structure and function (Martin et al., 2006; Nakamura et al., 2011; Stefanovic et al., 2014); and (iii) disrupt ER-Golgi vesicle trafficking (Cooper et al., 2006; Gitler et al., 2008) and mitochondria-associated ER membrane (Mercado et al., 2013; Guardia-Laguarta et al., 2014), which results in ER stress. Further supporting the α-syn species toxicity, CMA inhibition by either PD-linked α-syn mutants or dopamine-modified wild-type α-syn results in an accumulation of α-syn, but also of undegraded CMA-substrates, involved for instance in the regulation of neuronal survival through the degradation of the neuronal survival factor myocyte enhancer factor 2D (MEF2D; Yang et al., 2009).

Regarding the lysosome, LMP is one mechanism for the induction of certain cell death pathways. As mentioned above, disruption of lysosomal membrane provokes cell death through release of CTSs and other hydrolases from the lysosomal lumen to the cytosol. These lysosomal proteases can remain active at cytosolic pH and induce cellular damages by degradation of vital proteins or activation of caspases. In relation to PD pathophysiology, mechanistic studies using the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD have reported a lysosomal dysfunction, characterized by lysosomal depletion and autophagosome accumulation. Such lysosomal deficiency was secondary to abnormal LMP induced by Complex I inhibition-mediated ROS production (Dehay et al., 2010; Vila et al., 2011). Recent studies reported that the pro-apoptotic Bax protein, which mediates MOMP, is activated in PD patients (Bové et al., 2014). In experimental PD mouse model, Bax translocates to the lysosome and mediates LMP before MOMP (Bové et al., 2014). Interestingly, pharmacological inhibition of Bax-mediated LMP and MOMP results in an overall attenuation of MPTP-mediated cell death, even if the treatment is administered once pathogenic neuronal changes are already in motion (Bové et al., 2014), suggesting that the phenomenon at work is reversible.

One of the meeting points between α-syn and lysosome involves ROS. Recent reports suggest that α-syn oligomers can induce both MOMP and in particular LMP (Freeman et al., 2013; Stefanovic et al., 2014). α-Syn aggregation underlies a bidirectional relationship with ROS production. Specific α-syn oligomers increase ROS production, whereas oxidized α-syn inhibits fibril formation in favor to toxic species (Norris et al., 2003; Cremades et al., 2012). Hence, α-syn-mediated ROS production can lead to LMP, as previously characterized in PD, and subsequently to cell death. All these studies suggest that oxidative stress impact both lysosomes and α-syn aggregation. In the past few years, another piece has been added to the puzzle, suggesting that α-syn might potentially spread in a prion-like manner, from cell to cell and region to region. Although mechanisms of α-syn release are not yet elucidated, α-syn may be released by exocytosis in a calcium-dependent manner (Lee et al., 2005; Emmanouilidou et al., 2010), a phenomenon exacerbated after lysosomal inhibition (Alvarez-Erviti et al., 2011), subsequently enhancing disease progression and the lysosomal contribution to the pathology. Non-genetic factors, however, cannot be excluded as important risks to PD. This includes ageing for instance which remains the most compelling risk factor for PD. Ageing is also associated with mitochondrial and lysosomal impairments as well as ROS production (Dufour and Larsson, 2004; Mattson and Magnus, 2006), linking the several key events that occur in neuronal cell death in PD.

Of interest, pharmacological or genetic enhancement of autophagy has been shown to be beneficial in PD models. For example, in the MPTP-treated mouse model, pharmacological activation of ALP with the mammalian target of rapamycin (mTOR) inhibitor, rapamycin, attenuates neurodegeneration and lysosomal dysfunction (Dehay et al., 2010; Malagelada et al., 2010). Consistent with this approach, viral-mediated overexpression of ALP components, such as transcription factor EB (TFEB), LAMP2a or Beclin-1, provided neuroprotection in viral-mediated α-syn-overexpressing rodent models of PD (Spencer et al., 2009; Decressac et al., 2013; Xilouri et al., 2013). With regards to the development of therapeutic approaches, we must keep in mind that a balance needs to be maintained between boosting and inhibiting processes of autophagy. Indeed, autophagy has been shown to have both survival promoting and death promoting roles (Eskelinen, 2005). Hence, enhancement of lysosomal biogenesis or specific activation of late steps of the autophagy machinery might provide more successful approach compared to a broad activation of the whole autophagy machinery, potentially leading to a deleterious effect and eventually cell death. Increasing the ability of neurons under attack to degrade protein aggregates remains a promising strategy for PD.

CONCLUDING REMARKS

Seventeen years after its association with PD, α-syn is now considered as a central player in PD pathogenesis, linking genetic and idiopathic forms of parkinsonism. Two key elements strongly associate α-syn aggregation and lysosomal dysfunction: (i) aggregated or post-translationally modified forms of α-syn can directly or indirectly inhibit lysosomal function; and (ii) the occurrence of a lysosomal depletion in brains from PD patients as well as in several experimental models of PD. Consistent with these evidences, LB formation might be the result of the combination of both α-syn aggregation and lysosomal failure, as key components of autophagy and α-syn have been localized in LB. Altogether, this suggests that α-syn aggregation and lysosomal impairment, enhanced with ageing, could play a deleterious duet leading to dopaminergic cell death (**Figure 1**).

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Alpha-synuclein spreading in Parkinson's disease

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Formation and accumulation of misfolded protein aggregates are a central hallmark of several neurodegenerative diseases. In Parkinson's disease (PD), the aggregation-prone protein alpha-synuclein (α-syn) is the culprit. In the past few years, another piece of the puzzle has been added with data suggesting that α-syn may self-propagate, thereby contributing to the progression and extension of PD. Of particular importance, it was the seminal observation of Lewy bodies (LB), a histopathological signature of PD, in grafted fetal dopaminergic neurons in the striatum of PD patients. Consequently, these findings were a conceptual breakthrough, generating the "host to graft transmission" hypothesis, also called the "prion-like hypothesis." Several *in vitro* and *in vivo* studies suggest that α-syn can undergo a toxic templated conformational change, spread from cell to cell and from region to region, and initiate the formation of "LB–like aggregates," contributing to the PD pathogenesis. Here, we will review and discuss the current knowledge for such a putative mechanism on the prion-like nature of α-syn, and discuss about the proper use of the term prion-like.

Keywords: α-synuclein, spreading, aggregation, Parkinson disease, neurodegenerative diseases

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder of unknown origin mainly characterized by the loss of neuromelanin-containing dopaminergic neurons in the substantia nigra pars compacta (SN) and the presence of intraneuronal proteinaceous cytoplasmic inclusions called Lewy bodies (LB). One of the main protein components of the LB is the protein α-synuclein (α-syn). Accompanying LB (which are located in neuronal perikarya), gross distrophic neurites containing α-syn and ubiquitin inclusions and called Lewy neurites (LN) are common in PD pathology. Besides SN dopaminergic neurons, a significant number of other central and peripheral neuronal populations exhibit Lewy pathology (combination of LB and LN), phenotypic dysregulation, or degeneration in PD patients (Dickson, 2012).

UPDATE ON α-SYNUCLEIN AND PD

α-synuclein is a 14 kDa protein consisting of 140 amino acids which is localized to presynaptic terminals and the nucleus (Maroteaux et al., 1988), cytosol and in some cellular membranes, such as the mitochondria-associated membrane in the endoplasmic reticulum (ER; Guardia-Laguarta et al., 2014). To date, six different missense mutations – p.A53T, p.A30P, p.E64K, p.H50Q, p.G51D, p.A53E – in the gene encoding for α-syn (*SNCA*) have been identified to cause autosomal-dominant forms of PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Athanassiadou et al., 1999; Spira et al., 2001; Zarranz et al., 2004; Ki et al., 2007; Choi et al., 2008; Puschmann et al., 2009; Appel-Cresswell et al., 2013; Lesage et al., 2013). Although the exact function of α-syn remains unknown, substantial evidence suggest that α-syn function is related to its capacity to interact directly with membrane phospholipids, particularly highly curved membranes such as vesicles. In particular, α-syn seems to play a role in the vesicle trafficking during the neurotransmission release.

In aqueous solution α-syn does not have a defined structure and is normally referred as a natively unfolded protein. However, the α-syn protein adopts oligomeric and/or fibrillar conformations in certain pathological conditions (such as mutations in the SNCA gene, oxidative stress and post-translational modifications). Mounting evidence suggests that the pathological α-syn species include the post-translationally modified, mutant, oligomeric or aggregated forms. These pathological species may induce toxicity by several mechanism such as (i) disrupting the normal function of α-syn in neurotransmission release, where it may act as a negative regulator of DA release (Jenco et al., 1998; Abeliovich et al., 2000; Murphy et al., 2000; Cabin et al., 2002; Chandra et al., 2005; Larsen et al., 2006; Chen et al., 2013; DeWitt and Rhoades, 2013), (ii) impairing mitochondrial structure and complex I activity, as well as mitochondrial dynamics and mitophagy (Martin et al., 2006; Devi et al., 2008; Liu et al., 2009; Chinta et al., 2010; Kamp et al., 2010; Loeb et al., 2010; Nakamura et al., 2011), (iii) disrupting ER-Golgi vesicular transport, which results in toxic ER stress (Cooper et al., 2006; Gitler et al., 2008; Thayanidhi et al., 2010) and (iv) impairing the efficiency of some protein-degradation mechanisms (Martinez-Vicente and Vila, 2013), thereby interfering with the normal physiology of the cell, and eventually leading to cell injury and death. However, it is worth noting that two recent studies contend that some α-syn oligomers may also serve an important

physiological function as synaptic vesicle wranglers (Burre et al., 2014; Wang et al., 2014).

The notion that α-syn in PD may self-propagate and spread progressively between interconnected brain regions via a cell-to-cell transmission mechanism has been strongly promoted recently (**Table 1**). Braak et al. (2003) described the presence of pathological α-syn aggregates in different brain regions, such as caudal raphe nuclei, coeruleus–subcoeruleus complex and SN. Based on this finding, Braak et al. (2003) suggested the possibility that sporadic PD might progress in six stages that follow a caudo-rostral pattern. Although other groups have confirmed some of these PD stages (Bloch et al., 2006; Dickson et al., 2010; Halliday et al., 2012) not all sporadic PD cases follow this theoretical caudo-rostral pattern of progression (Burke et al., 2008; Alafuzoff et al., 2009). Moreover, this staging does not explain the absence of clinical symptoms in subjects who on autopsy have widespread α-syn pathology. Regardless of the validity of Braak staging, this model has the merit of showing that α-syn lesions in PD are not only present in the SN, but in several other brain areas including both the peripheral nervous system (PNS) and central nervous system (CNS). According to the Braak staging hypothesis, PD might originate outside of the CNS by a causative pathogen capable of entering the CNS by way of retrograde axonal and transneuronal transport, with misfolded α-syn being a possible candidate for such a pathogen. Supporting this idea, α-syn pathology is abundant in the peripheral autonomic nervous system (pANS) of patients with LB diseases (Gelpi et al., 2014). Interestingly, epicardial fat tissue obtained during cardiac surgery from patients without parkinsonism but with some premotor symptoms such as constipation and acting dreams, exhibited α-syn pathology (Navarro-Otano et al., 2013).

Soon after Braak's hypothesis, two groups independently reported that embryonic mesencephalic neurons grafted into the striatum of PD patients develop LB many years after grafting (Kordower et al., 2008; Li et al., 2008) suggesting a host-to-graft transmission of the LB pathology in the human brain. Following these findings, the terms "prion" and "prion-like" started being widely used to describe the potential pathogenic mechanism of the α-syn protein. In this scenario, α-syn could be released by living cells (via an active process such as exocytosis), or by dying cells into the surrounding extracellular milieu. Thereafter, grafted neurons could take up this released α-syn through different pathways, including endocytosis. Once inside the grafted neurons, the exogenous α-syn could act as a template that promotes misfolding of endogenously produced α-syn, ultimately leading to the formation of LB (Brundin et al., 2008). However, we believed that there are still few unsolved questions that should be answered before using confidently the term "prion" to describe the α-synuclein protein (**Table 2**). In this way, it is worth noting that a third group reported no LB pathology in a patient 14-year after graft transplantation (Mendez et al., 2008). The differences between the LB presence or not in the grafts could be associated with differences in the histology protocols used, the graft environment, the years post-grafting and/or individual differences between PD patients (Brundin et al., 2008).

CELL-TO-CELL TRANSMISSION OF α-SYNUCLEIN

All these previous histopathological findings in human samples suggested the transmission of α-syn between cells. The question remains "can α-syn really be secreted and internalized by cells?" Since α-syn lacks an ER signal sequence that would direct it to secretory pathways, it was initially though that α-syn was exclusively an intracellular protein. However, the finding that α-syn species (monomeric and oligomeric) can be detected in human plasma and cerebrospinal fluid (CSF; Borghi et al., 2000; El-Agnaf et al., 2003) suggested the idea that α-syn can be secreted. Currently, it is well known that α-syn can be secreted into the culture medium by several types of neuronal cells (El-Agnaf et al., 2003; Lee et al., 2005; Sung et al., 2005; Emmanouilidou et al., 2010; Danzer et al., 2011). Although the exact mechanism of α-syn release has not been fully elucidated, recent results point toward a non-classic secretory pathway. In particular, it seems that α-syn may be released by exosomes in a calcium-dependent manner (Lee et al., 2005; Emmanouilidou et al., 2010) and further exacerbated after lysosomal inhibition (Alvarez-Erviti et al., 2011b).

On the other hand, several studies demonstrated that α-syn can be internalized by cells (Sung et al., 2001; Zhang et al., 2005; Danzer et al., 2007, 2009; Luk et al., 2009; Nonaka et al., 2010; Waxman and Giasson, 2010), probably by a classical endocytic mechanism (Sung et al., 2001; Lee et al., 2008a; Hansen et al., 2011; Volpicelli-Daley et al., 2011) that could include dynamin-dependent receptor-mediated endocytosis (Desplats et al., 2009; Hansen et al., 2011). However, considering the size of α-syn fibrillar aggregates, receptor-mediated endocytosis, which requires specific interactions between ligands and cell-surface receptors, seems unlikely to be the principal mode of fibril internalization. Other mechanisms could potentially mediate the transcellular movement of cytosolic α-syn aggregates [e.g., tunnel-like structures connecting two cells, called nanotubes (Gousset et al., 2009)], although these have not been fully demonstrated. Finally, α-syn monomers could potentially enter cells via passive diffusion by interacting with membranes and lipids (Ahn et al., 2006; Lee et al., 2008a; Auluck et al., 2010).

Recently, *in vitro* studies demonstrated that synthetic recombinant preformed α-syn fibrils (PFFs) could act as a seed to induce the recruitment of endogenous soluble α-syn into insoluble pathologic aggregates in cells overexpressing α-syn (Luk et al., 2009; Hansen et al., 2011; Volpicelli-Daley et al., 2011). The formation of these α-syn aggregates within recipient cells leads to alterations in synaptic functions, compromising neuronal excitability and connectivity, and culminates in neuronal death.

One of the first *in vivo* studies demonstrating that α-syn can be spread via a cell-to-cell transmission mechanism was by Desplats et al. (2009). GFP-labeled mouse cortical neuronal stem cells were injected into the hippocampus of transgenic mice expressing human α-syn under the control of the Thy-1 promoter. Four weeks after transplantation, 15% of the grafted cells exhibited human α-syn immunoreactivity. Interestingly, few of these cells exhibited inclusion bodies within the cytoplasm. In a separate study, 5% of fetal post-mitotic

Table 1 | Summary of *in vivo* studies representing the major milestones in the α-synuclein-injected toxicity.

Inoculum	Injection site	Recipients	Reference
Central nervous system			
Symptomatic Tg M83 mice brain lysates	n.s.	Tg M83$^{+/+}$ mice	Mougenot et al. (2012)
Recombinant mouse α-syn Symptomatic Tg M83 mice brain lysates	Striatum	C57BL/6 J mice	Luk et al. (2012a)
Recombinant human α-syn Symptomatic Tg M83 mice brain lysates	Cortex Striatum	Tg M83$^{+/+}$ mice	Luk et al. (2012b)
Recombinant human and mouse α-syn Symptomatic Tg M83 mice brain lysates Insoluble fraction of DLB brains	SN	C57BL/6 J mice	Masuda-Suzukake et al. (2013)
Brain homogenates from Tg M83$^{+/+}$ Human brain homogenates from MSA patients	Parietal lobe	Tg (M83$^{+/-}$:GFAP-luc) mice	Watts et al. (2013)
Recombinant human and mouse α-syn	SN Striatum Ent. Cortex	C57BL/6 J mice	Masuda-Suzukake et al. (2014)
LB-purified from PD patients	SN Striatum	C57BL/6 J mice Non-human primates	Recasens et al. (2014)
Peripheral Nervous Sytem			
rAAV expressing human α-syn	Left vagus nerve	Rats WT	Ulusoy et al. (2013)
Recombinant human α-syn Human SN lysates from PD patient	Intestinal wall	Rats WT	Holmqvist et al. (2014)
Recombinant human α-syn	Olfactory bulb	C57BL/6J mice	Rey et al. (2013)
Human and mouse recombinant α-syn	Hindlimb muscle	Tg M83$^{+/+}$ mice M20 WT mice	Sacino et al. (2014b)

α-syn, α-synuclein; DLB, dementia with Lewy body; Ent. Cortex, entorhinal cortex; GFAP, Glial fibrillary acidic protein; LB, Lewy body; Luc, luciferase; MSA, multiple system atrophy; n.s., not specified; PD, Parkinson's disease; SN, substantia nigra; rAAV, recombinant adeno-associated virus; Str, striatum; Tg, transgenic; WT, wild-type.

Table 2 | Missing evidences or open questions about α-synuclein spreading in PD.

Open questions
What is the composition and structure of recombinant α-syn seeds, brain homogenates samples or LB-purified samples?
What are the α-syn species responsible for toxicity and spreading in recombinant α-syn seeds, brain homogenates samples or LB-purified samples?
Are there differences in biophysical or structural properties between α-syn species responsible for toxicity and spreading?
Does spreading implies infectivity?
Are α-syn species specific from a synucleinopathy to another? Is there a strain notion?
Are cofactors (intracellular or extracellular) necessary for self-propagation?
What is the contribution of the axonal transport in the spreading process?
Is glia involved in propagation to interconnected brain structures?
Is there a common pathway/pattern for tissue migration?
What is the mechanism of cell death in those α-syn spreading based models? Does the immune response play a role?
How to improve the reproducibility of recombinant α-syn seeds? α-syn assembly by PMCA or qRT-QuIC might overcome this obstacle.
Can we extrapolate the results obtained in α-syn spreading based models into human diseases?
Does the other neurodegenerative-associated proteins (Aβ, tau, huntingtin . . .) share the same spreading-toxic properties of α-syn?

α-syn, α-synuclein; Aß, amyloid-beta; LB, Lewy body; PMCA, protein misfolding cyclic amplification; qRT-QuIC, quantitative real-time quaking-induced conversion.

dopaminergic neurons grafted into the striatum of mice over-expressing human α-syn, exhibited human α-syn immunoreactivity 6 months after transplantation (Hansen et al., 2011), thus confirming the transfer of human α-syn from host-to-graft *in vivo*. In addition, this study also demonstrated that different forms of human α-syn, including monomers, oligomers and fibrils, could be taken up by neurons *in vivo* by endocytosis (Hansen et al., 2011). In addition, host-to-graft transmission of human α-syn has also been reported in rats (Kordower et al., 2011).

Once demonstrated that α-syn could be transmitted between cells, the next step was to explore the potential pathogenic effect of α-syn transmission *in vivo*. In this context, both synthetic and murine disease-associated forms of α-syn were able to induce a PD-like α-syn pathology *in vivo* (Luk et al., 2012b). Luk and colleagues reported that the intracerebral injection of brain homogenates derived from old α-syn transgenic mice (which exhibited α-syn pathology) into the neocortex and striatum of young asymptomatic transgenic mice induced a widespread accumulation of pathological α-syn throughout the anterior/posterior extent of the neural axis spanning the CNS, from olfactory bulb (OB) to the spinal cord. These effects were mostly observed by 90 days post-injection, although at 30 days post-injection some α-syn pathology was already evident. Similar results were obtained after the injection of synthetic recombinant α-syn PFFs, providing the first evidence that PFFs alone were sufficient to initiate and propagate the α-syn pathology *in vivo*. Furthermore, the inoculation of either symptomatic brain lysates or α-syn PFFs accelerated and increased the accumulation of α-syn in these transgenic mice and reduced their lifespan. Mougenot et al. (2012) reproduced part of these results. In this case, the injection of brain homogenates from symptomatic α-syn transgenic mice into the brains of healthy transgenic mice accelerated the characteristic clinical signs of paralysis observed in this mouse model and reduced the lifespan of injected animals. In addition, insoluble phosphorylated α-syn at Ser129 was also found in the brains of inoculated mice.

The pathological spreading of α-syn was also reported in wild-type (WT) mice (Luk et al., 2012a). The injection of synthetic recombinant α-syn PFFs into the striatum of WT mice induced a pathological time-dependent accumulation of endogenous α-syn that was associated with cell loss in the SN and impaired motor coordination. The formation of an LB/LN-like pathology in PFFs-inoculated mice occurred upstream of SN DA neuron loss, indicating that the α-syn pathology was sufficient to induce the cardinal behavioral and pathological features of sporadic PD. The injection of human and mouse PFFs directly into the SN (Masuda-Suzukake et al., 2013) or hippocampus (Sacino et al., 2014a) of WT mice also induced a time-dependent widespread accumulation of α-syn pathology, although no neuronal loss in the SN or motor impairment was found in this case. It is noteworthy that the α-syn spreading efficiency observed in different laboratories depends heavily on several factors which include the preparation of synthetic recombinant α-syn, the choice of the strain of mice (Sacino et al., 2014c) as well as the brain areas of inoculation (Masuda-Suzukake et al., 2014) and overall the possibility of a species barrier. Furthermore, Sacino et al. (2014c) raised an important point about the non-specific immunohistochemical staining of the Ser129-phosphorylated α-syn antibody (mAB81A). This antibody reacts with phosphor-Ser129 but also with phosphorylated neurofilament subunit L (NFL). To overcome this obstacle, this antibody has to be used cautiously associated with an optimizing protocol including (i) the use of very low antibody concentrations for minimal background; (ii) the confirmation with other phosphor-Ser129 α-syn specific antibodies and amyloid dyes such as Thioflavine S; and (iii) the combination with biochemical procedures to separate the proteins by size to detect phosphorylated α-syn.

More recently, Recasens et al. (2014) demonstrated that human α-syn species contained in PD-derived LB are pathogenic and have the capacity to initiate a PD-like pathological process, not only in rodents but also in non-human primates. Nigral LB containing pathological α-syn were purified from postmortem PD brains by sucrose gradient fractionation and subsequently inoculated into the SN or striatum of WT mice and macaque monkeys. In both mice and monkeys, intranigral or intrastriatal inoculations of PD-derived LB extracts resulted in progressive nigrostriatal neurodegeneration starting at striatal dopaminergic terminals. In LB-injected animals, exogenous human α-syn was quickly internalized within host neurons and triggered the pathological conversion of endogenous α-syn. At the onset of LB-induced neurodegeneration, host pathological α-syn diffusely accumulated within nigral neurons and anatomically interconnected brain regions. LB-induced pathogenic effects required both human α-syn present in LB extracts and host expression of α-syn. Similarly, the injection of brain homogenates from patients with other synucleinopathies, such as dementia with Lewy bodies (DLB; Masuda-Suzukake et al., 2013) and multiple system atrophy (MSA; Watts et al., 2013), triggered α-synuclein pathology in mice. While the DLB homogenate did not induce a glial response or neuronal loss, mice injected with MSA exhibited prominent astrocytic and microglial activation and developed progressive signs of neurologic dysfunction. These contradictory results concerning human α-syn-induced neurodegeneration might be explained by differences in: (i) mouse strain (WT vs. transgenic), (ii) injection site (SN vs. parietal lobe), and (iii) sample sonication (non-sonicated vs. sonicated). A further possibility is that different α-syn strains might exist in each disease (PD, MSA, and DLB), thus explaining the differences observed after the injection of each synucleinopathy sample. Supporting this concept, distinct α-syn strains generated through repetitively seeded fibrillization *in vitro* exhibited different seeding properties both *in vitro* (Bousset et al., 2013) and *in vivo* (Guo et al., 2013).

PERIPHERAL TRANSMISSION OF α-SYNUCLEIN PATHOLOGY TO THE BRAIN

While the studies mentioned above involved a direct intracerebral inoculation of pathological α-syn, other studies have addressed the possible transmission of α-syn pathology from the periphery to the brain. For example, recombinant adeno-associated virus (rAAV) serotype 2/6-expressing human WT α-syn has been injected into the left vagus nerve in the neck of rats (Ulusoy et al., 2013). This injection induced a strong expression of human α-syn in the medulla oblongata (MO), leading to a caudo-rostral spreading of the α-syn pathology into other interconnected brain regions, such as the pontine coeruleus-subcoeruleus complex, the dorsal raphe, the hypothalamus and the amygdala. In addition, α-syn accumulation present in the aforementioned areas was accompanied by morphological evidence of neuronal abnormalities (i.e., thread-like axons with irregularly spaced, densely labeled varicosities). Surprisingly, the transmission of α-syn did not reach the SN, and neuronal damage was not induced in this brain region for at least 18 weeks after the injection.

In another study, Brundin et al. (2008) examined if α-syn could transfer from the OB to other brain structures through

neuronal connections (Rey et al., 2013). To answer this question, different molecular species (monomers, oligomers composed of soluble high molecular weight species, and fibrils) of recombinant human α-syn were injected into the OB of normal mice. The authors reported that cells in different layers of the OB (i.e., the glomerular layer, mitral cell layer and granule cell layer) readily take up recombinant monomeric and oligomeric α-syn. Fibrillar α-syn was also taken up, but to a much lesser extent within the time frame of the experiments. Soon after the injection (1.5 h and 3 h), soluble and oligomer, but not fibrillar, α-syn species were detected in several interconnected brain regions, including the anterior olfactory nucleus, the frontal cortex, the tenia tecta, the olfactory tubercle, the periform cortex, the striatum and the amygdala. At these time points, few microglial cells in the OB, anterior olfactory nucleus and frontal cortex were positive for human α-syn. α-Syn in microglial cells was present only locally, and not in other brain regions 12 h after injection into the OB. In contrast, at later time points, α-syn was extensively detected in microglial cells, suggesting that microglia might clear the human α-syn released into the extracellular space by the neurons. Recently, a study from the group of Giasson reported that *in vitro*-generated PFFs induced α-syn pathology by a single peripheral intramuscular injection of α-syn in transgenic mice, associated with robust gliosis and motor impairments (Sacino et al., 2014b).

The gastrointestinal pathway has also been extensively studied. Pan-Montojo and colleagues reported that intragastric administration of the environmental toxin rotenone induced α-syn accumulation in both the enteric nervous system (ENS) and CNS following the same pattern of progression as hypothesized by Braak (Pan-Montojo et al., 2010). Firstly, they reported α-syn accumulations in ENS neurons as soon as 1–5 months after rotenone treatment. Next, they determined whether the local effect of rotenone on the ENS could lead to alterations in the synaptically connected ANS centers in the spinal cord and brainstem [i.e., in the intermediolateral nucleus in the spinal cord (IML) and dorsal motor nucleus of the vagus (DMV)]. Both the IML and DMV exhibited accumulation and aggregation of α-syn 1.5 and 3 months after rotenone treatment, although α-syn pathology in these areas was not associated with neuronal death. Interestingly, the SN also exhibited α-syn accumulation, phosphorylation and inflammatory signs 3 months after rotenone treatment. Unlike the DMV and IML, α-syn increments in the SN were associated with neuronal loss. After intragastric rotenone administration, pesticide was not detected in the blood or brain, and no inhibition of complex I activity in muscle or brain was found, suggesting that the reported alterations in the mentioned brain regions were not due to a systemic effect of rotenone. Remarkably, the rotenone-induced α-syn pathology was specific, as only neuronal subpopulations with direct connections to the ENS showed alterations, while nearby areas (e.g., striatum, cerebellum, and cortex) remained unaffected. This specificity together with the fact that the appearance of α-syn accumulations in the SN were only detected at the last treatment time-point, raised the possibility of a direct mechanism between cells being responsible for this pattern of progression of the α-syn pathology. To confirm this hypothesis, Pan-Montojo et al. (2012) severed some of the connecting nerves between the CNS and the gut, which delayed the appearance of motor symptoms after oral rotenone treatment. This treatment also stopped the progression of α-syn pathology into the IML and DMV, and prevented cell death in the SN (Pan-Montojo et al., 2012). Recently, Holmqvist et al. (2014) have demonstrated that both human α-syn present in the SN of PD patients and distinct recombinant α-syn forms (including monomers, oligomers and fibrils) can be transported via the vagal nerve to the CNS after the injection into the intestinal wall of WT adult rats.

α-SYNUCLEIN TRANSMISSION AND NEUROINFLAMMATION

The secretion of α-syn by neurons may not only induce toxicity once inside the cytoplasm of neighboring cells, but also in the extracellular space; this may activate glial cells and induce chronic inflammation (i.e., a common pathological feature of PD), thereby contributing to the progression of the pathology throughout the brain. Supporting this idea, glial cells (i.e., astrocytes and microglia) are able to take up and degrade synthetic recombinant α-syn aggregates even more efficiently than neurons (Lee et al., 2008b). Indeed, α-syn can be transmitted between neurons and glial cells *in vitro* (Lee et al., 2010; Alvarez-Erviti et al., 2011a). Interestingly, the exposure of neuron-derived α-syn induced an inflammatory reaction in rat primary astrocytes (Lee et al., 2010) and microglia (Zhang et al., 2005; Reynolds et al., 2008; Alvarez-Erviti et al., 2011a). The direct transfer of α-syn from neurons to astrocytes was demonstrated *in vivo* using transgenic mice overexpressing human α-syn under a neuronal promoter. In these transgenic mice, abundant human α-syn accumulation was observed not only in neurons but also in glial cells (Lee et al., 2010). Consistent with these results, recombinant α-syn oligomers and monomers injected into the neocortex of WT mice were taken up by oligodendrocytes (Reyes et al., 2014). Similarly, in rAAV-treated rats overexpressing human α-syn, embryonic oligodendrocytes grafted into the striatum were found to contain this human α-syn, thus further demonstrating the neuron-to-astrocyte transmission of α-syn (Reyes et al., 2014).

α-SYNUCLEIN AND SIDEKICKS

Recently, several studies have provided convincing evidence that this same self-propagating mechanism of the α-syn protein may be applicable to a wide range of neurodegenerative associated proteins, including Aβ, tau, huntingtin, superoxide dismutase 1 (SOD1) and TDP-43 (see Guo and Lee, 2014, for review). Each of these proteins (i.e., recombinant proteins or contained in brain lysates) have been shown to act as a template or seed that could efficiently recruit their soluble counterparts into elongating fibrils in cultured cells and/or living animals. Recently, Cicchetti et al. (2014) described the presence of mutant huntingtin (mHtt) in tissue grafted into the brains of three patients with Huntington 's disease (HD) who received their transplants 9–12 years before they died. Similarly to the embryonic mesencephalic neurons grafted into the striatum of PD patients which develop LB many years after grafting, the presence of mHtt in this graft tissue could be explained by the host-to-graft transmission of the neurodegenerative-associated protein Htt. However, it is worth noting that the mHtt in this study was localized

to the extracellular matrix of the transplant tissue, unlike the mHtt protein aggregates found within the non-grafted regions, which localized to the neurons and neuropil (Cicchetti et al., 2014).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Mounting evidence suggests the concept that α-syn may be responsible for initiating and spreading the pathological process in PD. Notably, cellular and animal models developed so far based on the transmission (or spreading) properties might allow to screen therapeutic approaches against α-syn pathology (Sato et al., 2014). Of interest, a recent study using the PFFs-based model of PD demonstrated that immunotherapy with antibodies specifically targeting misfolded α-syn is able to block the entrance and propagation of α-syn in neurons, and hence prevents the development of neuropathological abnormalities in the brain (Tran et al., 2014).

However, several important questions remain to be solved (**Table 2**): (i) it is currently unknown whether the pathological conversion of endogenous α-syn triggered by PD-derived material or recombinant α-syn fibrils actually occurs directly through a seeding process or indirectly as a general response to cellular stress; (ii) the association between pathological α-syn accumulation and neuron cell death remains so far correlative. In addition, there is no definitive evidence to support the idea that PD can be contagious from one person to another, as is characterized for prion diseases (Beekes et al., 2014). In this line, a retrospective, postmortem study of recipients of cadaver-derived human growth hormone (hGH) found no reported incidence of PD, although the donors of pituitary glands used for hGH preparation probably included people with PD, and pathological α-syn is frequently found in the postmortem pituitary glands of people with PD (Irwin et al., 2013). One of the possible experiments would be to isolate α-syn aggregates developed in PD-derived material or recombinant α-syn fibrils injected animals, and injecting again in a healthy animals. These experiments would allow us to differentiate between infectious and self-propagating properties. Some approaches should be tested to evaluate the transmission of these disorders between animals (mice and monkeys) in order to study species-barrier properties or the use of different administration routes (intracerebrally, intranasal or fluids). All these studies should answer the unavoidable question of infectivity and/or contagiousness, the last missing criterion that defines a prion disease. However, until the issues mentioned above around nature and mechanisms of α-syn prion-like properties are better understood, we believe that the term prion for α-syn has to be used and considered cautiously. A new term referring as self-propagating pathogenic protein for α-syn needs to emerge and this is a mechanism well worth considering.

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Midbrain catecholaminergic neurons co-express α-synuclein and tau in progressive supranuclear palsy

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Objective: To analyze the frequency and distribution of α-synuclein deposits in progressive supranuclear palsy (PSP).

Methods: The brains of 25 cases of pathologically confirmed PSP were evaluated with immunohistochemistry for α-synuclein and tau. Multiple immunofluorescent stains were applied to analyze the expression of tau and α-synuclein aggregates in catecholaminergic neurons. Patients' clinical symptoms were retrospectively recorded.

Results: Deposits α-synuclein in the form of typical Lewy bodies (LBs) were only found in two PSP cases (8%) that fulfilled the clinical subtype of PSP known as Richardson's syndrome (RS). LBs were present in the locus ceruleus (LC), substantia nigra pars compacta (SNc), basal forebrain, amygdala and cingulated cortex in a distribution mimicking that of Parkinson's disease (PD). Triple-immunolabeling revealed co-expression of α-synuclein and tau proteins in some tyrosine hydroxilase (TH)-positive neurons of the LC and SNc.

Conclusions: There is no apparent clinical correlation between the presence of LBs in PSP. Tau protein co-aggregate with α-synuclein in catecholaminergic neurons of PSP brains suggesting a synergistic interaction between the two proteins. This is in keeping with the current view of neurodegenerative disorders as "misfolded protein diseases".

Keywords: neurodegenerative diseases, atypical parkinsonian disorders, lewy bodies, multiple immunolabeling techniques, substantia nigra

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Introduction

Aggregation of α-synuclein in the form of Lewy bodies (LBs) is the histopathological hallmark of idiopathic Parkinson's disease (PD) and dementia with LBs (DLB; Spillantini et al., 1997). LBs are present in the 50% of Alzheimer's disease brains (AD; Hamilton, 2000) but they are less frequently found in other neurodegenerative diseases such as progressive supranuclear palsy (PSP; Abhinav et al., 2011). At present it is well known that protein aggregation phenomena represent a crucial pathobiological mechanism shared by neurodegenerative diseases such AD, PD, PSP and Huntington disease. Accordingly, these diseases have often been categorized as "misfolded protein diseases", since cross-seeding of misfolded proteins

apparently underlies a number of pathological events associated with different diseases (reviewed in Soto and Estrada, 2008; Cuanalo-Contreras et al., 2013; Morales et al., 2013).

PSP is the most common atypical parkinsonian disorder and is clinically characterized by prominent postural instability, vertical gaze supranuclear palsy, pseudobulbar palsy, cognitive impairment and levodopa unresponsiveness (Steele et al., 1964). The pathology of PSP is characterized by the accumulation of abnormal tau protein within neurons (neurofibrillary tangles) and glial cells (tufted astrocytes and coiled bodies) in the brain. The distribution of tau inclusions is mainly subcortical and the globus pallidus, subthalamic nucleus and substantia nigra are severely affected in most cases. Other subcortical nuclei are affected to varying degrees and cases of severe cortical degeneration have been also described (Litvan et al., 1996). Whether the presence of LBs in PSP represents a normal aging process or the coexistence with PD remains unclear (Uchikado et al., 2006).

The aim of the present study was to analyze the density and distribution of LBs in the brains of patients with PSP, to investigate the clinicopathological correlation of this association and to characterize the interaction of α-synuclein and tau in cases of PSP with LBs by means of multiple immunolabeling techniques.

Materials and Methods

Subjects

Twenty five cases (2005–2013) of pathologically confirmed PSP according to the revised NINDS criteria (Litvan et al., 1996) from the Navarra Biomed Brain Bank were studied. The establishment and operational rules of Navarra Biomed Bank are both under the supervision of a Local Clinical Ethical Committee in keeping with current Spanish legislation (Royal Decree 1716/2011). Informed written consent form was obtained in all cases. The clinical features of those cases with LBs were evaluated by retrospective review of medical records. The distribution of LBs was assessed with the Braak's PD staging scheme (Braak et al., 2003). For control purposes, the mesencephalon of two age-matched individuals (both males) without any known type of neurological disease were used.

Neuropathological Evaluation

The brains were dissected through the corpus callosum in the sagittal plane. The left cerebral hemisphere was immersed in a 10% saline solution of 10% formaldehyde for 4 weeks. According to the recommendation guide proposed by BrainNet Europe (Bell et al., 2008), multiple regions of the neocortex were examined including frontal cortex (middle frontal gyrus), superior temporal, inferior parietal, occipital and cingulate cortex, entorhinal cortex, hippocampus, amygdala, basal nucleus of Meynert, striatum, subthalamic nucleus, brainstem and cerebellum.

Routine workflow included immunohistochemical staining of 3–5 μm-thick paraffin-embedded sections, followed by counterstaining with hematoxylin-eosin.

Immunohistochemistry

Formalin-fixed sections (3–5 mm-thick) were mounted on slides and deparaffinized. After conducting a routine antigen retrieval protocol, sections were incubated overnight with either a mouse monoclonal antibody against α-synuclein (NCL-L-ASYN; Leica Biosystems) or with a mouse monoclonal antibody anti-human PHF-TAU (clone AT-8; Thermo Scientific). The reaction product was visualized using an automated slide immunostainer (Leica Bond Max) with Bond Polymer Refine Detection (Leica Biosystems Newcastle Ltd).

For semiquantitative analysis LBs density and Lewy neurites (LN) were scored as follows: 0 = absent, + = mild, ++ = moderate, +++ = severe and ++++ = very severe (McKeith et al., 2005). In the assessment LBs brainstem type and cortical were included. A PD neuropathological stage was assigned according to the staging scheme proposed by Braak. For this measure only the distribution of LBs in any brain region was taken into account instead of the LBs density.

Dual Colorimetric Immunohistochemistry

Briefly, a sequential dual immunostaining protocol was used for the simultaneous visualization of α-synuclein and tau. The colorimetric detection of α-synuclein was carried out firstly using a Bond Polymer Refine DAB detection kit (Leica) resulting in a brown precipitate. Next, the Bond Polymer Refine Red detection kit (Leica) was used to visualize the expression of tau protein by means of a red-colored precipitate.

Multiple Immunofluorescence and Confocal Microscopy

Multiple immunofluorescent stains were carried out in coronal sections through the mesencephalon comprising both the substantia nigra pars compacta (SNc) and the locus ceruleus (LC). Briefly, 5 mm-thick brain blocks were postfixed for 24 h in a buffered solution containing 4% paraformaldehyde. Once fixed, sections were cryoprotected with a solution made of 20% gelatin and 2% dimethylsulphoxide (DMSO) in 0.1 M PBS, pH 7.4. Next, frozen coronal sections (40 μm-thick) were obtained in a sliding microtome and collected in the cryoprotective solution as 5 series of adjacent sections.

Free-floating sections were incubated in a cocktail of primary antisera comprising 1:200 mouse anti-synuclein (Invitrogen, Ref. 08-1215), 1:200 rabbit anti-Tau (Dako-Sigma, Ref. A0024) and 1:50 goat anti-tyrosine hydroxilase (TH) (Santa Cruz, Ref. Sc-7847) overnight at room temperature. Sections were then incubated for 90 min at room temperature in a cocktail of alexa-tagged secondary antibodies, comprising 1:200 Alexa488-coupled donkey anti-mouse IgG (Invitrogen, A21202), Alexa546-coupled donkey anti-rabbit IgG (invitrogen, Ref. A31572) and Alexa 633-coupled donkey anti-goat IgG (Invitrogen, Ref. A21082). Stained sections were finally mounted in gelatine-coated slides, air dried at room temperature in the

TABLE 1 | Density and distribution of Lewy bodies.

Age/Sex	Brain weight		Cc	Hdg	Ec	A	nbM	SNc	LC	Rn	DMVn	PD Braak stage
84F	1050	LB	+	0	++	++	0	+	+++	+	+	4
		LN	0	+	0	+	+	+	++	0	+	
73M	1200	LB	+	0	+	+	+	+	+++	+	N.A.	4
		LN	0	+	0	0	+	0	+	+	N.A.	

Abbreviations: A, amygdala; Cc, cingulate cortex; DMVn, dorsal motor vagal nucleus; Ec, entorhinal cortex; Hdg, hippocampus dentate gyrus; LC, locus ceruleus; N.A., not available; nbM, basal nucleus of Meynert; PD, Parkinson's disease; Rn, raphe nucleus; SNc, substantia nigra pars compacta.

dark, dehydrated in toluene and mounted with Entellan (Merck).

Sections were inspected in a Zeiss 510 META confocal laser-scanning microscope. To ensure appropriate visualization of the labeled structures and to avoid false positive results, the emission from the argon laser at 488 nm was filtered through a band pass filter of 505–530 nm and color-coded in green. The emission following excitation with the helium laser at 543 nm was filtered through a band-pass filter of 560–615 nm and color coded in light blue. Finally, a long-pass filter of 650 nm was used to visualize the emission from the helium laser at 633 nm and color coded in red.

Results

Clinical Features of the PSP Patients

The first patient was an 84-year-old woman who underwent consultation at the Neurology Department complaining

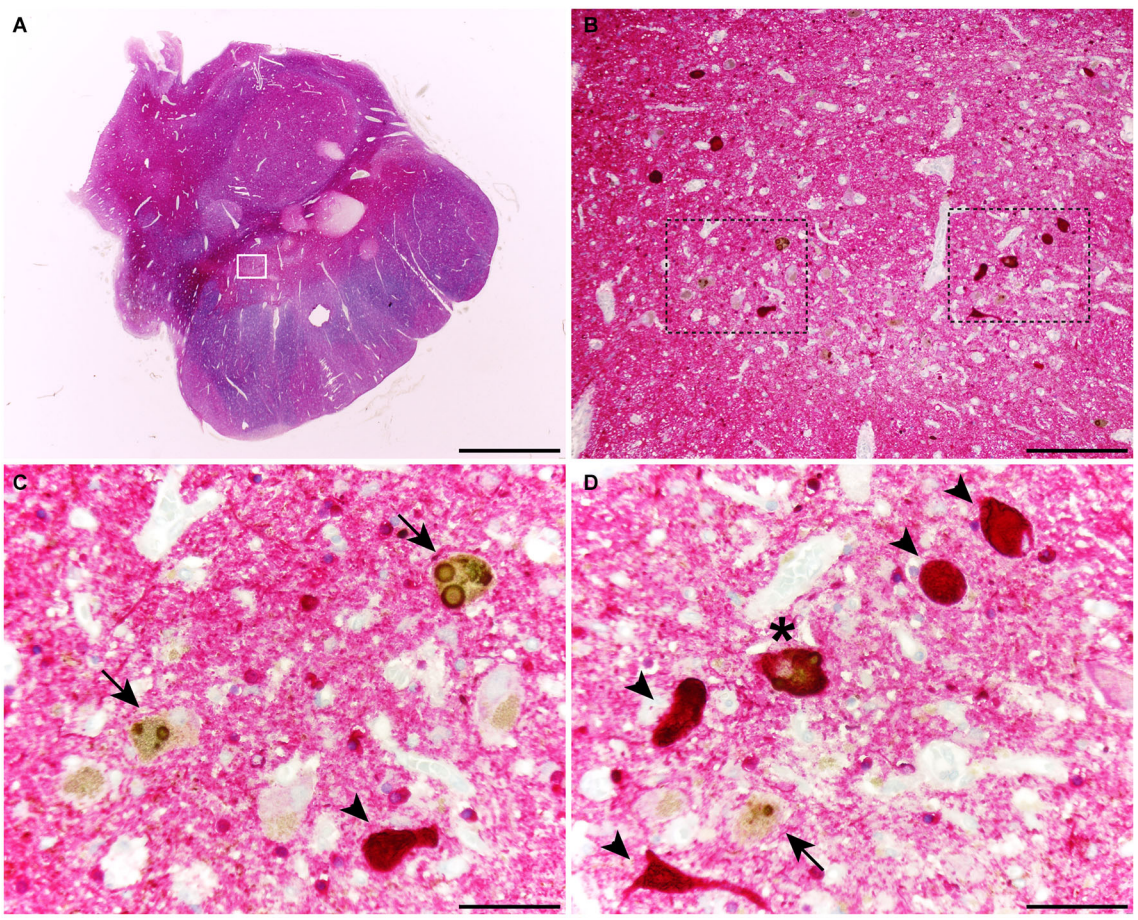

FIGURE 1 | Coronal section through the ventral mesencephalon showing the obtained dual stain for α-synuclein (TH; brown) and tau (red). At the level of the substantia nigra pars compacta (SNc), up to three different types of melanin-containing neurons were observed, comprising (i) brown-stained neurons containing Lewy bodies (LBs; arrows), (ii) red-stained neurons with tau immunoreactivity (arrowheads); and (iii) neurons showing both LBs and tau deposits (asterisks). Scale bar is 4,000 μm in **(A)**; 200 μm in **(B)** and 50 μm in the high-magnification insets shown in panels **(C)** and **(D)**.

of progressive gait unsteadiness, with postural imbalance and frequent falls reported over the previous year. She did not refer cognitive decline. Neurologic examination revealed facial inexpressiveness and mild hypophonia. Glabelar and sucking reflexes were present. Ocular motility was normal, generalized bradykinesia and rigidity were present with a rigid neck extension posture, deep tendon reflexes were brisk, gait was slow and unsteady with severe impairment of postural righting reflexes. Computed cranial tomography showed moderate cortical atrophy. A diagnosis of atypical parkinsonian disorder with early gait impairment suggestive of possible PSP was established and treatment with carbidopa/levodopa was initiated. One year later she referred impossibility in rising from a seated position and difficulty in language articulation. Her gait disorder progressed and she was wheelchair bound. Response to carbidopa/levodopa was absent. Moreover, a mild reduction of up gaze was found during the clinical examination. On subsequent visits, oculomotor disorder was established with blepharospasm, apraxia of eyelid closing and vertical gaze palsy. She died from aspiration pneumonia related to dysphagia 4 years and 2 months after the first visit.

The second patient was a 73 year old man complaining from memory loss and reduced talkativeness. Neuropsychological evaluation showed mild short-term memory loss, disturbance of executive functions, word finding difficulties and perseverative errors. The diagnosis of a lobar frontotemporal dementia was suspected. Two years later he developed progressive gait unsteadiness and frequent falls. On examination focal findings included reduction of upgaze, dysphasic language and slowness of gait with disequilibrium. A trial of carbidiopa/levodopa was unsuccessful. The diagnosis of probable PSP was suspected. On subsequent years he developed bulbar symptoms with dysartria, dysphagia and a behavior disorder with irritability, insomnia and occasional visual hallucinations (VH). He died from aspiration pneumonia 3 years and 9 months after the first visit.

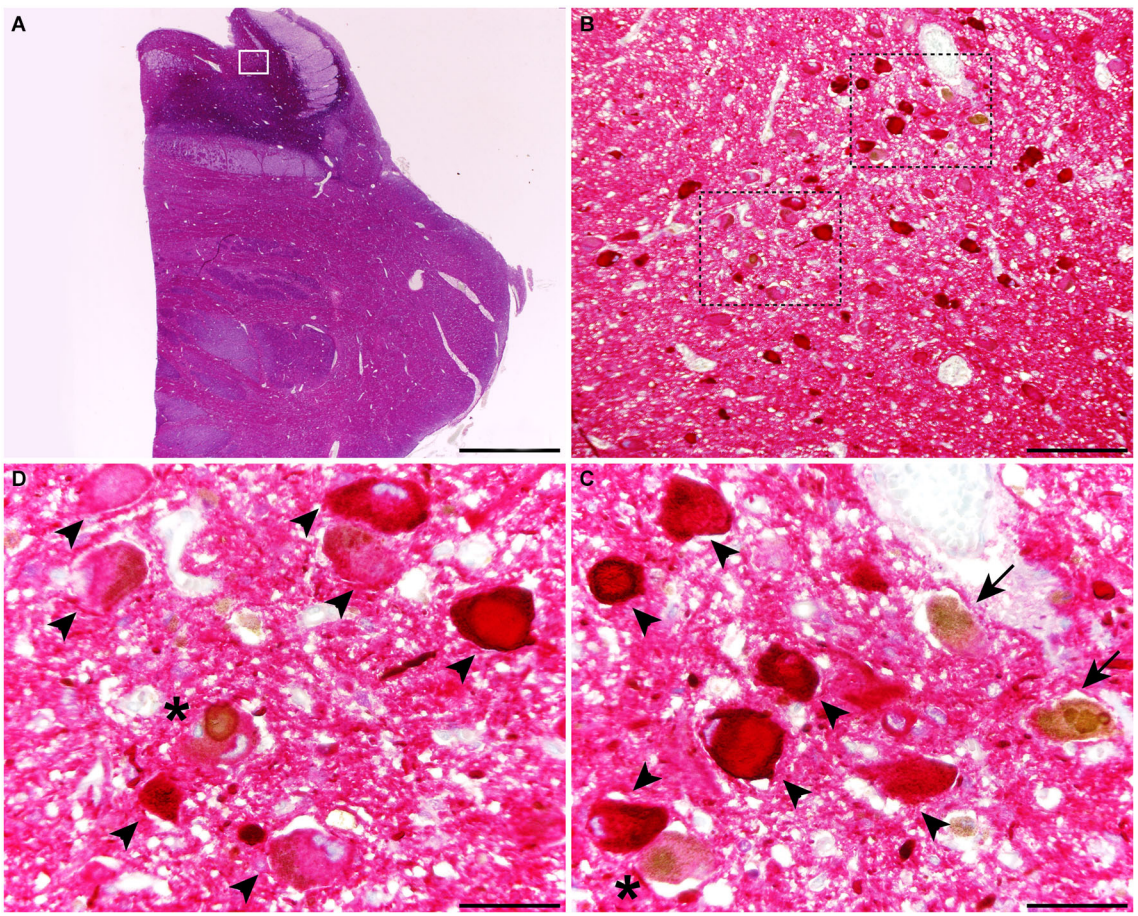

FIGURE 2 | Coronal section through the dorsal pons at the level of the Locus ceruleus (LC), stained for α-synuclein and tau. At the level of the LC, up to three different types of melanin-containing neurons were observed, comprising (i) brown-stained neurons containing Lewy bodies (LBs; arrows), (ii) red-stained neurons with tau immunoreactivity (arrowheads); and (iii) neurons showing both LBs and tau deposits (asterisks). Scale bar is 4,000 μm in **(A)**; 200 μm in **(B)** and 50 μm in the high-magnification insets shown in panels **(C)** and **(D)**.

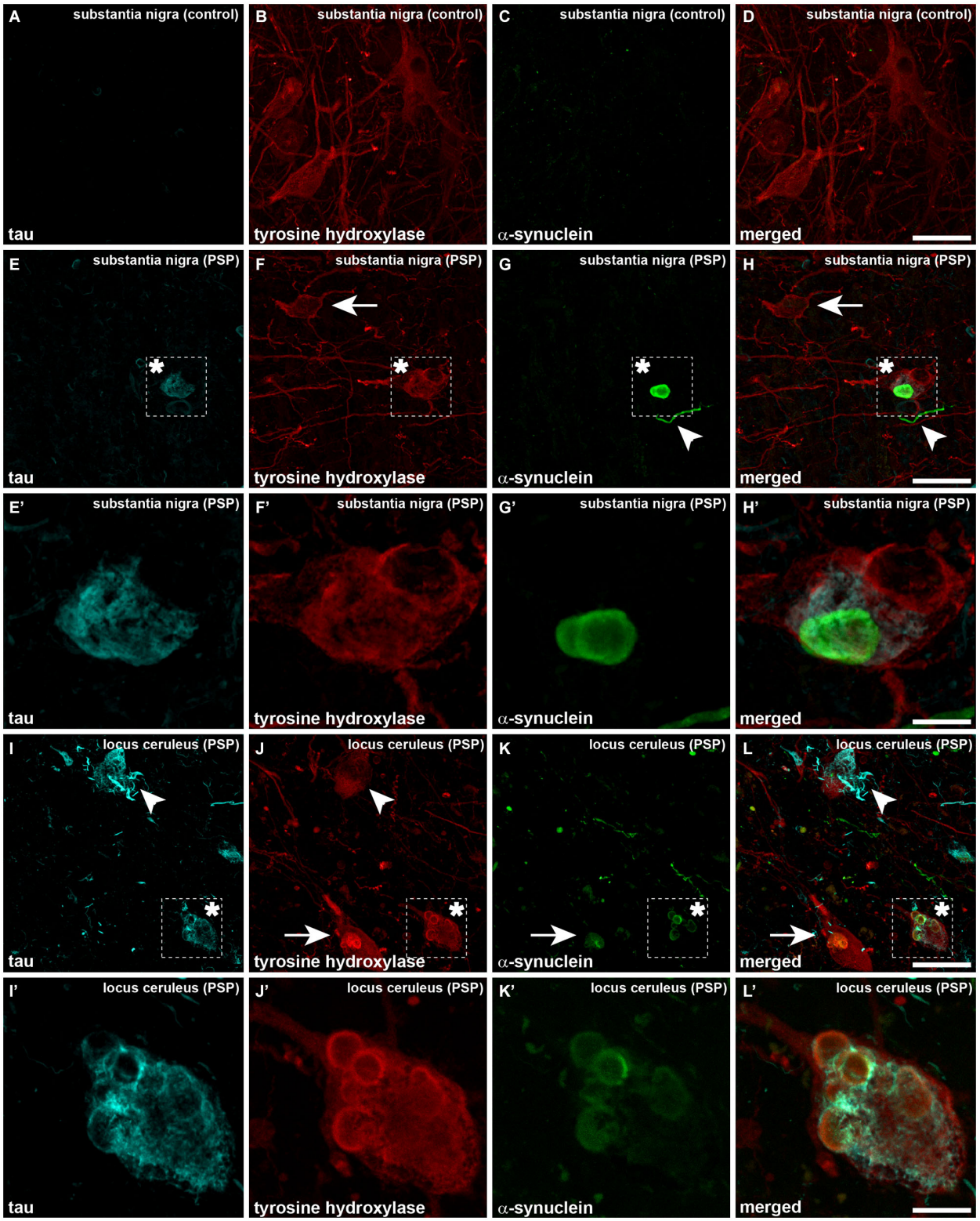

FIGURE 3 | Triple immunofluorescent detection of TH, α-synuclein and tau, as observed in a control case (A–D) as well as in the SNc and LC from a case diagnosed with PSP (E–L'). Neurons in the SNc from a control case are only immunoreactive for TH (red channel), without any noticeable immunoreactivity for tau and α-synuclein (**A** and **C**, respectively). When considering PSP cases at the level of the SNc and besides some neurons single-expressing TH (arrows), neurons containing both LBs and tau aggregates

(*Continued*)

FIGURE 3 | Continued
were clearly visible (asterisk). The presence of dystrophic neurites positive for α-synuclein is also often observed (arrowheads). The same holds true at the level of the LC. Panels **(I–L)** are low-magnification photomicrographs showing (i) TH+ neurons with tau aggregates (arrowheads), (ii) TH+ neurons with LBs (arrows); and (iii) TH+ neurons containing both LBs and tau immunoreactivity (asterisk). It is worth noting the presence of dystrophic neurites immunoreactive for either tau or α-synuclein. Panels **(I',J')** are high-magnification insets taken from **(I–L)** to better appreciate one TH+ neuron showing tau immunoreactivity and multiple LBs. Scale bar is 50 μm for low-magnification photomicrographs and 12.5 μm for insets.

Histopathological Findings

The presence of α-synuclein aggregates was only found in 2 patients from our cohort comprising a total of 25 cases available at our Navarra Biomed Brain Bank. In both cases the immunohistochemical detection of phosphorilated tau demonstrated numerous positive neuronal and glial fibrillary inclusions including tufted astrocytes and coiled bodies, as well as neuropil threads fulfilling the neuropathological criteria of PSP (Hauw et al., 1994; Litvan et al., 1996) such as dystrophic axons and neurites. The greatest concentration of tau pathology was found in the SNc and in the LC. The subthalamic nucleus, the dentate nucleus of the cerebellum and pontine nuclei displayed a moderate density of tau pathology. Mild density of tau deposits were found in the hippocampus/entorhinal cortex (without a preferential layer distribution), putamen, pallidum and amygdaloid complex. Neocortical sections showed rare tau-positive glial and neuronal inclusions.

Regarding α-synuclein deposits, protein aggregates in the form of LBs and LN were consistently found throughout the brain stem, with particular high incidence at the level of the LC in both cases, as shown in **Table 1**. Mild density of LBs and LN were found in the motor dorsal vagal nucleus, raphe nucleus and SNc. Case 1 showed moderate density of α-synuclein deposits in the entorhinal cortex and amygdale while case 2 contained scattered LBs and LN in those areas. Both cases displayed mild α-synuclein deposits in the hippocampus dentate gyrus, cingulate cortex and nucleus basalis of Meynert. No LBs nor LN were found in the subthalamic nucleus, striatum, pallidum, thalamus or neocortical regions.

When considering catecholaminergic nuclei such as the SNc and the LC, the dual colorimetric detection of tau and α-synuclein showed different types of neuromelanin-containing neurons, comprising (i) neurons with tau deposits; (ii) neurons with LBs (quite often with more than one LB); and (iii) neurons with both types of aggregates, i.e., tau and LBs (**Figures 1**, **2**). These three types of neurons were also observed in the conducted triple immunofluorescent stains, which also fully confirmed the monoaminergic nature of these neurons by showing that all these types of neurons were also positive for TH, both at the level of the SNc and the LC (**Figure 3**). Furthermore, it is also worth noting that a minimal number of TH+ neurons completely lacked any type of protein aggregates (**Figure 3**).

Discussion

Here, LBs were found in 8% of PSP brains, a percentage similar to what might be expected in series of age-matched normal controls (Tsuboi et al., 2001). The frequency of LBs in our PSP population was lower than in previous reports (**Table 2**). Besides case reports describing the incidental presence of LBs in PSP (Mori et al., 1986; Fearnley et al., 1991; Judkins et al., 2002; Abhinav et al., 2011) the percentages of LBs in necropsy series of PSP-diagnosed patients ranged between 10.7% to 31.5% (Tsuboi et al., 2001; Mori et al., 2002; Uchikado et al., 2006; Keith-Rokosh and Ang, 2008).

In the two PSP cases of the present study, LBs were widely distributed throughout the brainstem and cerebrum in a pattern that looks like what can be expected in PD. These similarities on the distribution of LBs comparing PD and PSP have also been already reported, although some controversies remain, since it has been described that LBs were only found in the amygdala of PSP patients (similarly to AD brains) and not in neither the SNc nor the LC nucleus (Tsuboi et al., 2001; Uchikado et al., 2006). The two PSP patients described in this paper presented clinical manifestations of the classical phenotype of PSP, originally described by Richardson which accounts for the most common clinical form of PSP. The Richardson's syndrome (RS) is characterized by an insidious onset and relentlessly progressive postural instability and falls, gait disturbance, supranuclear vertical gaze abnormalities, pseudobulbar palsy, rigidity in extension and a dysexecutive syndrome (Williams et al., 2005). The clinical significance of LBs cannot therefore

TABLE 2 | Studies analyzing the presence of Lewy bodies in progressive supranuclear palsy.

Reference	N (%)	α-synuclein immunohistochemistry	Doble immunolabeling techniques	Neuronal colocalization of tau and α-synuclein
Abhinav et al. (2011)	1	Yes	No	-
Keith-Rokosh and Ang (2008)	4 (12,5)	Yes	No	-
Uchikado et al. (2006)	29 (10,7)	Yes	Yes	Yes
Judkins et al. (2002)	1	Yes	No	-
Mori et al. (2002)	5 (31,5)	Yes	Yes	Yes
Tsuboi et al. (2001)	13 (12)	Yes	Yes	Yes
Gearing et al. (1994)	2	No	No	-
Fearnley et al. (1991)	1	No	No	-
Mori et al. (1986)	1	No	No	-

be fully ascertained, bearing in mind that LBs might represent an incidental finding without any clinical significance (Tsuboi et al., 2001). It is also worth noting that PSP cases with LBs previously reported in the literature had often received clinical diagnosis other than PSP (Keith-Rokosh and Ang, 2008).

The second patient developed VH, a common finding in patients with underlying LB pathology considered to be due to neuronal dysfunction specific to α-synuclein accumulation (Popescu et al., 2004). The brain of this patient contained isolated LBs in the amygdala and in the cingulated cortex. By contrast, the brain from the first patient showed a higher density of LBs in the amygdala but she did not developed VH. Although infrequently observed, VH may also be present in PSP brains without LBs (Bertram and Williams, 2012). The patient described by Compta et al. (2009) with clinical features of PD and VH and widespread phosphorylated tau deposits consistent with PSP, included severe tau pathology in the hippocampus and amygdala in the absence of LBs, therefore leading to the suggestion that the location of pathological lesions in the brain is the most important factor determining VH (Compta et al., 2009). Although it is tempting to speculate that the clinical heterogeneity seen in parkinsonian disorders may reflect the occurrence of combined pathology, it is very likely that the distribution of pathological deposits also is a key underlying factor in determining clinical differences.

The main limitation of the present study is represented by the fact that LBs were only found in two PSP patients, therefore minimizing potential clinico-pathological correlates. Nevertheless, it should be stressed that our data unequivocally showed the presence of tau and α-synuclein co-expression within single TH-positive neurons in both the SNc and the LC nuclei. Earlier data from the literature have reported tau and α-synuclein co-localization in very few neurons of the nucleus basalis of Meynert and in the LC, whereas this co-expression was never observed in the SNc (Uchikado et al., 2006). Furthermore, a kind of "globular" α-synuclein aggregates in just a few neurons with phosphorylated tau-positive cytoplasm at the level of the pontine tegmentum has also been reported elsewhere (Tsuboi et al., 2001).

The colocalization of tau and α-synuclein in the same neuron suggests an association between tau and α-synuclein aggregation that could be mediated by tau inclusions that promote the fibrillation of α-synuclein to form LBs in regions with abundant tau lesions (Popescu et al., 2004). Evidence of an interaction between tau and α-synuclein at a biochemical level has also been found. Brain α-synuclein accumulation determined by western blotting was above normal levels in the substantia nigra of some patients with PSP (Tong et al., 2010). Experimental studies have demonstrated that coincubation of tau and α-synuclein synergistically promotes fibrillization of both proteins in LBs (Giasson et al., 2003) and interactions between α-synuclein and tau at the cellular level cause disruption of cytoskeletal organization, axonal transport defects and aberrant synaptic organization that contribute to neuronal dysfunction and death (Roy and Jackson, 2014). Moreover, it is worth noting that protein aggregation develops naturally with age, although the question of whether this aggregation is cause or consequence of normal -or abnormal- aging remains to be fully elucidated (Cuanalo-Contreras et al., 2013).

Double-label studies demonstrated that in Pick disease -a 3R tauopathy-, LBs usually colocalize with tau-positive Pick bodies (Popescu et al., 2004). Regarding AD cases, in which tau is composed of a mixture of 3R and 4R tau isoforms, LBs typically colocalize with tau-positive neurofibrillary tangles especially in neuronal populations vulnerable to both neurofibrillary tangles and LBs, such as those in the LC and basal nucleus of Meynert (Ishizawa et al., 2003). Moreover, a potential α-synuclein and tau co-aggregation in neurons and neuritis from the olfactory bulb in AD patients has been more recently reported (Fujishiro et al., 2008).

We conclude that the presence of LBs in PSP patients may be viewed as a secondary phenomenon probably reflecting a synergistic effect between α-synuclein and tau. This phenomenon is more evident in populations of catecholaminergic neurons such as dopaminergic neurons in the SNc and noradrenergic neurons at the level of the LC. Finally, bearing in mind the low incidence of α-synuclein and tau co-localization in our series of PSP patients, we strongly believe that a direct clinical correlate to be induced by the co-aggregation of these two misfolded proteins is very unlikely.

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frontiers in Neuroanatomy

OPINION
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Habitual behavior and dopamine cell vulnerability in Parkinson disease

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The cardinal features of Parkinson disease (PD) often begin focally, typically in one limb, and may remain relatively restricted to one side of the body for many years. It is now well established that dopaminergic neurons in the ventro-lateral tier of the substantia nigra pars compacta (SNpc), which project mainly to the caudal putamen, are the first to degenerate in the initial phase of PD (Fearnley and Lees, 1991; Halliday et al., 2008; Blesa et al., 2010) indicating differential vulnerability. The caudal region of the striatum (dorsolateral striatum in rodents) has been associated with habitual (or automatic) behavior (Redgrave et al., 2010), consequently the differential loss of dopamine (DA) from this region provides the pathophysiological substrate for the early impairment of automatic movements (walking, writing, . . .) in early PD.

The brain has two major systems for controlling behavior: a goal directed mechanism (GD) and a mechanism mediating stimulus-response habits (**Figure 1**). The goal directed system entails conscious, voluntary control of actions aimed toward obtaining rewards or avoiding punishments. Action selection is determined by competitions between relative outcome values, i.e. if outcome A is more valuable than outcome B, then learned behavior that will lead to outcome A will be selected. Examples of goal-directed control would be: heading to the fridge or going to a restaurant when we are hungry, taking the elevator or taking the stairs back to the apartment. This goal-directed process engages the prefrontal cortex and dorsolateral striatum (Yin et al., 2004).On the other hand, the habitual system detects well-learned cues that have been associated with specific responses, and therefore elicit automatic stimulus-response behavior via the re-entrant loop that connect sensorimotor cortical areas with the posterior putamen (dorsolateral striatum in rodents) (Barnes et al., 2005). Habits are established gradually over time. They evolve after many repetitions of a task being performed under flexible goal-directed learning and depend heavily on the statistical regularities between specific stimuli and consequent responses. Examples of habitual control would be, walking, riding a bike or driving. The critical test for habits is that they are resistant to outcome-devaluation (Adams and Dickinson, 1981). Inappropriate habitual responses are frequently difficult to eradicate and have to be corrected by goal-directed interventions after they fail to achieve their original intention. In this Opinion article we put forward the hypothesis that a significant factor that confers vulnerability to the ventro-lateral tier of SNpc at the onset of PD may reside in the key functional role that these neurons play in the performance of habitual behavior, switching between habitual and goal-directed control, and engaging both goal-directed and habitual control when carrying out multiple tasks simultaneously.

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Functional Anatomy of Habitual vs. Goal Directed Behavior

The nigro-striatal system has two major components, the associative loop and the motor loop. The associative loop comprises the dorso-medial SNpc that projects to the head of the caudate

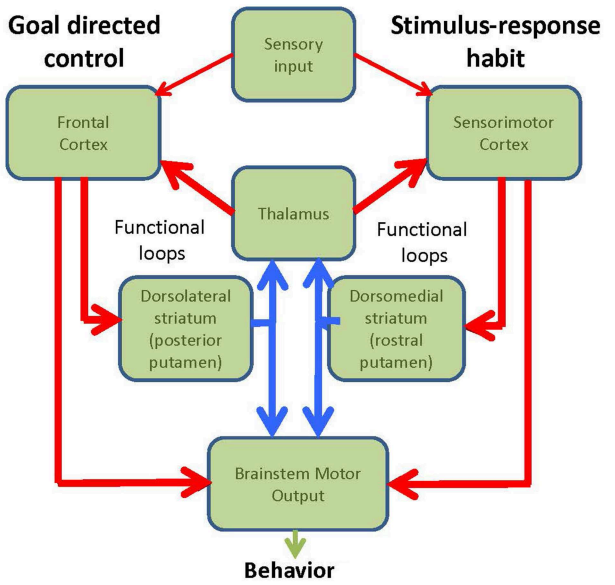

FIGURE 1 | Diagram of the functional loops involved in goal-directed and habitual behavior.

and rostral putamen, regions that have been associated with goal-directed behavior and executive functions (Yin and Knowlton, 2006). On the other hand, the ventro-lateral SNpc projects to the posterior putamen, which engages the sensorimotor circuits and habitual performance (Jog et al., 1999; Haber et al., 2000; Packard and Knowlton, 2002; Redgrave et al., 2010).

As humans, much of our thinking in daily life is made possible by engaging automatic or habitual control, e.g., walking, typing, etc…In fact, depending on the predictability of what we are doing, we frequently switch back and forth between goal-directed to habitual control. Goal-directed cognition (e.g. listening, talking) is often performed simultaneously while carrying out predictable sensorimotor tasks under habitual control (e.g. making tea, driving). Social interactions are characterized by the need to perform simultaneous and sequential activities while attending to multiple stimuli. We suggest that multi-tasking in humans could be an important vulnerability factor that puts the ventro-lateral subpopulation of dopaminergic neurons at greater pathological risk. Our hypothesis is that switching in and out of habitual control requires an unusually demanding anatomo-physiological network, which makes the dopaminergic neurons servicing sensorimotor territories of the basal ganglia especially vulnerable. We will argue that this represents a key factor for the neuronal degeneration associated with PD. As the ventro-lateral SNpc/caudal putamen (habitual system) becomes dysfunctional in PD, the goal-directed system has to be recruited to perform the previously automatic habitual tasks. This compensatory mechanism could have a "double-sword" effect by shifting the vulnerability to the now overloaded dopaminergic neurons in dorso-medial SNpc. This process could further propagate the neurodegeneration (Halliday and McCann, 2010).

Factors Associated with SNpc Degeneration

Several studies analyse and discuss the nigro-striatal features that have been associated with potential degeneration of dopaminergic SNpc cells in PD (Hirsch et al., 2013; Sulzer and Surmeier, 2013). However, most of the identified characteristics are shared with the more medially located DA cells that seem to be less vulnerable in PD. Here we summarize some of the more relevant anatomo-functional markers that have been suggested to confer vulnerability to nigro-striatal neurodegeneration, which is the preferential and initial site of significant neuronal death in PD. We acknowledge the major current interest in the evolution of synuclein deposits (typically giving rise to Lewy bodies) in different regions of the nervous system. However, the actual significance of such aggregates to explain symptoms and to cause neurodegeneration in PD is unsettled yet (van de Berg et al., 2012).

Neuromelanin

Interestingly, mammals generally show few melanized dopaminergic cells, which become more prominent in primate species and are particularly abundant in the human mesencephalon (Herrero et al., 1993). However, the presence of neuromelanin *per se* seems not to account for the specific vulnerability pattern of ventro-lateral tier, because not all brainstem (or anywhere else) pigmented neurons die in early PD (Gibb, 1992; Fedorow et al., 2005).

Mitochondrial Stress

Dopaminergic neurons are under a high mitochondrial oxidant stress (Surmeier et al., 2011). In fact, calcium entry through L-type channels during autonomous spiking, which characterizes this population, increases the vulnerability of SNpc dopaminergic neurons to the toxins 6-OHDA and MPTP; substances that are used to create animal models of PD (Chan et al., 2007). Thus, reduced complex I mitochondrial activity and elevated oxidant stress (Guzman et al., 2009) is a likely important factor in the PD-related pathogenesis of SNpc (Schapira, 2008; Blesa et al., 2015).

DA and Vesicular Transporters

The activity and distribution of the synaptic dopamine transport (DAT) protein represents an entry for neurotoxic substances such MPTP and 6-OHDA (Dauer and Przedborski, 2003). DAT shows a dorso-ventral gradient with the expression of higher levels of glycosylated (mature, highly functional) protein found in the vulnerable ventral SNpc (Reyes et al., 2013). However, these neurons did not show a uniform expression of glycosylated DAT (Reyes et al., 2013). Together with the fact that other midbrain areas show glycosylated DAT, makes it unclear how these small expression differences could account for the differential vulnerability observed between SNpc and VTA neurons or between dorsal and ventral SNpc DA neurons (Gonzalez-Hernandez et al., 2004). Alternatively, another possible vulnerability feature for these neurons is their decreased vesicular accumulation of DA57 (Liang et al., 1996;

Damier et al., 1999) and reduced levels of VMAT2 (Pifl et al., 2014). Failure to store DA into pre-synaptic vesicles appropriately would lead to higher cytoplasm levels of free dopamine and formation of cytotoxic free radicals. However, the extent to which this factor is able to account for the regional vulnerability of ventral midbrain DA neurons remains to be established.

Dopaminergic Striatal Axonal Arborization

The nigro-striatal projection exhibits one of the highest levels of divergent arborization (Matsuda et al., 2009; Bolam and Pissadaki, 2012). Thus, it has been estimated that a DA neuron that terminates in the rodent dorsal striatum has 102.165–245.103 synapses, while the number of synapse associated with corresponding neurons from the VTA are in the range of 12.351–29.644 (Bolam and Pissadaki, 2012). Importantly, in humans, the increase in the numbers of DA neurons (12.000 in rats vs. 382.000 in humans) has not kept pace with the striatal volume that these neurons innervate *(Vol str in* mm^3*: rats: 19.9*[30]*; humans: 6,280*[43]*)* (Bolam and Pissadaki, 2012). The inference is that the degree of divergence in humans must therefore be even greater, thereby creating a substantially greater metabolic and proteostatic load on the human DA cells that innervate the sensorimotor striatum (Dryanovski et al., 2013). It remains to be ascertained if the latero-medial gradient of neuronal loss exhibited in the SNpc in PD is paralleled by degree of arborization.

PD Etiopathogenesis Is Multifactorial

While the differential features summarized above may play a significant role in nigro-striatal neurodegeneration, current evidence strongly indicates a multi-factorial origin for PD. Thus, mutation of single genes (i.e. parkin, LRRK-2, DJ-1, synuclein) can lead to DA neuronal loss (with or without Lewy body aggregates), glucocerebrosidase (GBA) expression correlates not only with the risk of developing PD (Beavan and Schapira, 2013) but also with its progression (Brockmann et al., 2015). Moreover, several genetic loci have been associated with increased risk of developing PD (Nalls et al., 2014). Finally, several environmental, life-style habits and toxic exposure have also been associated with higher or lesser risk of developing PD (Ross and Abbott, 2014; Tanner et al., 2014; Tanner and Comella, 2015). Accordingly, it is unlikely that the origin of neurodegeneration in PD could be tight to a single pathogenic mechanism or event.

Here, we would like to stress some clinical observations. Thus, that at the onset of PD the neuronal cell loss is highly asymmetrical, and mainly affecting one unilateral sub-group of DA neurons that innervate sensorimotor territories of the caudal putamen. As a consequence, the motor deficit is typically restricted to just one body part. These specificities are difficult to explain by changes in cellular markers that are widespread and feature in a high proportion of neurons.

A more integrative explanation that might bridge the gap between specific molecular abnormalities and the selective vulnerability of SNpc is to consider the functional anatomy of the nigro-striatal system as a significant risk factor. Our suggestion is that the early loss of ventro-lateral tier neurons could be determined by their role in the acquisition and control of automatic movements, and the resultant switching between goal-directed and habitual modes. This suggestion is supported by recent data where it has been reported that DA neurons are engaged by multiple events in tasks that have cognitive and sensorimotor components (Matsumoto and Takada, 2013), in addition to events associated with reward prediction (Schultz et al., 1997). It seems then, that DA neurons can be subdivided into functionally separate subpopulations; the ones specially coding reward prediction errors that are concentrated medially in the VTA, and neurons located in lateral SNpc that are responsive to a much wider range of salient sensory events, including to those associated with reward (Matsumoto and Hikosaka, 2009; Matsumoto and Takada, 2013). In rodents, these data are supported by observations that SNpc neurons also signal the initiation or termination of self-paced sequential behavior (Jin and Costa, 2010). This start/stop related activity emerged with learning, was specific for particular actions, and did not reflect timing or movement speed related actions. These data further support our hypothesis that, ventro-lateral DA neurons are sensitive to and activated by numerous aspects of action performance, learning and task switching. This more frequent pattern of activation of lateral SNpc neurons could represent an additional if not fundamental metabolic load that, associated with the features summarized above, could confer specific vulnerability to these cells.

Parkinson's Disease as a Consequence of Human Behavior

Early in life, humans undergo an extended period of learning, during which a wide range sensorimotor, perceptual, cognitive and social skills, are acquired. Many of these skills have repetitive components, so after the first few decades, much of our behavior contains embedded fragments of automatic stimulus-response control. Many of these naturalistic automatic 'chunks' would, in a laboratory setting, turn out to satisfy formal criteria for stimulus-response habits—comparative insensitivity to outcome devaluation (Adams and Dickinson, 1981). An important consequence of establishing automatic habits is that it affords the possibility of undertaking multiple tasks simultaneously. These may consist of two or more automatic actions (walking and chewing gum), or a combination of an automatic and a goal directed action (walking and talking).

Becoming bipedal, thereby freeing the upper extremities to manipulate, allowed independent cognitive functions while doing things on the move. Consequently, the motor system in general, and the cortico-basal ganglia loops in particular, adapted to accommodate parallel processing. Dopaminergic activity, with its ability to modulate striatal excitability, plays an essential role in the initiation and expressions of self-paced stimulus-response. Accordingly, the dopaminergic nigro-striatal system becomes under increasing demand, especially the portion regulating habitual behavior. As a result, we believe, PD is the pathological result of increased functional demand on the nigro-striatal system produced by the evolutionary imperative for multitasking. Until recently this increased load had no apparent

negative consequences, perhaps because life expectancy was short and below the risk age for developing PD. However, this has changed drastically, and the anticipated increase in future life expectancy is likely only to exacerbate this situation.

Thinking ahead, we realize that an ultimate research goal would be to replicate or simulate comparable levels of multitasking in suitable animal models to test the proposed hypothesis. This will pose a significant experimental challenge. However, our specific aim will be to establish non-human primate and rodent models in which ventro-lateral dopaminergic neurons are put under long-term functional stress.

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frontiers in Neuroanatomy

GENERAL COMMENTARY
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Corrigendum: Habitual Behavior and Dopamine Cell Vulnerability in Parkinson Disease

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A corrigendum on

Habitual behavior and dopamine cell vulnerability in Parkinson disease
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Figure 1 of the article by Hernandez et al. (2015) contained a minor error, which we hereby rectify. In the original figure dorsolateral striatum (posterior putamen) is shown as part of the goal-directed loop while dorsomedial striatum (rostral putamen) is shown as part of the habitual loop. These boxes are switched. We therefore re-submit **Figure 1** with the correct boxes in the middle panel.

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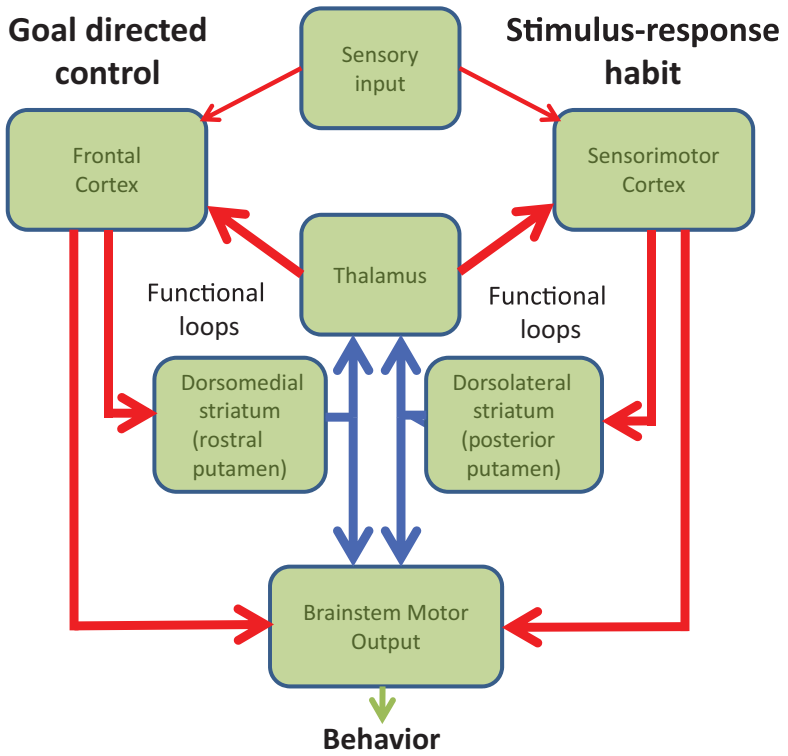

FIGURE 1 | Diagram of the functional loops involved in goal-directed and habitual behavior.