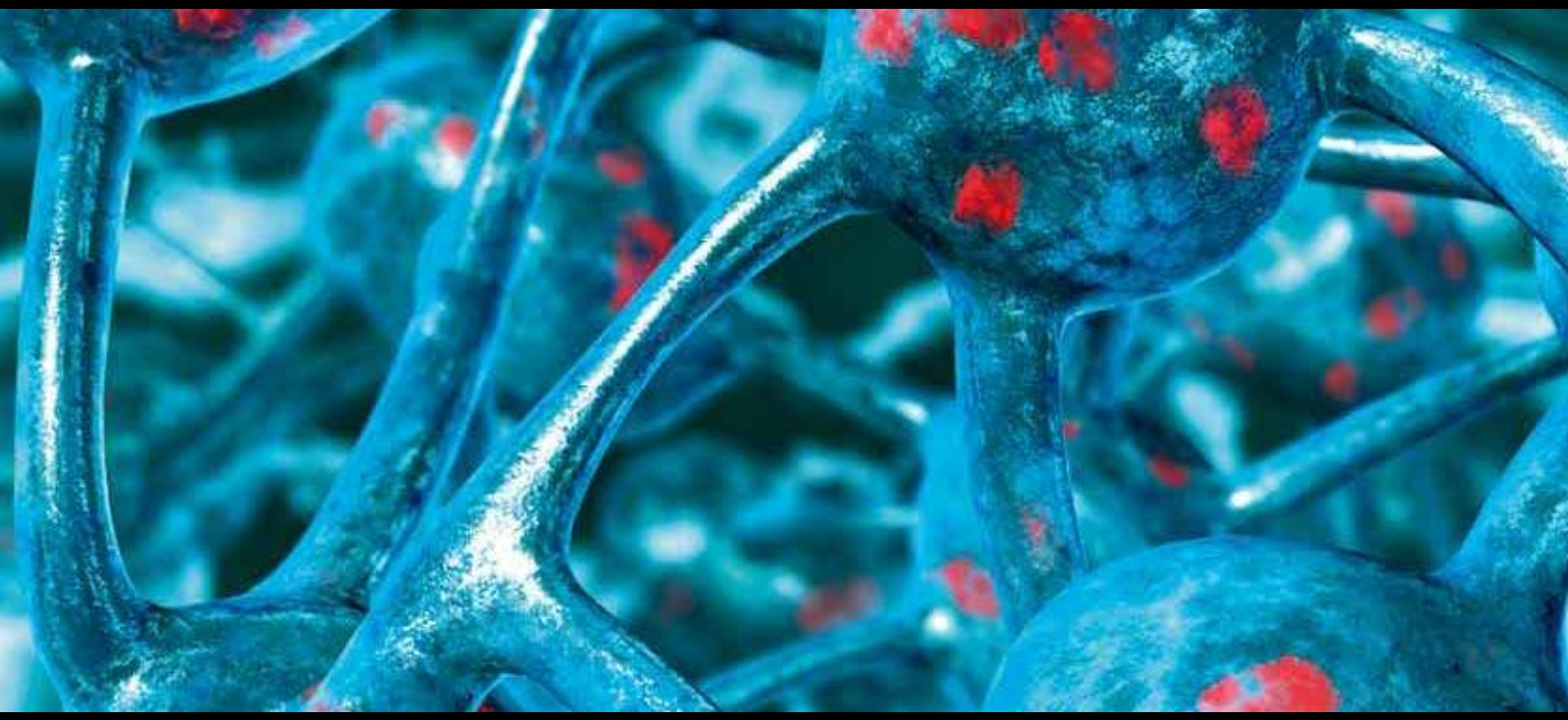
Animal Models of Parkinson's Disease

Guest Editors: Yuzuru Imai, Katerina Venderova, David S. Park, Huaibin Cai, and Enrico Schmidt



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Editorial Board

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Editorial

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Parkinson's disease (PD) is considered a multifactorial disorder, which is neuropathologically characterized by age-dependent neurodegeneration of dopaminergic neurons in the midbrain. Different neurotoxins including synthetic compounds, heavy metals, and dopamine itself have been proposed to be environmental risk factors of PD. Recent genome-wide genetic and mutational studies provide information on various genetic risk factors while microglial activation in the affected regions has emerged to be involved in the disease development as a local microenvironmental factor. A wide variety of animal models of PD substantially contribute to the understanding of these issues and the development of therapeutic approaches as an alternative to humans although none of them fully recaptures the symptoms and pathology of PD. This special issue is composed of 9 excellent reviews and 3 distinguished original articles that summarize the most recent progresses and ideas obtained from animal models in the pertinent field, while reporting the putative molecular mechanisms of neurodegeneration, therapeutic challenges and limitations using PD models, and generation of new versions of PD models.

The first review paper briefly outlines animal models of PD, covering toxin-induced and genetic models of vertebrate and invertebrate animals, in which characteristic features of each model are discussed.

Mishandling of monoamines including dopamine has been hypothesized to damage neurons. The second review paper describes mice with impaired functions of the vesicular monoamine transporter VMAT2, in which progressive loss of catecholamine-secreting neurons is observed. Such models may be potentially useful for the development of new therapeutic strategies, which would complement current dopamine replacement.

Neuropathological analysis of the postmortem PD brain tissues suggests that an adverse interaction with surrounding glia and other nonneuronal cells may be one of critical steps in neurodegeneration. The third review highlights endotoxin-induced inflammation models, in which activation of microglia and lymphocyte by a bacterial lipopolysaccharide deteriorates a healthy relationship with neurons.

Mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) gene have been identified to cause autosomal-dominant late-onset PD and are also implicated in sporadic PD. The neuropathological features of PD brain tissues with the *LRRK2* mutations are characterized by typical Lewy body pathology in the brainstem. The forth paper reviews a variety of *LRRK2*-related models.

Mutations and increased expression in the *α-synuclein* gene cause the development of early-onset familial PD. The formation of *α-synuclein* fibrils and aggregates, a main component of Lewy bodies and Lewy neurites, is considered a key process in the pathogenesis of PD and other synucleinophathies. Other genetic determinants include the genes for Mendelian forms of PD and susceptible genes. The following two papers focus on the potential of *Drosophila* genetic models to examine *α-synuclein* and other responsible genes.

Deep brain stimulation (DBS) by electrical pulses could be one of useful therapeutic avenues for PD. However, DBS's technique requires advancement and poor understanding of the mechanisms involved hinder application in clinical practice. The seventh review paper discusses the optimization of a rat PD model for DBS.

Hydrogen has turned out to reduce oxidative damage. The eighth paper introduces the neuroprotective effects of hydrogen on experimental animal models for PD and possible application in treatment and prevention of PD.

The last review explains the limitations of animal models, showing differences between humans and animals, and difficulties in interpretation of obtained results with animal models.

The first research paper investigates selective degeneration of dopaminergic neurons in the substantia nigra and associated motor dysfunction induced by inhalation of mixed manganese compounds on mice. This model could be instrumental for evaluating some aspects of a progressive loss of dopaminergic neurons. The second research paper examines the possible effects of testosterone on PD using a mouse model induced by 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration. The study suggests that loss of testosterone induces remodeling in the morphology of medium spiny neurons where dopaminergic neurons of the substantia nigra project although no interaction between testosterone and loss of dopaminergic neurons by MPTP administration is observed. The third research paper of this special issue addresses improvement of potential gene therapy to compensate for impaired complex I activity of the mitochondria using the yeast single-subunit NADH-ubiquinone oxidoreductase, NDI1. NDI1 is functionally able to replace complex I, activity of which is thought to be compromised in most of PD cases.

A decreased sense of smell is one of early signs of PD. Although degeneration of tyrosine hydroxylase-positive neurons in the olfactory bulbs is observed, the pathogenic mechanism underlying olfactory deficits is not well understood. The forth research paper addresses this issue using a rat model bearing the pathogenic α-synuclein.

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Review Article

Toxin-Induced and Genetic Animal Models of Parkinson's Disease

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Parkinson's disease (PD) is a common progressive neurodegenerative disorder. The major pathological hallmarks of PD are the selective loss of nigrostriatal dopaminergic neurons and the presence of intraneuronal aggregates termed Lewy bodies (LBs), but the pathophysiological mechanisms are not fully understood. Epidemiologically, environmental neurotoxins such as pesticides are promising candidates for causative factors of PD. Oxidative stress and mitochondrial dysfunction induced by these toxins could contribute to the progression of PD. While most cases of PD are sporadic, specific mutations in genes that cause familial forms of PD have led to provide new insights into its pathogenesis. This paper focuses on animal models of both toxin-induced and genetically determined PD that have provided significant insight for understanding this disease. We also discuss the validity, benefits, and limitations of representative models.

1. Introduction

Parkinson's disease (PD) is one of the most common chronic neurodegenerative disorders. It is characterized by a variety of motor (bradykinesia, rigidity, tremor, and postural instability) and nonmotor (autonomic disturbances and psychosis) symptoms. Although it can be diagnosed accurately, no therapeutic strategies can cure or completely block the progression of PD. Pathologically, PD is characterized by the severe loss of dopaminergic (DAergic) neurons in the pars-compacta nigra and the presence of proteinaceous α-synuclein inclusions, called Lewy bodies (LBs), which are present in neurons of the central nervous system (specific cortical regions, brain stem, and spinal cord), peripheral autonomic nervous system, enteric nervous system (ENS), and cutaneous nerves [1–3]. Similar to other neurodegenerative diseases, such as Alzheimer's disease, age is the major risk factor for PD although 10% of the people with the disease are younger than 45.

Although PD is regarded as a sporadic disorder, remarkably few environmental causes or triggers have been identified [4–6]. Pesticides and herbicides are the most likely candidates for environmental agents associated with the pathogenesis of PD. On the other hand, PD characteristics are seen in a number of familial motor disorders caused by different genetic factors. Animal models of neurodegenerative diseases, including PD, have in general been quite instructive in understanding their pathogenesis. Ideally, animal models of PD, whether induced by environmental risk factors (neurotoxins) or genetic manipulations, should faithfully reproduce the clinical manifestations (behavioral abnormalities), pathological features, and molecular dysfunctions characterizing the disease. Unfortunately, animal models rarely mimic the etiology, progression, and pathology of PD completely, and in most cases, only partial insight can be gained from these studies. Despite these difficulties, animal models are considered to be very helpful in the development of therapies to treat PD. In this paper, we discuss recently developed neurotoxin-induced and genetic model animals of PD.

2. Animal Models of PD Induced by Neurotoxins

PD is currently viewed as a multifactorial disease. Environmental exposures, particularly to pesticides, are thought to be involved in the pathogenesis of sporadic PD. Specifically, the herbicide Paraquat (PQ) and the fungicide Maneb (MB; manganese ethylene-bis-dithiocarbamate) have been associated with the incidence of PD [7, 8]. However, a causal

role for pesticides in the etiology of PD has yet to be definitively established. In animal models, PD-like disorders induced by neurotoxins or other chemical compounds have led to a better understanding of the pathophysiology of PD (Table 1).

3. 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)

In 1979 and 1983, MPTP was initially identified as a strong neurotoxin when heroin addicts accidentally self-administered MPTP and developed an acute form of parkinsonism that was indistinguishable from idiopathic PD [9, 10]. A detailed neuropathological study of MPTP-induced parkinsonism in humans showed severe neuronal degeneration in the substantia nigra and the absence of LBs [11]. The lack of LBs may have reflected the age of the patient and the duration of exposure to MPTP. The tragic results of MPTP poisoning in the heroin addicts led to the development of MPTP-induced rodent and nonhuman primate animal models of PD, which have proved extremely valuable [12–16]. The MPTP-exposed primates show good response to therapy with L-3,4-dihydroxy-L-phenylalanine (L-DOPA) and dopamine (DA) receptor agonists [15, 16]. However, rats are relatively insensitive to MPTP neurotoxicity compared with primates. Rats given MPTP at doses comparable to those used in mice do not show remarkable neurodegeneration [17, 18]. Only high doses of MPTP cause DAergic neurodegeneration in rats, indicating that complete blockade of the DA receptors is required for them to display signs of parkinsonism. Mice, like rats, are also less sensitive to MPTP than primates [19, 20].

This model also shows pathological changes in the ENS, as observed in PD. In PD, gastrointestinal (GI) dysfunction was hypothesized to depend on neuronal degeneration in the ENS that is similar to that seen in the CNS. Recent studies show that the administration of MPTP results in decreased tyrosine hydroxylase- (TH-) positive enteric neurons in mice, indicating that the MPTP model mice should be suitable for understanding the extranigral pathophysiology of PD [21, 22].

4. 6-Hydroxy-Dopamine (6-OHDA)

Like MPTP, 6-OHDA is a neurotoxin that has been successfully used in induction animal models of PD. 6-OHDA's strong neurotoxic effects were described by Ungerstedt in 1971, in a study presenting the first example of using a chemical agent to produce an animal model of PD [23]. Since 6-OHDA cannot cross the blood-brain barrier (BBB), systemic administration fails to induce parkinsonism. This induction model requires 6-OHDA to be injected into the substantia nigra, medial forebrain bundle, and striatum [24, 25]. The effects resemble those in the acute MPTP model, causing neuronal death over a brief time course (12 hours to 2-3 days).

Interestingly, the intrastriatal injection of 6-OHDA causes progressive retrograde neuronal degeneration in the substantia nigra and ventral tegmental complex (ST-VTA) [25–27]. As in PD, DAergic neurons are killed, and the non-DAergic neurons are preserved. However LBs do not form. Typically, 6-OHDA is used as a hemiparkinson model, in which its unilateral injection into the substantia nigra causes asymmetric motor behavior (turning, rotation) when apomorphine, a DAergic receptor agonist, or amphetamine, a dopamine releasing agent, is given systemically. In this model, the quantifiable motor behavior is a major advantage for screening pharmacological screening agents for their effects on the DAergic system and for testing cell replacement therapies [28–30].

5. Rotenone

Rotenone is a naturally occurring complex ketone pesticide derived from the roots of *Lonchocarpus* species. It can rapidly cross cellular membranes without the aid of transporters, including the BBB. Rotenone is a strong inhibitor of complex I, which is located at the inner mitochondrial membrane and protrudes into the matrix.

In 2000, Betarbet et al. demonstrated in rats that chronic systemic exposure to rotenone causes many features of PD, including nigrostriatal DAergic degeneration [31]. Importantly, pathological features match those seen in typical PD. For example, many of the degenerating neurons have intracellular inclusions that are morphologically similar to LBs. These inclusions also show immunoreactivity for α-synuclein and ubiquitin, like true LBs [31, 32]. The rotenone-administered model animals also reproduce all the behavioral and pathological features seen in the typical form of human PD. However, rotenone-injected rats without nigrostriatal DAergic neuronal loss demonstrate the same abnormal motor behaviors as those with such pathological features [32, 33]. This finding suggested that the abnormal behaviors of PD could depend, at least partly, on the damage to non-DAergic neurons in the nigrostriatal area. Furthermore, rotenone exposure also causes the loss of myenteric neurons in the rat [34].

6. Paraquat and Maneb

Because of its close structural similarity to 1-methyl-4-phenylpyridinium (MPP^+, the active metabolite form of MPTP), an herbicide, 1,1′-dimethyl-4,4′-bipyridinium, named paraquat has been suggested as a risk factor for PD [35]. The systemic administration of paraquat to adult mice results in a significant decrease in substantia nigra DAergic neurons, a decline in striatal dopamine nerve terminal density, and a neurobehavioral syndrome characterized by reduced ambulatory activity [36]. These data support the idea that paraquat crosses the BBB to cause destruction of the dopamine neurons in the substantia nigra, like MPP^+ [36]. The prolonged exposure to paraquat leads to a remarkable accumulation of α-synuclein-like aggregates in neurons of the substantia nigra pars compacta in mice [37]. Chronic exposure to paraquat also reduces the expression of the nicotinic acetylcholine receptor (nAChR) subunit $\alpha 3/\alpha 6\beta 2^*$

Table 1: Representative neurotoxin-induced mammalian models of Parkinson's disease.

Neurotoxin	Behavioral and pathological features	Molecular mechanisms
MPTP	(1) Parkinsonism (akinesia, rigidity, and tremor) with acute onset (2) Relatively less potent in rodents (3) Good response to L-DOPA and DA-agonists (4) Loss of TH-neurons (-fibers) and DA-content in nigrostriatal region (5) Loss of TH-neurons (-fibers) in ENS (6) α-Synuclein-positive inclusions (7) No typical LBs	(1) Easily crosses the BBB (2) Converted to MPP^+ in glial cells (3) Transferred into mitochondria by transporters (4) Inhibits electron transport chain complex I (5) Upregulation of iNOS, NADPH-oxidase, and ROS (6) Microglial activation
6-OHDA	(1) Intracerebral administration (2) Quantifiable locomotor abnormalities (rotation, akinsesia) (3) Good response to L-DOPA and DA-agonists (4) Loss of TH-neurons (-fibers) and DA-content in nigrostriatal region (5) No typical LBs	(1) Transferred into mitochondria by transporters (2) Inhibits electron transport chain complex I (3) Microglial activation
Rotenone	(1) Parkinsonism (bradykinesia, fixed posture, and rigidity) (2) Good response to L-DOPA and DA-agonists (3) Loss of TH-neurons (-fibers) and DA-content in nigrostriatal region (4) α-Synuclein-positive inclusions, resemblance to true LBs (5) Loss of myenteric neurons	(1) Easily crosses the BBB (2) Inhibits electron transport chain complex I (3) Upregulation of NADPH-oxidase (4) Microglial activation
Paraquat (+ Maneb)	(1) Parkinsonism similar to that of induced by MPTP (2) Loss of DA-content in nigrostriatal region (3) α-Synuclein-positive inclusions with long exposure	(1) Crosses the BBB by neutral amino acid transporter (2) Inhibits electron transport chain complex I (3) Reduction of nAchR-mediated DA release (4) Inhibits complex III (Maneb)

MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA: 6-hydroxy-dopamine; L-DOPA: L-3,4-dihydroxy-L-phenylalanine; TH: tyrosine hydroxylase; DA: dopamine; ENS: enteric nervous system; LB: Lewy body; BBB: blood-brain barrier; MPP^+: 1-methyl-4-phenylpyridinium; iNOS: inducible nitric oxide synthase; ROS: reactive oxygen species; nAchR: nicotinic acetylcholine receptor.

(the asterisk indicates the possible presence of additional subunits). Normally, the activation of both nAChR subtypes stimulates DA release in the striatum [38–40]. The injection of paraquat selectively reduces the $\alpha 3/\alpha 6\beta 2^*$-mediated DA release from the striatum in primates [41].

Manganese ethylenebis-dithiocarbamate (Maneb) is an organomanganese fungicide that is broadly used in agriculture and is a putative causative agent for PD. Surprisingly, Thiruchelvam et al. found that the neurotoxic effects of maneb or paraquat on the nigrostriatal DA system in mice are synergistically potentiated in combination [42]. Their report argued that this finding has important implications for the human risk of PD, because the marked geographical overlap in the estimated annual agricultural applications of paraquat and maneb means that people living in these areas may be exposed to the synergistic neurotoxicity of these two agents [42, 43].

7. Pathophysiological Mechanisms of DAergic Neurotoxins

All the representative neurotoxin-induced PD models described above show defective mitochondrial function, manifested by the inhibition of mitochondrial complex I or III. MPTP is a highly lipophilic agent. After its systemic administration, MPTP rapidly crosses the BBB. Once in the brain, MPTP is converted to 1-methyl-4-phenyl-2,3-dihydropyridium ($MPDP^+$) in glial cells (astrocytes) and serotonin neurons by monoamine oxidase B (MAO-B) and then spontaneously oxidizes to MPP^+ [44, 45]. Thereafter, MPP^+ is released into the extracellular space. Unlike MPTP, MPP^+ is a polar molecule that cannot freely enter DAergic neurons. Thus, a plasma membrane transport system is required. MPP^+ has a high affinity for dopamine transporter (DAT) as well as for norepinephrine and serotonin transporters [46, 47]. Once inside DAergic neurons, MPP^+ can accumulate in mitochondria and impair mitochondrial respiration by inhibiting complex I in the electron transport chain [44, 48], which induces the generation of reactive oxygen species (ROS). MPP^+ can also bind to vesicular monoamine transporters (VMATs), which help move selected materials into synaptic vesicles containing DA [49]. MPP^+ can also remain in the cytoplasm and interact with cytosolic enzymes [50].

Inducible nitric oxide synthase (iNOS) is also involved in the pathogenesis of MPP^+-induced parkinsonism in animal models. Increased iNOS has also been found in the substantia nigra of autopsied PD patients, indicating that NO overproduction is a feature of the human disease

[51, 52]. Excess NO could contribute to the formation of free radicals, which could damage DAergic neurons, leading to the development of PD symptoms. Mice null for iNOS show a resistance to neuronal damage by MPTP, and iNOS inhibitors protect against the degeneration of DAergic neurons in MPTP-treated mice [53, 54]. Furthermore, microglial cells can be activated by the formation of free radicals and iNOS-mediated damage, and thereby exacerbate the toxicity of MPTP [55–57]. Finally, MPTP can also upregulate NADPH-oxidase in the substantia nigra of mice [56], which is significant because NADPH-oxidase appears to be ubiquitously expressed in all brain regions and metabolizes molecular oxygen, generating superoxide as a product. In fact, MPTP toxicity is diminished in mice lacking functional NADPH-oxidase, indicating a pivotal role for superoxide ions in the neurotoxicity induced by MPTP [56].

The toxicity of 6-OHDA also involves mechanisms of oxidative stress. 6-OHDA can be taken up by DAergic neurons through DAT [58, 59]. Once transported into neurons, 6-OHDA is oxidized like DA. The oxidized molecule generates free radicals inhibits mitochondrial complex I and produces superoxide and hydroxyl radicals [58, 59]. It is not only toxic to the DAergic neurons but can also induce microglial activation [59].

Like MPTP, the pesticide rotenone is very lipophilic, crosses the BBB, and is distributed evenly throughout the brain [59, 60]. It can enter mitochondria, where it inhibits complex I of the electron transport chain with high affinity [59]. Interestingly, the inhibition of microglial activation by an antibiotic, minocycline, can attenuate the neurotoxicity of rotenone [61]. Gao et al. also showed that the neurotoxicity of rotenone is reduced in neuron-glia cocultures from NADPH oxidase-null mice [62]. The DA uptake of the neuron-enriched cultures was not affected by the addition of microglia from NADPH oxidase-null mice, the addition of microglia from wild-type (WT) mice significantly increased the sensitivity of DAergic neurons either from WT or knockout (KO) mice to rotenone neurotoxicity. These data indicate that microglial NADPH oxidase, but not neuronal NADPH oxidase, is responsible for the NADPH oxidase-mediated neurotoxicity of rotenone [62]. Paraquat mainly crosses the BBB through the neutral amino acid transporter [63–65]. Once in the brain, it is selectively taken up by the terminals of DA-containing neurons in the substantia nigra by the DAT, and it inhibits mitochondrial complex I [63]. Maneb contains a major active fungicidal component, manganese ethylene-bis-dithiocarbamate (Mn–EBDC). In a rat model in which Mn–EBDC is directly delivered to the lateral ventricles, Mn–EBDC causes selective DAergic neurodegeneration [66]. Mn–EBDC preferentially inhibits mitochondrial complex III [66].

8. Genetic Animal Models of PD

Although the etiopathogenesis (including environmental factors) of PD is not fully understood, the extensive examination of human postmortem material, the genetic analysis of patients, and the study of experimental animal models have shed significant light on the molecular mechanisms involved in its progression. However, since the number of patients with familial PD is extremely low compared to the number with sporadic PD, genetic studies in affected human families are very difficult. Therefore, the development of animal genetic models for PD is especially important, and such models provide an opportunity not only to investigate the genetic etiology of PD but also to identify new factors that could be invaluable in terms of diagnosis, drug design, and/or therapy [67, 68]. Even invertebrate animals, for example, *Drosophila melanogaster*, are useful models for surveys of human PD. While their numbers of neurons and glia are obviously much smaller than in rodents and primates, *Drosophila* have the same types of neuron-glia systems, and a great number of genes and molecular transduction pathways are conserved between *Drosophila* and humans.

In recent years, several genetic animal models of PD have been reported, including models for autosomal-dominant (AD) inheritance patterns. The genes manipulated in these models include *α-synuclein*, *leucine rich repeat kinase 2* (*LRRK2*), *ubiquitin carboxyl-terminal esterase L1* (*UCHL1*), and *high temperature requirement A2* (*HTRA2/Omi*) (Table 2). There are also models of autosomal-recessive (AR) inherited PD, which involve KO or knockdown genes for *parkin*, *DJ-1*, and *phosphatase and tensin homolog-* (*PTEN-*) *induced novel kinase 1* (*PINK1*) (Table 3). In addition, we will review a PD mouse model deficient in *nuclear receptor-related 1* (*Nurr1*), also named *nuclear receptor subfamily 4, group A, member 2* (*NR4A2*), which is a susceptibility gene for familial PD (Table 2).

8.1. α-Synuclein. *α-synuclein* was the first gene linked to an AD-type familial PD, called Park1. The identification of an *α-synuclein* mutation in this family revolutionized PD research, since α-synuclein is the main component of LBs, which are observed in the sporadic PD brain. This striking result strongly indicates that genetic and sporadic PD may share similar etiologies and that investigating α-synuclein-mediated pathogenesis in familial PD could uncover important information about sporadic PD. Three missense mutations of *α-synuclein*, encoding the substitutions A30P, A53T, and E46K, have been identified in familial PD [67–70]. Furthermore, the duplication or triplication of *α-synuclein* is sufficient to cause PD, suggesting that the level of α-synuclein expression is a critical determinant of PD progression [71, 72]. Even though no direct relationship between sporadic PD and α-synuclein expression has yet been shown, the existence of several polymorphisms in the promoter or 3′-UTR of the *α-synuclein* gene suggests that its expression level might be a risk factor [73–75].

Human α-synuclein is an abundant 140-amino acid presynaptic phosphoprotein involved in vesicle handling and neurotransmitter release. Mutations in α-synuclein that increase the propensity for misfolding are probably deleterious, because the misfolded forms are toxic, and they induce cell death *in vitro* [76, 77]. Among the variety of abnormal forms that mutant α-synuclein can adopt, protofibrils and fibrils seem to be the most toxic [77]. These demonstrations

Table 2: Autosomal-dominant PD models.

Gene	Animal	Manipulation	DA neuron loss	LB-like inclusions[1]	DA-responsive motor deficits[2]	References
a-synuclein (*PARK1*)	Nematode	Transgenic	Yes[§]	No	Yes	[79, 80]
	Fly	Transgenic	Yes	Yes	Yes	[78]
	Mouse	Transgenic	No	Yes[§] (PrP promoter)	Yes[§] (PDGFβ promoter)	[81–91]
	Rat	Transgenic	Yes	No	Yes	[92–95]
	Monkey	Transgenic	Yes	No	ND	[96]
UCHL1 (*PARK5*)	Mouse	Transgenic	Yes	No	Yes	[105, 106]
LRRK2 (*PARK8*)	Nematode	Transgenic	Yes	ND	ND	[116]
	Fly	Transgenic	Yes	No	Yes	[113–115]
	Mouse	Transgenic	No	No	Yes	[117–119]

DA, dopamine; LB, Lewy body; ND, not determined; PrP, prion; PDGFβ platelet-derived growth factor β.
[1]LB-like inclusions by definition contain filamentous α-synuclein.
[2]ND could include some degree of behavioral impairment in spontaneous and locomotor activity and in response to sensory stimulation.
[§]Controversial. The opposite result has also been shown.

Table 3: Autosomal-recessive PD models and other causative genes of PD.

Gene	Animal	Manipulation	DA neuron loss	LB-like inclusion[1]	DA-responsive motor deficits[2]	References
Parkin (*PARK2*)	Nematode	Knockout	No	No	No	[124]
	Fly	Knockout	Yes	No	Yes	[125, 126]
		Transgenic	Yes	No	Yes	[131, 132]
	Mouse	Knockout	No	No	ND	[127–130]
		Transgenic	Yes	Yes	ND	[133]
PINK1 (*PARK6*)	Fly	Knockout	Yes	No	Yes	[135, 136]
	Mouse	Knockout	No	No	ND	[137–139]
DJ-1 (*PARK7*)	Fly	Knockout	Yes	No	Yes	[144–148]
	Mouse	Knockout	No	No	ND	[149–151]
HtrA2/Omi (*PARK13*)	Fly	Knockout	No	No	No	[153]
	Mouse	Knockout	No	No	ND	[154, 155]
Nurr1 (*NR4A2*)	Mouse	Knockout	Yes	No	ND	[158–160]

DA, dopamine; LB, Lewy body; ND, not determined.
[1]LB-like inclusions by definition contain filamentous α-synuclein.
[2]ND could include some degree of behavioral impairment in spontaneous and locomotor activity and in response to sensory stimulation.

of α-synuclein toxicity *in vitro* led to the creation and extensive analysis of many *α-synuclein*-based animal models of PD.

Although flies (*Drosophila*) and nematodes (*C. elegans*) do not have complex nervous systems compared to vertebrates and do not express endogenous α-synuclein, they are useful for identifying genetic and pharmacological modifiers of *α-synuclein* and its product. In *Drosophila*, the overexpression of WT and mutated (A30P, A53T) human α-synuclein causes the age-dependent loss of dorsomedial DAergic neurons, an accumulation of LB-like filamentous inclusions with α-synuclein immunoreactivity, and compromised locomotor activity (climbing ability) [78]. In *C. elegans*, α-synuclein overexpression leads to accelerated DAergic neuronal loss and motor impairment [79, 80]. However, the neurons of these nematodes do not contain notable synuclein-containing inclusions.

Many different mouse lines that overexpress α-synuclein under various promoters have been generated in the last ten years, and most have been described in recent reviews [81–83]. Mice expressing α-synuclein containing two mutations (A30P + A53T) under the TH promoter show progressive declines in locomotor activity and the loss of substantia nigra neurons and striatal DA content [84, 85]. Similarly, mice overexpressing WT human α-synuclein under the neuron-specific platelet-derived growth factor β (PDGFβ) promoter show reduced TH immunoreactivity and DA content in the striatum and impaired motor performance [86]. Mice over-expressing WT human α-synuclein under another neuron-specific promotor, Thy1, show strong widespread expression in cortical and subcortical neurons, including the substantia nigra pars compacta, but no glial, spinal, or neuromuscular pathology [87–89]. These mice have an increased sensitivity to mitochondrial damage from low doses of MPTP [89]. Mice in which the mouse prion promoter (mPrP) is used to drive the expression of α-synuclein A53T show α-synuclein aggregation, fibrils and truncation, α-synuclein phosphorylation, ubiquitination, and progressive

age-dependent neurodegeneration, just as in humans [90, 91].

Several viral vectors, primarily lentiviruses and adeno-associated viruses (AAVs), have been used to drive exogenous α-synuclein. Because viral vector delivery requires stereotactic injections within or near the site of the neuronal cell bodies in the substantia nigra pars compacta, rats are generally used for these studies although the model has been reproduced in other rodents [92–95]. The overexpression of human WT or A53T mutant α-synuclein by AAVs in the SNc neurons of rats causes the progressive age-dependent loss of DA neurons, motor impairment, and α-synuclein-positive cytoplasmic inclusions [92]. Kirik et al. also overexpressed WT or A53T mutant α-synuclein in marmosets [96], in which the α-synuclein protein was expressed in 90%–95% of all substantia nigra DA neurons. The transduced neurons showed evidence of severe pathology, including α-synuclein-positive cytoplasmic inclusions, granular deposits, and loss of the TH-positivity.

It is particularly notable that the phenotypic outcome of α-synuclein overexpression in mice heavily depends on the promoter used to drive transgene expression. Unfortunately, most of these models fail to accurately mimic PD in that there is no progressive loss of DA neurons. The loss of TH-positive cell bodies in the substantia nigra does not necessarily indicate cell death. Despite the lack of overt degenerative pathology in the DA-positive neurons, obvious locomoter abnormalities due to degeneration of the nigrostriatal system and a lack of DA responsiveness are observed in the various mouse α-synuclein models. Thus, most of these lines are excellent models of α-synuclein-induced neurodegenerative disorders, such as PD.

Although mutated α-synuclein causes human familial PD, α-synuclein's physiological roles in PD are not fully understood. In KO mice of α-synuclein, neuronal development and the formation of presynaptic terminals are normal [97]. Moreover, double KO mice that lack α- and β-synuclein exhibit normal basic brain functions and survive to adulthood [98]. Thus, the loss of α-synuclein function is unlikely to play a role in the pathogenesis of α-synuclein-induced neurodegeneration. Meanwhile, α-synuclein KO mice show reduced rearing activity in the open field, decreased DA content in the striatum, and a decrease in the reserve pool of vesicles in the hippocampus [97, 99]. These results indicate that α-synuclein may play a regulatory role *in vivo*, possibly in the fine tuning of synaptic plasticity and/or vesicle maintenance. Interestingly, several lines of α-synuclein-null mice have a complete or partial resistance to the MPTP [100, 101]. Dauer et al. showed that this resistance is not due to abnormalities of the DA transporter, which appears to function normally in α-synuclein null mice [100]. These reports indicate that α-synuclein is not obligatorily coupled to MPTP sensitivity, but can influence MPTP toxicity on some genetic background.

8.2. UCHL1. A rare AD-inherited form of PD, PARK5, is caused by a missense mutation in the *UCHL1* gene. UCHL1 constitutes 1%-2% of the brain proteins and functions in the ubiquitin-proteasome system. The ubiquitin hydrolase activity of UCHL1 is important for freeing reusable ubiquitin monomers. The missense mutation in PARK5 causes an Ile93Met substitution in the UCHL1 protein (UCHL1Ile93Met), and this mutant was initially shown to have decreased ubiquitin hydrolase activity [102]. Interestingly, UCHL1 is detected in LBs in sporadic PD cases [103]. These findings initiated a debate on whether the Ile93Met mutation causes a gain of function (toxicity) or loss of function (deficiency).

The gracile axonal dystrophy (*gad*) mouse is an AR-mutant that shows sensory ataxia at an early stage, followed by motor ataxia. Saigoh et al. showed that these mice exhibit spontaneous intragenic deletion of the *UCHL1* gene and do not express the UCHL1 protein [104]. These mice do not show obvious pathological changes in the nigrostriatal DA pathway; in particular, there is no loss of DA cell bodies in the substantia nigra. Setsuie et al. generated UCHL1Ile93Met-overexpressing mice and reported a reduction in the DAergic neurons of the substantia nigra and of the DA content in the striatum [105]. These mice show behavioral and pathological phenotypes of parkinsonism at 20 weeks of age. Moreover, recently, Yasuda et al. performed a viral vector-mediated *α-synuclein* injection into the substantia nigra of the UCHL1Ile93Met transgenic mice [106]. These mice show a significantly enhanced loss of DA-positive cell bodies in the substantia nigra and of DA content in the striatum. The neurotoxicity is enhanced by PARK5-associated UCHL1Ile93Met mutant, but not influenced by the loss of UCH-L1 WT protein *in vivo*, indicating that the UCHL1Ile93Met toxicity results from a gain of function.

8.3. LRRK2. The *LRRK2* mutation is another type of AD-PD, called *PARK8*. LRRK2 is a large protein containing a serine/threonine kinase and a GTPase domain that is localized to membranous structures [107]. The frequency of the common LRRK2 Gly2019Ser mutation was 1% in patients with sporadic PD and, interestingly, 4% of patients with hereditary PD [108]. The risk of PD when the LRRK2 Gly2019Ser mutation was present was 28% at age 59 years, 51% at 69 years, and 74% at 79 years. The motor symptoms and non-motor symptoms of LRRK2-associated PD are more benign than those of idiopathic PD. In autopsied tissue, the LB pathology was present in a representative LRRK2 G2019S case, indicating that LRRK2 and α-synuclein share some pathogenic mechanisms [109]. Yet, LRRK2 may play a role in neuronal outgrowth and guidance, and its precise physiological function remains to be clarified [110].

dLRRK is a *Drosophila* orthologue of LRRK2, and it shows elevated expression in DA neurons of the head [111, 112]. Liu et al. overexpressed constructs with mutations similar to those found in patients (G2019S), in *Drosophila* [113]. The neuronal expression of LRRK2 or LRRK2-G2019S produces an adult-onset selective loss of DAergic neurons, locomotor dysfunction, and early mortality. However, the phenotype caused by the G2019S-LRRK2 mutant is more severe than that cause by the expression of equivalent levels of WT LRRK2. Treatment with L-DOPA improves

the mutant LRRK2-induced locomotor impairment but does not prevent the loss of TH-positive neurons. Some fly models that overexpress other LRRK2 mutations, such as I1122V, Y1699C, and I2020T, show similar results, in terms of an age-dependent impairment of locomotor activity that improves with DA stimulation, and the loss of DA neurons [113–115]. Moreover, in transgenic *C. elegans*, DA marker loss is greater in those expressing G2019S LRRK2 than WT LRRK2 [116].

Transgenic mice made using bacterial artificial chromosome (BAC) technology and expressing WT LRRK2, or the R1441G or G2091S mutation exhibit mild axonal pathology in the nigrostriatal DA projection [117, 118]. However, the conditional overexpression of neither WT LRRK2 nor its G2019S mutation causes degeneration of the DA-containing neurons [119]. Interestingly, although the LRRK2 conditional transgenic mice show minimal nigrostriatal pathologies, they exhibit a progressive age-dependent motor impairment that is improved by DA stimulation. LRRK2 involvement in the pathogenesis of PD may be limited, and other genetic and/or environmental factors are probably required to trigger DA neuronal degeneration.

LRRK2 KO mice are viable, have no major abnormalities, and live to adulthood, and there is no significant difference in the susceptibility of LRRK2-deficient and WT mice to MPTP [120]. In *LRRK2*-KO *Drosophila* models, differing results on the pathology of the DA neurons have been obtained [111, 121]. Lee et al. showed that *LRRK* loss-of-function mutants exhibited severely impaired locomotive activity [111]. Moreover, DAergic neurons in *LRRK* mutants showed a severe reduction in tyrosine hydroxylase immunostaining and shrunken morphology. Conversely, Wang et al. demonstrated that mutants lacking *dLRRK* kinase activity are viable with normal development and life span as well as unchanged number and pattern of DAergic neurons [121]. Nematode deletion mutants indicate that LRRK2 is dispensable for the development and maintenance of DA neurons [122].

8.4. Parkin. *Parkin* covers approximately 1.3 Mb of genomic DNA and is the causative gene for representative AR juvenile PD (*PARK2*). Mutations in *parkin* are not only a cause of familial PD but are also seen in 20% of young-onset sporadic PD cases [123]. Parkin is an E3 ubiquitin ligase that functions in the ubiquitin-proteasome system. The loss of parkin function is believed to result in abnormal accumulations of parkin's substrates. Springer et al. demonstrated that *pdr-1* (the nematode parkin homolog) mutants are viable and display no obvious morphological defects or alterations in motility, egg-laying behavior, brood size, or life span under standard growth conditions [124]. Moreover, the authors did not detect any effect of the mutations on the survival of the DA neurons in the worms. However, overexpression of the α-synuclein A53T mutation in *pdr-1* mutants leads to developmental arrest and lethality, indicating this *C. elegans* model recapitulates parkin insolubility and aggregation similar to several AR juvenile PD-linked *parkin* mutations [124].

Drosophila parkin-null mutants exhibit a reduced lifespan, locomotor defects (flight and climbing abilities), and male sterility [125, 126]. The locomotor defects derive from the apoptotic cell death of muscle subsets whereas the male sterile phenotype derives from a spermatid individualization defect at a late stage of spermatogenesis. Mitochondrial pathology is the earliest manifestation of muscle degeneration and a prominent characteristic of individualizing spermatids in parkin mutants. These mutants also display a decrement in the TH level and degeneration of a subset of DA neurons in the brain [126]. Several parkin-null mice have been generated and display motor and cognitive deficits including reduced locomotor activity and decreased spontaneous alteration in the T-maze; however, they show no substantial DAergic behavioral abnormalities [127–130]. Pathologically, KO mice exhibit slightly abnormal DA nigrostriatal and locus coeruleus noradrenergic regions [128, 129].

The overexpression of human mutant *parkin* in *Drosophila* causes an age-dependent, selective degeneration of DA neurons accompanied by progressive motor impairment [131, 132]. *Parkin-Q311X* mice also exhibit multiple late-onset and progressive hypokinetic motor deficits [133]. Stereological analyses revealed that the mutant mice develop age-dependent DA neuron degeneration in the substantia nigra and a significant reduction of the striatal DA level, accompanied by a significant loss of DA neuron terminals in the striatum. These results indicate that *parkin* mutants may play a pivotal role in the dominant-negative etiological mechanisms of PD.

8.5. PINK1. *PINK1* is another causative gene for the AR inherited PD called *PARK6*. PARK6 is the second most frequent early-onset AR PD. PINK1 is located in mitochondria and is a putative mitochondrial kinase, because it contains a conserved serine/threonine kinase domain with an N-terminal mitochondrial-targeting motif [134]. Thus, the PD-causative mutations of *PINK1* may cause loss of function. Park et al. and Clark et al. generated and characterized loss-of-function *Drosophila* PINK1 mutants [135, 136]. These flies exhibit male sterility, apoptotic muscle degeneration, defects in mitochondrial morphology, and increased sensitivity to multiple stresses, including oxidative stress.

Park et al. showed an age-dependent decrease in DA levels and a mild loss of DA neurons in these *Drosophila* mutants [135]. Notably, the PINK1 mutants share marked phenotypic similarities with parkin mutants. Parkin overexpression is able to rescue the mitochondrial defects found in PINK1, although the double mutants do not show an enhanced phenotype. PINK1 overexpression does not rescue parkin phenotypes. Together, the data indicate that parkin and PINK1 function, at least partly, in a common pathway, and PINK1 acts upstream of parkin. Whereas PINK1-deficient mice show age-dependent mitochondrial dysfunction, increased sensitivity to oxidative stress, decreased evoked DA release, and DA receptor agonist-responsive impairment of striatal plasticity, the number of DA neurons, the level of striatal DA, and the level of DA receptors are the same as in WT animals [137–139]. These phenotypes are similar to those of *parkin*-KO mice.

8.6. DJ-1. Deletion or point mutations in *DJ-1* have been identified in early onset AR PD (*PARK7*). DJ-1 plays a role as an antioxidant and chaperone, and it is expressed ubiquitously in the cytosol, mitochondrial matrix, and intermembranous space [140]. In vitro, downregulation or KO of the endogenous DJ-1 increases cells' vulnerability to oxidative stress and proteasome inhibition, implicating it in the cellular response to oxidative stress [141–143]. *Drosophila* possesses two different orthologs of the human DJ-1 gene, named *DJ-1α* and *DJ-1β*. While loss-of-function DJ-1β mutants have normal numbers of DA neurons, classical genetic analyses and RNAi experiments have yielded contradictory results regarding the function of DJ-1α in DA neuron maintenance [144–148]. However, DA neuron loss cannot be detected in DJ-1α/DJ-1β double-deletion mutants, which are also viable, fertile, and have a normal life span. Some studies have reported a loss of DA neurons upon acute RNA silencing of DJ-1α [147, 148].

Similar to α-synuclein and parkin KO mice, DJ-1 KO mice do not show major DA-agonist-responsive behavioral abnormalities or the loss of nigrostriatal DA neurons [149–151]. In particular, although the levels of striatal DA and DA receptors are unchanged, the evoked dopamine release from striatal slices is clearly reduced, most likely as a consequence of increased reuptake. DJ-1 mutant mice also show an increased sensitivity to MPTP [150]. This is rescued by restoring the DJ-1 expression in mutant mice, further indicating a role for DJ-1 in the oxidative stress response.

8.7. HtrA2/Omi. *HtrA2/Omi* has been identified as the causative gene for a rare inherited PD, *PARK13*. HtrA2/Omi has a PDZ domain in addition to a serine protease domain and is localized to the mitochondrial intermembrane space by its mitochondria-targeting sequence. Whitworth et al. have demonstrated a genetic interaction between *HtrA2/Omi* and *PINK1*, described below, by investigating the eye phenotype of double mutant flies [152]. Their study revealed that *HtrA2/Omi* acts downstream of *PINK1* and is independent of the *parkin* gene. Yet, Yun et al. indicated that *HtrA2/Omi* null fly mutants show neither mitochondrial morphological defects nor DAergic neuronal loss [153]. They also generated a *Drosophila HtrA2/Omi* mutant analogue to the human mutation G399S, which was identified in *PARK13* patients. HtrA2/Omi G399S retains a significant, if not complete, function of HtrA2/Omi, compared with protease-compromised versions of the protein, indicating that *HtrA2/Omi* is unlikely to play a pivotal role in PD pathogenesis or as an etiological factor. The targeted deletion of *HtrA2/Omi* in mice increases their sensitivity to stress-induced cell death [154, 155]. Animals lacking HtrA2/Omi display a progressive movement disorder similar to progressive akinesia, a rigidity syndrome, showing lack of coordination, decreased mobility, bent posture, tremor, and a decreased number of TH-positive striatal neurons [155].

8.8. Nurr1 (NR4A2). Nurr1 is a member of the nuclear receptor superfamily and is involved in the differentiation and development of nigrostriatal DA neurons. Le et al. identified two mutations in *Nurr1* associated with Parkinson disease (–291Tdel and –245T → G), which map to the first exon of *NR4A2* and affected one allele in 10 of 107 individuals with familial Parkinson disease [156]. Mutations in *Nurr1* alter the transcription of *TH* and the DA transporter, suggesting that alterations in Nurr1 may cause chronic DA alterations that could increase susceptibility to PD [157]. Nurr1 is essential for the development of the ventral mesencephalic DA neurons, because homozygous Nurr1-KO mice do not develop DA neurons in the substantia nigra and die soon after birth [158]. Heterozygous Nurr1-KO mice exhibit a significant decrease in rotarod performance and locomotor activities [159]. These phenotypes are associated with decreased DA levels in the striatum, decreased numbers of DAergic neurons, and a reduced expression of Nurr1 and DAT in the substantia nigra. Moreover, Le et al. reported that heterozygous Nurr1-KO mice show a significant decrease in the total number of TH-positive neurons in the substantia nigra and reduced DA in the striatum after MPTP administration [160]. Thus, these mice show a progressive DA phenotype that bears some resemblance to that found in α-synuclein-overexpressing and mutant mice. Therefore, *Nurr1*-knockdown mice may provide a good model for investigating the later stages of PD characterized by severe DA neuron loss.

9. Concluding Remarks

The symptoms of PD become apparent after more than 80% of the DA neurons have died. The rate of substantia nigral cell loss is assumed to be about 2,500 per year in normal people. The loss of DA function can be accelerated by exposure to neurotoxins and by molecular (genetic) abnormalities, leading to a fast and significant decrease in the number of DA neurons. Consequently, these pharmacological and/or genetic insults can cause early onset of PD. This scenario indicates that critical pathological changes could be initiated one or two decades prior to the onset of PD.

As described above, whether the causative factor is a toxic compound or a mutated gene, we have no perfect animal models of PD. So far, the neurotoxin-induced vertebrate models of PD are suitable for investigating disease-modifying therapies, since they have already proved predictive. Several genetic animal models of PD are useful for understanding the early processes of degeneration in the nigrostriatal DA system. In particular, transgenic α-synuclein animals are valuable for researching general toxicity effects and the mechanisms of α-synuclein pathology, as well as for confirming potential therapeutic strategies. Recently, causative mutations and risk factors for PD have been identified in more genes. The homozygous loss of function of *glucocerebrosidase* (*GBA*) causes Gaucher's disease whereas its heterozygous loss of function increases the risk of developing sporadic PD [161]. *ATP13A2* is causative for a juvenile onset AR hereditary PD with dementia (*PARK9*) [162]. Animal models of these mutations have not been described, but once they are available, they will undoubtedly shed new light on the mechanisms of PD.

Conflict of Interests

The authors declare no conflict of interest.

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Review Article

VMAT2-Deficient Mice Display Nigral and Extranigral Pathology and Motor and Nonmotor Symptoms of Parkinson's Disease

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Dopamine is transported into synaptic vesicles by the vesicular monoamine transporter (VMAT2; SLC18A2). Disruption of dopamine storage has been hypothesized to damage the dopamine neurons that are lost in Parkinson's disease. By disrupting vesicular storage of dopamine and other monoamines, we have created a progressive mouse model of PD that exhibits catecholamine neuron loss in the substantia nigra pars compacta and locus coeruleus and motor and nonmotor symptoms. With a 95% reduction in VMAT2 expression, VMAT2-deficient animals have decreased motor function, progressive deficits in olfactory discrimination, shorter latency to behavioral signs of sleep, delayed gastric emptying, anxiety-like behaviors at younger ages, and a progressive depressive-like phenotype. Pathologically, the VMAT2-deficient mice display progressive neurodegeneration in the substantia nigra (SNpc), locus coeruleus (LC), and dorsal raphe (DR) coupled with α-synuclein accumulation. Taken together, these studies demonstrate that reduced vesicular storage of monoamines and the resulting disruption of the cytosolic environment may play a role in the pathogenesis of parkinsonian symptoms and neurodegeneration. The multisystem nature of the VMAT2-deficient mice may be useful in developing therapeutic strategies that go beyond the dopamine system.

1. Introduction

Parkinson's disease (PD) is a devastating neurodegenerative disease and is characterized by a preferential loss of dopamine neurons. PD is distinguished by the cardinal symptoms of resting tremor, rigidity, bradykinesia, and postural instability [1–3]. The incidence of PD is positively correlated with age; there is a greater than 40-fold increase in prevalence between the ages of 55 and 85 [3]. Approximately 5–10% of PD patients have a familial form of Parkinsonism with either an autosomal dominant or autosomal recessive pattern of inheritance. These familial forms are characterized by an age of onset before 40 years and a slowly progressive course [4]. Pathogenic changes in PD are extensive and, in addition to the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and loss of striatal innervation, include degeneration of the norepinephrine (NE) neurons of the locus coeruleus (LC), serotonin (5-HT) neurons of the raphe nuclei, the dorsal motor nucleus of the vagus, and the peripheral autonomic nervous system, among others [3, 5, 6]. Furthermore, Lewy body pathology can also be found in the LC, nucleus basalis of Meynert, hypothalamus, cerebral cortex, and in components of the peripheral nervous system [2, 3, 7]. As the acknowledgement of pathology associated with PD expands, symptoms beyond the cardinal motor phenotype are also more commonly recognized, including hyposmia, sleep disturbances, gastrointestinal dysfunction, anxiety, depression, and autonomic disturbances [8, 9]. The onset of these nonmotor symptoms typically comprise a prodromal phase of the disease, which

can last anywhere from a few years to decades. These symptoms often play a large role in the quality of life and disease etiology, and highlight the need to be more vigilant as we look beyond a dopamine centric view and broaden our understanding of PD pathogenesis. In doing so, targets for therapeutic intervention may be revealed and provide a more comprehensive view of the disorder.

Abnormalities with monoaminergic handling and neurotransmission are associated with a number of neurological disorders, in addition to PD, such as schizophrenia, depression, and drug addiction. Although the etiopathogenesis of PD remains unclear, it has been hypothesized that the mishandling of DA as well as other monoamines could underlie disease development. In this regard, many researchers have proposed that the accumulation of cytosolic DA has the ability to induce cytotoxicity with age; however, the long-term toxicity of DA *in vivo* has only recently been firmly established [10]. Many chemical models of PD, such as 6-OHDA, manipulate the oxidative environment of dopaminergic neurons to induce cell death. The endogenous generation of reactive oxygen species (ROS), resulting from both metabolism of monoamines in the cytosol and autooxidation of monoamines, has been implicated as a mediator in the pathophysiology of PD [10, 11]. However, physiologically, neurons have many safeguards to maintain neuronal health and protect against degeneration.

The vesicular monoamine transporter 2 (VMAT2) is one such custodian that functions to regulate the cytosolic environment of the neuron, protecting it from endogenous and exogenous toxins. Localized on vesicular membranes in neurons, VMAT2 acts to accumulate cytosolic monoamines into synaptic vesicles after they have been synthesized from their precursors for regulated exocytotic release [12]. The sequestration of monoamines is important for maintenance of normal neurotransmission and also acts to keep intracellular levels of the monoamines below potentially toxic levels [13, 14]. VMAT2 is a 12-transmembrane domain H^+-ATPase antiporter, which uses an electrochemical gradient to drive transport; two protons are exchanged for one monoamine molecule [13, 15, 16]. VMAT2 has a similar selectivity for all monoamines and is present throughout the central nervous system and in the periphery in mast cells and platelets. Phylogenetically, VMAT2 is a member of the solute carrier protein family and the toxin-extruding antiporter (TEXAN) gene family, which includes bacterial resistance genes [17, 18]. Moreover, VMAT2 contains sequence homology and functional similarities to the major facilitator superfamily of drug resistance transporters; many researchers have hypothesized that VMAT2 has evolved to serve an analogous role in eukaryotic systems by providing a mechanism to sequester and clear toxins from the cell [19, 20]. Thus, vesicular sequestration serves a dual purpose: preventing the interaction of toxins with molecular machinery and limiting exposure of neighboring cells to the toxin. In fact, VMAT2 was partly identified via its ability to confer resistance to the dopaminergic toxin 1-methyl-4-phenylpyridinium (MPP+), which is commonly used to induce a Parkinsonian phenotype in mice [14]. The level of VMAT2 expression is essential to proper monoaminergic handling, as it regulates both the size of the vesicular monoamine pool and influences the availability of monoamines in the cytosol, influencing cellular susceptibility to oxidation [14]. The monoamines, particularly DA and norepinephrine (NE) have the ability to spontaneously oxidize in the cytosol, potentially damaging cellular machinery [21].

2. VMAT2 and PD

Evidence for the monoamine theory of PD surfaced as early as the 1950s but has not begun to be fully appreciated until recently. Reserpine, an inhibitor of vesicular monoamine transport, was first introduced as a potent antihypertensive drug [22]. Reserpine acts by depleting cells of their monoamine stores; however, it is not selective for the periphery and affects the central nervous system as well [22, 23]. Patients who took reserpine chronically began to display lethargy similar to that seen in depression, contributing to the monoamine hypothesis of affective disorders [22]. Furthermore, treatment with reserpine also reproduced many of the deficits observed in PD, including a decrease in locomotor activity, akinesia, L-DOPA responsive stride length, a depressive-like phenotype, and cognitive decline [24–27]. Acute depletion of monoamine stores was found to reproduce a similar symptom profile as mice dosed with the commonly used MPTP model of PD.

Theoretically, the loss of VMAT2 function within the neuron would be associated with a reduction in vesicular sequestration of monoamines, a concomitant accumulation of cytosolic monoamines, depletion of striatal monoamines, and the development of a Parkinsonian phenotype. It is thought that together with the dopamine transporter (DAT), VMAT2 may be able to modulate susceptibility to neurodegeneration [20, 28]. There has been much speculation about the role of VMAT2 in mediating efficient clearance of DA in those populations vulnerable to neurodegeneration [28, 29]. To this end, a positive correlation exists between VMAT2 expression levels and regions of the brain spared from Parkinsonian degeneration. For example, the caudate and putamen have higher DAT expression relative to VMAT2, consequently incurring more damage than other monoaminergic areas of the brain like the hypothalamus, which has high levels of VMAT2 relative to DAT [20]. *In vivo* imaging and postmortem-binding studies displayed marked reductions in VMAT2 immunoreactivity in the caudate, putamen, and nucleus accumbens of PD brains [30, 31]. Interestingly, a gain of function haplotype of VMAT2 was found to be protective against the development of PD in humans [32]. Despite these data, it is still unclear if reductions in VMAT2 are a contributor to or a consequence of PD.

VMAT2 has been directly implicated with a pathological hallmark of PD: α-synuclein. This key component of Lewy bodies has been found to bind and permeabilize vesicles, potentially causing leakage of monoamines into the cytosol [33]. This has been hypothesized to be mediated via a direct interaction between VMAT2 and α-synuclein, disrupting synaptic vesicle dynamics [34]. Moreover, overexpression of

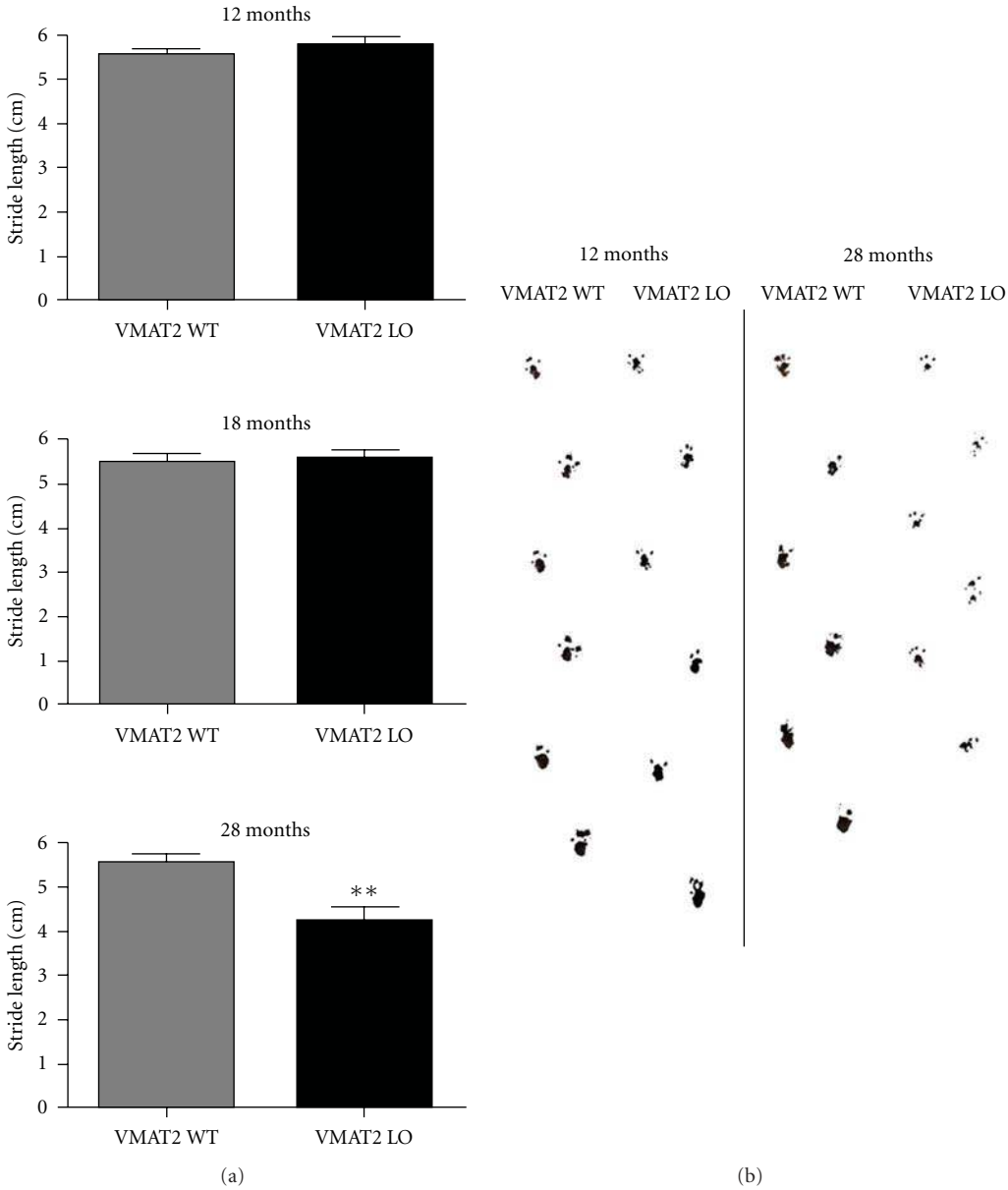

Figure 1: VMAT2-deficient animals display impaired stride length at older ages. (a) No deficits in forepaw stride length were apparent at 12 or 18 months of age in VMAT2-deficient mice. At 28 months of age, VMAT2-deficient mice display motor deficits as measured by inked paw stride length. Results represent average stride length (cm) ± SEM for 4–6 animals per genotype, $^{**}P < .01$. (b) Representative forepaw stride lengths for VMAT2 WT and deficient mice at 12 and 28 months of age.

α-synuclein causes the downregulation of VMAT2 protein *in vitro*, triggering increases in cytosolic DA and ROS [33, 35]. Taken together with evidence from oxidative stress studies, these data demonstrate that the perturbation of VMAT2 can create an environment conducive to PD-related cell damage and pathology.

3. Genetic Manipulation of VMAT2

3.1. VMAT2 Knockout Mice. In order to investigate the exact role of VMAT2 in monoaminergic signaling several lines of transgenic VMAT2 mice have been generated. Unfortunately, complete deletion of the VMAT2 gene resulted in an animal,

which moved little, fed poorly, and died within a few days after birth [36, 37], most likely a consequence of significantly reduced monoamine concentrations required for proper monoaminergic signaling. In light of this lethality, important information concerning the role of VMAT2 in monoamine neurotransmission was gleaned *in vitro*. For example, it was determined that VMAT2 is a key determinant of quantal release from monoaminergic cells, as levels were severely reduced or absent from transgenic cultures. Moreover, these data provided further evidence for the importance of presynaptic storage and release of monoamines for postsynaptic receptor responsiveness [36, 37].

Although the VMAT2 KO mice do not survive into adulthood, their creation also yielded mice that are heterozygous for VMAT2 (VMAT2 HT). Unlike the VMAT2 KO mice, the VMAT2 HTs are fully viable into adulthood, display a 50% reduction in VMAT2 expression, and were physiologically similar to their wildtype littermates [38]. Although reports have varied, overall the VMAT2 HTs appear to have a significant reduction in monoamines, perceived to be a consequence of reduced vesicular storage capacity [37–40]. Behaviorally, the VMAT2 HT mice perform normally in passive avoidance and locomotor activity tests, but display a depressive-like phenotype including anhedonia, locomotor retardation, and sensitivity to stress [38, 41]. This phenotype is ameliorated with the administration of antidepressants such as imipramine, fluoxetine, and bupropion, suggesting a combined involvement of all three monoamine neurotransmitters [41].

When challenged with various exogenous toxicants, the VMAT2 HTs begin to manifest deficits due to reduced vesicular storage capacity. Methamphetamine causes greater neurotoxicity in the VMAT2 HT mice compared to wild-type animals, with significant reductions in DA, DA metabolites, and DAT [42]. These findings were coupled with a less pronounced increase in extracellular DA, suggesting that cytosolic DA is the prevailing factor in the potentiation of methamphetamine toxicity observed in the mice [42]. Behaviorally, amphetamine produced enhanced locomotor activity but reduced reward as measured by conditioned place preference [38]. In addition to the amphetamines, VMAT2 HT mice were also found to be acutely more sensitive to the effects of the Parkinsonian drug MPTP. Presumably, due to their reduced capacity to sequester MPP+, VMAT2 HT mice undergo twice the dopaminergic cell loss observed in wild-type animals, accompanied by markers of striatal damage such as reductions in DA, DAT and increased glial fibrillary acidic protein (GFAP) mRNA [38, 39]. Although the VMAT2 HT mice did not display any overt signs of Parkinsonism or PD-like neuropathology, they do exhibit an increased susceptibility to MPTP toxicity and thus, researchers postulated that the mice may be useful in teasing out the mechanisms of L-DOPA toxicity. It was found that primary DA neurons harvested from VMAT2 HTs were more vulnerable to L-DOPA than wild-type neurons; decreased VMAT2 activity might attenuate L-DOPA efficacy by augmenting endogenous dopaminergic toxicity [43]. However, these results were not observed *in vivo* [40]. Despite the absence of a clear link between vesicular storage and L-DOPA-induced dopaminergic dysfunction, manipulating VMAT2 still produces an increased sensitivity to parkinsonian toxins and signs of depression, one of the most prevalent nonmotor symptoms associated with PD.

3.2. The VMAT2 Hypomorph Mouse. As investigators continued to ponder the role of VMAT2 in the pathogenesis of PD, further perturbation of the gene was necessary to produce a more profound disruption of monoamine storage than previously achieved with the VMAT2 HT mice. This perturbation was manifested in a line of mice that expressed only 5% of the VMAT2 protein. It is important to note that unlike the previous VMAT2 KO and HT mice, the KA1 line was created through gene targeting using a completely differently strain of mouse, which was found to be α-synuclein null [44]. Both the hypomorphic VMAT2 allele and the α-synuclein-null allele were both unintended consequences of an attempt to make VMAT2 knockout mice, but notably serendipitous to the PD field (see below). Unlike the VMAT2 KO mice, the KA1 mice are fully viable into adulthood with the absence of gross physical defects [45]. The survival of these KA1 mice allowed the examination of the effects of reduced vesicular storage over a lifetime, in addition to the study of the nuances of vesicular uptake mechanisms; whereas, both VMAT2 KO and chronically reserpinized animals are not amenable to studying the effects of aging on monoamine packaging defects.

Although no VMAT2 expression was detected in these mice through immunohistochemistry or in situ hybridization, residual VMAT2 was observed using western blotting approximating a 95% reduction [10, 45]. Consequently, there were general reductions in tissue levels of the major monoamines, DA, NE, and 5-HT reduced by 92%, 87%, and 82%, respectively, which became progressively worse with age, accompanied by increased monoamine turnover and reduced DA availability in terminal and cell body regions of the SNpc and ventral tegmental area (VTA) [45, 46]. In addition to the reduction of monoamines, the KA1 mice were also found to have altered striatal neurotransmission and signaling. Although levels of DAT mRNA, protein, and activity and D_1/D_2 receptor expression remained unchanged, electrically stimulated DA release was dramatically reduced by approximately 70% compared to age-matched wild-type animals [47, 48]. As demonstrated in the VMAT2 KO mice, a decrease in striatal DA release this dramatic is indicative of smaller vesicular DA stores due to a reduction in VMAT2 expression [47]. Considering that electrically stimulated DA release is absent in VMAT2 KO neurons, these data suggest considerable intraneuronal compensation for the 95% deficit in VMAT2 [37]. Moreover, due to the disproportionate decrease in DA release compared to the reduction in VMAT2 expression, it is possible to conceive that in wild-type neurons, not all VMAT2 protein is required to fill vesicles for exocytotic release; many transporters may, in fact, act as a reserve [47]. Additionally, even though no compensation was seen through changes in DA receptor expression, ablating VMAT2 by 95% did induce a supersensitization of the D_2/D_3 autoreceptors and downregulated phosphorylation of tyrosine hydroxylase (TH) at serine residues (Ser19, Ser31, and

Ser40), which are critical for catechol feedback inhibition [46]. Finally, the KA1 mice were found to downregulate substance P while upregulating enkephalin, allowing for the possibility of abnormalities in organization of DA-mediated signaling via both the direct and indirect pathways [45, 48]. Taken together, these data provide further evidence for the role of VMAT2 expression in regulating the size of both vesicular and cytosolic DA pools within the CNS, thus influencing extracellular neurotransmission [46, 47].

With the abundance of changes in striatal neurotransmission, the KA1 mice were tested for the presence of a behavioral phenotype that correlated with PD. As in reserpinized animals, reductions in VMAT2 in the KA1 mice cause a general decrease in locomotor activity [45]. At an early age, the KA1 mice demonstrated a significant impairment in motor coordination, independent of motivational factors, as measured by the challenging beam traversal and rotarod, which becomes progressively more severe with age [45, 46]. However, they exhibit normal reactivity in novelty place preference task [45]. As expected, the KA1 are exquisitely sensitive to acute doses of MPTP and amphetamine. When exposed to amphetamine, the KA1 mice display an increase in stereotypic behaviors and abnormalities in DA release [45, 47]. Similarly, the KA1 have a lower threshold to MPTP toxicity, demonstrating dopaminergic damage and locomotor deficits [45]. Conversely, when L-DOPA is administered, the KA1 mice exhibit locomotor hyperactivity and amelioration of deficits in motor coordination and balance [45, 46]. Interestingly, despite the presence of both striatal dopamine deficiency and a motor phenotype, when assessed for signs of Parkinsonian degeneration, no evidence of DA cell loss was found at any age [46]. However, as mentioned above, these mice contain a spontaneous chromosomal deletion spanning the α-synuclein gene locus [44–47]. The lack of this noteworthy gene may account for the absence of degeneration as cytosolic dopamine and other monoamines have been proposed to inhibit α-synuclein fibrillization by oxidatively ligating to α-synuclein [49, 50], thus retaining α-synuclein in its neurotoxic protofibril conformation. Assuming that protofibrils are the pathogenic species, a 95% decrease of VMAT2 in neurons should have lethal implications, causing the cytosolic auto-oxidation of catecholamines to increase, amplifying protofibril concentration. To answer the question more fully, it was necessary to introduce the α-synuclein gene into the mice with low VMAT2 expression.

3.3. Perfected Perturbation: VMAT2-Deficient Mice. Although the availability of the VMAT2 KA1 mice provided an extremely useful model with which to further examine the importance of DA handling, the complete ablation of such a ubiquitous protein such as α-synuclein severely limited the utility of these mice from the perspective of dopamine handling and PD pathogenesis. Fortunately, the Emory colony of KA1 mice contained animals that were heterozygous for both the VMAT2 and α-synuclein alleles. Through diligent breeding, all traces of the α-synuclein mutation were eliminated from the KA1 line of mice yielding the VMAT2-deficient mice. Consistent with previous reports of genetic and pharmacological reductions of VMAT2, striatal DA levels were reduced by 85% in VMAT2-deficient mice with a concomitant reduction in the metabolites, DOPAC and HVA; VMAT2-deficient mice also exhibited an age-dependent decline in DA [10]. Several intraneuronal compensatory mechanisms were also observed in the VMAT2-deficient mice including an increase in TH activity, increased DA turnover, and an age-dependent decline in DAT expression and activity [10]. Additionally, several markers of oxidative stress and damage were observed in the VMAT2-deficient mice. Although cysteinyl-DA was undetectable due to the reduced basal levels of DA and increased DA turnover, free cysteinyl-DOPA and DOPAC adducts were significantly increased at both 2 and 12 months of age; protein carbonyls and 3-nitrotyrosine did not manifest until 12 months of age, demonstrating that neuronal oxidative stress became progressively worse with age [10]. The chronic dysregulation of DA within VMAT2-deficient neurons began to contribute to neuronal degeneration in older animals, as evidence of cell death was seen through silver deposition and a progressive loss of TH-positive neurons within the SNpc [10].

Behaviorally, VMAT2-deficient mice exhibit many of the Parkinsonian motor phenotypes. Beginning at 2 months of age, VMAT2-deficient mice have general deficits in novelty-induced locomotor activity, which is L-DOPA responsive (Table 1) [10]. Interestingly, in the VMAT2-deficient mice it has been observed that the major motor deficits do not appear until 28 months of age, coinciding with the most severe nigral cell loss (Taylor and Miller, unpublished observations). Compared to age-matched wild-type littermates, VMAT2-deficient mice do not demonstrate a deficit in forepaw stride length until 28 months of age; this behavior is thought to mimic the shuffling gait observed in PD patients [51] (Figure 1). This behavior is also L-DOPA responsive, establishing that the motor phenotype is due to dopamine insufficiency. Combined with the dopaminergic characterization of these mice, these data reveal that reduced vesicular storage of DA is enough to induce Parkinsonian neurodegeneration.

Mounting evidence for degeneration of the locus coeruleus (LC) in human PD highlights the importance of expanding the focus of research from the nigrostriatal system in order to expose the deficits in other neurotransmitter systems [3, 7, 52–55]. Beginning at 18 months of age, the VMAT2-deficient mice displayed a mild reduction in TH staining in the SNpc and striatum, which increased moderately with age [10, 56]. More dramatic reductions in TH staining were observed in the locus coeruleus (LC) at 18, 24, and 30 months of age [56]. This pattern of neuronal loss was verified using unbiased stereological counts, demonstrating that neuronal loss in the LC precedes nigral loss in the VMAT2-deficient mice [56]. The LC of VMAT2-deficient mice undergoes a much more rapid decline from 12 to 18 months of age, with an overall 72% neuronal loss from 6-30 months of age [56]. The SNpc of the VMAT2-deficient mice does not start to degenerate until 18 months of age, with an overall 59% cell loss, similar to the loss observed in humans [56]. Taken together, these data suggest that, unlike other chemical and genetic models of PD, the LC undergoes

a much more severe degeneration than the SNpc in the VMAT2-deficient mice.

In classical PD, motor disturbances do not present clinically until approximately 70–80% of striatal dopamine and 40–50% of nigral cell bodies have already been lost; however, other nonmotor symptoms are evident before the onset of motor disturbances. These include, but are not limited to, hyposmia/anosmia, gastrointestinal disturbances, sleep abnormalities, autonomic dysfunction, anxiety, and depression [52, 57]. It is probable that other neurotransmitters such as NE and 5-HT significantly contribute to these symptoms, as both the LC and raphe nucleus have also been shown to degenerate in PD, in addition to the SNpc [3, 54, 55]. With the pathology observed in the major monoaminergic systems of the VMAT2-deficient mice, the presence of nonmotor phenotypes would not be unlikely.

Olfactory disturbances are one of the first nonmotor symptoms observed in PD; patients have demonstrated impairments in odor detection, differentiation, and identification [58–60]. Moreover, this nonmotor symptom is not responsive to traditional dopaminergic therapies [61]. When subjected to a battery of olfactory discrimination tests at various ages, VMAT2-deficient animals were unable to discriminate between two blocks (one scented with bedding from their home cage and one scented from the cage of a foreign animal of the same sex), and consequently displayed no preferential exploration of either block [62]. VMAT2 wild-type animals displayed preferential exploration of the foreign-scented block at all ages tested [62]. When challenged in a similar test of olfactory acuity using scents commonly used on the University of Pennsylvania Smell Identification Test (UPSIT), VMAT2-deficient mice again showed no preferential exploration of the novel scent as compared with water, whereas VMAT2 wild-type animals spent more time investigating the novel scent [62]. The olfactory deficit is not corrected by L-DOPA treatment in human PD patients, nor is it effective in our mice (Table 1). To ensure there was not a problem in general sensory perception, mice were tested for nonolfactory sensory deficits. VMAT2-deficient animals showed no deficits in response to tactile stimulation, quinine taste aversion, trigeminal nerve function, muscle strength, or visual acuity [62].

In order to investigate behavioral sleep disturbances in the VMAT2-deficient mice, sleep latency tests were conducted in VMAT2 wild-type and deficient mice during their circadian nadir. Beginning at 2 months of age, VMAT2-deficient mice show a shorter latency to behavioral signs of sleep compared to age-matched wild-type controls, which is responsive to an acute dose of L-DOPA (Table 1) [62]. The circadian activity of VMAT2-deficient animals is also significantly lower than that of age-matched wild-type controls at younger ages, but follows normal patterns compared to wild-type animals [62]. VMAT2-deficient animals were next behaviorally examined for gastric emptying at 2, 6, 12, and 18 months of age, as gastrointestinal dysfunction in PD occurs in over 70% of PD patients [9, 57]. Solid gastric emptying was significantly delayed overall in VMAT2-deficient mice, with an increased stool frequency, indicating a fair amount of gastrointestinal dysfunction in the VMAT2-deficient animals [62]. As in humans, an acute dose of L-DOPA did not ameliorate the gastrointestinal dysfunction observed in these animals (Table 1).

Table 1: Summary of L-DOPA responsive Parkinsonian symptoms.

Behavior	L-DOPA responsive in VMAT2-Deficient?	L-DOPA responsive in humans?
Olfactory Discrimination	No	No
Sleep Latency	Yes	No
Anxiety	Suggested	Variable
Depression	Yes	Variable
Gastrointestinal Dysfunction	No	No
Locomotor Activity	Yes	Yes*
Forepaw Stride Length	Yes	Yes*

*Falling, freezing of gait, and postural instability are all L-DOPA unresponsive.

Disruptions in DA, NE, and 5-HT neurotransmission, including degeneration of the LC and DR, have been found in PD patients with anxiety and/or depression; similar pathology has been observed in the VMAT2-deficient mice indicating the possibility for both anxiety-like and depressive-like phenotypes [9, 53, 62]. Moreover, the VMAT2 HT mice have been previously found to display a depressive-like phenotype [41]. Severe reduction of VMAT2 expression in the VMAT2-deficient mice was found to trigger both anxiety and progressive depressive behavior. VMAT2-deficient mice showed a significant increase in percentage of open arm time in the elevated plus maze at 4–6 months of age, while the increased immobility time in the forced swim and tail suspension tests did not occur until 12 months of age; suggesting that anxiety precedes depressive symptoms in VMAT2-deficient animals and that the depressive-like phenotype is progressive [62]. Additionally, a low dose of desipramine that had no effect in wild-type animals was able to normalize immobility times in VMAT2-deficient mice; similarly, an acute dose of L-DOPA was also able to ameliorate depressive-like symptoms in the VMAT2-deficient mice (Table 1) [62]. Despite the presence of many of the nonmotor symptoms associated with PD in the VMAT2-deficient animals, the animals have not yet been tested for cognitive deficits or presence of autonomic dysfunction. The involvement of multiple neurotransmitter systems and evidence from other mouse models with noradrenergic degeneration suggests that cognitive and cardiovascular deficit may also be present in the VMAT2-deficient animals [63, 64].

4. Conclusions

As the VMAT2-deficient mice have reduced levels of DA, NE, and 5-HT, L-DOPA responsive motor deficits, and almost the full constellation of nonmotor symptoms, mice with altered VMAT2 expression may represent a new model of PD that encompasses many of the motor and nonmotor symptoms, as well as the neurochemical pathophysiology (Figure 2) [10, 41, 45, 62]. Moreover, most current models

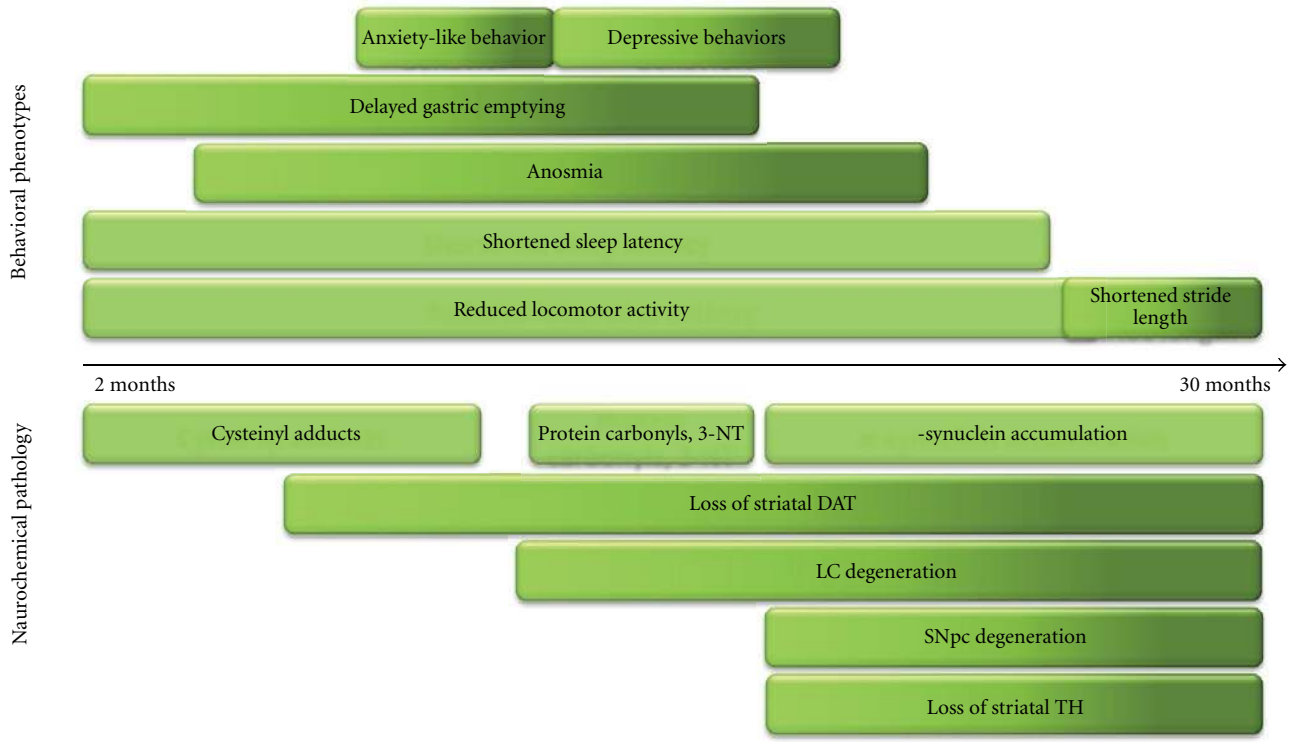

Figure 2: Timeline of Parkinsonian features observed in the VMAT2-deficient mice from 2–30 months of age. Symptoms or pathology indicated by a solid colored box did not increase in severity as the mice aged. All boxes end at the last age the symptom or pathology was measured. Behavioral phenotypes: reductions in locomotor activity and latency to behavioral signs of sleep were first observed at 2 months of age and were found to persist until their last measurements at 6 months of age and 18 months of age, respectively. Gastric emptying was first measured at 2 months of age, and increased in severity until the last time point at 18 months of age. Hyposmia began at 4 months, with full anosmia at 6 months of age and persisting until the last evaluation at 18 months. Anxiety-like behavior was first assessed at 4 months of age, persisting until 6 months of age. Even though depressive-like behaviors were measured at 4–6 months of age, presence of a depressive-like phenotype was not detected until 12 months of age, lasting until 15 months of age. Finally, reductions in forepaw stride length were not seen until 27 months, reaching full severity at 30 months of age. Neurochemical pathology: evidence of oxidative damage was first observed through the formation of cysteinyl adducts at 2 months, which were still present at 12 months. Protein carbonyls and 3-nitrotyrosine formation did not occur until 12 months of age. Accumulation of α-synuclein began at 18 months with evidence remaining until 24 months of age. Loss of striatal DAT expression measured immunohistochemically began at 6 months of age progressing in severity until 22 months of age. Reductions in striatal TH expression begin at 18 months of age, reaching maximal severity at 30 months. Degeneration of the LC starts at 12 months of age in the VMAT2-deficient animals, preceding nigral loss, which does not begin until 18 months of age.

of PD, genetic and chemical, represent a relatively short disease progression. The average lifespan of a mouse is two years; disease progression must reflect this because sporadic PD, like Alzheimer's disease, is a disease of aging. The VMAT2-deficient mice exhibit a high age dependency coupled with a progressive behavioral decline (Figure 2). The nigral and extranigral pathology combined with the motor and nonmotor symptoms in the VMAT2-deficient mice strongly argue that the underlying pathogenesis of human PD likely has some common features. For example, many of the PARK genes have been shown to disrupt proper recycling, trafficking, and release of vesicles. While the mode of vesicular disturbance may differ in individual PD cases, disrupted vesicular function, whether it is via storage or trafficking, of monoamines may represent a common pathogenic mechanism. These mice demonstrate that it is possible that PD pathogenesis represents more than altered DA homeostasis; a global disruption of monoamine storage and handling may be necessary to fully invoke the pathology associated with the disease. Utilizing the VMAT2-deficient mice as a new model of PD, could potentially lead to new adjunct therapeutic strategies, which complements current dopamine replacement therapy, improving the quality of life for many patients.

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Review Article

The Endotoxin-Induced Neuroinflammation Model of Parkinson's Disease

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Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra. Although the exact cause of the dopaminergic neurodegeneration remains elusive, recent postmortem and experimental studies have revealed an essential role for neuroinflammation that is initiated and driven by activated microglial and infiltrated peripheral immune cells and their neurotoxic products (such as proinflammatory cytokines, reactive oxygen species, and nitric oxide) in the pathogenesis of PD. A bacterial endotoxin-based experimental model of PD has been established, representing a purely inflammation-driven animal model for the induction of nigrostriatal dopaminergic neurodegeneration. This model, by itself or together with genetic and toxin-based animal models, provides an important tool to delineate the precise mechanisms of neuroinflammation-mediated dopaminergic neuron loss. Here, we review the characteristics of this model and the contribution of neuroinflammatory processes, induced by the *in vivo* administration of bacterial endotoxin, to neurodegeneration. Furthermore, we summarize the recent experimental therapeutic strategies targeting endotoxin-induced neuroinflammation to elicit neuroprotection in the nigrostriatal dopaminergic system. The potential of the endotoxin-based PD model in the development of an early-stage specific diagnostic biomarker is also emphasized.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by tremor, rigidity, bradykinesia, and postural instability, which result from the progressive loss of dopaminergic (DA) neurons in the substantia nigra [1]. The primary cause of PD is still unknown although aging seems to be a major risk factor.

Parkinson's disease displays racial differences as can be seen from recent studies which have shown that incidence of PD in African-Americans is lower than in Caucasian whites and Asians [2, 3]. Both environmental and genetic factor contribute to PD pathogenesis. Pesticides exposure (paraquat, organophosphates, and rotenone), rural living, farming, well water drinking, metals (manganese, copper, mercury, lead, iron, zinc, and aluminum), diet, head trauma, and infections have been proposed as potential risk factors [4–6]. Caffeine intake and smoking reduces the risk of PD [4, 5]. 10%–15% of all PD cases have a genetic component [7]. Fifteen chromosomal loci have been linked to PD [8]. Genes associated with PD are α-synuclein, parkin, ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), PTEN-induced putative kinase 1 (PINK1), DJ-1, and leucine-rich repeat kinase 2 (LRRK2 or dardarin) [6]. Recent data has shown the involvement of mitochondrial dysfunction in molecular cell death pathways in PD [9]. Moreover, some studies revealed that several PD-associated genes impact on mitochondrial integrity directly or indirectly, which provides a specific link between mitochondrial dysfunction observed in sporadic PD [10, 11]. α-syn, Parkin, PTEN-induced kinase 1 (PINK1), DJ-1, leucine rich repeat kinase 2 (LRRK2), and HTR2A were found to be localized in the mitochondria under certain conditions where they maintain mitochondrial integrity and morphology [11, 12]. Although mitochondria produce energy for cellular events, during catabolism, this organelle also produces reactive oxygen

species (ROS) that can cause oxidative damage, directly on mitochondrial enzymes, mitochondrial genome, and mitochondrial membrane permeability resulting in apoptosis. For neurodegenerative diseases, mitochondrial dysfunction is one of the hallmarks of pathogenesis caused by ROS inducing cell death [13]. Mitochondrial dysfunction and neuroinflammation may simultaneously induce neuronal cell death. Because mitochondria is the major source of ROS, and mitochondria can be easily affected by ROS [14, 15]. The α-synuclein mutation is autosomal dominant whereas the *parkin*, *DJ-1,* and *PINK1* gene mutations are autosomal recessive during inheritance. *LRRK2* is frequently mutated in late onset PD [16]. PD diagnosis is based on clinical findings, but there is no conclusive test for diagnosis yet [17]. The pathological hallmark of PD is selective loss of dopaminergic, neuromelanin-containing neurons in the pars compacta of the substantia nigra and presence of intraneuronal inclusions called the Lewy body [6]. Mechanisms involved in neurodegeneration in PD are protein misfolding, mitochondrial and ubiquitin-proteasome dysfunction, oxidative stress, inflammation, and apoptosis [18]. There is no current treatment in PD, but replacement of L-DOPA- is a viable therapeutic approach for arresting PD [19].

The current knowledge about pathogenesis of PD is still limited; therefore, the development of animal models is essential for better understanding of PD pathogenesis and the testing of new drugs [20]. An ideal animal model should mimic clinical and pathological features of the disease. Available animal models of PD can be divided into two categories: toxin-based and genetic [21]. 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) selectively destroy catecholaminergic neurons. Recent studies have shown that environmental toxins such as rotenone and paraquat induce progressive loss of DA neurons through inhibition of mitochondrial respiratory chain complex I [21]. Toxin-based animal models for PD are limited in that they do not model the slow and progressive loss of dopaminergic neurons and the decrease in generation of Lewy bodies [8].

Like toxin-induced models, genetic animal models of PD have contributed to the understanding of the disease. Knockout mice with deletion of *parkin*, *DJ-1* or *PINK1* genes have been generated [22–24]. Several transgenic mouse models of *α-synuclein* gene have been developed, including mice overexpressing α-synuclein [25], carrying the point mutations of α-synuclein [26] or knockout mice for α-synuclein [27]. Recently, conditional knockout models of PD have been generated. In MitoPark mice, the mitochondrial transcription factor A (TFAM) has been selectively deleted in dopaminergic neurons [21]. Loss of TFAM activity in MitoPark mice leads to impaired oxidative phosphorylation specifically in dopaminergic neurons [21].

There is some evidence that inflammation plays a major role in the pathogenesis of PD. Activated microglia were found in the striatum and the substantia nigra in PD [28, 29] and proinflammatory cytokine such as tumor necrosis factor (TNF), interleukin-1beta (IL-1β), interleukin-6 (IL-6), and inducible nitric oxide synthase (iNOS) are increased in cerebrospinal fluid of patients with PD [30, 31]. Epidemiological studies also support the role of inflammation in PD disease. A study found that the risk of PD was lower in persons who regularly took nonsteroidal anti-inflammatory drugs (NSAIDs) than in persons who did not take these drugs [32]. In addition, inflammation has a major impact on pathogenesis toxin-induced and even genetic models for PD [33, 34]. Due to the role of inflammation in PD, the need for purely inflammation-driven animal models has emerged. Firstly, an in vitro model developed by (lipopolysaccharide) LPS-induced neurotoxicity in mixed cortical neuron/glia cultures [35]. Later, an in vivo LPS-induced PD model was devoloped by Castaño et al. [36]. Since then, LPS-induced PD model has been widely accepted and used for understanding pathogenesis of PD and testing new drugs in the treatment of PD. In this paper, we will summarize the various in vivo LPS-induced PD models. Furthermore, we will highlight the combined models of LPS with toxin-induced or genetic models and pathogenesis of LPS-induced PD models. We have mentioned the contribution of LPS-induced PD models to studies of PD pathogenesis and to new drug development for the treatment of PD.

2. Neuroinflammation in Parkinson's Disease (Epidemiological Data, Toxin-Based Animal Models, Genetic Models, PET Imaging, and Peripheral Immune System in PD)

The process of neuroinflammation has been shown to be involved in PD by McGeer et al. in 1988. They have found that activated microglia and T-lymphocytes are present around the Substantia Nigra pars compacta (SNpc) of postmortem PD patients [28, 37, 38]. Followup studies have confirmed the presence of inflammation related enzymes iNOS and cyclooxygenase-2 (COX2) in SNpc.; Mogi et al. reported the increased levels of TNFα, β2-microglobulin, epidermal growth factor (EGF), transforming growth factor α (TGFα), TGFβ1, and interleukins 1β, 6, and 2 in striatum of PD brain at the molecular level [39–42]. When the cerebrospinal fluid and serum of PD patients were analyzed, IL-2, TNFα, IL-6, and RANTES levels were found to be increased [42–45]. Immunological studies have also shown the presence of activated (CD4+ CD45RO+) T-cells in serum of PD patients [46, 47]. In order to monitor activated microglia in the PD brain, [^{11}C](R)-PK11195, which is a marker of peripheral benzodiazepine binding sites that is selectively expressed by activated microglia, is used in PET studies [48, 49]. It has been found that the density of activated microglia is highest in clinically affected regions of the brain, supporting the fact that inflammatory responses by intrinsic microglia contribute to the progression of PD. All these studies show that activated microglia take part in PD pathogenesis; however, in most of the studies, late stages of PD brains were examined and involvement of microglia to the inflammation at early or late stages was mere speculations. Recent data from tissue culture studies, however, supports the notion that microglia contribution occurs in early stage PD [50, 51]. In addition to etiologic studies, the determination of risk factors for developing PD has been

tried. For genetic analyses, polymorphisms of TNFα, IL-1β, IL-1α, IL-6, and CD14 genes were analyzed, and association studies demonstrated that the polymorphisms are common among patients [52–57].

In l-Methyl-4-Phenyl-1,2,3,6-tetrahydropyridine (MPTP) models of PD, mitochondria complex I is inhibited and ATP levels decrease resulting in cell death. In this model, activated microglia and infiltration of activated T-lymphocytes were detected in brains of MPTP-treated animals [58–61]. In another model for PD, 6-hydroxydopamine (6-OHDA), cells are selectively killed by generation of free radicals and oxidative stress. Crotty et al. have recently shown a significant increase in number of activated microglia in 6-OHDA lesioned rats [62]. A study by Depino et al. concerning 6-OHDA lesioned rats did not find an increase in TNFα both on the mRNA and protein levels. An increase in IL-1β protein levels has not been detected whereas significant increase in mRNA levels of IL-1β has been detected [63].

3. Experimental Considerations

LPS is now well established as an effective initiator of DA neurodegeneration. The neurotoxic effect of LPS has been first demonstrated in cell culture-based *in vitro* models. The *in vitro* cell culture model of LPS-mediated neuroinflammation and neurotoxicity is based on the mesencephalic mixed neuron-glia culture system [64]. In vitro studies on rat mesencephalic cultures suggest that dopaminergic neurons are twice as sensitive to LPS as nondopaminergic neurons and that the toxicity of LPS occurs via microglial activation [65, 66]. As an economical and efficient system, *in vitro* studies are still valuable to explore the molecular mechanisms of LPS-mediated neurotoxicity and for screening candidate therapeutic compounds.

3.1. Characteristics and Versions of the Model. To extend the observations made in the *in vitro* LPS-mediated neuroinflammation model to a physiologically more relevant setting, the single intranigral LPS injection model has been developed in 1998 [36]. Compared with the *in vitro* LPS model, a single injection of low microgram quantities of LPS to the SN enables the comparison of the relative vulnerability to inflammatory damage of dopaminergic neurons in the SN versus those in the VTA, dopaminergic versus nondopaminergic neurons in the SN, and dopaminergic versus nondopaminergic neuronal projections in the corpus striatum [36, 51, 64]. Consistent with previous *in vitro* findings, an *in vivo* endotoxin model has shown that LPS-induced neurodegeneration is primarily observed in dopaminergic neurons and nondopaminergic neurons such as GABAergic neurons. SN are mostly spared by this process; microglial activation precedes dopaminergic neurodegeneration indicating a temporal relationship between glial activation and neurodegeneration, and finally LPS-induced microglial activation plays a more prominent role than astroglial activation in the release of various neurotoxic mediators that lead to dopaminergic neurodegeneration [64]. Acute intranigral or supranigral LPS injections (2 μg) produce a rapid activation of microglia (within 24 h) and loss of striatal dopamine (by day 4) accompanied by loss of SN DA neurons (within 21 days) [67, 68]. Injection of LPS to the SN results in an irreversible, but not progressive loss of the dopaminergic neurons in SNpc. While striatal DA is rapidly reduced, no further decline is seen during 1 year, indicating a permanent lesion but a lack of progression [69]. This model does not induce DA neuron death directly by activating microglia/monocytes. Although acute intranigral LPS administration produces rapid and intense microglial activation, microglia morphology reverts to normal form within 30 days, indicating a short-lived response and not a prolonged or progressive state of activation [70]. The successful demonstration of single intracerebral LPS injection induced dopaminergic neurodegeneration prompted further examination on whether a less intense and chronic period of inflammation in the SN would lead to a delayed and progressive nigrostriatal dopaminergic neurodegeneration. Indeed, chronic infusion of nanogram quantities of LPS to the SN via an osmotic minipump for two weeks induces significant glial activation accompanied by delayed, progressive, and preferential degeneration of SNpc dopaminergic neurons [71]. Although the SN is far more sensitive than the striatum to the inflammatory stimulus [69], intrastriatal or intrapallidal injection of LPS also induces neuroinflammation and dopaminergic neurodegeneration in rodents [72–76]. The Globus pallidus is an integral component of the basal ganglia that is important in regulation of movement. The intranigral LPS model has recently been established in mice [77]. Future research can be performed using knockout mice to study other potential mechanisms of neuroinflammation-induced neurodegeneration [77, 78]. Systemic inflammation has been suspected to influence the activities of the immune cells in the brain and consequently contributes to the chronic neurodegenerative process for diseases such as PD [79]. Systemic administration of LPS has been found to induce progressive degeneration of nigral dopaminergic neurons in male C57BL/6 mice [80]. Systemic LPS injection also induces apoptotic cell death in SN [81]. Interestingly, progressive dopaminergic cell loss occurs in mice given a single systemic exposure to LPS, which contrasts with the lack of progressive dopaminergic neuron loss in rats provided with a single, acute, intranigral LPS infusion [67, 69, 70].

Several experimental considerations including LPS strain, administration route and dosing of LPS, strain, gender, and age differences of experimental animals should be taken into account for the design of experimental setting in LPS-based PD model. As discussed above, administration route and location of LPS determine the characteristics of the LPS-based PD model. While a single intranigral or supranigral injection of LPS does not cause progressive dopaminergic cell loss, chronic infusion of endotoxin to SN or systemic LPS administration leads to a time-dependent progression in dopaminergic neurodegeneration. The degree of dopaminergic neurodegeneration is also concentration-dependent [51]. 14 days after injection of 0.1 μg to 10 μg LPS into the rat SN, TH-positive (TH+) neurons in the SN were decreased by 5%, 15%, 20%, 45%, 96%, and 99%, respectively [82]. The possible effect of the differences between LPS strains has not been evaluated to date.

3.2. Strain, Gender, and Age Differences. Although different mouse strains present striking differences in the extent of dopaminergic neurodegeneration induced by neurotoxin MPTP, injection of LPS to the SN region of Wistar, Fisher, or Sprague-Dawley rats have a similar loss of SNpc DA neurons [36, 51, 83]. Differences between rat strains have not been reported for the acute intracerebral LPS model.

Gender differences seem to be an important factor in the sensitivity to the LPS-induced dopaminergic neurodegeneration. C57BL/6 female mice are more resistant to systemic LPS than male mice [80, 81]. Repeated monthly LPS injections are required to cause both motor behavioral deficits and dopaminergic neuronal loss in female mice.

Several studies compared the detrimental effects of LPS on the nigrostriatal pathway and its behavioral consequences between young and aged animals. Four weeks after bilateral intrapallidal injection of LPS (10 μg), a greater loss of SNpc DA neurons in the older (16 months old) than the younger Fisher F344 rats (3 months old) with alpha-synuclein-positive intracellular inclusions in the SN dopaminergic neurons of the LPS-injected middle-aged rats could be observed [76]. While young rats recovered from LPS-induced locomotor deficits four weeks after intrapallidal LPS injection, aged rats failed to improve on measures of speed and total distance moved, which may be caused by microglial activation and proinflammatory cytokine expression [74]. In addition, greater nitration of proteins like alpha-synuclein occurred in the SN of elderly rats versus young rats, accompanied by higher expression level of iNOS. The Lewy body, a pathological hallmark of PD, contains nitrated alpha-synuclein, which is prone to oligomerization. These results imply that an exaggerated neuroinflammatory response that occurs with aging might be involved in the increase in prevalence of neurodegenerative diseases like PD [74]. One month after intrastriatal injection of LPS microglial activation, lipid peroxidation, ferritin expression, and total nigral iron content in aged rats significantly increased. In addition, LPS significantly altered the turnover ratio of HVA to DA [74]. Injection of LPS into the globus pallidus of young and middle-aged rats substantially decreased TH as can be evidenced by immunostaining in SNpc one month after injection [76]. Loss of TH expression was accompanied by increase iron and iron-storage protein ferritin levels in glial cells of the SN pars reticulata. Despite great increase in nigral iron levels, ferritin induction was less pronounced in older rats, suggesting the regulation of ferritin is compromised with age. Intrapallidal LPS injection also increased expression of alpha-synuclein and ubiquitin in TH(+) neurons of the SNpc. These findings suggest that pallidal inflammation significantly increases stress on dopaminergic neurons in the SNpc. Alterations in nigral iron levels may increase the vulnerability of nigral neurons to degenerative processes. Thus, an age-related increase in iron as well as susceptibility to inflammation may play an important role in PD-related neurodegeneration, as free radicals produced from the inflammatory response can become more toxic through increased ferrous iron catalyzed Fenton chemistry. This may enhance oxidative stress, exacerbate microglia activation, and drive the progression of PD [76].

3.3. Assessment of the Neuroinflammation, Neurodegeneration, and Their Effect. Several immunohistochemical, histological, biochemical, and behavioral parameters are used to evaluate the neuroinflammation and neurodegeneration in LPS-based PD models. Reduction of TH immunoreactivity is used as an index for dopaminergic cell death. The preferential degeneration of SNpc DA neurons was further corroborated by studies that employ fluorogold retrograde labeling of the striatonigral DA pathway prior to LPS injection.

Similar reduction of TH immunoreactivity and fluorogold-labelled neurons in the SN following LPS administration suggests dopaminergic cell death rather than downregulation of TH [68]. The number of TH(+) cells is determined using stereological analysis.

TH enzyme activity from striatal tissue can be measured as an indirect index of dopaminergic neurodegeneration. A single intranigral injection of LPS causes reduction in TH enzyme activity [36]. In vivo microdialysis can be used to measure changes in extracellular concentrations of dopamine and its metabolites in freely moving rats in response to administration of an endotoxin. In a recent study, dopamine metabolites in the dialysate obtained from the rat brain were measured by high-performance liquid chromatography (HPLC) using electrochemical detection [84]. Results showed that intrastriatal perfusion of different concentrations of LPS produced a dose-dependent decrease in the extracellular DOPAC output.

Intracerebral injections of LPS (5 or 10 μg) into the cortex, hippocampus, striatum, or SN of rats enhances the death of only SN DA neurons, possibly because microglial cell density in the SN is 4-5 times higher than in other regions [69, 71, 85]. LPS administration induces a rapid activation of microglia within hours as demonstrated by morphological transformation of OX-42-positive microglia. SN microglia became fully activated exhibiting the characteristic amoeboid morphology [71]. This is accompanied by intense expression of glial fibrillary acidic protein- (GFAP-) immunoreactive astrocytosis in the SN [68]. Double immunostaining of the tissue slices shows that iNOS and 3-nitrotyrosine (3-NT)-immunoreactive cells are predominantly microglia [70]. Activated microglia can even be found in the basal ganglia and brainstem of PD cases or in rodents using positron emission tomography (PET) with [^{11}C](R)-PK11195 [86–89]. To the best of our knowledge, in vivo PET imaging for the evaluation of microglial activation has only been used in intraperitoneally LPS-treated rats in a recent study by Ito et al. [90]. For this model, the authors have concluded that the intensity of peripheral benzodiazepine receptor signals in [(11)C]PK11195 PET may be related to the level of microglial activation rather than the number of activated microglia.

Neuroinflammation-mediated dopaminergic neuronal loss induced by LPS may also have functional significance as demonstrated by behavioral tests. Thirty days following supranigral LPS injection, rats show unilateral behavioral deficits as evidenced by ipsilateral circling following amphetamine administration [70]. Intrapallidal LPS injection causes permanently slowed locomotor activity in aged rats [76]. Automated movement tracking analyses has shown that young rats (3 months old) recovered from

LPS-induced locomotor deficits four weeks after intrapallidal LPS injection, yet older rats (16 months old) failed to improve on measures of speed and total distance moved. In contrast to MPTP and 6-OHDA, intranigral LPS administration does not produce behavioral dysfunction in early periods (1, 3, and 7 days after the lesions); however, LPS drastically increases HVA at the first time point, simulating features of the premotor phase of PD [91]. The combination of both systemic LPS and MPTP causes striatal DA and gait instability as revealed by reduced stride length in male C57Bl/6J mice at 4 months after injection [92].

3.4. Combined Models. In most environmental models for PD, a single neurodegenerative agent is introduced to cause nigrostriatal dopamine depletion. However, cell loss in human PD may often be caused synergistically by multiple toxins or vulnerabilities. Recent studies have also focused on the effects of LPS challenge in toxin-based and genetic models of PD. As discussed in Section 2, the findings of neuroinflammation are also observed in toxin-based and genetic models of PD. Increased mRNA and protein expression of both CD14 and TLR4 in the SN, but not in the caudate-putamen nuclei of mice treated with MPTP, in comparison to untreated animals, suggests that the endotoxin receptors are overexpressed in specific areas of the CNS during experimental PD [93]. Thus, the neurotoxin challenge may also cause a predisposition for the exacerbation of chronic neuroinflammation.

A recent study by Koprich et al. has shown that injection of a nontoxic dose of LPS into adult rat SNpc leads to microglial activation and increased levels of IL-1β, without causing death of dopaminergic neurons *in vivo*, but causing increased vulnerability for DA neurons to a subsequent low dose of 6-OHDA [94]. This exacerbation of 6-OHDA-induced neuronal loss by LPS appears to be partly mediated by IL-1β, since treatment with both LPS and IL-1 receptor antagonist rescued some of the dopaminergic neurons from 6-OHDA-induced death following LPS-induced sensitization to dopaminergic degeneration. Another recent study has shown that 6-OHDA injection into the adult rat striatum and subsequent nontoxic LPS injection into the SNpc cause an increased level of dopaminergic neuronal death and motor deficits compared with the administration of either toxin alone [95]. Thus, the initial insult causes priming of microglia, while the second insult shifts microglial activation towards a proinflammatory phenotype with increased IL-1β secretion. Specific IL-1β inhibition reversed these effects and nitric oxide (NO), a downstream molecule of IL-1β action, is partially responsible for the exacerbation of the neurodegeneration that has been observed [95]. The combination of systemic LPS and MPTP, but not either alone, causes striatal DA and gait instability in male C57Bl/6J mice about 4 months after injection [92]. MPTP alone acutely reduced striatal DA levels, but this effect was transient as striatal DA recovered to normal levels after 4 months. The nigrostriatal dopaminergic neurons can succumb to multiple toxic agents that independently may have only a transient adverse effect. The effect of methamphetamine (MA) dopaminergic toxicity, like MPTP toxicity, frequently cited as a model of PD, is potentiated by intrastriatal LPS administration [96]. This combined model leads to behavioral impairment and striatal dopaminergic deficits, but not to alteration in other monoaminergic systems including serotonin, norepinephrine, and histamine. The combination of striatal LPS and MA results in microglial activation limited to the nigrostriatal region. Furthermore, neuroinflammation, oxidative stress, and proapoptotic changes in the striatum are more accentuated with combined treatment of LPS and MA compared to individual treatments. In addition, cytoplasmic accumulation of alpha synuclein has been observed in the SN of mice treated with LPS and MA. L-Dopa treatment, also, significantly attenuates behavioral changes, and dopaminergic deficits can be induced by LPS and MA [96].

Inflammatory priming of the SN by LPS influences the impact of later neurotoxin exposure, and this process was called as neuroimmune sensitization of neurodegeneration [97].

Repeated injection with the herbicide paraquat causes oxidative stress and a selective loss of dopaminergic neurons in mice. In this model, the first paraquat exposure, though not sufficient to induce any neurodegeneration, predisposes neurons to damage by subsequent insults. Multiple toxin exposure may synergistically influence microglial-dependent DA neuronal loss and, in fact, pretreatment with one toxin may sensitize DA neurons to the impact of subsequent insults. Priming the SNpc neurons with LPS influences the impact of later exposure to paraquat [97]. LPS infusion into the SN-sensitized DA neurons to the neurodegenerative effects of a series of paraquat injections commencing 2 days later. In contrast, LPS pretreatment protects against some of neurodegenerative effects of paraquat when the pesticide is administered 7 days after the endotoxin, suggesting the importance of the time of exposure. These results suggest that inflammatory priming may influence DA neuronal sensitivity to subsequent environmental toxins by modulating the state of glial and immune factors, and these findings may be important for neurodegenerative conditions, such as PD [97]. Microglial activation acts as a priming event leading to paraquat-induced dopaminergic cell degeneration. A study by Purisai et al. elucidated the mechanism underlying this priming event. They found that a single paraquat exposure is followed by an increase in the number of cells with immunohistochemical, morphological, and biochemical characteristics of activated microglia, including induction of NADPH oxidase [98]. When initial microglial response was inhibited by the anti-inflammatory drug minocycline, subsequent exposures to the paraquat fail to cause oxidative stress and neurodegeneration. If microglial activation was induced by pretreatment with LPS, a single paraquat exposure suffices to trigger a loss of dopaminergic neurons. Moreover, mutant mice lacking functional NADPH oxidase are spared from neurodegeneration caused by repeated paraquat exposure [98].

The LPS-based model has also been combined with a genetic model of PD [77]. In mutant alpha synuclein (αSYN) transgenic mice, but not synuclein knockout mice, intranigral LPS administration led to neuroinflammation

associated with dopaminergic neuronal death and the accumulation of insoluble SYN aggregates as cytoplasmic inclusions in nigral neurons. Nitrated/oxidized SYN has also been detected in these inclusions. These results suggest that NO and superoxide release by activated microglia may be the mediator that links inflammation and abnormal αSYN in PD neurodegeneration [77]. Although loss-of-function mutations in the parkin gene cause early-onset familial PD, Parkin-deficient (parkin$-/-$) mice do not display the nigrostriatal degeneration pathway, suggesting that a genetic factor is not sufficient, and an environmental trigger may be needed to cause dopaminergic neuron loss. Upon administration of low-dose systemic LPS for prolonged periods, parkin$-/-$ mice display subtle fine-motor deficits and selective loss of dopaminergic neurons in the SN, suggesting that the loss of the Parkin function increases the vulnerability of the nigral DA neurons to inflammation-related degeneration [99].

4. Neuroinflammation Model of Parkinson's Disease Induced by Prenatal Exposure to Lipopolysaccharide

Parkinson's disease symptoms' typically manifest in late adulthood, after loss of dopaminergic neurons in the nigrostriatal system. Lack of heritability for idiopathic PD has implicated adulthood environmental factors in the etiology of the disease. However, compelling evidence from recent experimental studies has shown that exposure to a wide variety of environmental factors during the perinatal period (environmental toxins such as pesticides) and during the prenatal period (bacterial endotoxin LPS) can either directly cause a reduction in the number of dopamine neurons or cause an increased susceptibility to degeneration of these neurons with subsequent environmental insults or with aging alone [100] (Figure 1). A fraction of pregnant women suffer from vaginal or cervical bacterial infections, and there may be a risk for bacterial toxins including LPS to impact the fetal development. One of the potential targets for an endotoxin assault may be the developing nigrostriatal DA pathway [64]. The endotoxin model implies a role of proinflammatory cytokines, which may relate to epidemiological studies of early-life infectious agents and intrauterine infections.

The proinflammatory cytokine TNFα kills DA neurons and is elevated in the brains of patients with PD (Figure 1). LPS is a potent inducer of TNFα and both are increased in the chorioamniotic environment of women who have bacterial vaginosis during pregnancy. This suggests that prenatal maternal infection might interfere with the normal development of fetal DA neurons [101]. In utero exposure to LPS following a single injection of the endotoxin intraperitoneally (10 000 endotoxin units) into gravid Sprague-Dawley rats at embryonic day 10.5, a critical time point during embryonic dopaminergic neuron development, causes a significant reduction in the striatal DA and nigral dopaminergic cell number, accompanied by elevated levels of striatal and nigral TNFα in offspring sacrificed at 21 days, indicating that prenatal exposure to LPS not only creates a neuroinflammatory response but also disrupts the normal development of dopaminergic neurons [101]. Dopaminergic neuron loss is apparently permanent as it is still present in 16 months old animals [102]. In utero LPS exposure does not appear to affect dopaminergic neurons in the ventral tegmental area (VTA) or nondopaminergic neurons in the substantia nigra [101]. In contrast to TNFα, levels of IL-1β are not affected by prenatal LPS treatment [101]. LPS administration results in significant microglial activation and sustained elevation of TNFα in both the SN and the corpus striatum, even several weeks after the sole initial exposure, suggesting a persisting effect [103].

However, endotoxin-induced dopaminergic cell loss does not seem to progress as prenatal LPS reduces the baseline number of dopaminergic neurons in offspring, but the baseline remains stable once it has been established even beyond 16 months of age (similarly 20%–30% reduction in the number of SNpc dopaminergic neurons across studies and across ages) [100, 101, 103, 104].

In utero LPS exposure may predispose the nigrostriatal dopaminergic system of the pups to enhanced susceptibility to neurotoxins such as rotenone and 6-OHDA [103, 104]. Using male offspring at 3 months of age, Ling et al. has not been able to find any synergistic toxic effects of prenatal LPS and postnatal 6-hydroxydopamine (6OHDA) exposures [104]. In contrast, a subtoxic dose of neurotoxin rotenone (1.25 mg/kg/day, 14 days, intrajugular) injected at 18 months of age to female rats exposed prenatally to LPS, exerted a synergistic effect on dopaminergic cell loss, suggesting that a preexisting proinflammatory state can be a risk factor for environmental toxins [103]. One subtoxic rotenone dose did not directly lead to cell loss in these aged female rats. However, against the background of prenatal LPS exposure, cell loss was significant in the SNpc, displaying an interaction of prenatal exposure and adulthood challenges, which suggests that age and multiple environmental hits play a role. Dopaminergic cell loss was associated with decreased striatal DA and increased striatal dopaminergic activity ([HVA]/[DA]). Animals prenatally exposed to LPS exhibited a marked increase in the number of reactive microglia that was further increased by rotenone exposure. Prenatal LPS exposure also led to increased levels of oxidized proteins and the formation of α-Syn and eosin positive inclusions resembling Lewy bodies. These results suggest that exposure to low doses of an environmental neurotoxin like rotenone can produce synergistic dopaminergic neuron losses in animals with a preexisting proinflammatory state [103]. This supports the notion that PD may be caused by multiple factors and the result of multiple hits from environmental toxins. Yet, despite neuroinflammation, the progressive loss of dopaminergic neurons that characterizes PD is rarely seen in animals. In a recent study, 7-month-old male rats prenatally exposed to LPS were subjected to supranigral infusion of LPS and sacrificed after 2 or 12 weeks [105]. LPS infusion into animals prenatally exposed to LPS produced a neuroinflammatory response during the 14 days of LPS infusion that subsequently reverted to normal state over the next 70 days. In animals with preexisting inflammation (i.e., prenatal LPS); however, the acute changes seen were attenuated but the return to normal

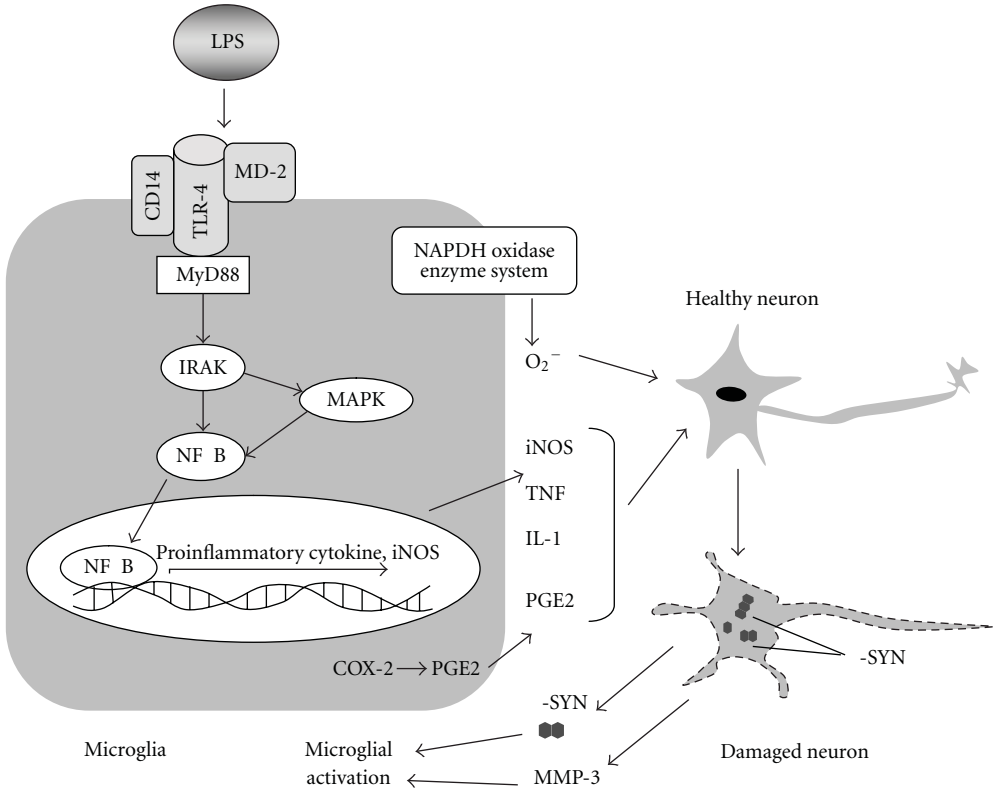

Figure 1: Simplified schematic representation of the link between LPS-induced microglial activation, inflammatory mediators, and dopaminergic neurodegeneration. Microglia respond to pathogens, proinflammatory cytokines, neuronal dysfunction, and cellular debris after injury or necrosis. These cells are at the forefront of the defence mechanisms that could set the conditions for repair or contribute to neuronal damage. Such equilibrium might depend on the expression and function of specific TLRs and how they are activated by endogenous and exogenous ligands and signals. Recognition of such signals lead to transcriptional activation of innate immune genes. Bacterial endotoxin LPS is a potent stimulator of macrophages, monocytes, microglia, and astrocytes causing release of various immunoregulatory and proinflammatory cytokines and free radicals. Neurons do not express functional TLR-4. Thus, LPS does not appear to have a direct effect on neurons, making it an ideal activator to study indirect neuronal injury mediated by microglial activation [64]. LPS binds to its intermediate receptor CD14 and in concert with TLR4 and accessory adaptor protein MD2 triggers the activation of kinases of various intracellular signaling pathways. The MyD88-dependent cascade initiates NFκB activation through the IKKs and/or the MAPK pathway, leading to the upregulated expression of proinflammatory cytokines (TNFα, IL-1β) and increased production of other inflammatory mediators (NO and PGE2, synthesized by iNOS and COX-2, resp.). These soluble mediators collectively damage nigral dopaminergic neuron. MMP-3 and αSYN released by stressed neurons aggravate microglial activation. Astrocyte, different activation states of microglia, peripheral immune cells, many molecules involved in intracellular signaling pathways, and crosstalk between TLR signaling pathway and NADPH oxidase enzyme system are not shown for the simplicity. Please see text for the abbreviations and the details of TLR signaling pathway.

state took much longer. Prenatal LPS exposure also causes a disturbance in the glutathione homeostasis in offspring brain, which renders dopaminergic neurons susceptible to secondary endotoxin insults in adulthood [106].

When rats, prenatally exposed to LPS, were evaluated at 4, 14, and 17 months, the progressive dopaminergic neuron loss was parallel to that of the controls suggesting that prenatal LPS exposure does not produce an accelerated rate of dopaminergic neuron loss [107]. Prenatal LPS exposure disrupted the dopaminergic system involving motor function, but this neurochemical effect was not accompanied by behavioral impairment, which is probably due to adaptive plasticity processes [108]. Prenatal LPS administration (100 μg/kg, i.p.) on gestational day 9.5 impairs the male offspring's general activity and decreases the striatal dopamine and metabolite levels in adulthood after an additional immune challenge [108]. Following prenatal LPS exposure, significant reductions in DA and 5-hydroxytryptamine (5-HT) levels were found in the frontal cortex, nucleus accumbens, striatum, amygdala, hippocampus, and hypothalamus of male offspring at 4 months of age [109]. The loss of DA and 5-HT were accompanied by a significant increase in homovanillic acid over DA and 5-hydroxyindoleacetic acid over 5-HT ratios in most tested areas. These data further validate prenatal LPS exposure as a model of PD, since DA and 5-HT changes are similar to those seen in PD patients.

The neonatal period is developmentally distinct from the gestational period, and exposures to endotoxin in either may lead to different consequences. In an *in vivo* study using a mouse model with nigrostriatal lesions, produced by the administration of MPTP, microglia activated by systemic LPS

were neurotoxic toward dopamine neurons in aged mice but unexpectedly neuroprotective in neonatal mice [110]. The inflammatory process in the brain, which is accompanied by changes in the levels of proinflammatory cytokines and neurotrophins, along with the presence of activated microglia, has recently gained much attention in the area of neurodegenerative diseases. Activated microglia produce either neuroprotective or neurotoxic factors. Many reports indicate that activated microglia promote degeneration of dopaminergic neurons in PD. On the other hand, there are several lines of evidence that microglia also have a neuroprotective function [111]. Microglia activated with LPS in the nigrostriatum of neonatal mice protect dopaminergic neurons against the neurotoxin MPTP whereas activated microglia in aged mice promote death of dopaminergic cells by MPTP. Recent findings suggest that the function of activated microglia may change *in vivo* from neuroprotective to neurotoxic during aging as neurodegeneration progresses in the PD brain [111]. These results suggest that the activated microglia in neonatal mice are different from those in aged mice, with the former having neurotrophic potential toward the dopamine neurons in the SN in contrast to the neurotoxic effect of the latter [112].

As discussed above, recent studies have begun to identify specific factors occurring as part of the in utero or perinatal environment that may predispose or even cause damage to the nigrostriatal system, suggesting that environmental factors early in life of an individual cause a predisposition to develop symptoms of PD. Interactions of prenatal environment, adulthood environment, gender, age, and genetic background may also modify this risk [100]. Recently, animal studies have been described that specifically consider the role of gestational exposures in disrupting the nigrostriatal system and each has implications for elaborating on our current understanding of the etiology of PD.

5. Cellular and Molecular Mediators of Endotoxin-Mediated Dopaminergic Neurodegeneration

Unlike the direct death of dopaminergic neurons caused by neurotoxins such as MPP^+ or 6-OHDA, endotoxin-mediated dopaminergic neurodegeneration seems to result from indirect neuronal death due to inflammatory reactions. Bacterial endotoxin LPS is capable of activating glial cells, predominantly microglia, to release a wide variety of proinflammatory and neurotoxic factors that include reactive oxygen and nitrogen species, proinflammatory cytokines, and lipid mediators [113]. A number of mechanisms by which inflammatory-activated microglia and astrocytes kill neurons have been identified in cell-culture studies [114]. Results from studies employing enzyme inhibitors, neutralizing antibodies, specific inhibitors of inflammatory signaling pathways, and knockout animals have identified these soluble factors and signaling molecules involved in microglial activation as major contributors to the endotoxin-mediated dopaminergic neurodegeneration [64].

The toll encoding gene has first been identified in Drosophila embryos, where it has a role in dorsoventral axis determination [115, 116]. Many organisms have multiple homologues of the Drosophila toll gene, which is very conserved among species [117]. In vertebrates, TLR (Toll-like receptors) recognize pathogen associated molecular patterns of bacteria, fungi, and viruses and play roles in host defense mechanism. TLR4 takes part in recognition of strongly conserved patterns of gram-negative cell wall components, LPS and discriminates indigenous from foreign molecules [118]. In TLR4 signaling, TLR4 must first associate with its extracellular binding partner, myeloid differentiation factor 2 (MD-2), before ligands can bind to the TLR4-MD-2 complex [119, 120]. The TLR4-MD-2-Ligand complex forms a heterodimer with another TLR4-MD-2 ligand complex and the signal is transferred to the TLR4's Toll/interleukin-1 receptor (TIR) domain. The signal is than further transduced via an unknown mechanism [118, 121]. The signal is then transmitted to two separate pathways which are the MyD88 path activating Nf-κB and Toll/IL-1 receptor also containing adaptor inducing IFN-β (TRIF) path. In the MyD88 path, MyD88 adaptor-like protein (Mal or TIRAP) mediates the TIR-TIR association between TLR4 and MyD88 [122]. Next, an interaction occurs between IL-1 receptor-associated kinase (IRAK) and MyD88. That interaction results in the activation of a cascade leading to the phosphorylation of Nf-κB transcription factors. This path results in activation of Activator Protein-1, RelA and p50 heterodimers and regulates expression of proinflammatory cytokines [123, 124]. In the other pathway, TRIF and TLR4 require an adaptor molecule called TRAM for signal transduction, which mediates endocytosis of the TLR4 receptor complex [125, 126]. TRIF forwards the signal after incorporation of TRAF3- or TRAF6-mediated adaptor molecules to either TRIF-binding kinase- (TBK-) IKK or RIP, respectively [127]. TBK-IKK terminates Interferon regulatory factor-3 dimerization and translocation into nucleus to induce IFN-β synthesis; in this way, TBK-IKK regulates cellular response to inflammation [128]. On the other hand, TRAF6 interacts with RIP and activates Nf-κB through TAK1, which operates the same as in the MyD88 pathway, causing late phase Nf-κB activation [127].

5.1. Nitric Oxide. Nitric oxide (NO) is an important messenger molecule in a variety of physiological systems. NO, a gas, is produced from L-arginine by different isoforms of NOS and takes part in many normal physiological functions, such as promoting vasodilation of blood vessels and mediating communication between cells of the nervous system. In addition to its physiological actions, free radical activity of NO can cause cellular damage through a phenomenon known as nitrosative stress [129]. Although many *in vitro* and *in vivo* studies support an involvement of NO in microglial-mediated dopaminergic neuronal death due to LPS-treatment, some studies suggested that NO is not involved [113]. For instance, the first in vivo study of the endotoxin-based PD model reported that the neurotoxic effect of LPS was not mediated by NO [36]. However, increasing evidence from recent studies supports for the notion that excessive production and accumulation of NO in the LPS-induced DA lead to neurodegeneration [64].

Intracerebral administration of LPS causes increase in the iNOS enzyme activity and NO production [130, 131]. Immunofluorescence and immunohistochemical analyses have revealed that iNOS is located in fully activated microglia having a characteristic amoeboid morphology [70, 132]. After intranigral LPS injection, iNOS mRNA levels and protein expression increase [132]. In Western blot analysis, iNOS has been shown to be induced in the SN after injection of LPS in a time- and dose-dependent manner [133]. The increase in iNOS expression inversely correlates with the TH immunolabeling and animals pretreated with a selective inhibitor of iNOS, N(G)-nitro-L-arginine methyl ester (L-NAME), exhibited complete protection against behavioral deficits induced by intrastriatal LPS injection [130]. Furthermore, LPS-induced loss of dopaminergic neurons is significantly inhibited by the administration of L-NAME [133]. Decrease in DA level and increase in cytochrome-c release and caspase-3 activation were significantly reversed with treatment of L-NAME [131]. Thus, increased NO availability subsequent to iNOS induction seems to play an important role in the initial phase of neurodegeneration. Hunter et al. have suggested that permanent expression of the iNOS plays a role in the progressive loss of dopaminergic neurons but not the initial loss induced by LPS [75]. Although the mechanism of NO-mediated neurodegeneration remains uncertain, it has been suggested that NO contributes to LPS-induced dopaminergic neurodegeneration through several mechanisms. NO has been shown to modify protein function by nitrosylation and nitrotyrosination, contribute to glutamate excitotoxicity, inhibit mitochondrial respiratory complexes, participate in organelle fragmentation, and mobilize zinc from internal stores [129, 134]. NO can react with superoxide radicals to form peroxynitrite radicals that are short-lived oxidants and highly damaging to neurons [64, 135]. Mitochondrial injury is prevented by treatment with L-N(6)-(l-iminoethyl)-lysine, an iNOS inhibitor, suggesting that iNOS-derived NO is also associated with the mitochondrial impairment [72]. NO inhibits cytochrome oxidase in competition with oxygen, resulting in glutamate release and excitotoxicity [114].

The main cellular source of NO in the CNS are microglia whereas astroglia constitute the main defense system against oxidative stress. However, under pathological or chronic inflammatory conditions, astroglial cells may also release neurotoxic mediators. Although the PD-associated gene DJ-1 mediates direct neuroprotection, the upregulation of DJ-1 in reactive astrocytes also suggests a role in glia [136]. The intracerebral LPS-based PD model is associated with a moderate reactive astrogliosis [70]. DJ-1 acts as a regulator of proinflammatory responses, and its loss contributes to PD pathogenesis by deregulation of astrocytic neuroinflammatory damage [137]. When treated with LPS, DJ-1-knockout astrocytes generate significantly more NO than littermate controls. The enhanced NO production in DJ-1(−/−) astrocytes is mediated by a signaling pathway involving reactive oxygen species (ROS) leading to specific hyperinduction of iNOS. These effects coincide with significantly increased phosphorylation of the p38 mitogen-activated protein kinase (MAPK), p38 inhibition, suppressed NO production, and iNOS mRNA as well as protein induction. DJ-1(−/−) astrocytes also induce the proinflammatory mediators COX-2 and IL-6 in high levels. Primary neuron cultures grown on DJ-1(−/−) astrocytes became apoptotic in response to LPS in an iNOS-dependent manner suggesting the neurotoxic potential of astrocytic DJ-1 deficiency [137]. These findings warrant *in vivo* confirmation.

5.2. Reactive Oxygen Species. A large body of evidence supports the involvement of oxidative stress in the pathogenesis of PD [134]. Besides NO, ROS generated by activated glia, especially microglia are major mediators of the DA neurodegeneration cause by inflammation [64]. ROS can cause lipid peroxidation, protein oxidation, DNA damage, and mitochondrial dysfunction. LPS-induced ROS production in microglia is mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multisubunit enzyme [114]. This complex is responsible for the production of both extracellular and intracellular ROS by microglia. Importantly, NADPH oxidase expression is upregulated in PD and is an essential component of microglia-mediated dopaminergic neurotoxicity. Activation of microglial NADPH oxidase causes neurotoxicity through two mechanisms. Firstly, extracellular ROS released from activated microglia are directly toxic to neurons. Secondly, intracellular ROS amplifies the production of several proinflammatory and neurotoxic cytokines and compounds such as TNFα, prostaglandin E2 (PGE2), COX-2, and IL-1β [138]. The activation of the phagocyte NADPH oxidase (PHOX) by cytokines, LPS, or arachidonic acid metabolites causes microglial proliferation and inflammatory activation; thus, PHOX is a key regulator of inflammation. Pharmacologic inhibition of NADPH oxidase provides protection against LPS-induced neurotoxicity and PHOX knockout mice are resistant to LPS-induced loss of SNpc dopaminergic neurons [139, 140]. Gene expression and release of tumor necrosis factor alpha was much lower in PHOX−/− mice than in control PHOX+/+ mice [140]. By injecting LPS into the striatum of wild type and Nox1 knockout mice, it has been shown that Nox1, a subunit of NADPH oxidase, also enhances microglial production of cytotoxic nitrite species and promotes loss of presynaptic proteins in striatal neurons [141]. Activation of PHOX alone causes no cell death, but when combined with expressed iNOS, it results in extensive neuronal cell death via the production of peroxynitrite [114]. The relationship between the signaling pathway downstream of TLR4, after LPS stimulation, and the activation of the oxidase remains elusive. Using mice lacking a functional TLR4, it has been demonstrated that TLR4 and ROS work in concert to mediate microglia activation [142]. Both TLR4(−/−) and TLR4(+/+) microglia display a similar increase in extracellular superoxide production when exposed to LPS. These data indicate that LPS-induced superoxide production in microglia is independent of TLR4 and that ROS derived from the production of extracellular superoxide in microglia mediates the LPS-induced TNF-α response of both the TLR4-dependent and independent pathway [142].

The integrin CD11b/CD18 (MAC1, macrophage antigen complex-1) pattern recognition receptor mediates LPS-induced production of superoxide by microglia [143]. MAC1 is a TLR4-independent receptor for the endotoxin LPS. MAC1 is essential for LPS-induction of superoxide in microglia, implicating that MAC1 acts as a critical trigger in microglial-derived oxidative stress during inflammation-mediated neurodegeneration. Interestingly, MAC1 mediates reactive microgliosis and progressive dopaminergic neurodegeneration in the MPTP model of PD, suggesting a role for this receptor in neurodegeneration [144]. Activated matrix metalloproteinase-3 (MMP-3) released from stressed dopaminergic neurons is also responsible for microglial activation and generation of NADPH oxidase-derived superoxide and eventually enhances nigrostriatal DA neuronal degeneration [145].

5.3. Proinflammatory Cytokines. Of the variety of cytokines that are released by LPS-activated glia, the proinflammatory IL-1β and TNFα may be the major cytokines involved in the LPS-induced dopaminergic neurodegeneration [64]. The contribution of these cytokines to neurodegeneration is supported by studies showing that neutralizing antibodies against TNFα or IL-1 markedly reduce the LPS-induced loss of nigral dopaminergic neurons [64]. Activated microglial cells in the SN are found in all animal models of PD and patients with the illness. Compared with astroglia or microglia, they appear to possess a larger repertoire of cytokine production [64, 113]. Elevated levels of TNFα in the cerebrospinal fluid (CSF) and the postmortem brains of PD patients as well as in animal models of PD implicate that proinflammatory cytokines significantly influence the pathophysiology of the disease [146]. TNFα has a pivotal role in mediating the loss of DA neurons in PD, which has been demonstrated using the endotoxin-based model. A sustained elevation of TNFα has been observed in the striatum and the mesencephalon of rats prenatally exposed to LPS [104]. Furthermore, in the chronic LPS nigral infusion model of PD, the loss of SNpc dopaminergic neurons, and the activation of microglia are significantly reduced by blockade of the soluble form of the TNFα receptor [146]. Systemic LPS administration results in rapid increase of TNFα in the brain, which remains elevated for 10 months [80]. Furthermore, LPS leads to microglial activation, to an increase in the expression of proinflammatory factors such as IL-1β, and NFκB p65, and to a progressive loss of nigral TH-immunoreactive neurons in wild-type mice, but not in mice lacking TNFα receptors [80]. Nontoxic doses of LPS also induce secretion of cytokines and predispose dopaminergic neurons to be more vulnerable to a subsequent low dose of neurotoxins such as 6-OHDA. Alterations in cytokines, prominently an increase in IL-1β, have been identified as being potential mediators of this effect that is associated with the activation of microglia [94, 95]. Administration of an IL-1 receptor antagonist results in significant reductions in TNFα and interferon gamma and attenuates the augmented loss of dopaminergic neurons caused by the LPS-induced sensitization to dopaminergic degeneration. Nigral injection of LPS in a degenerating SN exacerbates neurodegeneration and accelerates and increases motor signs and shifts microglial activation towards a proinflammatory phenotype with increased IL-1β secretion [95]. Importantly, chronic systemic expression of IL-1 also exacerbates neurodegeneration and causes microglial activation in the SN. It has been found by *in vivo* studies that NO is a downstream molecule of IL-1 action and partially responsible for the exacerbation of dopaminergic neurodegeneration, suggesting that IL-1 exerts its exacerbating effect on degenerating dopaminergic neurons by direct and indirect mechanisms [95].

Part of the challenge to sort out the contributions of individual cytokines to neurodegeneration may be a result of the complex interplay by various positive or negative feedback and feedforward loops among various cytokines, pro- and anti-inflammatory cytokines [64]. Microglial TNFα not only upregulates its own production in an autocrine fashion but also can further increase the surface expression of the neuronal TNFα cell death receptor (TNF p55 receptor) in a paracrine manner, thus exacerbating the LPS-induced neurotoxicity [64]. On the other hand, anti-inflammatory cytokines have been shown to reduce LPS-induced microglial activation and loss of SNpc dopaminergic neurons [147, 148]. The transforming growth factor beta 1 (TGF-β1), one of the most potent endogenous immune modulators of inflammation, exerts significant neuroprotection against LPS induction via its anti-inflammatory properties [147]. TGF-β1 inhibits the translocation of the cytosolic subunit p47phox of the LPS-induced PHOX from the cytosol to the membrane in cultured microglia. The molecular mechanisms of TGF-β1-mediated anti-inflammatory properties works via the inhibition of PHOX activity by preventing the ERK-dependent phosphorylation of Ser345 on PHOX's cytosolic subunit p47phox in microglia, thus reducing oxidase activities induced by LPS [147]. Using the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay and electron microscopy, Arimoto et al. have shown that intranigral injection of LPS causes marked microglial activation and a dose-dependent selective loss of dopaminergic neurons, which is mediated by apoptosis [148]. LPS injection leads to an increase in the mRNA expression of the proapoptotic proteins Bax, Fas, and the proinflammatory cytokines IL-1β, IL-6, and TNFα, while expression of the antiapoptotic gene Bcl-2 is decreased. Infusion of interleukin-10 (IL-10) by osmotic minipump protects against LPS-induced cell death of dopaminergic neurons. A corresponding decrease in the number of activated microglia suggests that the reduction in microglia-mediated release of anti-inflammatory mediators may contribute to the anti-inflammatory effect of IL-10 [148].

NFκB plays a key role in regulating neuroinflammation. Activation of NFκB depends on the phosphorylation of its inhibitor, IkappaB, by the specific IkappaB kinase (IKK) subunit IKK-beta. Compound A, a potent and selective inhibitor of IKK-beta, inhibits the activation of microglia, induced by nigral injection of LPS, and significantly attenuates LPS-induced loss of dopaminergic neurons in the SN [149]. Selective inhibition of NFκB activation affords neuroprotection by suppressing the activity of microglial NADPH oxidase

and by decreasing the production of ROS, and by inhibiting gene transcription of various proinflammatory mediators in microglia via IKK-beta suppression. Microglial activation may involve kinase pathways controlled by mixed lineage kinases (MLKs), a distinct family of mitogen-activated protein kinases, which might contribute to the pathology of PD. A potent MLK inhibitor, CEP-1347, inhibits brain TNFα production induced by intracerebroventricular injection of LPS in mice [150]. Coinjections of LPS with a p38 MAP kinase inhibitor to SN reduces iNOS and caspase-11 mRNA expression and rescues dopaminergic neurons in the SN [132]. Thus, LPS-induced dopaminergic cellular death in SN could be mediated, at least in part, by the p38 signal pathway leading to activation of inducible nitric oxide synthase and caspase-11.

5.4. Cyclo-Oxygenase-2 and Prostaglandin E2. Prostaglandins are potent autocrine and paracrine oxygenated lipid molecules that contribute appreciably to physiologic and pathophysiologic responses in brain and other organs [151]. Emerging data indicate that PGE2 plays a central role in neurodegenerative diseases. PGE2 signaling is mediated by interactions with four distinct G protein-coupled receptors, EP1-4, which are differentially expressed on neuronal and glial cells throughout the CNS, (here something is missing to make a sentence) [151]. EP2 activation has been shown to mediate microglial-induced paracrine neurotoxicity as well as to suppress the internalization of aggregated neurotoxic peptides in microglia [152]. PGE2 is produced at high levels in the injured CNS, where it is generally considered a cytotoxic mediator of inflammation. LPS upregulates the expression of COX-2 and increase the release of PGE2 in cultured microglia [64]. Intracerebral injections of LPS result in a significant upregulation of the striatal and nigral protein expression of COX-2 as well as the activation of microglia [153, 154]. Double labeling using immunohistochemistry identified that activated microglia rather than intact resting microglia are the main intracellular locations of COX-2 expression [64, 155]. *In vivo* pharmacological inhibition of COX-2 activity protects nigral dopaminergic neuronal loss and decreases microglial activation induced by intracerebral LPS injection, supporting the role of COX-2 in the pathogenesis of neuroinflammation-mediated neurodegeneration [153, 155, 156].

A local injection of LPS into the rat SN led to the induction of microsomal prostaglandin E2 synthase (mPGES)-1 in activated microglia [157]. Further *in vitro* and *in vivo* experiments with mPGES-1 knockout mice indicate the necessity of mPGES-1 for microglial PGE2 production. This study has shown that the activation of microglia contributes to PGE2 production through the concerted *de novo* synthesis of mPGES-1 and COX-2 at the sites of inflammation in the brain parenchyma. In contrast to that, a recent *in vitro* study suggests that mPGES-1 expression is not strictly coupled to the expression of COX-2 [158]. Activation of cultured spinal microglia via TLR4 produces PGE2 and causes NO release from these cells, showing that COX-PGE2 pathway is regulated by p38 and iNOS [159]. These findings emphasize that p38 in spinal microglia is a key player among inflammatory mediators, such as PGE2 and NO. *In vitro* experiments also indicate that microglial PGE2 plays an important role in astrocyte proliferation, identifying PGE2 as a key neuroinflammatory molecule that triggers the pathological response related to uncontrollable astrocyte proliferation [160].

5.5. Matrix Metalloproteinase-3. As discussed above (Section 5.2), the release of MMP-3 from apoptotic neurons may play a major role in degenerative human brain disorders, such as PD. The catalytic domain of recombinant MMP-3 induces the generation of TNFα, IL-6, IL-1β, and IL-1 receptor antagonist but not of IL-12 and iNOS, which are readily induced by LPS, in cultured microglia, suggesting that there is a characteristic pattern of microglial cytokine induction by apoptotic neurons [145]. MMP-3 activates the nuclear factor-kappaB (NFκB) pathway, and these microglial responses were totally abolished by preincubation with an MMP-3 inhibitor. MMP-3-mediated microglial activation mostly depends on ERK (extracellular signal-regulated kinase) phosphorylation but not on either JNK (c-Jun N-terminal protein kinase) or p38 activation. MMP-3-activated microglial cells caused apoptosis of neuronal cells in *in vitro* experiments. These results suggest that the distinctive signal of neuronal apoptosis is the release of the active form of MMP-3 that activates microglia and subsequently exacerbates neuronal degeneration [145]. The released active form of MMP-3, as well as the catalytically active recombinant from of MMP-3 leads to superoxide generation in cultured microglia [161]. MMP-3 causes dopaminergic cell death in mesencephalic neuron-glia mixed cultures of wild-type mice, but this is attenuated in the culture of NADPH oxidase subunit null mice (gp91(phox$-/-$)), suggesting that NADPH oxidase mediates the MMP-3-induced microglial production of superoxide and the following dopaminergic cell death. Moreover, in the MPTP model of PD, the nigrostriatal dopaminergic neuronal degeneration, microglial activation, and superoxide generation are largely attenuated in MMP-3$-/-$ mice. These results indicate that MMP-3 released from stressed dopaminergic neurons is responsible for microglial activation and generation of NADPH oxidase-derived superoxide and in turn exacerbates the nigrostriatal dopaminergic neuronal degeneration [161].

αSYN also induces the expression of MMP-3 in cultured microglia from rat [162]. The inhibition of MMP-3 significantly reduces NO and ROS levels and suppresses the expression of TNFα and IL-1β. Inhibition of MMP-3 also suppresses the activities of MAPK and transcription factors, NFκB and AP-1. The specific inhibitor of the protease-activated receptor-1 (PAR-1) and a PAR-1 antagonist significantly suppress cytokine levels, NO, and ROS production in αSYN-treated microglia, indicating that MMP-3 secreted by αSYN-stimulated microglia activate PAR-1 and amplify microglial inflammatory signals in an autocrine or paracrine manner [162]. *In vivo*, LPS injection into the SN of rats increases MMP-3 expression and activation suggesting that MMP-3 may participate in neuroinflammation-induced dopaminergic neurotoxicity [163]. These studies propose that the *in vivo* modulation of MMP-3 expression and

activity may provide the neuroprotection for dopaminergic neurons. Indeed, an antibiotic, doxycycline, shows neuroprotection for the dopaminergic system in a toxin-based model of PD and this appears to derive from antiapoptotic and anti-inflammatory mechanisms involving downregulation of MMP-3 [164].

5.6. Microenvironmental Changes and Intercellular Interactions. The CNS microenvironment plays a significant role in determining the phenotypes of both CNS-resident microglia and CNS-infiltrating macrophages. In this section, we summarize the microenvironmental changes such as astroglial responses, BBB alterations, and a wide range of intercellular interactions in the context of the endotoxin-based PD model.

5.6.1. Reactive Astrocytes and Parkinson's Disease. Astrocytes are the most abundant cell types in the CNS and participate in the local innate immune response triggered by a variety of insults. The role of astrocytes in the pathogenesis of PD is even less well understood than the one of microglia but they are known to secrete both inflammatory and anti-inflammatory molecules [165]. It has been proposed that astrocytes may play dual roles in PD [166]. Similar to microglial activation, star-shaped astrocytes transformed to reactive form have enlarged and thick bodies and respond to various stimuli, which coined the term reactive astrocytes [167]. Reactive astrogliosis is generally mild or moderate and rarely severely pronounced in autopsy specimens from the SN of PD patients [166]. Classic reactive astrocytes are observed in multiple system atrophy, progressive supranuclear palsy, and corticobasal degeneration, but not in PD cases; the extent of reactivity correlates with indices of neurodegeneration and disease stage [168]. Different subpopulations of astrocytes express disease-related proteins such as αSYN, parkin, and p-tau at different levels and in different combinations in different Parkinsonian syndromes but the roles of astrocytes in these conditions are not yet well defined [167, 168].

The role of astrocytes in the development of PD is still unknown and controversial. Astrocytes provide the optimal microenvironment for neuronal function by exerting active control over the cerebral blood flow and by controlling the extracellular concentration of synaptically released neurotransmitters [167]. Generally, astrocytes promote the survival and maintenance of dopaminergic neurons through secretion of various neurotrophic factors in the SN. The decreased levels of astrocyte-derived neurotrophic factors are at least in part responsible for DA neuronal death in PD [167]. Astrocytes become activated and synthesize pro- and anti-inflammatory cytokines, chemokines, antioxidants, neurotrophic factors, and prostanoids during neuroinflammation and neurodegeneration and interact with other immune competent cells. These mediators act as double-edged swords, exerting both detrimental and neuroprotective effects. For example, myeloperoxidase (MPO), a key enzyme in the generation of reactive nitrogen species (RNS), is upregulated in the midbrains of PD patients and MPTP treated mice [169]. This enzyme is localized within reactive astrocytes in MPTP-treated mice, and MPTP neurotoxicity is attenuated by ablation of MPO from the nigrostriatal pathway [167, 169].

5.6.2. Region-Specific Astroglial Responses in the Brain. Degenerative disorders of the brain often occur in a region specific fashion, suggesting differences in the activity and reactivity of innate immune cells. This may make astrocytes likely candidates to be responsible for region-specific incidence rates of neurological and neurodegenerative disorders. Cultured astrocytes from the cortex and midbrain already differ in their capacity and profile of cytokine expression under unstimulated conditions [170]. In response to LPS, both a region specific pattern of upregulation of distinct cytokines, and differences in the extent and time course of activation are observed. Thus, astrocytes reveal a region-specific basal profile of cytokine expression and a selective area specific regulation of cytokines upon LPS-induced inflammation [170]. The densities of astrocytes are much lower in the intact SNpc, compared with the cortex [171]. Furthermore, after LPS injection, damage to endothelial cells and astrocytes and the blood-brain barrier (BBB) permeability are more pronounced in the SNpc [171]. The *in vitro* responses of microglia and astroglia to inflammatory stimuli or environmental toxins also differ. Manganese significantly potentiates LPS-induced release of TNF-α and IL-1β in microglia, but not in astroglia [172]. These agents are more effective in inducing the formation of ROS and NO in microglia than in astroglia.

5.6.3. DJ-1, Oxidative Stress and Astrocytes. Recent findings support the developing view that astrocytic dysfunction, in addition to neuronal dysfunction, may contribute to the progression of a variety of neurodegenerative disorders. Thus, the treatments that support the beneficial aspects of astrocyte function may represent novel approaches targeting astrocytes to promote dopaminergic neurorescue. Although aging enhances the neuroinflammatory response and the alpha-synuclein nitration [73], the antioxidant capacity and glutathione metabolism of astrocytes are preserved from mature adulthood into senescence [173]. Thus, the oxidative stress seen in aging brains is likely due to factors extrinsic to astrocytes, rather than being caused by an impairment of the antioxidative functions of astrocytes. The PARK7 (DJ-1) gene, which has been implicated in some forms of early-onset, autosomal recessive PD, is apparently expressed mainly by the astrocytes in the human brain. Loss-of-function mutations lead to the characteristic selective neurodegeneration of nigrostriatal dopaminergic neurons. In addition to cell-autonomous neuroprotective roles, DJ-1 may act in a transcellular manner, being upregulated in reactive astrocytes in chronic neurodegenerative diseases, for example. In sporadic PD, and many other neurodegenerative diseases, reactive astrocytes overexpress DJ-1 whereas neurons maintain the expression at normal levels [136]. Since DJ-1 has neuroprotective properties and since astrocytes are known to support and protect neurons, DJ-1 overexpression in reactive astrocytes may reflect an attempt to protect themselves and the surrounding neurons against disease progression. Knocking down DJ-1 in astrocytes impairs

astrocyte mediated neuroprotection against rotenone [174]. DJ-1 is a ubiquitous redox responsive and cytoprotective protein with diverse functions. DJ-1 regulates redox signaling kinase pathways and acts as a transcriptional regulator of antioxidative genes. DJ-1 scavenges H_2O_2 by cysteine oxidation in response to oxidative stress and, thus, confers neuroprotection. Therefore, DJ-1 is an important redox-reactive signaling intermediate, controlling oxidative stress upon neuroinflammation and during age-related neurodegenerative processes such as PD [136]. However, the functional basis of neuroprotection elicited by DJ-1 has remained vague. DJ-1 stabilizes erythroid 2-related factor (Nrf2), a master regulator of antioxidant transcriptional responses, by preventing its association with the inhibitor protein Keap1 and by blocking Nrf2's subsequent ubiquitination [175]. Without intact DJ-1, Nrf2 protein is unstable, and transcriptional responses are thereby decreased both basally and after induction [175] though a recent study suggests that activation of the Nrf2 is independent of DJ-1 [176].

5.6.4. Nrf2/ARE Pathway and Parkinson's Disease. The expression of phase II detoxification and antioxidant enzymes is governed by a cis-acting regulatory element named the antioxidant response element (ARE). Nrf2 regulates genes containing the ARE element and is a member of the Cap'n'Collar basic-leucine-zipper family of transcription factors. Following activation, Nrf2 dissociates from Keap1, translocates to the nucleus, and binds to the ARE promoter sequences, as a part of the coordinated induction of a battery of cytoprotective genes including antioxidants and anti-inflammatory genes [177]. ARE-regulated genes are preferentially activated in astrocytes, which consequently have more efficient detoxification and antioxidant defense mechanisms than neurons. Astrocytes closely interact with neurons to provide structural, metabolic, and trophic support, as well as actively participating in the modulation of neuronal excitability and neurotransmission [177]. Therefore, alterations in astroglial function can modulate the interaction with surrounding cells such as neurons and microglia. Activation of Nrf2 in astrocytes protects neurons from a wide array of insults in different *in vitro* and *in vivo* paradigms, confirming the role of astrocytes in determining the vulnerability of neurons to deleterious stimuli [177]. Nrf2 has been shown to be important for protection against oxidative stress and cell death in toxin-based models of PD [177–181]. These findings remain to be confirmed in endotoxin-based models. Genetic data suggest that variation in Nrf2 gene NFE2L2 modifies the PD process, which provides another link between oxidative stress and neurodegeneration [182]. Nrf2 activating agents such as synthetic triterpenoids and sulforaphane are potential therapeutic targets for the prevention of neurodegeneration in PD [183–185].

5.6.5. Nrf2/ARE Pathway and Microglial Activation. The deficiency of Nrf2 results in an exacerbated inflammatory response and in microglial activation of the expression of the neurotoxin MPTP whereas inducers of Nrf2 downmodulate neuroinflammation [181]. Nrf2-deficient mice exhibit more astrogliosis and microgliosis, as determined by an increase in mRNA and protein expression levels for GFAP and F4/80, respectively, than wild-type mice. Inflammation markers, characteristic of classical microglial activation like COX-2, iNOS, IL-6, and TNF-alpha, are also increased. At the same time, anti-inflammatory markers, attributable to alternative microglial activation, such as FIZZ-1, YM-1, Arginase-1, and IL-4 are decreased [181]. These results demonstrate a role of Nrf2 in tuning the balance between classical and alternative microglial activation. The restoration of the redox balance may be a determinant in driving microglia back to the resting state. ROS generated by microglia could help to eliminate pathogens in the extracellular milieu and also to act on the microglia itself, altering the intracellular redox balance and functioning as a second messengers in the induction of proinflammatory genes. The modulation of microglial activation is a matter closely correlated with control of oxidative stress in this cell type and is crucial to restore its inactive state and modulate the inflammation in neurologic diseases [186]. Nrf2 is essential for the regulation of NADPH oxidase-dependent ROS-mediated TLR4 activation in macrophages [187]. Nrf2 activation by sulforaphane inhibits the inflammatory response to LPS in cultured rodent microglia [185]. These findings remain to be tested in the context of *in vivo* endotoxin-based PD models. Interestingly, LPS by itself is able to activate the cell's defense against oxidative and electrophilic stress, activating Nrf2 [185]. This mechanism may be a mediator of LPS preconditioning or endotoxin tolerance, a phenomenon which by prior exposure of innate immune cells like monocytes/macrophages to minute amounts of endotoxin causes them to become refractory to subsequent endotoxin challenges [188]. In contrast to the well-known protective effect of this phenomenon, in acute ischemic conditions, only one *in vitro* study has reported this benefit in dopaminergic neurotoxicity [189]. Further understanding the underlying mechanism of LPS preconditioning may open a new window for the treatment of PD.

Astroglial cells are also involved in the microglial modulation by Nrf2 [177]. These cells are known to play an important role in antioxidant defense and in modulating microglial activity in the CNS [165, 166]. Recently, astrocytes have been found to regulate excessive inflammation via induction of the microglial hemooxygenase-1 (HO-1) expression *in vitro* [190]. While pharmacological or genetic intervention on Nrf2 may provide a neuroprotective benefit, HO-1 does not protect or enhance the sensitivity to neuronal death in the MPTP model [191]. These results support the idea that the modulation of a master transcription factor may be a better strategy than targeting individual genes.

5.6.6. Blood-Brain Barrier Dysfunction and Peripheral Immune Cell Infiltration. The brain demands an adequate blood supply for the regulation of neuronal and synaptic function. To maintain concentrations of ions within narrow ranges as well as the adequate levels of metabolic substrates in various brain regions, neural milieu are strictly separated from circulatory spaces through BBB formation [167]. These unique biological structures are comprised of neurovascular

units such as brain capillary endothelial cells, pericytes, neurons, and astrocyte end-feet. Endothelial cells tightly connect at junctional complexes such as adherens junctions, tight junctions, and gap junctions confer low paracellular permeability. Pericytes and astrocytes regulate hemodynamic neurovascular coupling, microvascular permeability, matrix interactions, neurotransmitter inactivation, neurotrophic coupling, and angiogenic as well as neurogenic coupling through close proximity with neurons [167, 192]. Although there is no clear evidence as to whether these altered neurovascular circumstances are responsible for the loss of dopaminergic neurons in PD, several studies on PD patients and animal models suggest a pathogenic linkage between BBB disruption and dopaminergic neuronal death [167]. PET and histological studies on PD patients revealed BBB dysfunction in the midbrain of PD patients [193]. In addition, increased BBB permeability has been observed in the MPTP and the LPS models for PD [194]. These studies suggest that the disruption of the BBB has a relationship with neuronal cell death and neuroinflammation in PD [167]. There is also a direct correlation between the location of IgG immunoreactivity-a, a marker for disruption of neurodegenerative processes, including the death of nigral dopaminergic cells and reactive astrocytes. A precise spatial correlation also exists between disruption of the BBB and 3-nitrotyrosine immunoreactivity [194]. LPS-activated microglia can induce the dysfunction of the BBB in an *in vitro* coculture system with rat brain microvascular endothelial cells and microglia [195]. In the presence of LPS-activated microglia, tight junction proteins are fragmented, and barrier disintegrity and dysfunction induced by LPS-activated microglia are blocked by an NADPH oxidase inhibition, suggesting that LPS activates microglia to induce dysfunction of the BBB by producing ROS through NADPH oxidase.

Recent studies have shown that the dysfunction of the BBB combined with the infiltration of peripheral immune cells plays an important role in the degeneration of dopaminergic neurons [167]. However, these molecular and cellular changes are not specific to the PD, since they are also implicated in the pathogenesis of other neurodegenerative diseases [196]. The neuroinflammation may contribute to the infiltration of peripheral immune cells and leakage of the BBB into the SN. Various peripheral immune cells, such as T-cells, B-cells, microphages, and leukocytes infiltrate into the SN region in the LPS and MPTP models [167, 171, 197]. CD11b and MPO double-positive neutrophils infiltrate the SNpc following LPS injection [197]. MPO(+) neutrophils observed in SNpc express iNOS, IL-1β, COX-2, and monocyte chemoattractant protein-1 (MCP-1). In intact rodent brain, the densities of microglia are similar in SNpc and cortex [197]. In addition, the densities of astrocytes are much lower in the intact SNpc, compared with the cortex. However, LPS injection induces microgliosis and causes neutrophil infiltration into the SNpc, but not into the cortex [171]. The extent of neutrophil infiltration appears to be correlated with neuronal damage. The loss of neurons in the SNpc is significantly reduced in neutropenic rats versus normal rats following LPS injection. Furthermore, after LPS injection, damage to endothelial cells and astrocytes and increased BBB permeability are more pronounced in the SNpc. Excessive neutrophil infiltration, lower astrocyte density, and higher BBB permeability following LPS exposure contributes to severe inflammation and neuronal death in the SNpc compared with the cortex [171].

The links between T-cell immunity and the nigrostriatal neurodegeneration are supported by laboratory, animal model, and human pathologic investigations [198]. The presence of T-lymphocytes in the midbrain of PD patients suggests that the potential role of infiltrated peripheral cells is a factor of the PD pathogenesis [199]. Recently, Brochard et al. have reported that numerous CD4 and CD8 positive cells are detectable in postmortem PD patients [200]. The infiltration of CD4+ lymphocytes into the brain also contributes to the neurodegeneration in the MPTP model for PD [200]. Specifically, invading T-lymphocytes contribute to neuronal cell death via the Fas/FasL cell death pathway, implicating the emerging role of the adaptive immune system in the pathogenesis of PD [201].

The adoptive transfer of CD3-activated CD4+CD25+ regulatory T-cells (Tregs) is known to suppress immune activation and maintain immune homeostasis and tolerance. In MPTP-treated mice, it protects the nigrostriatal system from degeneration through suppression of microglial oxidative stress and inflammation [202]. Tregs also attenuates Th17 cell-mediated nigrostriatal dopaminergic neurodegeneration in the MPTP model [203]. In addition, these cells suppress nitrated αSYN-induced microglial ROS production and NFκB activation supporting the importance of adaptive immunity in the regulation of PD-associated microglial inflammation [204]. Taken together, these studies provide a rationale for future immunization strategies in PD [198].

Accumulating evidence suggests that the penetration of immune cells into the brain plays an important role in the degeneration of dopaminergic neurons in PD. Further understanding of the cellular and molecular mechanisms responsible for trafficking of immune cells from the periphery into the diseased CNS may be the key to targeting these cells for therapeutic intervention in PD [196].

In addition to glia-neuron crosstalk, multiple cell-to-cell interactions and immune regulations, critical for neuronal homeostasis, also influence immune responses [198, 205]. Microglia can be activated by MCP-1, which is expressed by dopaminergic neurons and can interact with its receptor CCR2 on microglial cells. The neuroimmune regulatory proteins CD47 and CD200 inhibit macrophage and microglia activation through binding to their receptors SIRPalpha and CD200R, expressed on phagocytes [206]. Upon stress, nigral dopaminergic neurons secrete MMP-3 and α-SYN, which activates microglial and astroglial cells [145, 207]. As disease progresses, secretions from α-SYN-activated microglia can engage neighboring glia cells in a cycle of autocrine and paracrine amplification of neurotoxic immune products. Astrocytes differentially regulate neutrophil functions through direct or indirect interactions between the two cell types [208]. Many of these established interactions between different cell types involved in neuroinflammation have been demonstrated *in vitro* and remain to be confirmed *in vivo*.

Dissecting the molecular determinants of complex interplay between CNS cells and immune cells in the context of the endotoxin-based PD model will give the possibility to test novel therapeutic strategies to promote restoration of injured nigrostriatal dopaminergic neurons.

6. Therapeutic Approaches

The endotoxin-induced neuroinflammation model for PD is a purely inflammation-driven model. However, all clinical and pathological features of PD can be observed in this model. Therefore, the LPS-induced model can be used to search for novel treatment strategies for the therapy of PD. In this section, we summarize known neuroprotective molecules, which have been tested using the LPS-induced PD models.

COX-2 is a rate-limiting enzyme in prostaglandin synthesis. Experimental and epidemiological evidence supports the protective role of COX-2 inhibition in PD. COX-2 is upregulated in SN both in the PD and in the MPTP model [209]. Pharmacological inhibition of COX-2 or the knockout of the COX-2 gene provides resistance to MPTP *in vivo* [209, 210] and to 6-OHDA-induced dopaminergic toxicity *in vitro* [211]. There is epidemiological evidence that the use of some NSAIDs lowers the incidence of PD [212]. On the other hand, according to meta-analyses of NSAID studies in PD, ibuprofen shows a slight protection against PD whereas aspirin and acetaminophen did not show any protective effects [213, 214]. Hunter et al. used the COX-2 inhibitor Celecoxib (Celebrex) in LPS-induced PD animal model for the first time. They were able to show that Celecoxib protects dopaminergic neurons by decreasing inflammation and by restoring mitochondrial function in the intrastriatal LPS-induced PD model [153]. Using the intranigral LPS rat model, Sui et al. [155] have shown that another COX-2 inhibitor, meloxicam, diminishes the activation of OX-42 positive microglia and reduces the loss of dopaminergic neurons in the SNpc. Clinical studies suggest that inhibition of COX-2 may cause side effects such as trombogenic cardiovascular diseases [156, 215]. In order to avoid potential side effects of COX-2 inhibition, new drugs have been targeted for dual inhibition of COX-2 and lipoxygenase (LOX) [156]. Dual inhibitor of COX-2 and 5-LOX has been shown to lower gastrointestinal side effects. Moreover, combination of the two inhibitors achieves a more potent neuroprotection than usage of single inhibitors [216]. Li et al. tested the dopaminergic neuroprotective effect of COX, LOX, and the combination of COX and LOX inhibitors in the intrastriatal LPS-induced animal model for PD. They found that the dual COX and LOX inhibitor, phenidone, is better than COX or LOX inhibitors alone for suppressing LPS-induced neurotoxicity [156].

Dexamethasone is a potent anti-inflammatory drug that has been tested in the intranigral LPS-induced PD model [67, 133]. These studies have shown that dopaminergic degeneration and microglial activation induced by LPS can be prevented by administration of dexamethasone [67, 133]. Dexamethasone also decreases the exacerbating effect of LPS during neurodegeneration induced by 6-OHDA [95].

Experimental and epidemiological evidence supports the protective role of nicotine in PD. Epidemiological studies have confirmed that there is an inverse correlation between cigarette smoking and the incidence of PD [217]. *In vitro* nicotine pretreatment inhibits LPS-induced TNF-α release in murine-derived microglial cells via the α-7 nicotinic receptor [218]. These results suggest that nicotine could protect dopaminergic neurons in the animal model of PD. Indeed, Park et al. have shown that nicotine significantly decreases the release of TNFα and the dopaminergic neuronal loss induced by LPS stimulation. Both effects were blocked by α7-nicotinic acetylcholine receptor blockers [219].

Peroxisome proliferators activated receptor (PPAR-γ) is a nuclear receptor that regulates transcription of various genes. It has been shown that the PPAR-γ agonist inhibits cytokine secretion in microglia and macrophage-like cells [220]. Hunter et al. have shown that a PPAR-γ agonist, pioglitazone, provides neuroprotection by decreasing inflammation and restoring mitochondrial function. Pioglitazone administration partially reduces the LPS-induced striatal dopamine loss and the TH-positive cell loss in the SN [153].

Minocycline is a semisynthetic tetracycline that exerts anti-inflammatory activities [221]. Minocycline significantly reduces the SN microglial activation induced by intranigral LPS administration [194]. Minocycline prevents the LPS-induced increase of mRNA levels of proinflammatory cytokines and diminishes the production of peroxynitrites [194].

Naloxone, an opioid receptor competitive antagonist, has been found to reduce microglial activation-mediated DA neurodegeneration in mouse cortical neuron-glia cocultures [64]. Systemic infusion of naloxone protects dopaminergic neurons against inflammation-mediated degeneration and decreases microglial activation *in vivo* through inactivation of NADPH oxidase [139, 222].

The neuroprotective effects of statins in CNS disorders such as experimental autoimmune encephalomyelitis, stroke, and Alzheimer's disease have been previously described [223–225]. Selley has shown that oral administration of simvastatin attenuates the depletion of dopamine DOPAC and HVA inhibits the formation of 3-nitrotyrosine and the production of TNFα in mice treated with MPTP [226]. Simvastatin has also been tested in the intranigral LPS-induced PD [227] and the LPS perfusion model [228]. Simvastatin prevents the loss of dopaminergic neurons and astrocytes induced by LPS in both models [227, 228]. Simvastatin increases BDNF expression [228], which may support neuronal and astroglial survival.

Osteopontin (OPN) is a glycosylated phosphoprotein that has first been identified in 1986 in osteoblasts [229]. OPN is constitutively expressed in most tissues, including the brain [208]. Iczkiewicz et al. have shown that OPN is constitutively present in dopaminergic neurons, in the SN, and that its expression is decreased in the MPTP model of PD and in patients with PD [230]. It has been reported that the intranigral injection of LPS enhances expression of OPN [231]. These results suggest that OPN may have a regulatory role in neuroinflammation. One peptide fragment of OPN contains the arginine-glycine-aspartic acid (RGD) domain

that has been associated with the neuroprotective effects of OPN [232]. Iczkiewicz et al. have tested RGD containing peptide fragments of OPN in the LPS-induced PD model. They found that the RGD containing peptide fragment of OPN protects against LPS-induced TH positive cell loss and alters gliosis in the rat SN [233].

Urocortin is a neuroprotective agent that is structurally related to the corticotrophin releasing factor (CRF) [234–236]. Abuirmeileh et al. have used urocortin for the treatment of the LPS-induced PD model. They have shown that urocortin reduces nigrostriatal damage induced by LPS and that this effect of urocortin is mediated by CRF_1 receptors [237–239].

7. Conclusion

Parkinson's disease (PD) is the second most common neurodegenerative disease with increasing incidence worldwide. Although the pathogenesis of PD remains elusive, accumulating evidence from many studies on animal models and patients shows that the pivotal role of microglial activation along with neuroinflammatory processes contribute to the initiation and progression of the nigrostriatal dopaminergic neurodegeneration in PD. In addition to that, recent studies have proposed that the BBB dysfunction combined with the infiltration of peripheral immune cells into the CNS plays an essential role in the degeneration of nigral dopaminergic neurons. Thus, using a purely inflammatory experimental model induced by the administration of the bacterial endotoxin, LPS, provides a valuable tool for the *in vivo* modeling of the characteristics of progressive dopaminergic neurodegeneration associated with neuroinflammation. Except for the acute direct administration of LPS to the nigral region, other modified forms of the model, including the prenatal one, realistically simulate the slow and progressive dopaminergic neuronal loss and permanent neuroinflammation. Furthermore, the combination of endotoxin-based PD models with genetic and toxin-based models is fruitful for the delineation of the complex interactions among the environmental and genetic factors and inflammatory processes involved in PD. Many experimental variables including sex, age, and strain of the animals have the potential to significantly perturb the functional and pathologic outcomes. These methodological issues should be considered in respect to the studies.

Several novel techniques, such as *in vivo* imaging of microglial activation, are waiting to be applied in the endotoxin-based model of PD. Molecular studies from the domains of transcriptomics, proteomics, and microRNomics will be valuable to gain in potential diagnostic markers for the disease [240]. Since the inflammatory responses precede the neurodegeneration and the motor dysfunctions, alterations of the immune parameters, both in CSF and blood, are likely to be useful as early diagnostic markers. The major challenge in this area is the enhancement of the specificity and sensitivity of the potential markers. Despite intensive research, the mechanisms of neuroinflammation-mediated nigral neurodegeneration are poorly understood. Whether neuroinflammation is a consequence or a cause of nigral neuronal loss is still unknown. Neuroinflammation seems to be a trigger of the initiation of neurodegeneration and progressive neurodegeneration continuously aggravates chronic neuroinflammatory processes. In this context, the stimulation of TLR4 by endogenous ligands released by injured dopaminergic neurons may contribute to this vicious circle [241].

In vivo imaging and molecular studies will also extend our understanding of the complex interplay between CNS and immune cells. Especially, the novel links between neuroinflammatory processes, oxidative stress, and Nrf2/ARE pathways that are mainly based on data from toxin-based models of PD should be confirmed by the endotoxin based model.

Based on the recent data, adaptive immune responses along with innate immunity are important mediators of neuroinflammation-mediated dopaminergic neurodegeneration. Recent evidence suggests that the importance of nonautonomous pathological mechanisms are involved in PD, which are mostly mediated by activated microglia and peripheral immune cells. Thus, the harnessing of the immune system by immunomodulating drugs or by immunisation aiming at the downregulation of immune responses remains promising future therapeutic options. Immune parameters will also be indispensable for the monitoring of therapeutic responses.

Abbreviations

MPTP:	1-Methyl-4-Phenyl-1,2,3,6-tetrahydropyridine
DOPAC:	3,4-dihydroxyphenylacetic acid
6-OHDA:	6-hydroxydopamine
ARE:	Antioxidant response element
BBB:	Blood-brain barrier
CNS:	Central nervous system
CSF:	Cerebrospinal fluid
COX-2:	Cyclo-oxygenase-2
EGF:	Epidermal growth factor
ERK:	Extracellular signal-regulated kinase
DA:	Dopamine
GFAP:	Glial fibrillary acidic protein
HO-1:	Hemooxygenase-1
HPLC:	High-performance liquid chromatography
HVA:	Homovanillic acid
5-HT:	5-hydroxytryptamine
IKK:	IkappaB kinase
IRAK:	IL-1 receptor-associated kinase
iNOS:	Inducible nitric oxide synthase
IFN-β:	Interferon-beta
IL-2:	Interleukin-2
IL-6:	Interleukin-6
IL-10:	Interleukin-10
IL-1β:	Interleukin 1β
JNK:	c-Jun N-terminal protein kinase
LPS:	Lipopolysaccharide
MMP-3:	Matrix metalloproteinase-3
MPP^+:	1-methyl-4-phenylpyridinium
MA:	Methamphetamine

MLKs: Mixed lineage kinases
TFAM: Mitochondrial transcription factor A
MD-2: Myeloid differentiation factor 2
Mal or TIRAP: MyD88 adaptor-like protein
PHOX: NADPH oxidase
L-NAME: N(G)-nitro-L-arginine methyl ester
NADPH: Nicotinamide adenine dinucleotide phosphate
NO: Nitric oxide
Nrf2: Nuclear factor erythroid 2-related factor
NFκB: Nuclear factor-kappaB
PD: Parkinson's disease
PPAR-γ: Peroxisome proliferator-activated receptor
PGE2: Prostaglandin E2
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
Tregs: Regulatory T cells
SN: Substantia nigra
SNpc: Substantia nigra pars compacta
SNpr: Substantia nigra pars reticulata
TUNEL: Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
TH+: Tyrosine hydroxylase-positive
TLRs: Toll-like receptors
TIR: Toll/interleukin-1 receptor
TRIF: Toll/IL-1 receptor containing adaptor inducing IFN-β
TGFα: Transforming growth factor-alpha
TGF-β1: Transforming growth factor-beta 1
TBK: TRIF-binding kinase
TNFα: Tumor necrosis factor-alpha
TH: Tyrosine hydroxylase
VTA: Ventral tegmental area.

Conflict of Interest Disclosure

The authors declare no competing financial interests.

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Review Article

Models for LRRK2-Linked Parkinsonism

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Parkinson's disease (PD) is a progressive neurodegenerative movement disorder characterized by the selective loss of dopaminergic neurons and the presence of Lewy bodies. The pathogenesis of PD is not fully understood, but it appears to involve both genetic susceptibility and environmental factors. Treatment for PD that prevents neuronal death progression in the dopaminergic system and abnormal protein deposition in the brain is not yet available. Recently, mutations in the leucine-rich repeat kinase 2 (LRRK2) gene have been identified to cause autosomal-dominant late-onset PD and contribute to sporadic PD. Here, we review the recent models for LRRK2-linked Parkinsonism and their utility in studying LRRK2 neurobiology, pathogenesis, and potential therapeutics.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder with movement, cognitive, and emotional dysfunction, affecting 2% of the population over the age of 60 years [1]. PD is characterized by tremors, rigidity, bradykinesia/akinesia, and postural instability resulting from the loss of dopamine neurons in the substantia nigra and other regions of the brain [2–5]. The pathological hallmark of PD is the presence of proteinaceous cytoplasmic inclusions termed Lewy bodies [5, 6]. PD is similar to other neurodegenerative diseases in that it presents with neuronal cell death and protein aggregation, though the relation between them is uncertain [6, 7]. The pathogenesis of PD remains incompletely understood, but it appears to involve both genetic susceptibility and environmental factors. Treatment for PD that prevents neuronal death progression in the dopaminergic system and abnormal protein deposition in the brain is not yet available.

Recently, mutations in the LRRK2 gene have been identified to cause autosomal dominant PD and contribute to sporadic PD [8–10]. To date, more than 50 variants including at least 16 disease-causing mutations have been reported [11–22]. This paper highlights the recent models for LRRK2-linked Parkinsonism and their utility in studying LRRK2 neurobiology, pathogenesis, and potential therapeutics. For other aspects of LRRK2 please refer to several recent excellent review papers [23–26]. Due to the length of this review, we apologize that we did not include all LRRK2 publications.

2. LRRK2 Gene and Protein

The LRRK2 gene spans a genomic region of 144 Kb, with 51 exons encoding 2527 amino acids. The *LRRK2* mRNA is expressed throughout the brain and other organs [9]; *in situ* hybridization in mice reveals that expression predominates within regions of the basal ganglia, which are associated with motor dysfunction in PD, and within nonmotor areas such as the hippocampus [27–31]. The *LRRK2* gene is conserved across species from invertebrates to human. *Caenorhabditis elegans* and *Drosophila melanogaster* each have only one *LRRK2* ortholog [9].

The LRRK2 protein contains several predicted domains (Figure 1) including Roc (Ras in complex proteins, belonging to the Ras/GTPase family), COR (C terminal of Roc), LRR, a leucine-rich repeat, consisting of twelve repetitions of a 22–28 amino acid motif, MAPKKK, a protein kinase catalytic domain which may be involved in serine/threonine phosphorylation, a WD40 domain and ankyrin repeats. The LRR and WD40 domains may be involved in protein-protein interactions [32]. The LRRK2 protein is expressed in all tissues examined, although at low levels. In the brain, LRRK2 is expressed in neurons, astrocytes, and microglia.

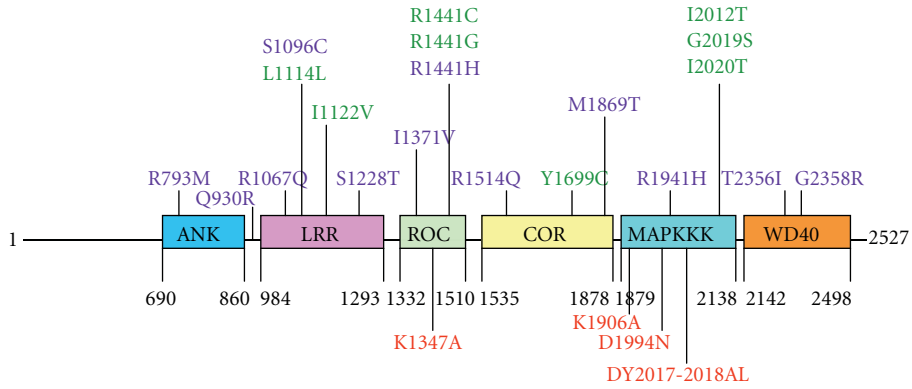

Figure 1: *LRRK2 domain structure and PD-linked point mutations.* The predicted domain boundaries are indicated by the residue numbers beneath. The position of the putatively pathogenic amino acid substitutions are shown in purple. Substitutions segregating with PD are shown in green. The kinase null and no-GTP binding alterations are shown in red.

Recent studies have detected LRRK2 in specific brain regions including the cortex, striatum, hippocampus, cerebellum and in dopaminergic neurons of the substantia nigra [30, 31, 33, 34]. However, the expression levels of LRRK2 in the dopaminergic neurons of the SNpc are very low. LRRK2 protein can be detected in Lewy neurites [35] and in Lewy bodies of sporadic PD [36]. In the subcellular level, it was found mainly in the cytoplasm and associated with lipid rafts, lysosomes, endosomes, mitochondria, and Golgi transport vesicles [9, 30, 33, 34, 37–39]. Several studies show that LRRK2 is enriched at the membrane of cells [30, 31, 40, 41] and that the membrane-associated fraction of LRRK2 may display greater kinase and GTP-binding activities than cytosolic LRRK2 [41]. Another study shows the recruitment of LRRK2 to the endosomal-autophagic pathway suggesting the functional involvement of LRRK2 in this pathway [42, 43].

Patients with LRRK2 mutations typically have a relatively late onset of PD with asymmetric rest tremor, bradykinesia, rigidity, and a good response to L-DOPA treatment [9, 44]. The pathological heterogeneity of affected individuals examined ranges from pure nigral degeneration without Lewy bodies to nigral degeneration associated with Lewy bodies, widespread Lewy bodies consistent with diffuse Lewy body disease, or neurofibrillary tau-positive tangles [8, 9, 45, 46]. Point mutations have been identified in almost all of the predicted domains of LRRK2 (Figure 1) [1, 21, 22, 47–49]. The most common mutation, G2019S, contributes to 5-6% of autosomal-dominant PD [50, 51] and 1-2% of sporadic PD [52]. The distribution of mutations across several different LRRK2 domains, the lack of deletions or truncations, and the dominant pattern of inheritance, are consistent with a gain-of-function mechanism for LRRK2-associated PD.

The normal function of LRRK2 is still unclear. Loss-of-function studies indicate that the *Drosophila* LRRK2 homologous protein (*CG5483*) is critical for the integrity of dopaminergic neurons in the fly [53] and Zebrafish LRRK2 homology is important for neuronal development [54]. Suppression of LRRK2 with siRNAs or a dominant inhibitory allele leads to increased neurite process length and complexity [55]. Based on the multidomain structure and various identified LRRK2 mutations, LRRK2 is predicted to serve as an upstream central integrator of multiple signaling pathways that are crucial for proper neuronal functioning. The presence of LRR and WD40 (protein interaction domains) and Roc and MAPKKK (enzymatic domains) within LRRK2 suggests that this protein may serve as a scaffold for the assembly of a multiprotein signaling complex. LRRK2 associates with various protein partners that are involved in several cellular pathways including chaperone machinery, cytoskeleton arrangement, protein translational machinery, synaptic vesicle endocytosis, the MAPK signaling cascades, ubiquitin/autophage protein degradation pathways, and other unidentified processes [23].

3. *In Vitro* Models and LRRK2 Biology

Studies using *in vitro* models (Table 1) reveal that LRRK2 is a kinase and a GTPase and identify various interaction partners, suggesting that LRRK2 may play important roles in protein aggregation and neuronal degeneration.

3.1. LRRK2 Kinase Activity. *In vitro* studies demonstrate that LRRK2 is predominately a serine/threonine protein kinase, which can phosphorylate itself and a generic substrate, myelin basic protein (MBP) [39, 55–60]. A LRRK2 variant with three potential sites of autophosphorylation altered to alanines (T2031A, S2032A and T2035A), does not display autophosphorylation activity and cannot phosphorylate the generic substrate, MBP [61, 62]. Further *in vitro* studies demonstrate that S2031 and T2032 are the critical residues required for LRRK2 autophosphorylation, and T2035 is important for catalytic activity, but does not serve as a phosphate acceptor [58]. Additional studies show that dimeric LRRK2 undergoes intramolecular autophosphorylation and that an intact C-terminus is required for kinase activity [61]. One recent report shows that T1343 also is an autophosphorylation site [63]. Moreover, S910 and S935 are also potential phosphorylation sites that may be involved in 14-3-3 proteins binding with LRRK2 [64–66]

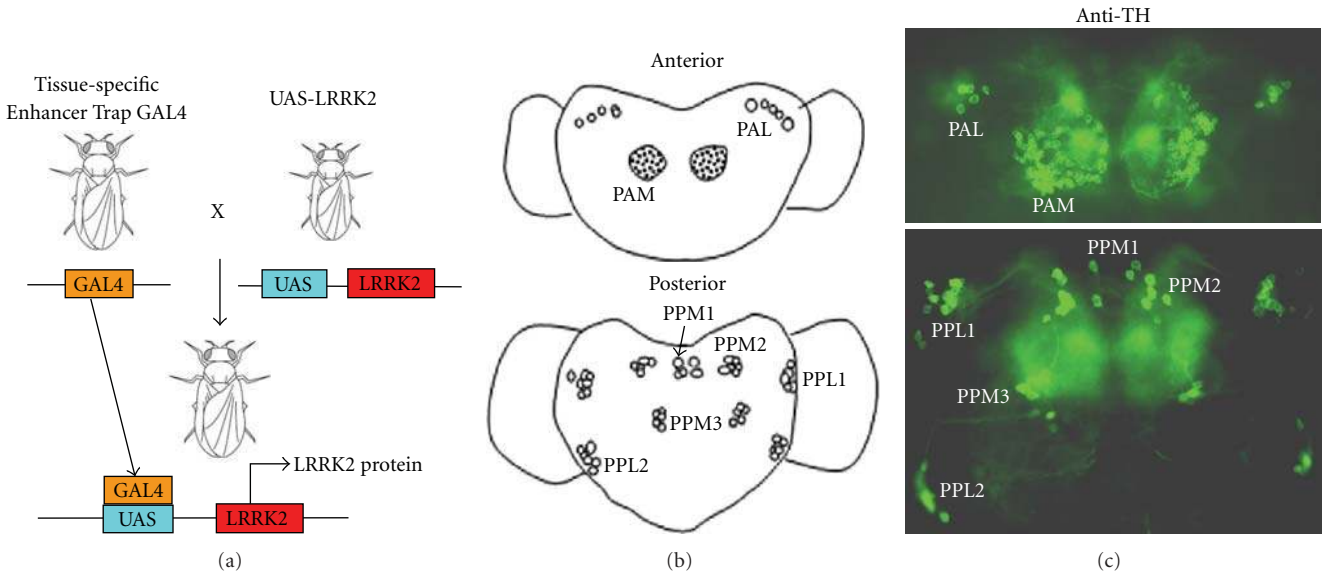

Figure 2: *UAS/GAL4 system and fly brain dopaminergic neurons*. A. Diagram of GAL4/UAS system to illustrate that tissue-specific expression of GAL4 leads to transcriptional activation of LRRK2. B. Diagram of DA neuron clusters in the medial and lateral areas of the adult fly brain as in previous publications [34, 67]. Five clusters: PPM1 (unpaired), PPM2 (paired), PPM3 (paired; protocerebral posterior medial), and PPL1 and PPL2 (paired; protocerebral posterolateral) on the posterior side. (*Center*) Two DA clusters: PAL (protocerebral anterolateral) and PAM (paired anterolateral medial) on the anterior side. C. Images of whole-mount-immunostaining of dopaminergic neurons in a adult fly brain using anti-TH antibodies followed by green fluorescent-conjugated second antibody detection.

Several pathogenic mutations of LRRK2 in PD have been found within the protein kinase domain active segment (e.g., G2019S), suggesting that these mutations may cause pathology through altering the kinase activity of LRRK2 [23]. The results from the most common mutation G2019S support this notion to increase LRRK2 kinase activity in assays to measure autophosphorylation or phosphorylation of generic substrates [39, 55–59]. However, controversy remains regarding whether other PD mutations alter LRRK2 kinase activity. For example, several studies demonstrated that the I1122V, R1441C, R1441G, R1514Q, Y1699C, and I2020T familial PD linked mutations of LRRK2 increased kinase activity [35, 37, 57, 67, 68]. Additionally, other mutations either did not influence or inhibit kinase activity [59, 60, 69]. Currently, LRRK2 kinase assays use *in vitro* autophosphorylation or phosphorylation of generic substrate or a phosphopeptide. Accordingly, the kinase activity results of some mutants vary among various laboratories, in part due to lack of sensitivity in the kinase assay of choice and various expression constructs. Identifying a physiologic substrate of LRRK2 and resolving the question of whether pathogenic mutations affect phosphorylation of this substrate is critically important to determine the mechanism by which LRRK2 induces PD see Figure 2.

To date, the physiological substrate(s) of LRRK2 remains unclear [23]. A search for proteins that are phosphorylated by the PD-linked mutant LRRK2-G2019S using rat brain extracts reveals that moesin is a substrate [59]. Moesin is a protein that anchors the actin cytoskeleton to the plasma membrane. Denatured moesin is efficiently phosphorylated by LRRK2 at Thr558, the residue previously identified as an *in vivo* phosphorylation site that regulates the ability of moesin to bind actin. LRRK2 also phosphorylates ezrin and radixin, which are involved in moesin binding actin [59]. Collapsing response mediator protein-2 (CRMP-2) has also been identified as a weak LRRK2 substrate, which is involved in the regulation of growth cones, microtubule dynamics and neurogenesis [70]. Recent reports also show that 4E-BP [71] and mitogen-activated protein kinase can also be phosphorylated by LRRK2 [72–74]. Additional studies are required to establish the physiological significance of these proteins as LRRK2 substrates.

3.2. LRRK2 GTP Binding and GTPase Activity. LRRK2 is a member of the recently defined ROCO family [75] and harbors a GTP-binding regulatory domain (ROC-COR) [76, 77]. LRRK2 is a GTP/GDP-binding protein, as measured by specific binding to GTP-agarose and radio-labeled GTP [57, 60, 76]. Both wild-type and PD-linked mutant LRRK2 bind to GTP and GDP. LRRK2-K1347A, which bears a mutation that alters the predicted GTP-binding site, does not appreciably bind to GTP and reduces kinase activity. This finding is further confirmed by the recent report showing that the crystal structure of the LRRK2 ROC domain in complex with GDP-Mg (2+) at 2.0-A resolution [78]. The crystal structure displays a dimer of the ROC domain. Two PD-associated pathogenic residues, R1441 and I1371, are located at the interface of two monomers that may alter the ROC dimerization and regulate LRRK2 GTPase and/or kinase activity. LaVoie's recent study further suggests that LRRK2 dimerization is associated with membrane binding and increased GTPase activity [41]. Familial-linked mutations in

Table 1: LRRK2 *in vitro* cell models.

Genes	Cell type	Toxicity	Protein aggregation	Kinase activity	GTPase activity	References
WT, R1441C, Y1699C, G2019S	HEK293T SH-SY5Y Primary neurons	+	ND	ND	ND	[79]
WT, I2020T	HEK293	ND	ND	+	ND	[38]
WT, G2019S, R1441C	HEK293 SH-SY5Y	ND	ND	+	ND	[39]
WT, G2019S, I2020T	Primary neuron	+	+	+	ND	[55]
WT, R1441C, Y1699C, G2019S	SH-SY5Y	+	+	+	ND	[56]
WT, G2019S, G2019S-K1906A, G2019S-D1994N, G2019S-DY2017-2018AL, WT-K1347A, G2019S-K1347A	HEK-293 SH-SY5Y Primary neuron	+	ND	+	+	[57]
WT, K1906M, G2019S, R1441C, R1441G, I1371V, I1122V, R1514Q, Y1699C, G2385R, I2012T, I2020T	HEK293FT SH-SY5Y Primary neuron	+	ND	+	+	[60]
WT, G2019S, A2016T, WT/A2016T, G2019S/A2016T, R1441C, Y1699C	HEK-293 Swiss-3T3 Human lymphoblastoid cells	ND	ND	+	ND	[80]
WT, T1343G, K1906M, T2035A, R1398Q	HEK-293 Neuro-2a	ND	ND	–	ND	[76]
WT, R1441C/G, T1398N	HEK-293T	ND	ND	ND	+	[67]
WT, G2019S	HEK-293 Primary neuron	ND	ND	+	ND	[81]

WT: wild type; ND: not determined.

LRRK2 within the ROC and COR domains (I1371V, R1441C, R1441G, and Y1699C) appear to increase GTP-binding as measured by binding to GTP-agarose, whereas mutations outside these domains did not affect GTP binding compared with wild-type LRRK2 [60]. However, other studies have shown that R1441C mutation do not increase GTP binding [67, 69].

The ROC domain of *LRRK2* shares sequence homology with all five subfamilies of the Ras-related small GTPase super family (Ras, Rho, Rab, Sar/Arf and Ran) and contains conserved motifs for GTPase activity. Three independent groups have demonstrated that LRRK2 has intrinsic GTPase activity and undergoes intrinsic GTP hydrolysis [67–69, 82, 83]. The purified full-length LRRK2 has only weak GTPase activity, suggesting that if it is active in the cell it may require accessory proteins. Notably, the ROC domain of LRRK2 is sufficient for its intrinsic GTPase activity. LRRK2 binds and hydrolyzes GTP similarly to other Ras-related small GTPases. Based on *in vitro* assays, R1441C/G and Y1699C PD-linked mutations appear to decrease in the rate of GTP hydrolysis compared to the wild-type LRRK2, suggesting that these mutants spend more time in the activated GTP-bound state [69, 78, 84].

Several studies have demonstrated that GTPase domain activity may regulate LRRK2 kinase activity [57, 58, 67, 82] since GTP binding stimulating LRRK2 kinase activity [58, 67] although there is still some evidence against the GTP binding activation model [85]. It is hypothesized that LRRK2, like other Ras-related GTPases, may serve as a molecular switch to regulate diverse cellular functions by cycling between GTP-bound (active) and GDP-bound (inactive) conformations. Based on the putative dimeric structure of LRRK2, it is predicted that the dimeric ROC or ROC-COR domains act as binary switches to regulate kinase activation [78, 84]. In this model, at the GTP-bound conformation, the dimerization of ROC or ROC-COR domains further induces self-association of the kinase domains, thus allowing for autophosphorylation and subsequent activation of

downstream kinase activity [61, 78, 84]. LRRK2 may regulate its own activity, as well as perhaps fulfilling a signaling role by regulating other proteins in the cell [41, 61, 86–88]. Multiple reports have shown that the kinase domain of wild-type LRRK2 phosphorylates several sequences within the GTP-binding ROC domain [61, 63, 89, 90], suggesting that the kinase domain may also regulate overall LRRK2 function. The PD-linked mutations do not identically display the same kinase or GTP domain activities, suggesting that there may be some interesting mechanistic differences between different mutations in the same domain, however the caveat that these observations could also be due to methodological differences between assays [91]. Nevertheless, mutations may prompt the protein to enter a GTP-bound state or slow the protein's return to the GDP-bound state.

3.3. Mutant LRRK2 Induces Toxicity. Patients with LRRK2 mutations exhibit neuronal degeneration in the brain [8, 9, 45–47]. PD-associated mutations of LRRK2 induce cell toxicity in multiple cell lines and rodent primary neurons with reduction of cell viability ranging from 10–40% (Table 1). Expression of mutant LRRK2 variants (I1122V, R1441C, Y1699C, G2019S, and I2020T) strikingly decreases neuronal cell viability by 2–5-folds. However, overexpression of wild type LRRK2 does not significantly decrease cell viability [55–58, 60, 79, 92].

Mutant LRRK2-mediated cell toxicity appears to involve apoptotic mechanisms as measured by TUNEL staining and caspase activation [57, 58, 79]. The mitochondria-dependent apoptotic pathway, in which cytochrome *c* is released and caspase-3 is activated, is thought to mediate mutant LRRK2 toxicity in neuronal cells. This seems to be dependent on Apaf1, a scaffold protein participating in apoptosome formation [83]. Another study has also shown that LRRK2 interacts with the death adaptor Fas-associated protein at the death domain (FADD), which may play a role in apoptotic neuronal death [93]. Since kinase activity is a critical component of LRRK2, significant efforts have been made to determine whether kinase activity is responsible for LRRK2 toxicity. Abolishing LRRK2 kinase activity diminishes the toxicity of all PD mutants tested in cell culture [56, 57]. Genetic alterations of LRRK2 with D1994N (a predicted proton acceptor) abolishes the predicted active site, K1906A (a ATP binding site) abolishes a putative ATP-binding site, and/or DY2017-2018AL altering the predicted DYG kinase active conserve motif significantly reduces LRRK2 kinase activity. Importantly, these constructs reduce mutant LRRK2-induced neuronal degeneration [56, 57]. Additionally, one study has shown that overexpression of the kinase domain, the ROC-COR-kinase fragment or the ROC-COR-kinase-WD40 fragments containing G2019S and R1441C mutations can reduce cell viability [94]. A recent report further supports this notion that inhibitors of Raf kinase GW5074, sorafenib and Raf inhibitor IV inhibit LRRK2 autophosphorylation and MBP phosphorylation result in reducing mutant LRRK2 toxicity [95].

R1441C and Y1699C mutants are associated with reduced LRRK2 GTPase activity [68, 69, 82, 83] suggesting that GTPase activity may contribute to LRRK2 toxicity. In addition, the K1347A alteration abolishes GTP binding and reduces kinase activity thereby reducing the mutant LRRK2-induced neuronal toxicity in cell culture [57, 60]. A recent study shows that the cytotoxic effect of ROC-ROC-kinase fragment in yeast was increased in a GTPase dead background or after the induction of R1441C mutation, which reduced GTPase activity [83]. This toxicity can be reduced by introduction of GTPase stimulating alterations (T1343G/R1398Q or R1398L). However, in cell culture, only the augmentation of the toxicity effect caused by ROC-ROC-kinase fragment can be replicated [83]. Thus, the contribution of GTPase domain activity in LRRK2 toxicity still warrants further investigation.

Recent studies have also shown that deletion of the LRR and WD40 domains can rescue G2019S and/or R1441C-LRRK2-induced toxicity [92, 96], likely via kinase activity as the deletion of the WD40 domain or even shorter C-terminal sequences renders LRRK2 kinase inactive [59, 96]. Given that the LRR and WD40 are putative protein-protein interaction domains, it is suggested that LRRK2 protein interactions may also contribute to its toxicity. However, this needs further study. Several pathogenic mutations (I1122V, R1441C, Y1699C, G2019S, and I2020T) increase the tendency of LRRK2 to form inclusion bodies [33, 56] suggesting that LRRK2 kinase activity may also contribute to protein aggregation [56, 79]. Together, these findings suggest that LRRK2 protein kinase activity plays an important role in both neuronal degeneration and protein aggregation, but the cellular pathways underlying these functions need further study.

3.4. LRRK2 Interaction Partners and Potential Cellular Pathways. There is a growing number of LRRK2 interaction partners that are identified and involved in several cellular pathways including chaperone machinery, cytoskeleton arrangement, protein translational machinery, synaptic vesicle endocytosis, the MAPK signaling cascades, ubiquitin/autophage protein degradation pathways, and other unidentified processes (Table 2).

LRRK2 interacts with proteins involved in chaperon pathways including Hsp90, Hsp90//p50^{cdc37}, HSp60, Hsp 70, and the c-terminal Hsp70 interacting protein (CHIP) [38, 81, 88, 97–99]. The Hsp60 interacts with recombinant human LRRK2 kinase domain in *E. coli*, and Hsp90/p50^{cdc37} interacts with full-length LRRK2 in mammalian cells [38, 58]. These chaperone proteins may help to maintain the proper folding of LRRK2. The HSP90/p50^{cdc37} chaperone complex binds to LRRK2 and may assist with the activation of other protein kinases [38]. In these studies, the Hsp90/p50cdc37 proteins do not serve as substrates but rather associate as chaperones assisting in proper folding and activation of the kinase. It has been shown that inhibition of Hsp90 disrupts the association of this chaperone with LRRK2 leading to proteasomal degradation of LRRK2, suggesting that Hsp90 inhibitors may be useful therapeutically to limit mutant LRRK2-mediated toxicity in neurons [81, 98]. CHIP binds ubiquitinates and promotes the ubiquitin proteasomal degradation of LRRK2 [98]. Overexpression of CHIP protects against mutant LRRK2-induced toxicity

Table 2: LRRK2 potential interaction proteins.

Pathway	LRRK2 fragment	Link with LRRK2	Method	References
Apoptosis	Full length	FADD	Co-IP (HEK293T cells, mouse brain)	[93]
	Full length	TRADD, RIP1	Co-IP (HEK293T cells)	[93]
Synaptic vesicle endocytosis	Full length LRR	Rab5b	YTH, pulldown, Co-IP	[111]
MAPK signaling	Full length, COR, Kinase domain	MKK3	Co-IP (HEK293T cells)	[72, 73]
	Full length	MKK4	Co-IP (HEK293T cells)	[72]
	Full length, COR, Kinase domain	MKK6	Co-IP (HEK293T cells), *C. elegans*	[72, 73]
	Full length, COR, Kinase domain	MKK7	Co-IP (HEK293T cells)	[72, 73]
	Full length	JIP1–3	Co-IP (HEK293T cells)	[74]
	Full length	JIP4	Co-IP (HEK293T cells)	[74]
Chaperone machinery	Full length, Kinase domain, N-term	Hsp90	Co-IP (HEK293T cells, mouse brain), YTH	[81, 88, 97–99]
	Full length, Kinase domain,	$p50^{CDC37}$	Co-IP (HEK293T cells, mouse brain)	[38, 81]
	Full length, ROC, N-term	CHIP	Co-IP (HEK293T cells, mouse brain), YTH	[98, 99]
cytoskeleton	Full length, ROC	a-tubulin	pulldown	[102]
	Full length, ROC	b-tubulin	Co-IP (HEK293T cells, mouse brain), pulldown	[102, 105]
	Full length	EF1A	Co-purification (insect cells), Co-IP (HEK293T cells)	[113]
	—	moesin	*in vitro, in vivo*	[59, 101]
	Full length, ROC-COR	DVL1/2/3	YTH, Co-IP (HEK293T cells)	[109]
	Full length	Sgg/GSK3b	*Drosophila*	[114]
	Full length	Actin cytoskeleton proteins	QUICK, Co-IP (NIH3T3 cells)	[115]
Protein translation	—	4E-BP	*in vitro, Drosophila*	[71, 116]
PD related proteins and others	Full length, COR	Parkin	Co-IP (HEK293T cells, SH-SY5Y cells, primary neurons),	[79]
	Full length	14-3-3 isoforms	Co-IP (HEK293T cells, Swiss 3T3 cells, mouse brain, kidney, spleen)	[64–66, 80]

FADD: Fas-associated protein with death domain; TRADD: tumor necrosis factor receptor type 1-associated death domain protein; LRR: leucine-rich repeat; YTH: yeast two-hybrid; ROC: Ras of complex protein; COR: C-terminal of ROC; MKK: mitogen activated protein kinase kinase; JIP: JNK interacting protein; Hsp: Heat shock protein; CHIP: C-terminus of Hsp70 interacting protein; EF1A: elongation factor 1α; DVL: dishevelled family of proteins; Sgg: glycogen synthase kinase 3β homolog Shaggy; QUICK: quantitative immunoprecipitation combined with knockdown.

whereas knockdown of CHIP exacerbates toxicity mediated by mutant LRRK2 via reducing degradation of LRRK2 proteins.

LRRK2 associates with various cytoskeleton proteins including alpha/beta-tublin, F-actin, moesin-related ezrin-radixin-moesin (ERM) family members, and the dishevelled family proteins [100], suggesting that LRRK2 may play a critical role in the regulation of microtubule and actin dynamics. LRRK2 associated with actin dynamics is evidenced by the following studies. MacLeod et al. first associated LRRK2 with the maintenance of neuronal process [55] and demonstrated that the neurons expressing the G2019S mutation but not wtLRRK2 had shorter neurites. Suppression of LRRK2 expression by shRNAs led to an increase in neurite length. Moreover expression of G2019S mutation led to tau-positive inclusions, which also colocalized with tau in these inclusions [55]. Biochemical studies show that LRRK2 phosphorylates denatured moesin and associates with other actin-binding ERM proteins: ezrin and radixin [59]. Further studies indicate that LRRK2 may connect with actin dynamics through phosphorylation of ERM proteins [101]. In developing *LRRK2* G2019S neurons, the numbers of pERM and F-actin enriched filopodia were significantly increased, which correlates with the retardation of neurite outgrowth in these neurons. Conversely, the levels of pERM and F-actin within the filopodia of *LRRK2* knockout neurons were significantly decreased and neurite outgrowth was promoted. These observations suggest a physiological link between LRRK2 and pERM in neuron development and neurite outgrowth [100].

Increasing evidence links LRRK2 with microtubule dynamics. For instance, LRRK2 colocalizes [38, 102] and interacts with tubulin through the LRRK2 ROC domain [102, 103]. LRRK2 phosphorylates β-tubulin at Thr107 in mouse brain, and this phosphorylation is significantly enhanced by G2019S mutation [104]. *In vitro* studies shows that tubulin phosphorylation by LRRK2 enhances microtubule stability in the presence of microtubule-associated proteins [105]. Moreover, levels of soluble β-tubulin are dramatically decreased in brains of LRRK2 expression mice [103, 106] and are significantly increased in the brains of LRRK2 KO mice [105]. The maintenance of microtubule dynamics is critical for neuronal development, axonal trafficking as well as synaptic formation and maintenance. The G2019S-enhanced tubulin phosphorylation may thus result in deregulation of microtubule dynamics that may in turn interfere with proper neuronal function [105]. Microtubules and microtubule-axonal transport has been reported to play a critical role in maintaining Golgi structure and integrity [107, 108]. Increased fragmentation of the Golgi apparatus was reported in transgenic mice overexpressing LRRK2, and this strongly suggests that the enhancement of tubulin polymerization affects the organization of microtubule in neurons leading to Golgi disruption [103]. Other studies also show that LRRK2 interacts with the dishevelled family of phosphoproteins (DVL1-3) and Rab5b suggesting that the interactions may play an important role in axon guidance and maintaining synaptic function [109, 110] by modulating the endocytosis of synaptic vesicles, further supporting a role for LRRK2 in trafficking [111]. Further investigation still remains to determine whether LRRK2 kinase and GTPase activities are involved in regulation of microtubule and actin dynamics in neuron development, neurite outgrowth and trafficking.

LRRK2 associates with proteins in other kinase cascades. LRRK2 kinase domain shares homology with MLKs and RIPKs, which are involved in signaling events in response to cellular stress insults. Similar to MLKs, LRRK2 has been shown to bind MKK3, 6 and 7 and to phosphorylate MKK3, 4, 6 and 7 [72, 73]. LRRK2 also interacts with the JNK-interacting proteins (JIPs) 1–4 which are scaffolding proteins that bring together MKKs and MAPKs activating the downstream kinases, JNK and p38 [74]. However, it is still unclear whether all the PD-linked mutations alter the interactions with MKKs, JIPs and their linked kinase cascades in PD pathology. Our unpublished data show that genetic or pharmacological suppression of JNK pathway suppressed PD-like Parkinsonism in LRRK2 transgenic flies. In addition, LRRK2 may also interact with ERK1/2 MAPK pathway since the ERK inhibitor U0126 can rescue LRRK2 G2019S-induced neurite shortening and cytotoxicity in culture cells [112]. A report also shows that LRRK2 may interact with oxidative stress via ERK phosphorylation [94]. Like RIPK1, LRRK2 interacts with FADD to induce death signaling resulting in caspase activation and apoptosis [93]. Taken together, these studies suggest that LRRK2 may act as an upstream kinase and interact with multiple cellular stress and cell death signaling pathways.

LRRK2 also associated with other PD-linked proteins. Co-immunoprecipitation studies have shown that LRRK2 associates with the PD-associated protein parkin [79] although there is a conflicting report using a different tagged LRRK2 construct that can not co-IP with parkin [110]. But further Drosophila studies including our own observations show that parkin suppressed LRRK2-induced PD-like phenotypes, suggesting that parkin is associated with LRRK2 *in vivo* [117, 118]. Although LRRK2 cannot directly bind α-synuclein, DJ-1, or pink-1, there are genetic interactions between LRRK2 and these genes in Drosophila [79, 118], C. elegans [119, 120], cell cultures [121] and mouse models [122]. This is illustrated by studies showing that expression of mutant LRRK2 promotes α-synuclein pathology in mice [103]. Since LRRK2 interacts with 14-3-3 proteins [64–66], which also interact with α-synuclein and negatively regulate cell death pathways, it is suggested that LRRK2 may indirectly interact with α-synculein via other proteins such as 14-3-3 to converge in PD. Given LRRK2 is a large and complex protein, further identification and characterization of LRRK2 interaction partners and their linked pathways is necessary to decipher the main functional roles of LRRK2 in PD pathogenesis.

4. Animal Models for LRRK2-Linked Parkinsonism (Table 3)

4.1. LRRK2 Drosophila Model. Drosophila melanogaster is an excellent model organism for studying pathogenesis and therapeutics of neuronal degenerative diseases [123, 124].

Use of the fly system has led to the unveiling of molecular and cellular pathophysiology of neurodegeneration, and has potential in discovering novel drug targets for long-sought therapeutics. Fly models have been successfully used to study the roles of α-synuclein, parkin, pink-1, DJ-1 and stress factors as well as provide important insights into disease pathogenesis [125–138]. Approximately 75% of the disease-related loci in humans have at least one *Drosophila* homologue, indicating a high degree of conservation from flies to human [139]. Adult fly brains have 13 dopaminergic neuron clusters with more than 1000 neurons that can be labeled with antityrosine hydroxylase (TH) antibodies, as illustrated in Figure 1. The fly has one homologue (CG5483) of human LRRK2. Several groups have generated transgenic or loss-of-function mutants LRRK2 fly models using UAS-GAL4 system (Table 3). This system takes advantage of the findings that the yeast GAL4 transcription factor binds very specifically to an upstream activation sequence (UAS). LRRK2 transgenes can be expressed either in various tissues or in a small group of specific cells under the control of the given promoter (*promoter-GAL4*).

Loss-of-function mutant studies indicate that *CG5483* protein is critical for the integrity of fly DA neurons [53] and control of synaptic overgrowth [140]. *Drosophila* lines expressing either fly *LRRK* (*dLRRK*) [53, 71] or human *LRRK2* [117, 118, 141] resemble some features of LRRK2-linked Parkinsonism. Inactivation of dLRRK kinase activity is not essential for fly development [142]. Although the neurochemical and behavioral phenotypes of these LRRK2 flies differ considerably from various groups. Transgenic expression of *Drosophila* wild-type LRRK2 homology protein (CG5483) and a mutation (R1069C) corresponding to the human "R1441C" mutation does not show any significant defects [53]. However, this mutation in the context of *Drosophila CG5483* may not be as pathogenic as the same R1441C change in the context of the human LRRK2 patients. Alternatively, the expression level of this mutant allele may not reach the pathology threshold in the fly. Overexpressing the human wild-type LRRK2 and the most common mutant form LRRK2-G2019S led to a selective loss of dopaminergic neurons in the brain, early mortality and locomotor impairment as reported by our group [141]. Moreover, LRRK2-G2019S increased autophosphorylation activity and caused a more severe parkinsonism-like phenotype than did wild-type LRRK2. Treatment with L-DOPA improved the mutant LRRK2-induced locomotor impairment, but did not prevent the loss of dopaminergic neurons, similar to what is seen in LRRK2-linked human PD. In support of this line of findings, several groups [117, 118, 141] have shown loss of dopamine and of dopaminergic neurons accompanied by behavioral deficits in their LRRK2 fly models. Coexpression of human parkin in LRRK2 G2019S-expressing flies provides significant protection against DA neurodegeneration that occurs with age or in response to rotenone [117]. Imai et al. reported that both human LRRK2 and the Drosophila orthologue of LRRK2 phosphorylate eukaryotic initiation factor 4E-(eIF4E-) binding protein (4E-BP), a negative regulator of eIF4E-mediated protein translation is a key mediator of various stress responses and suggest that 4E-BP may be a potential LRRK2 substrate [71]. Tain et al. have shown that loss of the Drosophila LRRK2 homolog activated 4E-BP and is able to suppress Pink-1 and parkin pathology [153]. Additionally, a recent study reports that LRRK2 interacts with 4E-BP at the postsynapse, whereas LRRK2 phosphorylates and negatively regulates the microtubule (MT-) binding protein Futsch at the presynapse [140].

LRRK2 also interacts with the microRNA (miRNA) pathway to regulate protein synthesis. Drosophila e2f1 and dp messenger RNAs are translationally repressed by let-7 and miR-184, respectively. Pathogenic LRRK2 antagonizes these miRNAs, leading to the overproduction of E2F1/DP, previously implicated in cell cycle and survival control and shown here to be critical for LRRK2 pathogenesis. LRRK2 associates with Drosophila Argonaute-1 (dAgo1) or human Argonaute-2 (hAgo2) of the RNA-induced silencing complex (RISC). In aged fly brain, dAgo1 protein level is negatively regulated by LRRK2. Furthermore, pathogenic LRRK2 promotes the association of phospho-4E-BP1 with hAgo2. These studies suggest that deregulated synthesis of E2F1/DP caused by the miRNA pathway impairment is a key event in LRRK2 pathogenesis [154]. With an outstanding battery of genetic tools for gene manipulation as well as the ability to carry out large-scale genetic screens inexpensively and rapidly for mutations affecting the disease process, the LRRK2 fly model provides a powerful tool to screen for LRRK2 interaction partners and LRRK2 substrates. Furthermore, the LRRK2 fly model can be used to conduct preclinical therapeutic screens to prevent neuronal loss and to rescue locomotor dysfunction in PD.

4.2. LRRK2 Caenorhabditis Elegan Models. LRK-1 is the *Caenorhabditis elegans* ortholog of human LRRK2, and transgenic as well as deletion mutants have been created in the worm [73, 119, 120, 143, 144, 155]. In LRK-1 deletion mutants, synaptic vesicle proteins mislocalize to both presynaptic and dendritic endings in neurons, suggesting that LRK-1 is involved in determining polarized sorting of synaptic vesicle proteins to axons by excluding these proteins from the dendrite-specific transport machinery in the Golgi [145]. In Wolozin et al.'s earlier studies, overexpression of wild-type and LRRK2 (G2019S) in *C. elegans* was protective against rotenone toxicity, whereas knockdown of endogenous LRK-1 by RNAi promoted toxicity, suggesting a role for LRRK2 in mitochondrial regulation [144]. In contrast, a recent study shows that the transgenic *C. elegans* overexpressing human LRRK2 wild type, R1441C and G2019S in dopaminergic (DA) neurons causes age-dependent DA neurodegeneration, behavioral deficits, and locomotor dysfunction that are accompanied by a reduction of dopamine levels *in vivo*. In comparison, R1441C and G2019S mutants cause more severe phenotypes than the wild-type protein. Interestingly, treatment with exogenous dopamine rescues the LRRK2-induced behavioral and locomotor phenotypes. In contrast, expression of the GTP-binding defective mutant, K1347A, or knockout of the *C. elegans* LRRK2 homolog, LRK-1, prevents the LRRK2-induced neurodegeneration and behavioral abnormalities. These results provide strong support for

Table 3: LRRK2 animal models.

Transgene	Loss of TH positive neurons	Lewy body	Motor deficits	Suitability for testing disease modifying therapy	Reference
Drosophila model					
$LRRK^{WT,}$ $LRRK^{R1069C}$ (R1441C) $LRRK^{P1}$, $LRRK^{ex1}$ (loss-of-function line)	ND	ND	+	ND	[53]
dLRRK(−/−), dLRRK(+/−), dLRRK RNAi, dLRRK Tg, R1069G(R1441G), Y1383C(Y1699C), I1915T(I2020T)	+	ND	+	ND	[71]
dLRRKe03680, dLRRK-WT, dLRRK- I1915T, dLRRKdf	ND	ND	+	ND	[140]
WT, G2019S, Y1699C, G2385R	+	ND	+	+	[117]
hLRRK2(WT), hLRRK2(I1122V), hLRRK2(Y1699C), hLRRK2(I2020T)	+	ND	+	+	[118]
WT, G2019S	+	ND	+	ND	[141]
dLRRK-WT, dLRRK-mutant(e03680)	+	ND	ND	Maybe	[142]
Caenorhabditis elegans model					
LRRK2+: *lrk-1(km17),* LRRK2+: *lrk-1(km41),* G2019S+: *lrk-1(km41)* LRRK2+: *lin-15(765ts)* R1441C+: *lin-15(765ts)* G2019S+: *lin-15(765ts)* K1347A+: *lin-15(765ts)*	+	ND	+	ND	[119]
wlzIs2(WT), km4, N2(WT)	ND	ND	ND	ND	[73] (Interact with MKK6)
wlzIs1(WT), wlzIs2(WT), wlzIs3(G2019S), wlzIs4(G2019S), wlzIs5(R1441C), wlzIs6(KD), wlzIs7(R1441C/KD), wlzIs2 : wlzIs4(LRRK2/DAT::GFP)	+	ND	ND	+	[143]
LGI, lrk-1(tm1898, km41); LGII, pink-1(tm1779); LGX, lqIs4 (ceh-10::gfp, lin-15(n765)), N2	ND	ND	ND	ND	[120] (Interact with PINK1)
N2(WT), N2(G2019S)	ND	ND	ND	+	[144]
LRK-1-K1726A(hLRRK2-I2020T) LRK-1-I1877T)(hLRRK2-I2020T)	ND	ND	ND	ND	[145] (*trans*-Golgi network)
Rodent model					
BAC(WT)	+	−	−	ND	[27]
BAC(G2019S)	ND	ND	+	ND	[146]
$LRRK2^{R1441G}$ BAC	+	−	+	Maybe	[147]
R1441C KI	ND	ND	+(AMPH-induced)	−	[148]
BAC(WT) BAC(G2019S)	+	ND	ND	Maybe	[149]
BAC(WT) BAC(G2019S)	+	ND	+	ND	[150]
WT, A53T, G2019S, KD, A53T/LRRK2WT, A53T/LRRK2G2019S, $LRRK2^{-/-}$	ND	+	ND	ND	[103]
LRRK2 null	−	ND	ND	ND	[151]
$LRRK2^{-/-}$	−	+(Kidney)	ND	ND	[122]
HSV-WTHSV-G2019SHSV-G2019S/D1994A	+	ND	ND	+	[152]
LRRK2 conditional G2019S	ND	ND	ND	Maybe	[81] (Hsp90 and LRRK2 stability)

WT: wild type; ND: not determined; GFP: Green fluorescent protein; KI, knocking in.

the critical role of GTPase/kinase activity in LRRK2-linked pathologies [119].

4.3. Zebrafish Model. Zebrafish have a homolog of human LRRK2 (XM_682700). The blockage of zebrafish LRRK2 (zLRRK2) protein by morpholinos caused embryonic lethality and severe developmental defects such as growth retardation and loss of neurons. In contrast, the deletion of the WD40 domain of zLRRK2 by morpholinos targeting splicing did not induce severe embryonic developmental defects; rather it caused Parkinsonism-like phenotypes, including loss of dopaminergic neurons in the diencephalon and locomotion defects. These neurodegenerative and locomotion defects could be rescued by overexpressing zLRRK2 or hLRRK2 mRNA [54]. The zLRRK2-ΔWD40 deletion also caused a significant reduction and disorganization of axon tracts, more prominently in the midbrain. These studies suggest that zLRRK2 may play an important role in neuronal development and provide a useful small vertebrate model for PD research.

4.4. LRRK2 Rodent Models. The LRRK2 protein expressed in mice shares 86% homology with the human protein (Genbank: NM-25730). Several groups generated LRRK transgenic and knockout models but they are not very robust PD models (Table 3). LRRK2 transgenic mice show some neurochemical and behavioral abnormalities but lack selective loss of dopaminergic neurons in substantia nigra [95, 103, 146–150]. Knockout of LRRK2 in mice also lack the obvious abnormality in DA neurons in brains [122, 151].

Conditional expression of LRRK2 WT and LRRK2 G2019S failed to exhibit neurodegeneration of DA neurons, but LRRK2 was expressed at low levels in DA neurons due to the use of the calcium/calmodulin dependent protein kinase II (CamKII) promoter [81, 103]. When the R1441C mutation is expressed under the control of the endogenous regulatory elements, by knock in of the R1441C mutation, there is no degeneration of DA neurons, but they show reductions in amphetamine-(AMPH-) induced locomotor activity [148]. Bacterial artificial chromosome (BAC) transgenic mice expressing LRRK2 WT, LRRK2 R1441G, LRRK2 G2019S have some evidence of neurodegeneration [147, 150], which is demonstrated by measuring the dopamine content after pharmacologically blocking the dopamine uptake. Li et al. report $LRRK2^{R\,1441G}$ BAC transgenic mice display hyperphosphorylation of tau and motor deficits. Two groups recently report that G2019S Lrrk2 BAC mice display abnormal dopamine neurotransmission as evident by a decrease in extracellular dopamine levels [149, 150]. However, Li et al. shows that the wild-type LRRK2 BAC mice revealed increases in dopamine release thereby contributing to hyperactivity phenotypes, while Melrose et al. shows wild-type LRRK2 mice also decrease dopamine levels but a bit less than G2019S-LRRK2 BAC mice. Moreover, they later also show that G2019S-LRRK2 BAC mice display changes in localization and increased phosphorylation of microtubule binding protein tau, suggesting that LRRK2 may impact tau processing [150].

Mutations in α-synuclein and Leucine-rich repeat kinase 2 (LRRK2) are linked to autosomal dominant forms of Parkinson's disease (PD). Recently, Lin et al. shows that there is a potential pathophysiological interplay between these two PD-related genes by generating a double transgenic mouse model coexpressing both human α-synuclein and LRRK2 genes [103]. Overexpression of LRRK2 alone did not cause neurodegeneration but the presence of excess LRRK2 greatly accelerated the progression of neuropathological abnormalities developed in PD-related A53T α-synuclein transgenic mice. Moreover, LRRK2 promoted the abnormal aggregation and somatic accumulation of α-synuclein in A53T mice, which likely results from the impairment of microtubule dynamics, Golgi organization, and the ubiquitin-proteasome pathway. Conversely, genetic ablation of LRRK2 preserved the Golgi structure and suppressed the aggregation and somatic accumulation of α-synuclein, thereby delaying the progression of neuropathology in A53T mice. These findings suggest that overexpression of LRRK2 enhances α-synuclein-mediated cytotoxicity [103]. Currently, there are no mouse models that overexpression mutant LRRK2 in parkin, pink-1 or DJ-1 knockout backgrounds.

LRRK2 knockout (KO) mice [151] are viable, have no major abnormalities and live to adulthood. Moreover, there is no significant difference in the susceptibility of LRRK2 KO and wild type mice to MPTP suggesting that LRRK2 may play a minor role in the development and the survival of DA neurons. Alternatively, the roles of LRRK2 may be compensated by LRRK1 since LRRK1 shares high homology with LRRK2 and is expressed in the brains. However, a recent study shows that there is an age-dependent kidney abnormality in LRRK2 KO mice. The kidneys of these mice, develop striking accumulation and aggregation of α-synuclein and ubiquitinated proteins, and may be involved in the autophagy-lysosomal defects [122]. The kidneys also display apoptotic cell death, oxidative damage and inflammatory response, suggesting that LRRK2 may play an important peripheral role during aging at least in kidney.

Most of the current LRRK2 transgenic mice have abnormalities in the nigrostriatal system, such as stimulated DA neurotransmission, decrease dopamine levels, or behavioral deficits, which probably represent some of the earliest neuronal dysfunction that is set in motion by pathogenic LRRK2 mutations. Reasons are not clear why mouse LRRK2 transgenic models do not exhibit more substantial pathology and why LRRK2 KO mice do not display abnormality in nigrostriatal system. It may relate to the fact that LRRK2 mutations in humans are only partially penetrant and that there may need to be other genetic and/or environmental hits that are required for degeneration of DA neurons. The BAC and knock in models express mutant LRRK2 during development and thus there may be compensatory mechanisms in the mouse that prevent loss of DA neurons by LRRK1 or other genes with the similar functions. Current mouse LRRK2 models can be used for early mechanism studies of LRRK2 but are less than ideal to test the neuronprotection therapies. The rodent models need to be improved by combining other PD risk factors, or by other approaches to express LRRK2 in the nigrostriatal

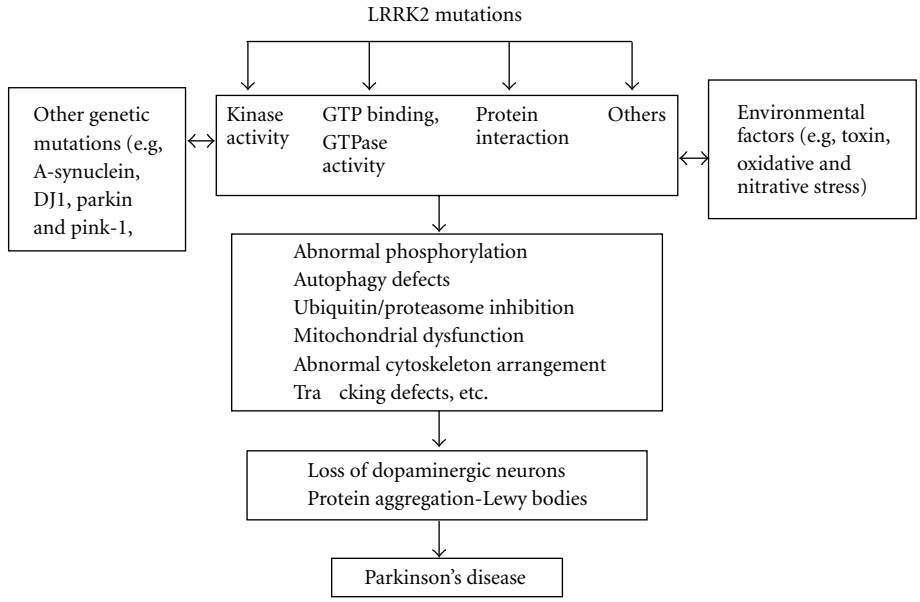

Figure 3: Potential pathways associated with LRRK2 in PD.

system. A recent promising mouse model is using AAV-mediated expression of mutant LRRK2 in middle brain causes remarkable dopaminergic neuron degeneration [152], which can be potentially used to test protective therapeutics of LRRK2-linked diseases.

5. Conclusion Remarks

In summary, the current findings in LRRK2 indicate that kinase activity and GTPase domain activity are the key components of LRRK2 functions and are associated with LRRK2-induced neuronal degeneration. Mutations within LRRK2 may potentially perturb protein conformation or protein-protein interactions with accessory proteins necessary for kinase and GTPase domain activity. It is important to note, however, that the increase in kinase activity seen with PD-associated mutations of LRRK2 must be interpreted with caution until these observations are confirmed with physiologically relevant substrates. Nevertheless, these current findings in LRRK2 kinase and GTPase are consistent with a model in which LRRK2 cycles between an active and an inactive conformation potentially integrating multiple signaling pathways and subsequently lead to protein aggregation and neurodegeneration. LRRK2 may also serve as a scaffold protein to recruit other signaling molecules through its protein-protein interaction domains. Thus, LRRK2 kinase, GTPase domain and scaffold activities may function together with other PD-related players to elicit disease pathology as depicted in Figure 3.

In addition, mutant LRRK2 may directly or indirectly interact with environmental factors and other genetic PD causes to converge on the pathways that induce protein aggregation and neuronal death. These interactions may occur at various levels, such as altering LRRK2 GTP-binding, GTPase and/or kinase activity, modulating LRRK2 kinase substrates, or influencing the function of LRRK2 interaction partners among others yet to be identified. Thus, identifying the putative LRRK2-interacting proteins, physiological substrates of LRRK2 kinase, regulators and downstream effectors of LRRK2 GTPase, as well as establishing how mutations lead to the familial and sporadic forms of PD through interactions between genetic factors and environmental toxins will likely provide crucial insights into the pathways involved in PD pathogenesis. Such investigations will facilitate the development of LRRK2 cell and animal models as well as enable the formulation of novel pharmacological interventions for the treatment of PD. The current findings in LRRK2 are beginning to pave the way for better-designed therapeutic options. The discovery of chemical inhibitors of LRRK2 kinase and GTPase domain activities may likely involve optimizing strategies that prevent dopaminergic neuron degeneration and to treat LRRK2-linked PD. Recently, several groups already report some potential LRRK2 kinase inhibitors in preventing neuronal death [85, 95, 121]. With more research into the genetics and biochemistry of LRRK2 and more LRRK2 animal models available, identifying LRRK2 kinase and GTPase domain inhibitors might lead us to effective new therapeutic approaches for the treatment of PD.

Abbreviations

PD: Parkisnon's disease
LB: Lewy body
LRRK2: Leucine-rich repeat kinase 2
PAL: Protocerebral anterior lateral
PAM: Paired anterolateral medial
PPL: Protocerebral posterior lateral
PPM: Protocerebral posterior medial
TH: Tyrosine hydroxylase.

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Review Article

α-Synuclein Transgenic *Drosophila* As a Model of Parkinson's Disease and Related Synucleinopathies

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α-Synuclein (α-Syn) is a major component of protein inclusions known as Lewy bodies, which are hallmarks of synucleinopathies such as Parkinson's disease (PD). The α-Syn gene is one of the familial PD-causing genes and is also associated with an increased risk of sporadic PD. Numerous studies using α-Syn expressing transgenic animals have indicated that α-Syn plays a critical role in the common pathogenesis of synucleinopathies. *Drosophila melanogaster* has several advantages for modeling human neurodegenerative diseases and is widely used for studying their pathomechanisms and therapies. In fact, *Drosophila* models expressing α-Syn have already been established and proven to replicate several features of human PD. In this paper, we review the current research on synucleinopathies using α-Syn *Drosophila* models and, moreover, explore the possibilities of these models for comprehensive genetic analyses and large-scale drug screening towards elucidating the molecular pathogenesis and developing therapies for synucleinopathies.

1. Introduction

Protein inclusions known as Lewy Bodies (LBs) are one of the hallmarks of Parkinson's disease (PD), in which the major component is now known to be α-synuclein (α-Syn) [1, 2]. LBs are found in the substantia nigra in PD and also more extensively in other brain regions in other synucleinopathies including multiple system atrophy and dementia with Lewy bodies (DLB) [3, 4]. The α-Syn encoding gene, SNCA, is the first gene in which missense mutations such as A30P and A53T were found to cause familial PD [5, 6]. Furthermore, the multiplication mutations of α-Syn gene were also found to cause familial PD [7]. Most importantly, single nucleotide polymorphisms (SNPs) of α-Syn have been reported to associate with an increased risk of sporadic PD, which comprises the majority of PD patients [8–11]. α-Syn expression has been experimentally shown to mimic several aspects of PD in transgenic animals, such as motor dysfunction, α-Syn aggregation/accumulation, and neurodegeneration [12–14]. These phenotypes are manifested not only by mutations in the α-Syn gene but also by overexpression of wild-type α-Syn [15], indicating that α-Syn plays a critical role in the common pathogenesis of synucleinopathies.

Drosophila melanogaster, commonly known as the fruit fly, has been recognized as a powerful organism for modeling human neurodegenerative diseases [16]. At least ~75% of human disease genes have *Drosophila* homologues [17]. Using *Drosophila* for modeling human neurodegenerative diseases has various advantages as follows: (1) analysis of gene functions *in vivo*, (2) rapid generation cycle (10–14 days) with a short life span (50–60 days), (3) suitability for genetic analysis, (4) abundant genetic information, and (5) little labor and cost-effective to maintain fly stocks (Table 1). In fact, *Drosophila* models of several neurodegenerative diseases including PD, Alzheimer's disease, and the polyglutamine diseases have already been established and have successfully provided valuable insights into the elucidation of pathomechanisms and development of therapies for these diseases.

Feany and Bender first developed transgenic *Drosophila* models expressing either wild-type or familial PD-linked mutants (A53T and A30P) of human α-Syn [12]. These

Table 1: Advantages of using *Drosophila* for modeling human neurodegenerative diseases.

(1) Analysis of gene functions in vivo
At least ~75% of human disease genes have *Drosophila* homologues.
(2) Rapid generation cycle with a short life span
10–14 days from embryo to adults.
Average life span is ~50–60 days.
(3) Suitable for genetic analyses
Stock centers maintain a variety of mutant fly libraries as public resources.
Various genetic screening methods have been established.
(4) Abundant genetic information
Whole genome sequence is available.
(5) Little labor and cost-effective to maintain fly stocks
Transgenic flies can be established at low cost.
Mutant flies are available from public stock centers at low cost.
Only small space is required for their maintenance.

α-Syn expressing flies replicate several features of human PD, including (1) locomotor dysfunction, (2) LB-like inclusion body formation, and (3) age-dependent loss of dopaminergic neurons and are therefore widely used for studying the molecular pathogenesis of α-Syn-induced neurodegeneration in not only PD but also synucleinopathies. In this paper, we will discuss what has been revealed in the pathogenesis of synucleinopathies using α-Syn *Drosophila* models, focusing on "misfolding and aggregation of α-Syn", "posttranslational modifications of α-Syn", and "oxidative stress" (Table 2).

2. Misfolding and Aggregation of α-Synuclein

Recent accumulating evidence has implicated that misfolding and subsequent aggregation of α-Syn play a central role in the pathogenesis of synucleinopathies [37]. Indeed, α-Syn has been demonstrated to be aggregated and deposited as inclusion bodies in flies expressing either wild-type or mutant α-Syn (A53T and A30P), the latter of which has accelerated aggregation propensity. Recently, Karpinar et al. showed that structurally-engineered α-Syn mutants with an increased propensity to form soluble oligomers exhibit enhanced neurotoxicity in *Drosophila* [18]. Moreover, a recent study demonstrated that histone deacetylase 6 (HDAC6) suppresses α-Syn-induced dopaminergic neuron loss and locomotor dysfunction by reducing α-Syn oligomers and instead promoting inclusion formation in α-Syn flies, further supporting a critical role of toxic oligomers in α-Syn-induced neurodegeneration in the pathogenesis of synucleinopathies [19].

Protein quality control systems function as a defense mechanism against protein misfolding and aggregation, which consist of molecular chaperones and protein degradation systems [38]. Molecular chaperones assist proper protein folding and hence are considered as essential proteins for protecting cells against the detrimental effects of the misfolding and aggregation of proteins such as α-Syn. Most molecular chaperones are induced upon heat stress to promote the refolding of misfolded proteins, and hence they are called heat shock proteins (HSPs) [39]. On the other hand, once proper protein folding has been altered, the resulting misfolded and aggregated proteins must be eliminated by their degradation. Two major protein degradation systems are the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system [40]. UPS degrades short-lived and misfolded proteins through selective deubiquitination of substrate proteins and their targeting to the proteasome, whereas the autophagy-lysosome system is a nonselective bulk degradation system for long-lived and misfolded proteins, which involves engulfment of substrate proteins into the autophagosome and their delivery to the lysosome. The role of molecular chaperones and protein degradation systems in protecting against α-Syn misfolding in the pathogenesis of synucleinopathies has been investigated using α-Syn *Drosophila* models.

2.1. Molecular Chaperones. As molecular chaperones are expected to protect against protein misfolding and aggregation, their roles in the pathogenesis of PD have been investigated so far [41]. Extensive colocalization with LBs has been demonstrated for several HSPs [42], and expression levels of HSPs have been reported to be elevated in synucleinopathy brains [43]. HSP70 has been shown to inhibit α-Syn aggregation *in vitro* [44], and HSPs, such as HSP27 or HSP70, have been reported to protect against α-Syn-induced neurotoxicity in cultured cells and transgenic mice [45, 46], suggesting an important role of HSPs in PD pathology.

Indeed, Auluck et al. demonstrated that coexpression of HSP70 ameliorated the toxicity of α-Syn to dopaminergic neurons without changing the number of inclusions [20]. They also confirmed that coexpression of Hsc4.K71S, a dominant negative form of *Drosophila* HSP70, accelerated dopaminergic neuron loss in α-Syn expressing flies. Furthermore, they subsequently showed that geldamamycin, an Hsp90 inhibitor and heat shock transcription factor 1-activator compound, protects against neurotoxicity through induction of Hsp70 in α-Syn flies [21]. Taken together, these results confirmed that the molecular chaperone HSP70 suppresses α-Syn toxicity *in vivo* by using the *Drosophila* system.

2.2. Protein Degradation. The UPS and the autophagy-lysosome system can degrade misfolded proteins, and impairment of these systems has been reported to cause neurodegeneration [40, 47]. Furthermore, the UPS has been suggested to coordinate with the autophagy system to eliminate misfolded proteins. Lee et al. have shown protective effects of the UPS on α-Syn-induced toxicity using cell culture and *Drosophila* models [22]. A cell culture-based study indicated that K48-linked polyubiquitination is protective against α-Syn-induced toxicity in a UPS-dependent manner. In α-Syn flies, coexpression of ubiquitin has been shown to suppress loss of dopaminergic neurons and locomotor dysfunction and to extend life-span. These

Table 2: Summary of studies on α-Syn-induced neurodegeneration using *Drosophila* models.

Mechanisms/modifiers of α-Syn toxicity	Effect	Findings	References
α-Syn expression		α-Syn expression causes dopaminergic neuron loss, LB-like inclusion body formation and locomotor dysfunction in *Drosophila* (wild-type < familial PD-linked mutants).	[12]
Misfolding and aggregation			
α-Syn oligomer formation	Enhance	α-Syn mutants which tend to form oligomers enhance α-Syn toxicity.	[18]
HDAC6	Suppress	Expression of HDAC6 reduces α-Syn oligomers and suppresses α-Syn toxicity.	[19]
HSP70	Suppress	Expression of HSP70 reduces α-Syn toxicity, and a dominate negative form of HSP70 enhances toxicity.	[20]
Geldanamycin	Suppress	Geldanamycin induces HSP70 expression and suppresses α-Syn toxicity.	[21]
Ubiquitin	Suppress	Expression of ubiquitin reduces α-Syn toxicity.	[22]
Cathepsin D	Suppress	Deficiency of cathepsin D enhances α-Syn-induced neurodegeneration.	[23]
Posttranslational modifications			
α-Syn phosphorylation at Ser129	Enhance	A phosphomimic S129D α-Syn mutant enhances α-Syn toxicity and a phospho-resistant S129A α-Syn mutant reduces toxicity.	[24]
α-Syn phosphorylation at Tyr125	Suppress	Expression of shark increases α-Syn Y125 phosphorylation and reduces α-Syn toxicity. Blocking of Y125 phosphorylation enhances toxicity.	[25]
α-Syn C-terminal truncation	Enhance	Expression of C-terminal truncated α-Syn (1–120) enhances α-Syn aggregation and toxicity.	[26]
α-Syn cleavage by Calpain I	Enhance	Calpain I-cleaved α-Syn fragments were identified in the brains of α-Syn flies as well as PD/DLB patients.	[27]
Oxidative stress			
Reactive oxygen species	Enhance	Hypoxia-induced oxidative stress enhances α-Syn toxicity, and expression of superoxide dismutase suppresses toxicity.	[28]
Dopamine	Enhance	Decreased dopamine levels by tyrosine hydroxylase RNAi reduces α-Syn toxicity.	[29]
Glutathione metabolism	Suppress	Defect of glutathione metabolism genes enhances α-Syn toxicity and expression of glutathione metabolism genes suppresses toxicity.	[30]
Nicotinamide	Suppress	Nicotinamide suppresses α-Syn toxicity through improvement of oxidative mitochondrial dysfunction.	[31]
Polyphenols	Suppress	Grape extracts containing various polyphenols suppress α-Syn toxicity.	[32]
Other PD-causing genes			
Parkin	Suppress	Expression of Parkin suppresses α-Syn toxicity.	[33–35]
PINK1	Suppress	Expression of PINK1 suppresses α-Syn toxicity.	[36]

results suggest that UPS-mediated degradation of α-Syn is a potential therapeutic approach for synucleinopathies including PD.

Cathepsin D (CathD) is a major lysosomal aspartyl protease and its defect results in fatal neurodegenerative diseases [48]. CathD has been shown to efficiently degrade recombinant α-Syn in *in vitro* experiments, and knockdown of CathD in cultured cells increased α-Syn levels, indicating a role of CathD in α-Syn degradation [49]. Using α-Syn expressing flies, Cullen et al. demonstrated that a CathD defect enhanced α-Syn-induced neurodegeneration *in vivo* [23]. CathD knock-out mice have also been shown to facilitate insoluble α-Syn accumulation and α-Syn-induced neurotoxicity, confirming that CathD may protect neurons against α-Syn-induced toxicity through degradation.

3. Posttranslational Modifications of α-Synuclein

Posttranslational modifications including phosphorylation, ubiquitination, or C-terminal truncation of α-Syn have been observed in LBs in the postmortem brain of synucleinopathy patients [37]. *In vitro* studies suggest that these modifications can accelerate oligomerization or aggregation of α-Syn.

Accordingly, the role of posttranslational modifications of α-Syn on toxicity has been studied using α-Syn expressing flies.

3.1. α-Synuclein Phosphorylation. Phosphorylation at Ser129 has been identified in α-Syn deposited as LBs in synucleinopathy brains [50]. To explore the pathological role of this phosphorylation *in vivo*, accumulation and phosphorylation of α-Syn was studied in flies expressing wild-type or mutant α-Syn. Indeed, α-Syn accumulated in these flies was phosphorylated at Ser129 as reported in human patients, and the order of the degree of phosphorylation was A53T > A30P > wild-type [51]. Mutagenesis studies demonstrated that the phosphomimic S129D mutant increases α-Syn-induced toxicity, whereas the phospho-resistant S129A mutant reduces the toxicity accompanied with an increased number of inclusion bodies [24]. Furthermore, GPRK2 has been shown to be responsible for the α-Syn phosphorylation in *Drosophila*. These studies revealed that Ser129 phosphorylation plays an important role for α-Syn-induced neurotoxicity and inclusion body formation.

Chen et al. recently reported that Tyr125 of α-Syn is also phosphorylated in α-Syn expressing flies [25]. This phosphorylation occurs at a young age but diminishes during the aging process in both humans and flies. They showed that soluble oligomers of α-Syn were increased by phosphorylation at Ser129 and decreased by phosphorylation at Tyr125. In addition, blocking Tyr125 phosphorylation increased α-Syn toxicity. Taken together, these studies suggest that α-Syn toxicity in synucleinopathies results from an imbalance between the detrimental action of Ser129 phosphorylation by accelerating toxic oligomer formation and a neuroprotective action of Tyr125 phosphorylation by suppressing oligomer formation.

3.2. α-Synuclein Truncation. Truncated small species of α-Syn have been detected in purified LBs and insoluble fractions from synucleinopathy brains [52, 53], suggesting that truncation of α-Syn contributes to aggregation and LB formation. Several studies have implicated that C-terminal truncation of α-Syn accelerates its aggregation [54, 55], and the NAC domain (residues 61–95) of α-Syn has been demonstrated to be essential for α-Syn aggregation *in vitro* [56, 57]. Indeed, flies expressing α-Syn with an NAC domain deletion (α-Syn Δ71–82) did not show any loss of dopaminergic neurons with no evidence of α-Syn aggregation, confirming an essential role of the NAC domain in α-Syn aggregation and toxicity *in vivo* [26]. On the other hand, expression of C-terminal truncated α-Syn (α-Syn 1–120) resulted in increased α-Syn aggregation and significantly greater loss of dopaminergic neurons than wild-type in *Drosophila*, suggesting a potential role of the C-terminal region of α-Syn in suppressing aggregation.

α-Syn has been shown to be a substrate for proteolytic cleavage by calpain *in vitro*, which is one of a family of intracellular calcium-dependent proteases [58, 59]. The calpain-cleaved α-Syn species exhibit a similar molecular size to truncated α-Syn fragments that have been shown to promote aggregation and to enhance toxicity [54, 55, 60]. Dufty et al. have identified calpain I-cleaved α-Syn fragments in the brains of human PD/DLB patients as well as α-Syn expressing flies using a specific antibody [27]. These results suggest that calpain I-mediated cleavage of α-Syn may be involved in the disease-linked aggregation of α-Syn in synucleinopathies.

4. Oxidative Stress and Antioxidants

Oxidative stress has been believed to play a central role in the progression of neurodegenerative diseases although its relationship with α-Syn toxicity has not been well elucidated. Dopaminergic neurons of α-Syn expressing flies have been shown to be sensitive to hyperoxia-induced oxidative stress [28]. Importantly, overexpression of Cu/Zn superoxide dismutase rescued both the dopaminergic neuron loss and locomotor dysfunction in mutant α-Syn flies. The same group also demonstrated that reduction of dopamine levels by RNAi silencing of the tyrosine hydroxylase gene decreases the neurotoxicity in α-Syn expressing flies, implying that dopamine which produces reactive oxygen species might be involved in the α-Syn-induced neurotoxicity through oxidative stress [29]. These results suggest that oxidative stress plays a significant role in the pathogenesis of PD *in vivo*.

Trinh et al. examined the involvement of the phase II detoxification pathway, specifically glutathione metabolism, in α-Syn-induced neurotoxicity in *Drosophila* models [30]. They found that the loss-of-function gene mutations affecting glutathione metabolism pathways enhance dopaminergic neuron loss in α-Syn expressing flies. Moreover, the dopaminergic neuron loss can be rescued by genetic or pharmacological interventions that increase glutathione biosynthesis or glutathione conjugation activity, suggesting that oxidative stress is involved in α-Syn-induced neurotoxicity and that induction of the phase II detoxification pathway may be a potential therapy for synucleinopathies.

In addition, feeding Nicotinamide, the principal form of niacin (vitamin B3), has been shown to improve the motor dysfunction in α-Syn expressing flies through improvement of oxidative mitochondrial dysfunction [31]. Grape extracts, which contain various polyphenols and exhibit scavenging effects on reactive oxygen species, also showed a significant improvement in locomotor function and average lifespan in α-Syn flies [32].

5. Association with Other PD-Causing Genes

Loss of function gene mutations of Parkin, an E3 ubiquitin ligase, is responsible for a rare familial form of PD, autosomal recessive juvenile Parkinsonism, which develops typical Parkinsonian symptoms as a result of midbrain dopaminergic neuron loss, but usually lacks LBs [61]. Although a direct molecular interaction between Parkin and α-Syn remains controversial, several studies have shown that coexpression of Parkin rescues α-Syn-induced dopaminergic neurodegeneration and motor dysfunction in α-Syn flies.

These studies suggest that up-regulation of Parkin expression may provide a novel therapy for PD [33–35].

Mutations in the PTEN-induced putative kinase 1 (PINK1) gene cause another form of autosomal recessive PD [62]. PINK1 has been shown to be located in mitochondria and is thought to be involved in cellular protection. Overexpression of PINK1 has been shown to rescue loss of climbing ability and neurodegeneration induced by α-Syn expression in *Drosophila* [36]. Furthermore, it has been suggested that Parkin and PINK1 function in a common pathway in maintaining mitochondrial integrity and morphology, as demonstrated using *Drosophila* models [63, 64].

6. Genomics and Proteomics Studies

One of the advantages of using *Drosophila* models in studying human diseases is the easiness to handle numerous samples at one time, which can provide us with reliable amounts of data for unbiased statistical analyses. In addition, shortness of their life span makes it convenient to perform time course analyses in relatively short time periods.

Scherzer et al. performed expression profiling analysis of α-Syn A30P flies at different disease stages using microarray and found that expression of genes involved in lipid processing, energy production, and membrane transport is significantly altered by α-Syn expression [65]. Xun et al. performed proteomic analysis of α-Syn flies at different disease stages using liquid chromatography coupled with mass spectrometry [66, 67]. They found cytoskeletal and mitochondrial protein changes in the presymptomatic and early disease stages in the α-Syn A30P expressing flies [66]. They further reported dysregulated expression of proteins associated with membrane, endoplasmic reticulum, actin cytoskeleton, mitochondria, and ribosome in the presymptomatic α-Syn A53T flies, consistent with the α-Syn A30P flies [67]. These unbiased genomics and proteomics studies especially in the presymptomatic α-Syn flies will provide us with further insight into pathomechanisms and potential therapeutic targets of synucleinopathies.

7. Concluding Remarks

As described above, α-Syn *Drosophila* models have been widely employed to uncover the molecular pathogenesis of synucleinopathies (Table 2). Most of the results reviewed here have indeed been confirmed in transgenic mouse models expressing α-Syn. As we described in the introduction, *Drosophila* is a powerful *in vivo* model to study human neurodegenerative diseases with various advantages (Table 1), especially its short life span since human neurodegenerative diseases gradually appear and progress in middle-late ages.

Genetic analyses using α-Syn expressing flies have revealed pathological associations between α-Syn and various synucleinopathy-related genes and have provided novel insights into the molecular pathogenesis of synucleinopathies. *Drosophila* models of other neurodegenerative diseases such as the polyglutamine diseases have also been established, and numerous comprehensive genetic screenings have been conducted and have elucidated previously unknown pathomechanisms, taking advantage of the characteristics of *Drosophila* [68]. Similarly, comprehensive genetic screenings using *Drosophila* models will further lead to the elucidation of the pathomechanisms of synucleinopathies including PD in the future.

On the other hand, *Drosophila* models are also suited for drug screening. Indeed, L-DOPA and dopamine agonists have been shown to exert therapeutic effects against α-Syn-induced neurotoxicity using α-Syn flies [69]. In addition, HDAC inhibitors such as sodium butyrate or SAHA, and SIRT2 inhibitors have been identified as novel therapeutic agents that protect against α-Syn-induced neurotoxicity using *Drosophila* [70, 71]. In the future, novel therapeutic candidates for synucleinopathies are expected to be developed by extensive large-scale drug screening using *Drosophila* models.

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Review Article

Drosophila Models of Parkinson's Disease: Discovering Relevant Pathways and Novel Therapeutic Strategies

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Parkinson's disease (PD) is the second most common neurodegenerative disorder and is mainly characterized by the selective and progressive loss of dopaminergic neurons, accompanied by locomotor defects. Although most PD cases are sporadic, several genes are associated with rare familial forms of the disease. Analyses of their function have provided important insights into the disease process, demonstrating that three types of cellular defects are mainly involved in the formation and/or progression of PD: abnormal protein aggregation, oxidative damage, and mitochondrial dysfunction. These studies have been mainly performed in PD models created in mice, fruit flies, and worms. Among them, Drosophila has emerged as a very valuable model organism in the study of either toxin-induced or genetically linked PD. Indeed, many of the existing fly PD models exhibit key features of the disease and have been instrumental to discover pathways relevant for PD pathogenesis, which could facilitate the development of therapeutic strategies.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting more than 1% of the population over age 60. Clinically, it is characterized by locomotor defects such as muscle rigidity, bradykinesia, postural instability, and tremor. The principal neuropathology that gives rise to these motor defects is the progressive and selective loss of dopaminergic (DA) neurons in the Substantia nigra pars compacta, which causes a deficiency of brain dopamine content. Another pathological hallmark of this disorder is the presence of cytoplasmic inclusions in the surviving DA neurons called Lewy bodies (LBs), which are mainly composed of α-Synuclein and ubiquitin among other proteins [1, 2]. However, it has been shown that such structures are not present in some genetic forms of PD.

Although the majority of PD cases are sporadic and are probably caused by a combination of risk factors like the aging process, genetic propensity, and environmental exposures, few environmental triggers have so far been identified. Weak associations between PD and exposure to environmental toxins or herbicides and pesticides have been reported [2], and several toxin-induced PD models have been developed [3]. However, epidemiological studies have also demonstrated the contribution of genetic factors in the pathogenesis of PD. Indeed, during the last decade, several loci whose mutations are causative of rare familial forms of the disease have been identified, which account for 5%–10% of all PD cases. These genes include *α-synuclein*, *parkin*, ubiquitin C-Terminal hydrolase-1 (*UCHL-1*), *DJ-1*, phosphatase and tensin homolog (PTEN)-induced kinase 1 (*PINK1*), leucine-rich repeat kinase 2 (*LRRK2*), *Omi/HtrA2*, *ATP13A2,* and glucocerebrosidase (*GBA*) [4–14]. However, it is noteworthy to mention that the relevance of some of them to PD is currently under debate [15]. Despite this, studies of the function of PD-linked genes have provided important insights into PD pathogenesis and have demonstrated that three types of cellular defects are mainly involved in the formation and/or progression of the disease: abnormal protein aggregation, oxidative damage, and mitochondrial dysfunction [16]. Due to the limitations of human genetic analysis, most of these studies have been performed in

model organisms, including mice, fruit flies, and worms as well as in cell culture. Indeed, there are currently many cellular and animal models of PD either genetic or toxin-based. Cellular models can be easily used for molecular, biochemical, and pharmacological approaches, but they can lead to misinterpretation and artefacts. In contrast, animal models allow studying a cellular process in the context of a whole organism and are thus more reliable. Despite this, it is also remarkable that none of the existing PD animal models recapitulate all PD symptoms, including those developed in mice [17].

In such a scenario, the fruit fly Drosophila has emerged as a valuable model for studying mechanisms of human neurodegenerative diseases, including PD. Although fruit flies seem to be completely unrelated to humans, fundamental cellular processes as well as many genes and signalling pathways are conserved between both organisms. Moreover, most of the genes implicated in familial forms of the disease have at least one fly homolog [18]. In addition, flies are capable of performing complex motor behaviours such as walking, climbing, and flying and their brain is complex enough to make these behaviours relevant to humans. The availability of very potent genetic tools that are impractical in mammals, their rapid growth and reproduction, and the fact that it is cheap and easy to maintain in the laboratory are features that make Drosophila an ideal model system to address novel biological questions including those relevant to human health [19–21]. Indeed, studies of genes involved in familial PD as well as the development of toxin-based models of PD in Drosophila have made significant contributions to our understanding of the disease [15, 22, 23]. Here, we have attempted to provide a comprehensive review on existing Drosophila models of PD, which have revealed valuable insights into potential pathogenic mechanisms and have been used to target modifiers of PD pathology by genetic or pharmacological interference.

2. Toxin-Induced Models of PD in Drosophila

As indicated above, familial PD cases are extremely rare, which suggests that environmental factors or gene-environment interactions play a predominant role in the development of sporadic PD. For that reason, several studies have been performed to model PD-associated neuron loss by neurotoxin intoxication in animals, the most popular parkinsonian neurotoxins being 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat [3, 24]. In general, toxin-induced PD models do not recapitulate the process of progressive neuron loss and the protein aggregation in LBs, due to the acute nature of the neurotoxin treatment [15], but they have been useful to support the notion that alterations in mitochondrial biology are essential for the development of PD [25]. Indeed, mitochondria are central to the actions of the above-mentioned toxins, which preferentially injure DA neurons. In Drosophila, several studies have shown that pharmacological treatment could be used to model sporadic PD. First, chronic exposure to the pesticide rotenone, a mitochondrial complex I inhibitor, recapitulated key aspects of sporadic PD in Drosophila since it resulted in neurodegenerative and behavioural defects [26]. Indeed, rotenote-treated flies showed dose-dependent motor deficits quantified by a negative geotaxis test, which is commonly used to perform locomotor ability analyses in Drosophila, as well as selective loss of DA neurons in all the brain clusters. In a different study, paraquat exposure caused reduced lifespan in flies as well as movement disorders such as resting tremors, bradykinesia, rotational behaviours, and postural instability, which mirror PD symptoms. These complex set of locomotor phenotypes were overall quantified by a negative geotaxis test. The authors also demonstrated that such phenotypes were caused by selective loss of DA neuron clusters [27]. Thus, both studies robustly modelled environmental toxin-induced PD in Drosophila and provide useful tools for studying the mechanism of DA neurodegeneration. Drosophila models of MPTP- or 6-OHDA-induced Parkinsonism have not been established so far.

3. Drosophila Models of Familial PD

The discovery of several genes affected in familial forms of PD has provided a new tool for PD modelling. Indeed, many PD animal models have been generated based on gene mutations that are linked to the disease including Drosophila [15, 17, 19–21, 23, 28]. Although Drosophila PD models cannot recapitulate fully the phenotypic and pathologic features of human PD patients, loss of DA neurons and locomotor defects have been observed in most of them. Moreover, they have offered the advantage of identifying evolutionary conserved pathways and cellular processes relevant to PD pathogenesis.

Different approaches have been used to generate PD models in Drosophila. In some cases, no Drosophila orthologs of a specific PD-linked gene do exist. Then, the model is generated by misexpression of the human gene either in its wild-type or mutant form, which is usually achieved by using the GAL4/UAS system [29]. Widely used in Drosophila genetic studies, this system allows time- and tissue-specific misexpression of any gene of interest in flies. Alternatively, when an ortholog of the human gene is present in the Drosophila genome, loss-of-function (LOF)/knockdown alleles of the gene can be generated by different genetic techniques, including RNAi. Moreover, misexpression of the corresponding gene can also be carried out. In general, misexpression of either human or Drosophila PD-related genes is performed when the PD forms associated to them have a dominant inheritance. In that case, Drosophila PD models are established using GAL4 drivers specific of the nervous system or of other tissues, like eyes or wings, in which a possible phenotype can be easily identified without affecting fly survival. LOF/knockdown alleles are phenotypically characterized when the PD forms associated to the corresponding genes have a recessive inheritance. By using any of these strategies, several Drosophila PD models based on different PD-linked genes have been generated. Examples of phenotypes obtained in these models are shown in Figure 1.

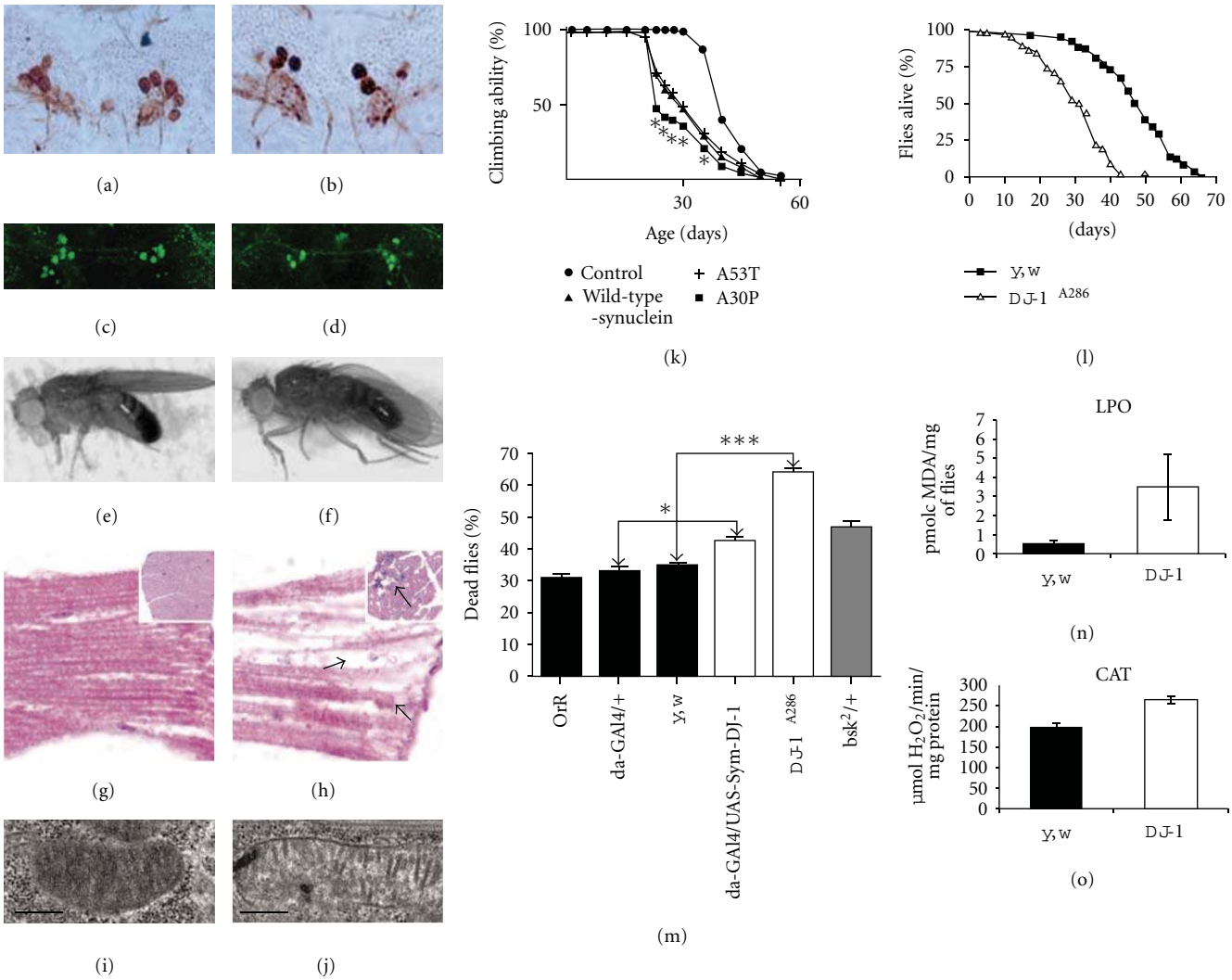

Figure 1: Representative phenotypes found in different Drosophila PD models. (a)–(d) DA neuron loss detected in Drosophila adult brains by immunostainings with anti-TH antibody, which specifically recognizes these neurons, in paraffin sections (a, b) or whole-mount brains (c, d). A reduction in the number of DA neurons is observed in both *Ddc-GAL4/DJ-1α RNAi* (b) [50] and *Ddc-GAL4/UAS-α*-Synuclein (d) [51] brains when compared to age-matched *Ddc-GAL4/+* controls (a, c). (e)–(j) Examples of phenotypes observed in *parkin* LOF mutants (f, h, j) compared to controls (e, g, i). They include downturned wings (f), muscle degeneration (h), and abnormal mitochondrial morphology (j) [52]. (k) Premature loss of climbing ability in transgenic flies expressing wild-type, A30P, and A53T mutant forms of α-Synuclein [34]. (l) Reduced lifespan of *DJ-1β* mutants compared to *y, w* control flies cultured under the same conditions. (m) Elevated sensitivity to paraquat stress in *DJ-1α* and *DJ-1β* mutant flies, represented by calculating the percentage of dead flies after feeding 15 mM for 18 h [53]. (n)–(o) Quantification of oxidative stress levels in 1-2-day-old *DJ-1β* mutants and age-matched *y, w* control flies. *DJ-1β* mutants show an increase in lipid peroxidation (LPO) product malondialdehyde (MDA) (n). Catalase (CAT) enzymatic activity is also increased (o) [54].

3.1. α-Synuclein. It encodes a small protein whose physiological function remains to be elucidated. However, mutations in the *α-synuclein* gene such as amino acid substitutions (A30P, E46K, and A53T), duplications, and triplications are causative of dominantly inherited forms of PD [4, 30–32]. Interestingly, α-Synuclein is one of the major structural components of LBs [33]. The first fly PD model was generated by overexpression of transgenes encoding either wild-type or mutant forms of human α-Synuclein in all Drosophila neurons since the Drosophila genome does not contain a clear *α-synuclein* homolog [34]. This resulted in an age-dependent and selective (complete or near complete) loss of DA neurons in the dorsomedial clusters (DMC) of the brain and formation of fibrillar α-Synuclein inclusions as well as a progressive loss of climbing ability, thus reproducing key PD features. Although several discrepancies regarding DA neuron loss upon *α-synuclein* overexpression were reported in subsequent studies [35, 36], associated to the different sensitivity of the methods used for DA neuron detection, recent analyses have confirmed that phenotype [37–39]. DA neurons were initially detected in paraffin-embedded brain sections stained with a specific marker (anti-Tyrosine hydroxylase (TH) antibody) (Figures 1(a) and 1(b)), but subsequent analyses were performed in whole-mount brain

preparations by confocal microscopy (Figures 1(c) and 1(d)). It has been proposed that while in paraffin-embedded sections only healthy DA neurons can be detected, some fluorescence is still observed in degenerating DA neurons. In any case, this fly model has been instrumental to decipher the neuropathological effects of the α-Synuclein protein as well as the regulation of aggregate formation. It has been demonstrated that inhibition of endoplasmic reticulum (ER)-Golgi trafficking and oxidative stress induction are major components of α-Synuclein-dependent toxicity [37–40]. Moreover, quantitative proteome analyses performed either on wild-type, A30P, or A53T α-Synuclein overexpressing flies at different disease stages revealed that deregulated proteins are primarily associated with membrane, endoplasmic reticulum, actin cytoskeleton, mitochondria, ribosome, cellular metabolism, and signalling [41–44]. Regarding α-Synuclein aggregation, overexpression of truncated forms of α-Synuclein in flies led to discover a central hydrophobic region of the protein which is essential for its aggregation as well as sequences C-terminal to residue 120 that have a more moderate role in influencing both aggregation and toxicity [45]. Moreover, several posttranslational modifications seem to regulate aggregation and toxicity of α-Synuclein. While phosphorylation of this protein at serine 129 is prominent in PD and influences α-Synuclein DA toxicity [46], phosphorylation at tyrosine 125 inhibits toxic oligomer formation and decreases with aging [47, 48]. These data suggest that α-Synuclein neurotoxicity in PD and related synucleinopathies may result from an imbalance between different C-phosphorylation events on the protein, regardless of the impact of such modifications on the normal function of α-Synuclein [48, 49].

3.2. Parkin. Mutations in the *parkin* gene were originally identified in families with autosomal recessive juvenile Parkinsonism (ARJP) [5]. It is the second most commonly affected PD gene and encodes a ubiquitin ligase associated with proteasomal degradation [55–57]. Since this gene is well conserved in Drosophila, several groups generated *parkin* null mutants in order to understand its biological role in flies. Although these mutants are viable, loss of Drosophila *parkin* function results in mitochondrial defects, degeneration of indirect flight muscles, hypersensitivity to oxidative and environmental stress, male sterility, reduced lifespan, partial lethality, and severe defects in both flight and climbing abilities [52, 58, 59]. It seems that oxidative stress, perhaps as a consequence of mitochondrial dysfunction, is a major determinant of those phenotypes [52, 60, 61]. Furthermore, *parkin* seems to be essential for the morphology, function, and integrity of several clusters of DA neurons in the Drosophila brain [59, 62]. Thus, fly *parkin* mutants recapitulate some key features of ARJP, suggesting that the mechanisms of DA neurodegeneration in mutant flies could resemble those underlying DA neuron loss in ARJP. It was proposed that loss of *parkin* function may lead to accumulation of one or several of its numerous substrates in the brain thereby resulting in ER stress, which in turn may lead to DA neuron death [28]. Regarding this, there are two studies in Drosophila which suggest that abnormal accumulation of Parkin substrates in Parkin-deficient DA neurons could be one of the causes of neurodegeneration. First, overexpression of human Parkin-associated endothelin-like receptor (PAEL-R), a Parkin substrate protein [63], in flies induces DA neuron loss in the DMC [64]. However, no Drosophila ortholog of this Parkin substrate has been described. We also demonstrated that targeted expression of Septin 4, the Drosophila ortholog of the human Parkin substrate CDCrel-1 [57], in DA neurons also causes age-dependent disruption of DA integrity in the DMC [65]. Since this neurotoxicity was dependent on *parkin* function and both proteins were able to interact in vitro, our results suggest that Septin4 could be a genuine substrate of Parkin in Drosophila [65]. This was the first study showing that accumulation of a Parkin substrate in flies could account for DA neurodegeneration in Drosophila *parkin* mutants [65].

It is interesting to mention that overexpression of mutant but not wild-type human *parkin* in flies also led to progressive degeneration of DA neurons from several clusters accompanied by a progressive motor impairment. These data suggested a possible dominant mechanism underlying the pathological phenotypes caused by mutant *parkin* in Drosophila, which could directly exert neurotoxicity in vivo [66, 67].

3.3. PINK1. Mutations in *PINK1* are also associated with recessive Parkinsonism. This gene encodes a putative serine/threonine kinase with a mitochondrial targeting sequence [8]. A recent study has demonstrated that the kinase domain faces to the cytosol, where its physiological substrates may reside [68]. The Drosophila *PINK1* gene encodes a protein that contains the same domains as its human counterpart, and fly *PINK1* models of PD were generated by transposon-mediated mutagenesis and RNAi [69–72]. Interestingly, *PINK* mutant flies shared marked phenotypic similarities with *parkin* mutants. They also exhibited male sterility, muscle degeneration, hypersensitivity to oxidative stress, mitochondrial defects, reduced lifespan, and DA neuronal degeneration accompanied by locomotor defects. Indeed, genetic analysis demonstrated that *PINK1* and *parkin* are functionally related. They showed that *parkin* overexpression rescued *PINK1* mutant phenotypes, whereas *PINK1* overexpression had no effect on *parkin* LOF phenotypes [69, 70]. These observations suggested that *PINK1* and *parkin* function in the same pathway, with *parkin* acting downstream of *PINK1*, and it seems that this pathway is conserved between flies and mammals [73]. Several studies have demonstrated that both fly genes regulate different aspects of mitochondrial physiology, thus explaining the mitochondrial morphological defects observed in Drosophila *PINK1* and *parkin* mutants. By means of genetic interactions, they illustrated a role of the PINK1/Parkin pathway in the regulation of the mitochondrial remodelling process in the direction of promoting mitochondrial fission and/or inhibiting fusion in Drosophila muscle and neuronal tissues [74–77]. However, these results also suggested that both genes are not core components of the mitochondrial dynamics machinery since LOF of key regulators of this process causes lethality and, as indicated above, *PINK1* and *parkin* mutants are viable.

Thus, it has been proposed that they probably regulate additional aspects of mitochondrial function that also impact mitochondrial morphology [76]. Interestingly, these results contrast with a human cell-based study which demonstrates that the PINK1/Parkin pathway promotes mitochondrial fusion in mammals [78]. One explanation for this discrepancy may be the existence of species-specific differences although the final conclusion is that in both systems there is a disrupted balance between mitochondrial fusion and fission [77]. Furthermore, it has been shown that PINK1 directly phosphorylates Parkin to control its translocation to the mitochondria [78]. Recent studies suggest that Parkin, together with PINK1, modulates mitochondrial trafficking, especially to the perinuclear region, a subcellular area associated with autophagy [79] and that PINK1 accumulation on mitochondria is both necessary and sufficient for Parkin recruitment to such organelles. These findings provide a biochemical explanation for the genetic epistasis found between *PINK1* and *parkin* in Drosophila and support a model in which PINK1 signals mitochondrial dysfunction to Parkin, and Parkin promotes their elimination [79, 80].

Genetic interaction experiments in flies also revealed putative additional components of the PINK1/Parkin pathway like Rhomboid-7 and Omi/HtrA2 [81, 82]. It seems that Rhomboid-7, a mitochondrial protease, could act as an upstream component of the pathway that may cleave the mitochondrial target motif of PINK1 thus allowing its activity not only in the mitochondria but also in the cytosol [81]. Besides, *Omi/HtrA2* was identified as a possible regulator of the PINK1/Parkin pathway, acting downstream of *PINK1* in Drosophila [82]. In contrast, another study showed that *Omi/HtrA2* does not play any role in the PINK1/Parkin pathway [83]. Although *Omi/HtrA2* sequence variations have been associated with an increased risk for PD [11, 84], its involvement in the disease is still controversial [12]. Additional work in Drosophila suggested that *PINK* deficiency also affects synaptic function in neurons, as the reserve pool of synaptic vesicles is not mobilized during rapid stimulation [85].

3.4. DJ-1. Mutations in the *DJ-1* gene are associated with rare familial recessive forms of PD [7]. *DJ-1* encodes a highly conserved protein belonging to the ThiJ/PfPI superfamily of molecular chaperones [86]. Although originally identified as an oncogenic factor [87], DJ-1 is a ubiquitous redox-responsive cytoprotective protein with diverse functions that, particularly in its oxidized form, has been recognized as a biomarker for cancer and neurodegenerative diseases [88]. Several cysteine residues in the DJ-1 protein can be oxidized with exposure to oxidative stress agents, being cysteine 106 critically required for DJ-1 to protect against oxidative damage both in vivo and in vitro [89, 90]. It has been shown that DJ-1 regulates redox signaling kinase pathways and acts as a transcriptional regulator of antioxidative gene batteries [91], but also acts as a redox-sensitive RNA-binding protein [92]. In contrast to mammalian species, two *DJ-1* orthologs do exist in Drosophila, *DJ-1α* and *DJ-1β*. While *DJ-1α* expression is restricted to the male germline, *DJ-1β* is ubiquitously expressed as its human counterpart [93, 94]. In order to explore the contribution of *DJ-1* in PD pathogenesis, we and others generated different Drosophila PD models by mutating these genes [50, 53, 93–95]. Those studies have revealed that flies mutant for *DJ-1α*, *DJ-1β*, or both are viable but exhibit enhanced sensitivity to toxins that induce oxidative stress such as H_2O_2, paraquat or rotenone, supporting that DJ-1 exerts a protective role against oxidative stress damage [50, 53, 93–95]. Consistent with this, we examined *DJ-1β* mutant flies for the extent of oxidative damage finding that *DJ-1β* loss of function results in cellular accumulation of reactive oxygen species (ROS) in adult brains, elevated levels of lipid peroxidation, and an increased catalase enzymatic activity [54]. It was also demonstrated that both the aging process and oxidation challenge promote overoxidation of DJ-1β at cysteine 104 (analogous to cysteine 106 in human DJ-1), a modification that could irreversibly inactivate the protein [90]. Consistent with this, aged flies showed further vulnerability to oxidative stress [90]. This suggests that the protective function of DJ-1 against oxidative stress could be progressively lost through aging, thus increasing the risk of DA neuron loss, since they are prone to oxidation. Despite this, only two studies have shown that targeted knockdown of *DJ-1α* via RNAi in flies resulted in age-dependent loss of DA neurons in the DMC [50, 53]. In addition, flies mutant for *DJ-1α* and *DJ-1β* showed reduced lifespan and locomotor defects [53, 95]. Although initial studies did not examine the *DJ-1* mutant flies for mitochondrial pathology that could account for these phenotypes, a recent analysis has demonstrated that *DJ-1* inactivation leads to mitochondrial dysfunction in an age-dependent manner not only in flies but also in mice [96]. Indeed, flies double mutant for *DJ-1α* and *DJ-1β* manifest additional phenotypes that reflect mitochondrial dysfunction such as reduced ATP levels and defects in spermatogenesis [96]. Interestingly, all these defects resemble those found in *parkin* and *PINK1* mutants (see Sections 3.2 and 3.3). Consistent with this, the study provides evidence that *DJ-1* interacts with the PINK1/Parkin pathway in Drosophila, and suggests that *DJ-1* acts downstream of, or in parallel to, *PINK1* for proper mitochondrial function [96]. Cell culture studies revealed that a pool of DJ-1 is localized to the mitochondria [89, 97]. Thus, all these results suggest that *DJ-1*, *parkin*, and *PINK1* may act in common biological processes that are critical for mitochondrial function and that *DJ-1* dysfunction may lead to PD pathology through distinct molecular mechanisms.

3.5. LRRK2. Mutations in *LRRK2* are likely the most common genetic cause of PD and are associated with a dominant form of the disease [9, 10]. It encodes a large and complex protein containing several independent domains, including a GTPase domain and a kinase domain able to exhibit a GTP-dependent phosphorylation activity [98]. The exact mechanism by which *LRRK2* mutations cause PD is still unclear. Most disease-associated mutations of LRRK2 have been shown to increase its kinase activity and thereby its toxicity, but there is significant variation among different mutations which can even reduce its kinase activity or exhibit a tendency to aggregate [99–101]. In order to understand the

mechanisms of *LRRK2*-induced pathology, several groups have used Drosophila to model *LRRK2*-linked Parkinsonism. Expression of either wild-type or mutant forms of human LRRK2 in flies has led to inconsistent results, especially regarding neurodegeneration [102–106]. While one group did not obtain any significant defect in the tissues analyzed, including muscles and DA neurons [102], other studies reported photoreceptor and/or DA neuron loss by LRRK2 overexpression as well as locomotor impairments [103–106]. Moreover, it was shown that human LRRK2 expression sensitized flies to environmental toxins such as rotenone [106]. Interestingly, LRRK2-overexpression phenotypes in fly eyes and DA neurons were modified in a complex fashion by a concomitant expression of *PINK1*, *DJ-1*, or *parkin*, suggesting a genetic interaction between these PD-relevant genes [106]. Regarding this, co-immunoprecipitation assays performed in cell culture already demonstrated that LRRK2 interacts with Parkin but not with α-Synuclein, DJ-1, or Tau in human cells [107]. Disparate results have also been obtained when ablating endogenous *LRRK2* expression in flies [102, 104, 108]. Several studies showed that flies lacking *LRRK2* function showed no changes in DA neuron numbers and patterns thus indicating that the gene is dispensable for the survival of DA neurons in this organism [104, 108]. However, one study reported that DA neurons in *LRRK2* LOF mutants show a severe reduction in tyrosine hydroxylase immunostaining and shrunken morphology, implicating their degeneration, and exhibit a severely impaired locomotive activity [102]. Different results have been also obtained when exposing those mutants to oxidative stress agents. While *LRRK2* mutants encoding a truncated form of the protein were selectively sensitive to hydrogen peroxide, but not to paraquat, rotenone and β-mercaptoethanol [108], *LRRK2* deficient (by transposon insertion or chromosome deletion), or *LRRK2* RNAi animals were shown to be significantly more resistant to hydrogen peroxide-induced stress [104]. Interestingly, this study also provided genetic and biochemical evidence that the Drosophila LRRK2 kinase modulates the maintenance of DA neurons by regulating protein synthesis, since it can phosphorylate initiation factor 4E-binding protein (4E-BP), a negative regulator of eukaryotic protein translation implicated in mediating the survival response to various physiological stresses [109–111]. Its phosphorylation relieves its inhibition of protein translation which could be detrimental when unregulated in times of stress. This would explain why flies expressing pathogenic forms of LRRK2 exhibit enhanced sensitivity to oxidative stress agents while flies lacking LRRK2 activity are resistant [104]. Consistent with this, it has been recently demonstrated that LRKK2 interacts with the microRNA pathway to regulate protein synthesis [112]. It is interesting to mention that a genetic interaction between 4E-BP (*Thor*) and *parkin/PINK1* has also been found, because its loss of function in *Drosophila* significantly reduces *parkin* and *PINK1* mutants viability while 4E-BP overexpression is sufficient to suppress the phenotypes described in these mutants [113]. Thus, these results support a general role of deregulated protein translation in PD. Besides, a recent study has shown that LRRK2 also phosphorylates the forkhead box transcription factor FoxO and enhances its transcriptional activity, not only in Drosophila but also in humans [114]. They also demonstrated that *hid* and *bim*, which encode two cell death molecules regulated by FoxO, are responsible for LRRK2-mediated cell death suggesting that they are key factors during the neurodegeneration in *LRRK2*-linked PD [114]. In summary, it seems that the higher kinase activity exhibited by LRRK2 mutations could cause DA neuron loss by affecting different cellular processes.

4. Using Drosophila Models to Study Molecular Mechanisms Underlying PD

The main goal of establishing animal models of human diseases is to provide new insights into their pathogenic mechanisms. To address this, Drosophila offers a wide variety of genetics tools. One of them is the possibility to perform genetic screens, which allow genome-wide analyses of genetic interactions based on the dominant modification of a given phenotype obtained by loss or gain of function of the gene of interest. Besides, a candidate gene approach can also be performed, in which only those genes that are suspected to be related to the PD-linked gene are assayed for modifications of the phenotype. Both strategies have allowed identifying components of multiple signaling pathways involved in PD pathogenesis. As seen in section 3, some PD-related phenotypes obtained in the fly models are not externally visible as is the case of DA neurons loss. Genetic interaction assays and genetic screens based on such phenotypes are often unaffordable and time consuming. Then, other phenotypes caused by mutations of the PD-related gene, which are easy to score and quantify, are used in the assays. Here, we report several examples of the identification of genes and signaling pathways involved in PD pathogenesis by means of genetic interaction assays performed in flies (see Table 1). Similar genetic experiments have been performed to determine functional relationships among some of the PD-related genes (see Section 3).

In order to identify the molecular mechanisms underlying the pathology associated with loss of function of fly *parkin* (see Section 3.2), a genetic screen for modifiers of the partial lethality phenotype of Drosophila *parkin* mutants was performed. This study identified an LOF allele of the *glutathione S-transferase S1* (*GstS1*) gene as the stronger enhancer of that phenotype [115]. Consistent with this, it was found that reducing *GstS1* activity was able to enhance DA neuron loss in *parkin* mutants while *GstS1* overexpression significantly suppressed that phenotype [62]. Since members of the GST family have been involved in detoxification of ROS [121], these data suggested a connection between *parkin* and oxidative stress response. This hypothesis was confirmed when analyzing the transcriptional profile of *parkin* mutant flies, which showed that an elevated percentage of deregulated genes in the mutants have functions related to oxidative stress response [115].

The importance of glutathione metabolism on DA neuron survival was also demonstrated in a posterior study based on a candidate gene approach. It showed that LOF mutants of genes involved in glutathione synthesis (*Eip55E* and the

Table 1: Signaling pathways and molecular processes involved in PD pathogenesis that have been identified by using Drosophila PD models.

Pathway/process	Drosophila model	Interacting genes/toxins	References
Oxidative stress	*parkin*	*GstS1*	[62, 115]
		Paraquat	[58]
	α-synuclein	*GstS1, Eip55E* and *Gclm*	[39]
		MsrA/Eip71CD	[38]
		Sod	[40]
	DJ-1α/β	Paraquat	[53, 90, 93–95]
		Rotenone	[93]
		H_2O_2	[50, 94]
	LRRK2/4E-BP	Paraquat, H_2O_2	[104]
	PINK1	*Sod*	[72]
		Rotenone	[69]
		Paraquat	[69, 72]
PI3K/Akt signaling	*DJ-1α/β*	*PTEN, Dp110*	[50]
Ras/ERK signaling	*DJ-1α/β*	*Ret, rl*	[116]
JNK signaling	*parkin*	*bsk, hep, puc*	[59]
DA metabolism	Paraquat	*ple, Pu, Catsup*	[27]
	parkin	*VMAT*	[66]
Mitochondrial structure and function	*PINK1*	*parkin*	[69, 70, 74–76, 78]
TOR signaling	*parkin/PINK1*	*4E-BP*	[82]
Removal of excess or toxic protein forms	*α-synuclein*	*Hsp70*	[117]
		ubiquitin	[51]
		dHDAC6	[118]
		SIRT2	[119]
		ctsd	[120]
	parkin	*PAEL-R*	[64]
		Sept4	[65]

Gcl-modifying subunit, Gclm) or glutathione conjugation pathways (*GstS1*) enhanced DA neuron loss of α-Synuclein-overexpressing flies while their overexpression suppressed that phenotype. Those genes were previously isolated in a genetic screen using a yeast model of α-synucleinopathy [37, 122]. The results obtained in this study indicated that α-Synuclein toxicity inversely correlates with the abundance of glutathione and GstS1 and suggest a role for Phase II detoxification pathway in PD pathogenesis [39]. Several studies have also dealt with the importance of α-Synuclein oligomers removal from the DA neuron cytoplasm to keep their integrity. The finding that progressive loss of DA neuron integrity produced by α-Synuclein overexpression is preventable in flies through directed expression of Hsp70 strongly suggested that eliminating toxic forms or excess of the protein could be central to prevent neuron damage [117]. Recently, coexpression of ubiquitin has been shown to rescue DA neuron degeneration and locomotor dysfunction in α-Synuclein-overexpressing flies. This neuroprotection is dependent on the formation of lysine 48 polyubiquitin linkage which is known to target protein degradation via the proteasome [51] and suggests that an increase of α-Synuclein targeting for degradation is able to reduce its toxicity. The involvement of histone deacetylase 6 (dHDAC6) in α-Synuclein toxicity was also analyzed [118], due to its role on sensing ubiquitinated aggregates and consequently activating chaperones expression, facilitating aggresome formation, and determining the fate of ubiquitinated proteins [123–125]. The authors found that knocking down the *dHDAC6* gene on α-Synuclein-overexpressing flies increased the amount of α-Synuclein oligomers while decreased the number of cytoplasmatic inclusions and DA neurons, indicating that dHDAC6 protects DA neuron integrity via promoting α-Synuclein inclusion formation [118]. These results support the role of LB as a successful defense against the concentration of toxic protein forms. Interestingly, inhibition of another protein of the histone deacetylase family, Sirtuin 2 (SIRT2), was also found to protect against α-Synuclein toxicity in *Drosophila* [119]. Finally, another study reported that deletion of the *ctsd* gene, which encodes the lysosomal protease Cathepsin D, promoted the retinal degeneration observed when in α-Synuclein overexpressing flies, suggesting that this protease may act as a facilitator of α-Synuclein-degrading activity [120].

DA neuron degeneration is one of the most distinguishing features of PD. For this reason, it seemed reasonable that genes involved in cell survival/death could have a role in PD pathogenesis. One study tackled this question by performing genetic interaction assays between *DJ-1α* and candidate genes or signaling pathways previously implicated in cell survival. This study led to identify genes in the PI3K/Akt signaling pathway as specific modifiers of the *DJ-1α*-associated cell death phenotype. Consistent with the genetic interaction results, they found that PI3K/Akt signaling regulates cellular ROS levels and that *DJ-1α* downregulation leads to PI3K/Akt signaling impairment. The same effect was observed in *parkin* mutants, thus suggesting a common molecular event between the two models [50]. These results are in contrasts with those obtained in a recent study that reported no interaction between *DJ-1α/β* and PI3K/Akt in the fly eye [116]. The authors described an interaction between *Ret*, a potent activator of both PI3K/Akt and Ras/ERK pathways, and *DJ-1α/β* in *Drosophila*. However, this interaction in the fly eye seems to be mediated by Ras/ERK [116]. The discrepancies could be due to the different systems used on each study, although further work would be necessary to uncover the real connection between *DJ-1α/β* and PI3K/Akt signaling. A relationship between *parkin* and other apoptosis signaling pathways has also been reported [59, 126]. These studies showed that *parkin* LOF mutants exhibit JNK pathway activation in DA neurons and that downregulation of this pathway is able to rescue the DA neuron loss phenotype observed in these mutants [59]. Genetic interactions between *parkin* and members of the JNK pathway also suggested that *parkin* is a negative regulator of this pathway and that this regulation is driven by a reduction in *basket* transcriptional levels [59, 126].

Several genetic studies in Drosophila have also shown that variations in genes regulating dopamine homeostasis, which are conserved in humans but not known to be associated with familial PD, can modify the neurodegeneration phenotype observed in the PD models and alter susceptibility to paraquat, a known environmental PD risk factor [27]. Although it has been extensively discussed, no agreement on the beneficial/toxic effect of this molecule on DA neuron survival and consequently on PD patients has been achieved. Some *in vitro* studies suggest that treatment with L-dopamine, the most common palliative pharmacological compound used in PD patients, could be toxic to DA neurons due to the activation of oxidative cascades produced by an increase in dopamine levels [127–129]. Moreover, an elevation of dopamine synthesis in response to a variety of stressors may expose DA neurons to high levels of oxidative stress [130–132]. In such a scenario, it has been shown that hyperactivated dopamine synthesis in Drosophila *cathecolamines up* (*catsup*) mutants, which might be expected to place the organism under high levels of oxidative stress, is instead able to provide protection against the effects of paraquat exposure. In contrast, compromised dopamine synthesis enhances susceptibility to paraquat-induced oxidative stress [27], thus indicating that sensitivity to paraquat might be modified by variations in genes that regulate dopamine synthesis and metabolism. Moreover, other study has shown that overexpression of the *Drosophila* vesicular monoamine transporter (VMAT), which regulates cytosolic DA homeostasis, partially rescues the degenerative phenotypes caused by overexpression of human *parkin* mutants while its knockdown exacerbates these phenotypes [66]. These result indicate that Parkin-induced neurotoxicity results from the interaction of mutant human *parkin* with cytoplasmic dopamine.

5. Using Drosophila PD Models to Identify Potentially Therapeutic Compounds

Both the genetic and toxin-induced Drosophila PD models represent a promising system for therapeutic compound identification. Indeed, during the last decade the effect of several compounds has been analyzed on behavioural, neurodegenerative or biochemical phenotypes of such models leading to the identification of potentially therapeutic compounds that could alleviate PD symptoms (see Table 2). Although candidate compounds have been always used in these studies, they open the possibility of performing high throughput compound screens which will be undoubtedly useful for finding new drugs that could alleviate PD symptoms.

The first published study about compound treatments in a Drosophila PD model reported the effects of drugs commonly used for treating PD on the locomotor phenotype of *α*-Synuclein expressing flies and showed that some of them were able to suppress that phenotype [133]. Subsequently, and given the ability of increased chaperone activity to counteract *α*-Synuclein toxicity [117], the effect of Geldanamycin (GA), an antibiotic able to interfere with Hsp90 activity and activate stress response, was assayed over *α*-Synuclein expressing flies [35, 134]. Notably, feeding these flies with GA protected DA neurons against *α*-Synuclein induced degeneration, and this protection was driven by an increase in Hsp70 levels [134]. Inhibitors of the histone deacetylase SIRT2 also showed a protective effect against *α*-Synuclein toxicity [119].

Other studies have been also performed in several Drosophila PD models to look for potentially therapeutic compounds directed to reduce oxidative stress damage. As explained previously, the study of *α*-Synuclein toxicity in flies led to the identification of Phase II detoxification pathway as a possible target for therapeutic treatment [39]. In fact, feeding *α*-Synuclein-expressing flies or *Drosophila parkin* mutants with pharmacological inducers of that pathway like sulforaphane or allyl disulfide suppresses the neuronal loss of both PD models [39]. These findings raise the possibility that these and perhaps other chemical inducers of Phase II detoxification pathway may represent potential preventive agents for PD. Besides, it has been shown that dietary supplementation with *S*-methyl-*L*-cysteine (SMLC) inhibits the locomotor and circadian rhythm defects caused by ectopic expression of human *α*-Synuclein in Drosophila [38]. SMLC participates in the catalytic antioxidant mechanism involving Methionine sulfoxide reductase A (MSRA), one of the enzymes that catalyze the oxidation of the amino acid methionine to methionine sulfoxide, a reversible reaction

Table 2: Potentially therapeutic compounds able to modify different phenotypes in the Drosophila PD models.

Pathway/process	Compound treatment*	Drosophila model	Modified phenotype/s	References
Oxidative stress	Sulforaphane and allyl disulfide	*parkin*	DA neuron number	[39]
		α-synuclein	DA neuron number	[39]
	S-methyl-*L*-cysteine	*α-synuclein*	Locomotor activity	[38]
	Polyphenols	*α-synuclein*	Lifespan, locomotor activity	[135]
		Paraquat and iron	Locomotor activity	[136]
	α-tocopherol	*DJ-1β*	Lifespan	[54]
		PINK1	Ommatidial degeneration	[72]
	SOD	*PINK1*	Ommatidial degeneration	[72]
	Melatonin	*DJ-1β*	Lifespan	[54]
		Paraquat	Locomotor activity	[27]
		Rotenone	Locomotor activity, DA neuron number	[27]
	Bacopa monieri leaf extract	Paraquat	Oxidative markers levels	[137]
Oxidative stress/inflammatory process	Minocycline	*DJ-1α*	DA neuron number, dopamine levels	[138]
	Celastrol	*DJ-1α*	DA neuron number, dopamine levels, locomotor activity, and survival rate under oxidative stress conditions	[138]
TOR signaling	Rapamycin	*parkin/PINK1*	Thoracic indentations, locomotor activity, DA neuron number, and muscle integrity	[82]
Removal of excess or toxic protein forms	Geldanamycin	*α-synuclein*	DA neuron number	[35, 134]
Zinc homeostasis	Zinc chloride	*parkin*	Lifespan, locomotor activity, and percentage of adulthood survivors	[139]

* All treatments were administered as dietary complement.

that has been postulated to act protecting cells from oxidative damage. Furthermore, grape extract supplementation has been shown to recover locomotor ability and lifespan in *α*-Synuclein-expressing flies. It is known that grape extracts contain several polyphenols, compounds with antioxidant properties [135]. Other Drosophila PD models in which treatments with antioxidant compounds have been shown to be beneficial are those involving the *DJ-1α* and *DJ-1β* genes [54, 138]. Compounds with antioxidant and anti-inflammatory properties such as celastrol and minocycline conferred potent DA neuroprotection in RNAi *DJ-1α* mutants [138]. We have also recently demonstrated that chronic treatments with antioxidant compounds are able to modify the lifespan phenotype of *DJ-1β* mutant flies, thus suggesting that oxidative stress plays a causal role in such phenotype [54].

It is known that rapamycin is a small molecule inhibitor of TOR signaling that has been shown to lead to 4E-BP hypophosphorylation in vitro and in vivo [140, 141]. Notably rapamycin administration was able to suppress all pathologic phenotypes in *park* and *PINK1* mutants. Moreover, this suppression was found to be 4E-BP-dependent, since the administration of rapamycin to *parkin* and *Thor* or *PINK1* and *Thor* double mutants was completely unable to suppress these phenotypes [113]. Since 4E-BP activity can be manipulated by small molecule inhibitors such as rapamycin, this pathway represents a viable therapeutic target for PD treatment. Moreover, it has been recently suggested that *parkin* mutants, apart from the described phenotypes, also present altered zinc homeostasis. This is supported by the fact that dietary zinc supplementation in the form of zinc chloride increased lifespan as well as the percentage of *parkin* mutant flies reaching adulthood while this supplemented diet was deleterious to control flies [139].

Since most PD cases are sporadic and could be associated to different environmental agents, it is also essential the use of toxin-induced Drosophila PD models to assay the beneficial effects of candidate compounds. Polyphenol administration was also found to exert a beneficial effect on flies exposed to paraquat and iron, protecting, rescuing, and restoring the impaired locomotor activity caused by exposure to those agents [136]. Other antioxidant compounds such as melatonin have also been found to rescue locomotor deficits and DA neurodegeneration in flies exposed to rotenone [26]. Similarly, it has been recently reported that oxidative perturbations, measured by different oxidative markers,

induced by paraquat exposure in *Drosophila* are mitigated by treatment with leaf extracts of *Bacopa monieri*, an Indian herb with attributed neuroprotective functions [137].

6. Conclusions

As reported in this review, Drosophila has emerged as a very valuable model organism to study PD. Although it is impossible to fully recapitulate the key neuropathologic and clinical features of human PD in a single model organism, many of the existing PD models in Drosophila exhibit key features of the disease and have provided insights into PD pathogenesis. Either toxin-induced PD models or models based on mutations in genes that are linked to familial PD have provided the proper context by which conserved signaling pathways and molecular processes relevant to the disease are discovered and compounds able to suppress PD-related phenotypes in flies are discovered as well. Indeed, Drosophila PD models represent a promising system for the identification of new genes that could be involved in PD susceptibility/development as well as of therapeutic compound that could be relevant to alleviate PD symptoms in humans.

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Review Article

Optimizing a Rodent Model of Parkinson's Disease for Exploring the Effects and Mechanisms of Deep Brain Stimulation

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Deep brain stimulation (DBS) has become a treatment for a growing number of neurological and psychiatric disorders, especially for therapy-refractory Parkinson's disease (PD). However, not all of the symptoms of PD are sufficiently improved in all patients, and side effects may occur. Further progress depends on a deeper insight into the mechanisms of action of DBS in the context of disturbed brain circuits. For this, optimized animal models have to be developed. We review not only charge transfer mechanisms at the electrode/tissue interface and strategies to increase the stimulation's energy-efficiency but also the electrochemical, electrophysiological, biochemical and functional effects of DBS. We introduce a hemi-Parkinsonian rat model for long-term experiments with chronically instrumented rats carrying a backpack stimulator and implanted platinum/iridium electrodes. This model is suitable for (1) elucidating the electrochemical processes at the electrode/tissue interface, (2) analyzing the molecular, cellular and behavioral stimulation effects, (3) testing new target regions for DBS, (4) screening for potential neuroprotective DBS effects, and (5) improving the efficacy and safety of the method. An outlook is given on further developments of experimental DBS, including the use of transgenic animals and the testing of closed-loop systems for the direct on-demand application of electric stimulation.

1. Introduction

1.1. History. One of the well-established therapeutic interventions in neurological and psychiatric disorders, especially in the late stages, is the high frequency electrical stimulation of neuronal structures in the depth of the brain, named by convention "deep brain stimulation (DBS)". This method has developed from different lines of experimental and clinical investigations and technical innovations:

(1) stereotactic surgery,

(2) ablative brain surgery with tissue excision, thermocoagulation or cryolesioning,

(3) portable and implantable cardiac pacemakers.

The first experiments with stereotactic interventions in the brain date back to the 1920s when Hess in Zurich stereotactically implanted depth electrodes in freely moving cats. In the 1940s, Spiegel et al. in Philadelphia performed the first stereotactical operations in the human brain [1]. The pioneers of ablative brain surgery were Moniz and Scoville. Both were so-called psychosurgeons who tried to treat psychiatric disorders, mainly schizophrenia, by excising or destroying certain brain areas. Their method went through its ups and downs with the climax being the subsequently obsolete prefrontal leucotomy in the 1930s. However, thalamotomy, pallidotomy, lobectomy, cordotomy, dentatomy, and other ablative operations were also applied to treat movement disorders, pain, and epilepsy. For example,

in the 1950s, Hassler et al. [2] performed more than 300 stereotactic operations in patients with movement disorders, such as athetosis, torsion dystonia, tremor, and PD. They applied the coagulation of various subcortical, mainly pallidal and thalamic, structures and included acute electric stimulation with different pulse shapes and frequency to ensure an exact location of the electrode tip. Thereby, they found a clear target and frequency dependence of the stimulation effect on tremor, hyperkinesias, and rigidity. For example, stimulation of the inner pallidum with frequencies up to 10 Hz increased the tremor, but stimulation with frequencies from 25 to100 Hz decreased the tremor. With the improvement of surgical techniques and the introduction of implantable pulse generators (Medtronic, Minneapolis, MN, USA) in the 1950s, ablative surgery became a chronic electrical stimulation treatment, and DBS was born. Milestones of its application in central disorders were the therapeutic trials for the treatment of the following:

(1) pain and epilepsy by Bechtereva et al. in Leningrad [3],

(2) torticollis spasmodicus by Mundinger in Freiburg [4],

(3) dyskinesia by Siegfried et al. in Zurich [5],

(4) essential tremor and PD by Benabid et al. in Grenoble [6].

Despite the rapidly increasing application of DBS in clinical practice, its mechanisms of action remain poorly understood. Technical improvements and parameter optimization depend mainly on an empiric trial-and-error strategy. However, the electric stimulation of neurons affected by DBS acts according to the general rule of excitability, that is, according to an exponential strength-duration relationship [7]. Two major parameters characterize this relationship. These parameters were first defined 100 years ago by Lapicque to facilitate the comparison of excitability (excitation thresholds) between different objects [8]. The parameters are "rheobase" and "chronaxie", which are coordinates on the strength-duration curve for a stimulus. In neurons, the rheobase is the minimal current amplitude of an almost infinite duration that triggers an action potential, whereas chronaxie represents the shortest duration of an electrical stimulus having an amplitude equal to twice the minimum amplitude required for excitation. Therefore, the rheobase is half the current that needs to be applied for the duration of chronaxie.

1.2. Current Clinical Application. The spectrum of neuropsychiatric diseases treated by DBS, either routinely or in clinical studies, has expanded very rapidly (for review, see [9–13]). However, only the following 4 indications are approved for treatment with DBS by FDA/CE certification:

(1) essential tremor with stimulation of the ventrointermediate (VIM) thalamic nucleus [14],

(2) PD with stimulation of the subthalamic nucleus (STN) or the globus pallidus internus (GPi), a region that is analogous to the entopeduncular nucleus (EP) of the rat [15],

(3) dystonia with stimulation of the GPi for torticollis spasmodicus and generalized dystonia [16],

(4) treatment-resistant obsessive-compulsive disorder (OCD) with stimulation of the internal capsule anterior limb [17].

For the extension of approved indications for DBS, it is necessary to do the following:

(1) to define new target regions for specific indications,

(2) to optimize electrodes and stimulation parameters for specific target regions.

The largely unsolved questions regarding clinical DBS are the exact mechanisms of action of the method and the guidelines for the selection of optimal electrodes and optimal stimulation parameters. The overall aim is to achieve maximum therapeutic efficacy with a minimum of adverse side effects and energy draw. This requires basic studies under defined and reproducible conditions with repeated access to tissue samples in the neighborhood of the electrode tip, which can only be realized in animal model systems. Because PD occurs worldwide and it is the most frequent degenerative movement disorder, experimental investigations have focused on animal models of this disease [18]. These models have been most commonly established in rodents.

1.3. Animal Models. Animal models for the study of the pathogenetic mechanisms and new therapies for human movement disorders and psychiatric diseases, such as OCD, have traditionally been induced by neurotoxins, acting selectively on neurons affected by human diseases. Examples of the most common toxic models for the study of DBS are the following:

(1) the hemi-PD-like disorder induced in rats or mice by unilateral intracerebral injection of 6-hydroxydopamine (6-OHDA) [19] or a carotid MPTP injection in primates [20],

(2) the PD-like disorder induced by an intravenous injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice or primates [21, 22],

(3) the essential tremor-like disease by the intraperitoneal injection of the monoaminooxidase (MAO)-A inhibitor, harmaline, in mice [23, 24],

(4) an OCD-like disease induced by the subcutaneous injection of quinpirole in rats [25–29].

This paper focuses on optimization strategies for DBS using the 6-OHDA-induced hemi-Parkinsonism model in rats; this animal model has several advantages.

(1) The neurotoxin 6-OHDA exerts high selectivity for dopaminergic neurons, which are destroyed by reactive oxygen mechanisms in the substantia nigra pars compacta (SNc) either after a direct injection of

the toxin into this structure or after its retrograde transport from injected dopaminergic projections in the medial forebrain bundle (MFB) or the striatum (caudate putamen (CPu) of the rat) to the soma in the SNc. Therefore, a central pathophysiological feature of human PD, the selective dopaminergic denervation of the striatum, is reproduced resulting in similar electromyographic and gait abnormalities seen in PD patients [30–33].

(2) This model is the most widely used paradigm for PD research and is exceedingly well characterized on the molecular biological, histological and functional level. It allows for a direct comparison of data with the majority of experimental PD studies worldwide.

(3) The therapeutic effects of DBS on the core symptoms of PD, such as rigidity, hypokinesia, tremor, postural instability, and cognitive impairment, can easily be monitored using a broad spectrum of behavioral tests that can analyze single and complex motor and cognitive functions by investigating a wide variety of behaviors including the following:

(i) drug-induced rotation,
(ii) accelerated rotation on a treadmill,
(iii) ladder rung walking,
(iv) beam balance,
(v) postural balance,
(vi) asymmetric limb use in a transparent cylinder,
(vii) stepping movement,
(viii) lateralized response in a corridor task test,
(ix) free explorative movement in an open field, radial maze, and water maze,
(x) vibrissae-elicited forelimb placing,
(xi) paw reaching or pellet grasping on a staircase,
(xii) attention and impulsivity in a 5-choice serial reaction time recorder.

For details of the 6-OHDA-induced hemi-Parkinsonism model and other relevant animal models of PD, see [34].

To create an optimal experimental design for animal studies and to avoid unnecessary animal experiments with DBS, computational simulation and modeling possess great potential. *In silico* calculations allow for the prediction of influences of DBS parameter changes on electric field properties with increasing precision, the consequences of electrochemical processes at the interface between the electrodes and surrounding nervous tissue and electrical nerve cell activity.

2. Numerical Analysis of Electric Field Effects

To understand the effects of DBS, the question of its mechanism can be addressed at the cellular level by asking what structures are actually being stimulated or inhibited, axons, or cell bodies. This question has already been debated at the time when DBS has first been applied in the clinic [35]. However, only long after the first successful application of DBS in patients this question became a subject of numerical analysis using finite element modeling [36, 37]. The numerical analysis of electric field effects aims at describing the distribution of the stimulated neurons around the DBS electrode based on the inhomogeneous current density and field distributions in the stimulated brain tissue. The induced transmembrane potential and, alternatively, the so-called "activation function", are considered the major determinants for neuronal stimulation [38, 39]. A correct description of the distributions of both parameters calls for the invocation of the influence of inhomogeneous and anisotropic brain tissue properties [40, 41]. Anisotropies and inhomogeneities at the structural level are introduced by ionic conductivity and the permittivity patterns in the brain tissue. It can be assumed that membrane structures influence these properties in different ways. Although ion currents will mainly flow in parallel to membrane planes, displacement (capacitive) currents may flow perpendicularly to bridge membranes because of the high area-specific capacitance of these thin layers. Nevertheless, capacitive membrane bridging will probably play a significant role only in the high-frequency components of DBS pulses above 10 kHz [42]. For this reason, it seems justified to consider the anisotropic properties only for ionic currents. Because such properties are hard to obtain, global brain data for the anisotropy of water diffusion obtained from NMR measurements are used to describe the anisotropy of brain tissue [41]. Nevertheless, the frequency-dependent spreading of the stimulation signal in the brain tissue at the cellular level is not easy to describe. Such models require the correct description of cellular geometries and exist for tissues with a much simpler structure, such as the skin [43]. The electrochemical electrode properties, cell membranes, cytoplasmic structures and interstitial media form frequency filters that change the amplitude and frequency spectrum in the stimulated tissue depending on the electrode distance. These properties and the anisotropic properties at the cellular level are usually not considered, mainly because of the differences in the size of the cells and the DBS electrodes. Nevertheless, a major challenge for the transformation of human stimulation conditions into animal models is caused by this size difference. The size influences the maximally applicable voltage (or current) at which membrane poration and tissue damage are still avoided [44, 45]. In the following discussion, the major relationships of this limiting DBS parameter to the electrode size, medium conductivity, cell constant, and the local shape of the stimulation electrode are considered.

For a cubical cell confined by two square electrodes, the resistance, R, is given by Ohm's law when electrode effects are neglected

$$R = \frac{U}{I} = \frac{dE}{I} = \frac{dE}{iA}, \tag{1}$$

where U, I, d, E, and i stand for the voltage across the cell, the current through the cell, the electrode distance and area ($A = d^2$), and the current density in A/m^2, respectively.

(Please note that the vectorial properties of the parameters are neglected for simplicity.) The resistance can also be expressed by the cell geometry and the specific conductivity, σ

$$R = \frac{d}{\sigma A} = \frac{1}{\sigma \gamma}, \tag{2}$$

with $\gamma = d$ being the cell constant of the cubical cell, that is, the geometry factor relating the electrode impedance to the specific medium conductivity, σ. Although γ has been derived for a cubical chamber, it can easily be generalized to any cell geometry when medium anisotropies and electrode processes are neglected [39]. Combining (1) and (2), we get the general relationship of field strength and current density in a homogeneous medium

$$E = \frac{i}{\sigma}. \tag{3}$$

In the following discussion, a spherical electrode suspended in an homogeneous medium of conductivity σ will be considered. This model correctly describes the influence of electrode size on the cell constant, γ, and the interrelationships of the applied voltage, electrode current, field strength, and current density at the electrode surface and the distribution of these parameters in the surrounding medium. The resistance of a setup with two concentric spherical electrodes of distance x is (Figure 1)

$$R = \frac{r_{\text{cnt}} - r_{\text{el}}}{4\pi\sigma r_{\text{cnt}} r_{\text{el}}} = \frac{x}{4\pi\sigma(r_{\text{el}} + x) r_{\text{el}}}, \tag{4}$$

where r_{cnt} and r_{el} are the radii of the counter- and the inner electrodes, respectively. The two limiting cases of this model are two electrodes with comparable radii, that is, electrode areas of $A = 4\pi r_{\text{el}}^2$ leading to (compare to (2))

$$R_{(r_{\text{cnt}} \approx r_{\text{el}})} = \frac{x}{4\pi\sigma r_{\text{el}}^2} = \frac{x}{\sigma A}, \tag{5}$$

and a counterelectrode at an infinite distance. We obtain

$$R_{x \to \infty} = \frac{1}{4\pi\sigma r_{\text{el}}} = \frac{1}{\sigma\gamma}, \tag{6}$$

with the cell constant of $\gamma = 4\pi r_{\text{el}}$ for a spherical electrode. This situation is comparable to a unipolar stimulation with the counterelectrode being located in the stimulator case.

Applying Ohm's law to (6), expressing the electrode current by the current density at the electrode's surface and using (3) leads to

$$R_{x \to \infty} = \frac{1}{4\pi\sigma r_{\text{el}}} = \frac{U}{4\pi i_{\text{el}} r_{\text{el}}^2} = \frac{U}{4\pi\sigma E r_{\text{el}}^2}. \tag{7}$$

For the field strength at the surface of the electrode E_0, we obtain:

$$E_0 = \frac{U}{r_{\text{el}}} = \frac{RI}{r_{\text{el}}} = \frac{I}{4\pi\sigma r_{\text{el}}^2} = \frac{i_0}{\sigma}. \tag{8}$$

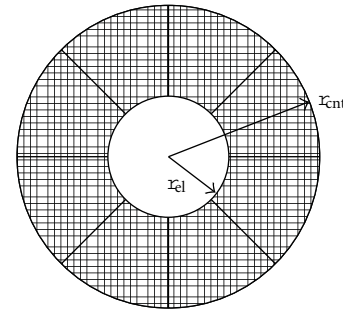


Figure 1: Distribution of the electric field between two concentric spherical electrodes. Electric field lines span the distance between the stimulation electrode of radius r_{el} and the counterelectrode with radius r_{cnt}. The medium between the electrodes has a conductivity of σ.

Expressing I by the current density at the electrode surface, we obtain (3). The field strength at distance x from the electrode is

$$E(x) = \frac{r_{\text{el}} U}{(r_{\text{el}} + x)^2} = \frac{I}{4\pi\sigma(r_{\text{el}} + x)^2}. \tag{9}$$

Equation (8) shows that not only the voltage or current applied to an electrode but also its surface curvature determines the medium field strength. Assuming that field strength, cell size, and orientation determine the induced transmembrane potential, which is one of the possible determinants of neuronal stimulation, (8) and (9) imply a number of conclusions.

(i) Induced transmembrane potentials above approximately 1 V, which are believed to cause membrane poration and cell damage, may occur especially at small electrodes.

(ii) Nerve tissue in the vicinity of high electrode curvatures, that is, blunt electrode edges, and the like, is especially vulnerable to electric cell damage.

(iii) Assuming that the redox-like processes at the electrode surfaces generate a constant voltage (overpotentials, see [42]) at the electrode-medium interface, the voltage portion required to overcome the overpotentials increases for smaller electrodes. This makes smaller electrodes more vulnerable to the precision of electrode machining, that is, electrode size, metal burs, and the like. Assuming that neurons are stimulated by induced transmembrane potentials in a range from 5 to 500 mV, a linear dependence of the induced transmembrane potential on the field strength [44, 45] suggests a reach of 10 r_{el} into the tissue.

(iv) Analysis of the inhomogeneous current density distributions at the electrode surfaces allows for the localization of probable hot spots of metal corrosion and the erosion of the insulating parts.

Numerical calculations of electric potentials, electric fields, and current densities around DBS electrodes can be

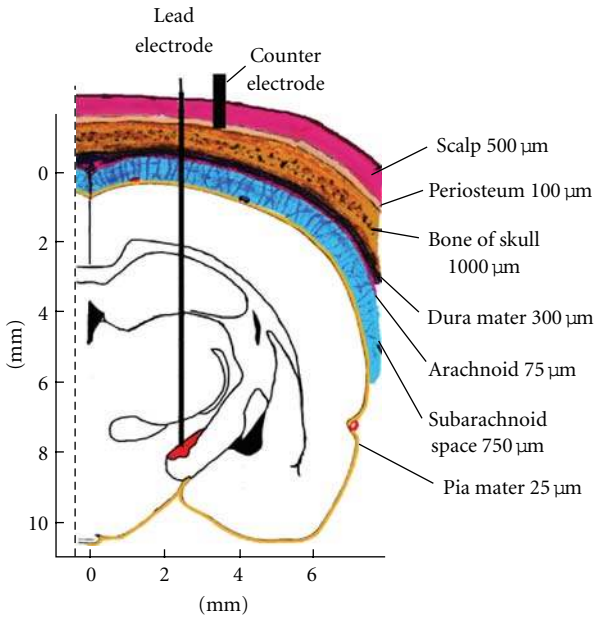


Figure 2: Electrode placement in a brain slice of a rat at bregma: −3.60 mm/interaural: 5.40 mm illustrating that the insulated electrode shaft penetrates several layers of different dielectric properties, that is, the scalp, bone of skull, dura mater, subarachnoid space, and brain tissue. For unipolar lead electrodes, the counter electrode is placed subcutaneously directly on the skull at a distance of more than 20 mm. The red structure at the tip of the electrode is the STN.

performed when dimensions and electric properties of the tissues that surround the electrodes are taken into account. Figure 2 shows a schematic frontal view of a brain slice of a rat, where a DBS electrode is placed in the STN.

A simplified numerical model for a unipolar DBS electrode in this brain slice is depicted in Figure 3. It features the major geometric properties of a rat head as shown in Figure 2. Figure 3(a) specifies the dimensions of the model on which numerical calculations with COMSOL based. We differentiated between high density/low conductivity tissue, that is, bone of skull, dura mater and arachnoid, and low density/high conductivity tissue and fluids, that is, pia mater, gray and white matter, and cerebrospinal fluid, which have different electric properties. Because of a lack of data on dielectric properties of rat tissues, the properties of the respective human tissues were assumed at a frequency of 130 Hz for the simulation in COMSOL. The rectangular DBS stimulation pulse can be modeled by a Fourier series with a basic frequency of 130 Hz. Because the Fourier coefficients of the signal are reduced for frequencies above 3 kHz, Table 1 contains values for 130 Hz, 1 kHz, and 3 kHz ([42]; for reference values see: http://niremf.ifac.cnr.it/tissprop/htmlclie/htmlclie.htm#atsftag). Figure 3(b) shows the calculated potential distribution around a stimulation electrode for use in a rat model (see Figure 8) across this brain model for an input voltage of 1 V.

Figure 3(b) demonstrates that the potential rapidly drops in the immediate vicinity of the electrode tip. Please note that there is a potential drop at the interface between brain and bone which is hardly visible at this resolution. Figure 4 shows the calculated distributions of electric potential, electric field, and current density around a cylindrical unipolar DBS electrode tip.

Figure 5 presents the comparison of simulated potential distributions between a cylindrical unipolar electrode (radius: 100 μm; see Figure 8) and a spherical unipolar electrode according to Figure 1 with the counterelectrode at an infinite distance. For a high consistency of the analytical and the numerical results and to reproduce the potential distribution around the cylindrical electrode at a distance of 400 μm, the center of the spherical electrode had to be positioned in the base of the cylinder and its radius had to be adjusted to ~86.6 μm. The comparison suggests that the presented analytical solution for a unipolar spherical electrode can be used for estimating the field and potential distributions around a stimulating electrode.

Numerical analyses have become very sophisticated in that they nowadays couple finite element models of the electrodes and surrounding medium with cable models of myelinated axons to predict the volume of activated tissue as a function of stimulation parameter settings and electrode design [46]. The combination of numerical modeling and experimental characterization of the voltage distribution generated by DBS in the brain provides information on the quality of the models regarding spatial and temporal characteristics of the voltage distribution generated by DBS electrodes [47]. By increasing the complexity of the model from an electrostatic, homogenous, and isotropic model to one that explicitly incorporates the voltage drop and capacitance of the electrode-electrolyte interface, tissue encapsulation of the electrode, and diffusion-tensor-based 3D-tissue anisotropy and inhomogeneity (see Section 3), it has been shown that the simpler models substantially overestimate the spatial extent of neural activation [48].

3. Electrochemical Considerations in the Context of DBS

Electrode processes are inherent when applying an electric field via a metal electrode in contact to an electrolyte-containing medium such as brain tissue. Electrochemists have been dealing with the properties of electrodes and electrode processes beginning in the 19th century [49]. Comprehensive overviews are given in textbooks, for example, Vetter [50] and Atkins [51]. Serious consideration should be given to the choice of electrode materials and stimulation parameters in experimental animal models of DBS. As described above, simply downscaling electrodes designed for use in humans to the size of animal brains is not possible. Most reports on the postmortem analyses of tissue integrity do not find signs of tissue damage after continuous DBS application in patients [52–54]. However, a newer report demonstrates histological alterations induced by electrode implantation and electrical stimulation [55].

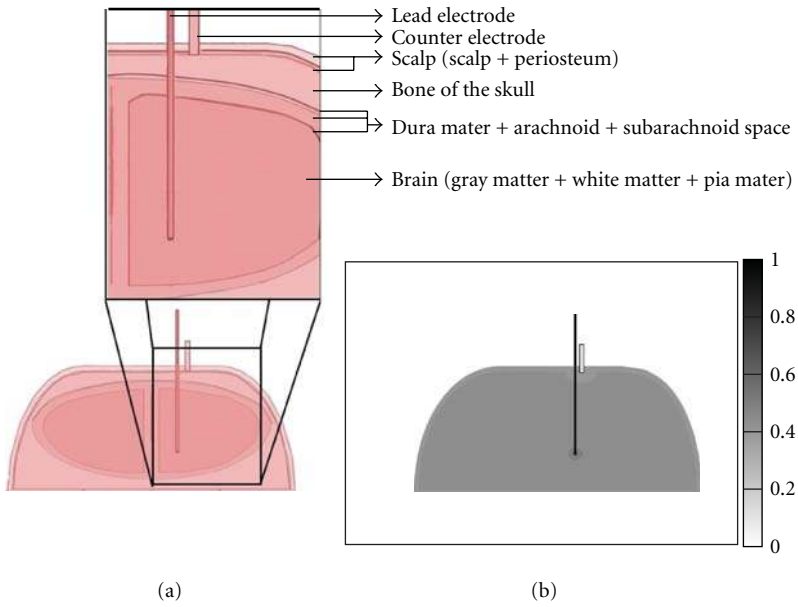


Figure 3: COMSOL simulation. (a) Tissue layers and dimensions for the COMSOL calculation around a DBS electrode (radius: 100 μm; see Figure 8) in the STN of a rat brain using dimensions depicted in Figure 2. Tissues of similar dielectric properties are summarized by arrows. (b) COMSOL simulation of electric potential in the cross-section depicted in (a). For simplicity reasons, the values of gray matter at 130 Hz from Table 1 were used for the tissue assumed as "brain".

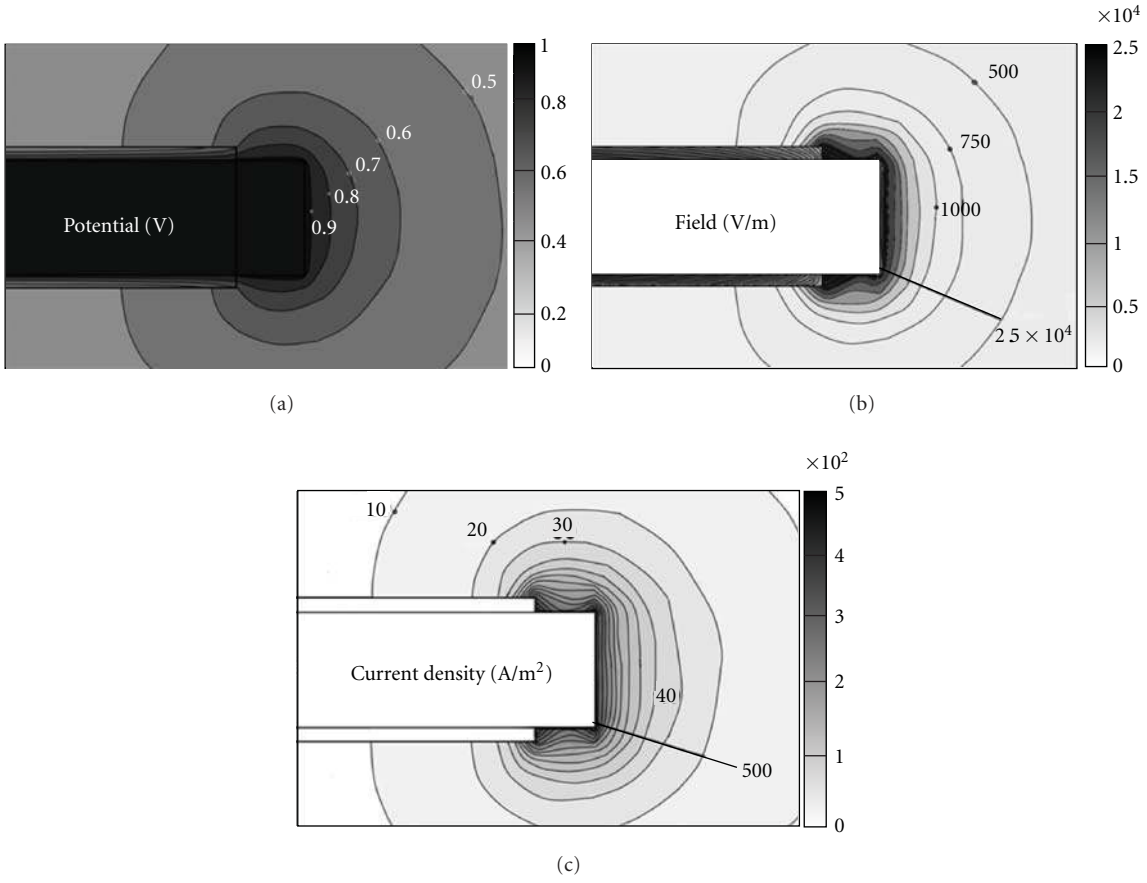


Figure 4: Numerical calculations of (a) the electric potential, (b) the electric field and (c) the current density around a cylindrical unipolar electrode (radius: 100 μm; see Figure 8) in the STN for an input voltage of 1 V.

Table 1: Dielectric properties of human tissues relevant to numerical simulations of DBS at different frequencies.

Tissue	At 130 Hz		At 1 kHz		At 3 kHz	
	Conductivity (S/m)	Relative permittivity	Conductivity (S/m)	Relative permittivity	Conductivity (S/m)	Relative permittivity
Brain gray matter	0.0915	2463000	0.0988	164060	0.10565	66831
Brain white matter	0.0590	1069500	0.0626	69811	0.0650	30133
Cerebrospinal fluid	2	109	2	109	2	109
Dura	0.5006	15276	0.5008	5344	0.5010	2360
Skull bone	0.0201	5355	0.0202	2702	0.0203	1246
Scalp	0.0005	42909	0.0007	32135	0.0009	30569

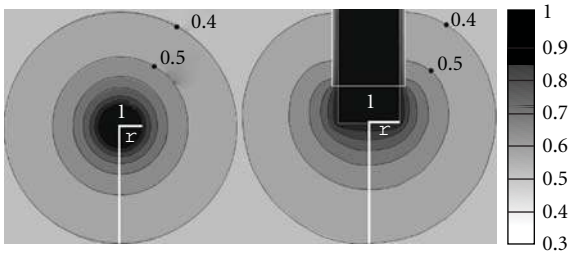


Figure 5: Simulated potential distributions of spherical ($r \sim 86.6\,\mu$m, cell constant $\gamma = 1.09$ mm) and cylindrical ($r = 100\,\mu$m, cell constant $\gamma = 1.00$ mm) electrodes.

In contrast, experimental DBS in rat models has often been accompanied by tissue damage, especially during long-term stimulation [56]. This might be the reason why many studies on DBS in rats were restricted to short-term stimulation. However, a recent study showed that tissue damage may also occur during short-term stimulation [57].

These contrasting findings in animals and patients may have various underlying reasons, such as smaller electrode size and blunt edges (higher curvatures), which both result in high field strengths in the vicinity of the electrodes and in higher local current densities leading to more intense local electrode reactions. Electrode reactions and the use of less inert electrode materials, for example, nonnoble metals, result in potentially toxic products, including denatured proteins, gas, dissolved metal ions, and erosion products of the insulating materials. Electrochemical reactions due to energy dissipation at the interface of stimulation electrodes to the surrounding tissue are unavoidable [42]. The degree of tissue damage is determined by the electrode materials. Nonnoble metals, such as stainless steel, may deposit iron ions in the tissue [57]. Metal ions are a potential source of protein-denaturation and the formation of new antigenic determinants leading to immune reactions [58]. Iron is especially known for its cytotoxicity [59]. The degradation of organic compounds and the evolution of gas, such as hydrogen and chlorine, are nonphysiological processes that change the properties of the extracellular fluid. These changes cause neuronal damage [60].

There are a number of parameters that have to be considered when applying electric fields in living tissue. One problem is that no ideally nonpolarizable electrodes, that is, electrodes of the 2nd kind, can be used under experimental or clinical stimulation conditions [42, 51, 61]. Polarizability is the reason for overpotentials. The shape, that is, the amplitudes of the Fourier components of the applied signal, determines the overpotentials that are dissipated in electrode processes (see below). Although electrodes for human use are driven in a constant-voltage mode, constant-current stimulation with square-topped fields is typically used in animal models (Figure 6). In constant-current mode, the electrodes are driven by a voltage function that corrects for energy dissipation by electrode processes [62]. A very important parameter influencing stimulation efficiency is the impedance of the tissue surrounding the electrode. This impedance changes shortly after electrode implantation and over time. An electrically insulating glial sheath forms around the stimulation electrodes in patients [52, 63] and in laboratory animals [64]. This sheath is presumably responsible for the increase of electrode impedance after DBS surgery [65, 66]. Finite element models have identified the thickness and conductivity of the encapsulation layer around the electrode contact and the conductivity of the bulk tissue medium as the main determinants of altered electrode impedance and found an approximately 50% reduction in the volume of activated tissue using typical DBS settings [67]. However, one study reported a time-dependent decrease of impedance after DBS surgery [68]. Recently, a glial cell culture system has been developed to model the impedance changes after electrode implantation [69]. Because electrode impedance is highly frequency dependent, changes in stimulation parameters that result in a change in the Fourier content may result in changes in stimulation efficiency [42, 61].

The rectangular stimulation pulse in Figure 6, as it is used in animals, is comprised of its basic frequency and higher harmonic frequencies, that is, its Fourier content [42]. Thus, if we assume a smooth function for the frequency dependence of the impedance for the harmonic, low amplitude signals, the impedance for every frequency can be calculated from Ohm's law applied to the voltage and current values. Accordingly, it should be possible to calculate the effective electrode impedance from the RMS values of voltage and current for a pulse signal that contains a Fourier spectrum of frequencies. Nevertheless, even for a harmonic signal, the impedance depends on the signal

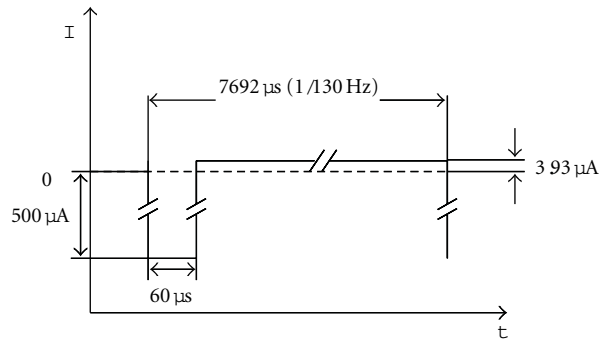


Figure 6: Stimulation pulse as commonly used in the rat model. Please note that the negative stimulation pulse is charge-compensated by the subthreshold positive current between stimulation pulses.

amplitude at every given frequency. Moreover, the charge-carrier transition from electronic currents in the electrode metal to the ionic current in the medium will lead to a nonlinear current voltage relationship and the generation of harmonic frequencies [70]. These complex electrode properties are usually described to include a constant-phase element (CPE; see [42]).

Models to describe this nonlinearity include redox processes requiring a certain activation energy for the charge-carrier transitions and electrochemical reactions, which are summarized under "overpotentials". An additional problem arises from the fact that the nonlinear current transfer function at a given frequency and electrode site (e.g., with a certain curvature) will be influenced by the current induced by the other harmonics or a DC-offset; that is, these currents may contribute to the activation energy required for the charge-carrier transitions at the frequency considered.

In principle, these interrelationships have to be accounted for in models that aim at calculating optimum stimulation parameters that are tailored to the individual patient. Although the situation is not as complicated for the larger electrodes used in humans, which avoid blunt edges and the approach is welladvanced [48], there is too little information on all of the necessary parameters in animal models of DBS where the nonlinear electrode properties play a stronger role (see above).

4. Effects of Experimental DBS on Neuronal Activity

Originally, DBS was seen as a functional ablation because of the similarity of its clinical effect to surgical ablation, which suppresses or inhibits the stimulated nucleus. Several neuronal mechanisms of inhibition at the site of and more remote from DBS have been considered. First, direct effects occur as a result of the field application to the neural membrane and result in regions of depolarization and hyperpolarization along each neural process [71, 72]. Therewith, DBS induces alterations in somatic voltage-gated currents that concertedly block neural output at the electrode (depolarization block). In particular, the persistent Na^+ current (I_{NaP}) is fully blocked, the Ca^{2+}-mediated responses are strongly reduced, suggesting a T- and L-type Ca^{2+} current depression, whereas the hyperpolarization-activated cationic current (I_h) is not affected [73]. However, DBS may hyperpolarize local neuronal cell bodies and dendrites directly [37, 72] or indirectly, given the elevated extracellular K+ levels in experimental Parkinsonism [74], which might interfere with normal activity and generate abnormal activity in neural networks [75]. Second, DBS may elicit indirect effects by activating axon terminals that make synaptic connections with neurons near the stimulating electrode (synaptic inhibition). Experimental and modeling results have shown that afferent inputs have a low threshold for activation during extracellular stimulation [76–80]. Given the large predominance of inhibitory presynaptic terminals in the STN and GPi, their release could locally reduce neuronal activity [81]. Indeed, *in vivo* [79, 82–86] and *in vitro* [73, 87–89] neural recordings in the stimulated nucleus show decreased activity during and/or after DBS. In contrast, this finding was not confirmed recently by microelectrode recordings in human STN when stimulation was delivered via an actual DBS macroelectrode [90]. Third, on a systemic level, the synaptic transmission of the efferent output of stimulated neurons may fail as a result of transmitter depletion, which results in synaptic depression or functional deafferentiation [91, 92].

However, evidence is accumulating for the activation (excitation) of the DBS-stimulated nucleus with subsequent transmission throughout the network. When computer algorithms are used to remove stimulus artifacts, DBS of the STN in primates increases activity in the GPi during stimulation [93]. In turn, this may induce the modulation of pathological activity in the whole network [94]. Recordings from the efferent target nuclei provide the most pertinent neural data on the effects of DBS. In contrast to the above-mentioned studies, *in vivo* recordings in efferent nuclei indicate that the output of the stimulated nuclei is increased by DBS [95–97]. This is possible despite somatic inhibition because action potential initiation from extracellular stimulation occurs in the axon [72, 98]. In general, cathodic stimuli generate membrane depolarization in regions near the electrode and membrane hyperpolarization in regions that flank the region of depolarization. The first few nodes of Ranvier are typically depolarized by the stimulus pulse because of the short internodal spacing of the axon compared to the spatial distribution of the field generated by DBS electrodes [37]. There is also early neurophysiological evidence of the occurrence of such phenomena [99–101]. The second effect of extracellular stimulation that supports the decoupling of activity in the axon and cell body during DBS is the activation of transsynaptic inputs in the close surrounding area of the soma (see above). In particular, because DBS-induced action potential initiation occurs in the axon, the efferent output of neurons suprathreshold for direct activation by the applied field is relatively unaffected by the transsynaptic inhibition, and the majority of local cells within 0.2 mm of the electrode will generate efferent output at the stimulus frequency when the therapeutic stimulation parameters are

used [37]. This "driven" axonal activity replaces spontaneous intrinsic firing with the exogenously induced patterns [102]. DBS, as an extracellular stimulation, is expected to activate subsets of both afferent and efferent axons, leading to antidromic spikes that collide with the ongoing spontaneous spikes and orthodromic spikes that evoke synaptic responses in target neurons. The cellular basis of this interaction between the anti- and orthodromic spikes is unknown, but this mechanism could converge at the level of the STN axon initial segment where spontaneous firing in STN neurons begins [103]. In addition, neurons subthreshold for direct excitation will exhibit suppression of their intrinsic firing patterns that are regulated by stimulation-induced transsynaptic inputs.

It still is a matter of debate regarding which of the effects of DBS is therapeutically effective and how DBS alleviates motor symptoms. There are at least three viable hypotheses. First, pathological GPi activity is inhibited (see above). Second, STN and GPi DBS induces the regularity of GPi activity [96], thereby reducing misinformation in the pathologically noisy GPi signal and abnormal stochastic resonance [93]. DBS may regularize the pathological synaptic activity of basal ganglia output structures [104] in addition to increasing the firing rate of fibers projecting from the site of stimulation [37, 95, 96, 105]. This regularized GPi activity may reduce thalamic error rates (a surrogate for Parkinsonian symptoms) [106] and increase the fidelity of thalamic neurons [107]. This view is experimentally supported by small changes in GPi firing rates in comparison to changes in regularity and bursting activity in response to DBS [96, 104, 108]. Third, DBS activity induces resonance amplification of the information signals in the basal ganglia-thalamus-cortex system necessary for normal movement. Indeed, there are multiple oscillators within this system at many different frequencies, although the main or average frequency is approximately 130 pps [109], and DBS resonates with normal intrinsic oscillators [110]. Basal ganglia oscillations in local field potentials in the 11–30-Hz range are antikinetic [7, 111–113]; reductions in STN oscillations in this frequency range are correlated with clinical improvement [114, 115], and DBS in this frequency range worsens motor performance [116, 117]. Oscillations in the range of 70 Hz are thought to be prokinetic because they are lost in Parkinsonism [7, 113, 118] and restored by levodopa treatment [116, 117].

5. Biochemical and Functional DBS Effects

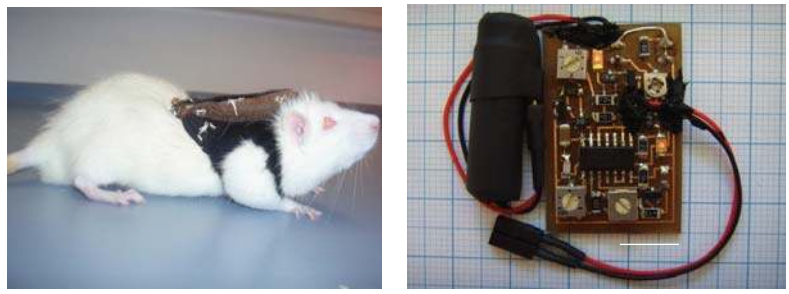
Effects of experimental DBS on neuronal activity are also reflected in changes of neurotransmitter release. Microdialysis studies show an increase in striatal dopamine (DA) release, an activation of striatal DA metabolism and an activation of striatal tyrosine hydroxylase (TH) activity [119–122]. Furthermore, an enhanced glutamate release in the rat entopeduncular nucleus (EP), the rat analog to the human GPi, during STN stimulation, indicating a facilitated activity of the STN during stimulation [105, 123] and an increased GABA release of pallidal origin in the SNr [124] were demonstrated. These findings are consistent with electrophysiological and theoretical data that suggest an excitation of axons (see Section 4). The described effects may explain the immediate effects of DBS, such as the alleviation of tremor by stimulation of the VIM nucleus of the hypothalamus. However, they cannot readily explain the delayed effects, such as the reduction of rigidity within seconds to a few minutes, the alleviation of hypokinesia after hours or days, the effect of STN DBS on tremor within seconds to days or the effect of GPi DBS on dystonia with a delay of days to weeks. Also, carryover effects can be observed. For example, hypokinesia returns only slowly after the cessation of DBS. These clinical observations suggest that electrical stimuli are translated into network reorganization or effects at the gene expression level.

Gene expression studies indicate that STN DBS may reverse a 6-OHDA lesion-induced increased expression of *glutamate decarboxylase-(GAD) 67* mRNA in the EP and in the substantia nigra pars reticulata (SNr) [125]. GAD catalyzes the synthesis of gamma-aminobutyric acid (GABA).

Care should be taken when DBS studies are performed in healthy animals because the data may not equal those acquired in Parkinsonian rats. In a microarray study, mRNAs of *synaptic vesicle protein 2b (Sv2b)* and *ubiquitin-conjugating enzyme E2B* are upregulated by DBS in healthy rats but downregulated by DBS in lesioned rats [126]. Sv2b is involved in synaptic vesicle exocytosis and thus, neurotransmitter release [127]. E2B plays a role in DNA repair [128] and is required for neurite outgrowth [129]. STN DBS, performed for 2 h in healthy rats, induced an increase in striatal TH activity without changes in TH gene expression determined by a TH activity assay and RT-PCR analysis [122]. In contrast, a microarray analysis combined with real-time PCR and immunohistochemistry showed an upregulation of TH gene expression, but not of TH-positive neurons or TH-positive fiber density, by STN DBS in 6-OHDA-lesioned rats [126]. Apparently, DBS effects are altered by an imbalance in the basal ganglia network caused by a 6-OHDA lesion.

We also found a DBS-induced downregulation of *calcium/calmodulin-dependent protein kinase-type IIA (CaMKIIa)* and *Homer1* in 6-OHDA lesioned rats [126]. Both genes are involved in glutamate neurotransmission [130–132]. In addition, we have found an upregulation of *insulin-like growth factor 2 (IGF2)* and *insulin-like growth factor-binding protein 2 (IGFBP2)* [126]. As these molecules play a role in postnatal neurogenesis in the hippocampus of mice [133] one could speculate that their upregulation after DBS could indicate a reorganization of the basal ganglia circuitry. An expression of immediate−early genes, for example, *c−fos*, has been found at the mRNA level [125] with *c−fos* being also induced by L−DOPA treatment in dopamine−denervated marmosets [134] and by immunohistochemistry [135] after STN DBS. The immunohistochemical study demonstrated an upregulation of c−Fos, c−Jun, and Krox−24 not only in the STN but also in the projection areas of the STN [135].

Functional studies, however, require animals that are awake and freely moving. Because of the above-mentioned methodological problems, the latter studies are scarce.



(a) (b)

Figure 7: Chronic instrumentation of a freelymoving rat. (a) Rat with a portable stimulator in a backpack; (b) stimulator purchased from the company Rückmann and Arndt, Berlin, Germany. Scale bar in (b): 10 mm.

Darbaky et al. [56] demonstrated an improvement of motor, but not cognitive, functions in 6-OHDA lesioned rats with STN DBS using platinum electrodes connected to a stimulus generator via a swivel. Other studies have found a reversal of limb-use asymmetry and an improvement in treadmill locomotion in 6-OHDA lesioned rats during STN-DBS [136, 137]. The development of instrumentation for freelymoving animals, such as an implantable microstimulation system [64] or a carry-on stimulator (described herein, see Figure 7), promises many more data on functional improvements. Using an implantable microstimulation system, Harnack et al. [138] demonstrated a preservation of dopaminergic nigral neurons in a 6-OHDA rat model with progressive Parkinsonism using chronic STN-DBS.

A role for BDNF is suggested by the results of chronic (14 d) DBS in freely moving 6-OHDA rats, which showed a protection of SNc neurons, arguing for beneficial functional effects of DBS in the early phase of PD [139].

6. Optimization Strategies for Experimental DBS

Optimization of DBS aims at (1) achievement of optimum electric coupling without nerve cell damage (2) adjustment to the treatment of different neurological and psychiatric diseases by finding the most effective target and (3) defining optimum stimulation parameters for the specific target. This multivariate testing requires long-term *in vivo* experiments in the animal model with (a) the systematic investigation of DBS effects under various stimulation conditions; (b) recording of motor and cognitive functions, and (c) analysis of the nervous tissue in the electrode environment on the cellular and molecular level. A prerequisite for such studies is the establishment of a disease model with chronically instrumented freely moving animals. This strategy will facilitate clinical treatment with highest efficacy and the lowest adverse side effects.

6.1. Chronic Instrumentation of Freely Moving Animals. The implementation of an animal model for the research on movement disorders not only requires adequate tests themselves but it also has to allow for the animals to express their natural locomotor behavior to not dismantle their drive for motion and to not change their routines. In the past, external stimulators constrained the animals, because the connecting cables were easily twisted by rotational movements. Also, the large appliances fixed to the animal restricted movements. Thus, such experiments were strongly limited in time. To date, basically three experimental designs allow for long-term experiments. First, housing the rat in an open cage and connecting a cable through the open cage top directly to the animal allows for most movements although it may not solve the rotation problem under all circumstances [139, 140]. Alternatively, animals are housed in cages with open tops allowing the tubes and cables to be connected to a swivel on top [141]. The swivel provides the cables with an additional degree of freedom and can also be set to read the rotation of the animal. A second option, being most promising for long-term animal experiments, is the implantation of the stimulator. This requires a small apparatus with low weight at the expense of a shorter battery life. Stimulation parameters can be adjusted from outside of the animal [64]. As a third option, the animal permanently carries the whole instrumentation in a backpack (Figure 7). This allows the device to be significantly larger and better accessible compared to the implantable device. Also, the battery may be exchanged for longer stimulation. In summary, this option combines the advantages of options 1 and 2, because (1) the surgical intervention is much less extensive compared to the implantation of the whole device and (2) the animal can move without constraints. This improved freely moving animal model is suitable for measuring classical drug-induced rotation because problems of the restraining cable and tube torsion do not arise.

6.2. Electrode Material and Stimulation Parameters. DBS in rodents requires electrodes that are thinner than those for humans, but it must be stable enough to pierce through the tissue without bending to ensure correct electrode placement. In addition to electrochemical problems arising from these dimensions (see Section 3) stability is an issue limiting the use of platinum/iridium electrodes for testing different electrode tip shapes or multipolar concentric alignments of electrodes. However, corrosion followed by tissue damage occurs when using stainless steel electrodes (see Section 3). Keeping in mind that the stimulation parameters can vary

in many different aspects, such as electrode polarity, current amplitude, and pulse width and frequency, and concerning the standard algorithms that are commonly used for an efficient DBS in humans, we can think about the comparative testing of several simple electrode designs for experimental DBS. One design implies a unipolar cathodic DBS-pulse with a counter electrode underneath the skin for a safe and simple current application just like the common setting used for human therapy with Medtronic devices where the counter electrode is part of the implantable pulse generator (IPG) case. Such an electrode made from platinum/iridium is depicted in Figure 8. Alternative settings consist of bipolar electrodes that can be designed in two different ways:

(1) one concentric bipolar electrode with two concentric contact surfaces, or;

(2) two separate, unipolar electrodes merged together at a region-specific distance.

The unipolar stimulation generates a nearly spherical field distribution, whereas bipolar electrodes produce a more focused field with higher effects in the space between the two electrodes, especially close to the electrode tips and edges. In both cases, the amplitude can be adjusted very precisely in small intervals in analogy to the Medtronic devices. With higher amplitudes, the distributed field increases and can affect structures at a distance from the electrodes, allowing for more neural elements to be stimulated. In the case of DBS of the STN, this may primarily concern the zona incerta and substantia nigra. Newly designed electrodes include sectorial or spot electrodes with a laterally directed field driven in the unipolar or bipolar modes. Such high-perimeter electrodes may increase the variation of current density on the electrode surface, decrease power consumption for the stimulation of axons and reduce the costs and risks of replacement of depleted stimulators [142].

Because of the inverse exponential function describing the interdependence of pulse width and amplitude reflected by the parameters rheobase and chronaxie (see Section 1 for an explanation of these historical items), it is obvious that with higher current amplitudes (i.e., field strength) the pulse width may be lowered nonetheless exciting the surrounding structures of the electrode sufficiently. To protect the treated subject from severe side effects, the stimulation amplitude has to be set as high as needed to reach the most benefit but as low as possible not to exceed the threshold that causes damage by electrochemical reactions and the unintentional excitation of nontarget structures. Chronaxies for DBS effects have been estimated to be around 65 μs for thalamic and around 75 μs for pallidal stimulation [143]. In STN DBS, pulse width seems to have minor influence on the improvement of clinical signs. However, higher pulse widths can be used successfully in pallidal stimulation or in the stimulation of thalamic structures, such as the VIM nucleus.

Although the frequencies of a therapeutic effect of DBS are mainly found in a range higher than 100 Hz, this parameter has also to be adjusted for specific areas and pathways. For example, stimulation of the PPN requires a lower stimulation frequency of 20–60 Hz [144–150]. Because animal models should mimic the clinical situation as closely as possible, we usually apply the human standard of 130 Hz for STN stimulation in the hemi-Parkinsonian rats as a compromise between power consumption and clinical efficacy, regarding this parameter as being of minor importance for strategies to optimize DBS.

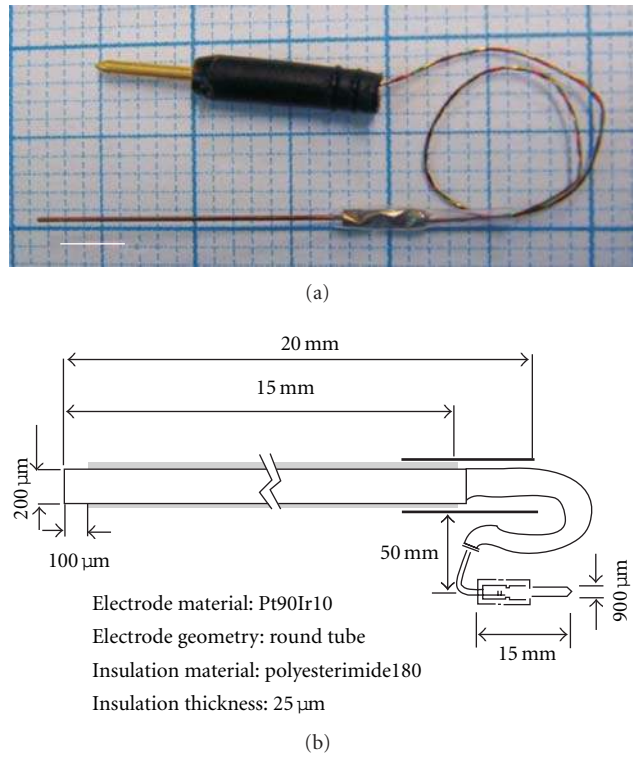


Figure 8: Photograph (a) and scheme (b) of a custom-made unipolar electrode (POLYFIL, Zug, Switzerland) with the pole made from platinum/iridium (PtIr) for experimental DBS in freely moving chronically instrumented rats. Scale bar in (a): 5 mm.

6.3. Closed-Loop Systems. One of the major challenges for future improvements of the DBS technology is the implementation of feedback modulation in so-called closed-loop systems involving built-in sensing capabilities. They were first realized for the treatment of epilepsy by taking advantage of EEG recordings for the controlled delivery of DBS to the seizure focus. For this purpose, originally nonimplantable bedside systems have been used, which are meanwhile substituted by implantable automatic devices, such as the responsive neurostimulator (RNS) lead system (NeuroPace, Mountain View, CA, USA) [151–154].

Further progress results from improved stimulation protocols that aim at desynchronizing the pathological oscillations of neuronal activity [155]. They require pulse generators capable of simultaneously recording physiological parameters and providing adapted stimuli. Basically,

Table 2: Experimental DBS with indications and target regions under study.

Indication	Target region	References
Parkinson's disease, progressive supranuclear palsy	Pedunculopontine (PPN) nucleus	[144–146, 148, 149]
Tremor types other than essential and Parkinsonian tremor (Holmes tremor, dystonic tremor, thalamic tremor, essential writer's tremor, and neuropathic tremor)	Ventrointermediate (VIM), ventral oralis (Vo) and anterior and posterior nucleus thalami, and subthalamic nucleus (STN)	[156–159]
Huntington's disease	Globus pallidusinternus and externus (Gpi and Gpe)	[160, 161]
Alzheimer's disease	Fornix/hypothalamus	[162]
Thalamic pain and poststroke fixed dystonia	Posterior limb of internal capsule	[163, 164]
Central nociceptive pain syndromes (ischemia, hemorrhage, multiple sclerosis, spinal cord, and injury)	Periaqueductal/periventricular gray matter (PAG/PVG)	[165]
Peripheral neuropathic pain (postzoster neuralgia, radiogenic plexus lesion, phantom pain, postdissectomy syndrome, chronic radiculopathy, and carcinoma pain)	Ventroposterolateral/ventroposteromedial (VPL/VPM) nucleus thalami, ventrocaudal (Vc) nucleus thalami, medial lemniscus, and PAG/PVG	[165, 166]
Epilepsy	Anterior and centromedian nucleus (AN and CMN) thalami, mammillary body (MB) hypothalamic and mamillothalamic tract, STN, hippocampus, caudate nucleus (CN), and cerebellum	[167, 168]
Obsessive-compulsive disorder	Anterior limb of internal capsule (ALIC), STN, ventral caudate, inferior thalamic peduncle, nucleus accumbens (NAc), and ventral capsule/ventral striatum (VC/VS)	[25–29, 169–171]
Depression	Subcallosal cingulated gyrus, inferior thalamic peduncle, NAc, VC/VS	[29, 172–174]
Gilles de la Tourette syndrome	Centromedian-parafascicular (Cm-Pf) and Vo complex thalami, Gpi, and NAc	[17, 29]
Minimally conscious state	Central thalamus	[175]

three different methods of desynchronizing stimulation with putative therapeutic impact have been developed:

(1) coordinated reset stimulation,

(2) nonlinear delayed feedback stimulation,

(3) multisite coordinated delayed feedback stimulation [155].

These methods will ultimately contribute to the optimization of the DBS technology in the clinical practice too.

6.4. Novel Target Regions and Indications. The expanding spectrum of neuropsychiatric diseases tested for the putative therapeutic effects of DBS requires a high flexibility of stimulation parameters. Efforts are also directed toward the search for suitable target regions. Nevertheless, most of these efforts follow a trial-and-error strategy. Of special interest, clinical problems of cognitive impairment and late-stage PD may be alleviated by DBS with modified frequencies and the targeting of the PPN. For example, impaired working memory is improved by the low-frequency (25 Hz) stimulation of the PPN [176]. In severe cases of late-stage PD with postural instability and freezing of gait, dual stimulation of the PPN with 25 Hz and of the STN with 60 Hz reveals a higher synergistic effect compared to STN-DBS or PPN-DBS alone [177]. The PPN is also targeted to reduce falls [148] and reaction times during motor tasks in PD [149]. Chronic low-frequency stimulation (25 Hz) of the PPN has been shown to restore functional connectivity [150]. Interestingly, a modification of DBS, using the dorsal column of the spinal cord as the target, enables functional recovery in chronic bilaterally 6-OHDA-lesioned PD rats [178, 179]. However, this could not be confirmed in initial clinical studies on PD patients [180]. Application of DBS in the centrum medianum-parafascicularis(Cm-Pf) complex for patients in a vegetative state is controversial as patients respond poorly if at all [181, 182]. A survey of potential candidates for DBS beyond movement disorders that are already approved for clinical DBS, such as PD (see Section 1.2), is given in Table 2. Notably, the survey by no means claims completeness. However, future studies will probably reduce the number of appropriate target regions of DBS for diverse indications. Therefore, an optimization concerning appropriate targets for any indication will be achieved.

6.5. Transgenic Disease Models. Drawbacks of the toxic animal models described in Section 1.3 are differences in the genetic background (healthy animals versus genetically susceptible patients) and in the pathogenetic mechanisms, whereby the models only partly mirror the pathogenesis and therapeutic response of the human diseases. In this situation, the transgenic technology has several advantages.

It provides a potentially unlimited number of animals that either lack or overexpress genes that have been identified as pathogenetically relevant risk genes in humans. For example, by introducing mutated candidate genes, transgenic models have been generated for the following:

(1) PD with α-synuclein (PARK1 gene) [183],

(2) Huntington's disease with huntingtin (HTT gene) [184],

(3) dystonia with torsinA (DYT1 gene) [185].

Of advantage is also the possibility for the exploration of certain details of the mechanisms of action of DBS using gene-targeted animals. For example, adenosine A1 receptor-mutant mice (knockout or null mice) have contributed to the elucidation of the role of adenosine for the suppression of essential tremor by DBS [24].

7. Conclusions and Outlook

Despite new and promising developments in the field of transgenic animal technology, the conventional 6-OHDA hemi-PD rat model is still suitable for the investigation of various aspects of experimental DBS, such as the analysis of electrochemical processes at the electrode/tissue interface and of molecular and cellular changes in the tissue surrounding the stimulating electrode. For optimization, new electrode materials and modified surface structures are investigated in combination with computational simulation and numerical electric field calculations. Also, new target regions are tested for effects of DBS on motor and cognitive functions assessed by specific behavioral tests. The final aim is the improvement of the efficacy and safety of DBS in clinical practice. Future investigations will concern the following issues, among others:

(1) optimization of pulse shape (f-content) to reduce adverse effects (such as electrode reactions and cell damage) to the system,

(2) technical improvements with smaller, rechargeable and sensor-containing DBS devices that enable current steering and closed-loop stimulation [154],

(3) desynchronization of pathological oscillatory excitations [155],

(4) a combination of fiber optic and optogenetic technology for the stimulation of selected neuronal populations [186],

(5) transgenic and primate animal models of movement disorders for the further elucidation of the mechanisms of action of DBS and for the more precise targeting of specific cell types by DBS [187],

(6) an individualized combination of therapies of DBS and medication,

(7) innovations such as microstimulation via brain-machine interfaces [188] and electrical microarray implants (NeuroNexus Technologies, Ann Arbor, MI, USA), which are being tested in animal models of human diseases.

These investigations will not only allow for a deeper insight into DBS mechanisms but also provide significant therapeutic benefit for patients with neuropsychiatric diseases, in particular in movement disorders such as PD [18].

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Review Article

Therapeutic Effects of Hydrogen in Animal Models of Parkinson's Disease

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Since the first description of Parkinson's disease (PD) nearly two centuries ago, a number of studies have revealed the clinical symptoms, pathology, and therapeutic approaches to overcome this intractable neurodegenerative disease. 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) are neurotoxins which produce Parkinsonian pathology. From the animal studies using these neurotoxins, it has become well established that oxidative stress is a primary cause of, and essential for, cellular apoptosis in dopaminergic neurons. Here, we describe the mechanism whereby oxidative stress evokes irreversible cell death, and propose a novel therapeutic strategy for PD using molecular hydrogen. Hydrogen has an ability to reduce oxidative damage and ameliorate the loss of nigrostriatal dopaminergic neuronal pathway in two experimental animal models. Thus, it is strongly suggested that hydrogen might provide a great advantage to prevent or minimize the onset and progression of PD.

1. Introduction

The central pathological feature of PD was loss of neurons in substantia nigra pars compacta (SNpc). DA depletion by the loss of dopaminergic neurons in SNpc is a primary symptom of PD [1]. PD is one of the most common neurodegenerative and progressive diseases, along with Alzheimer's disease (AD) [2, 3]. In these last two decades, many lines of evidence have emerged to suggest that oxidative stress is closely related to the onset and the progression of PD and AD.

Using neurotoxins in experimental animal models, an enormous number of studies have been undertaken to develop neuroprotective drugs against PD. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) was found to be a by-product of the chemical synthesis of a meperidine analog with potent heroin-like effects [4, 5]. MPTP has the ability to induce PD-like pathology and has been used in various species including nonhuman primates, and rodents. Among the neurotoxic mechanism of MPTP, mitochondrial impairment is highly associated with oxidative damage and related neurodegeneration; the detailed mechanism and the linkage between oxidative damage and neurodegeneration are discussed in this review. Although MPTP-induced PD model animals are regarded as the best reproducible model, another neurotoxin, 6-hydroxydopamine (6-OHDA; 2,4,5-trihydroxyphenylethylamine), is also used for toxin-induced animal model of PD [6].

Many trials have focused on the reduction of oxidative stress as a therapeutic strategy because oxidative stress is regarded as one of the major risk factors in the onset of PD as mentioned above. However, there are still no known antioxidant drugs which are clinically used to prevent PD. Here, the neurotoxic mechanism of MPTP which induces Parkinsonian pathology and behavior, and how molecular hydrogen prevents them, is discussed in this review.

2. Acute and Chronic PD Model Induced by MPTP

MPTP is a protoxin which is high lipophilic molecule, and can penetrate the blood-brain barrier (BBB) after systemic administration. After crossing the BBB, MPTP is readily

converted to 1-methyl-4-phenylpyridinium ion (MPP^+), an actual toxin which can lead to dopaminergic neurodegeneration [7]. MPTP conversion to MPP^+ is dependent on the activity of monoamine oxidase B (MAO-B) by a two-step reaction. First, MPTP is converted to the intermediate 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$), catalyzed by MAO-B [8]. Then, unstable $MPDP^+$ dissociates to MPP^+ and MPTP [9, 10]. Conversion of MPTP to $MPDP^+$ occurs in glial cells and serotonergic cells, not in dopaminergic cells. Dopaminergic neurons exhibit a high-affinity uptake process of MPP^+ through the dopamine transporter, which allows the neurotoxin MPP^+ to cause selective dopaminergic neuronal loss [11]. Inside the neurons, MPP^+ accumulates in the mitochondrial matrix, whose uptake is driven by mitochondrial transmembrane potential gradient [12, 13]. MPP^+ impairs mitochondrial respiration by inhibiting the multisubunit enzyme complex I of the mitochondrial electron transport chain [14, 15]. Inhibition of complex I causes two early and major events: ATP depletion and the buildup of reactive oxygen species (ROS). Complex I activity appears to be decreased by more than 50% to induce nonsynaptic mitochondrial ATP depletion. *In vitro* studies also revealed that mitochondria which are isolated from whole brain require 70% inhibition of complex I activity for ATP depletion. However, *in vivo* MPTP administration causes only a transient 20% reduction of ATP levels in mouse striatum and midbrain [16]. *In vitro* experiments with synaptic mitochondria show that exceeding a threshold of 25% inhibition of complex I results in significant ATP depletion [17]. These findings may imply that synaptic mitochondria show a better correlation with both complex I inhibition and ATP depletion than those in somatic mitochondria. This may answer the question why dopaminergic neurodegeneration shows retrograde degeneration from striatal nerve terminals, which are rich in synaptic mitochondria.

The extents of loss of dopaminergic neurons and behavioral alteration vary depending on differences in the protocol of MPTP administration. Acute administration (20 mg/kg, 3 or 4 times at 2 hours interval) can reduce ~70% of nigral dopaminergic neurons and ~90% of striatal nerve terminal fibers 7 days after administration when the loss of nigral neurons is stable [18] (see Table 1). Up to 10% of MPTP-administered involuntarily die within 24 hours because of cardiovascular side effects, not of dopaminergic neuronal loss, and mice were immobilized until 24 to 48 hours. Striatal MPP^+ level was increased and peaked ~3 hours after the last administration of MPTP. In subacute injection model, MPTP is administrated once a day at 30 mg/kg for 5 consecutive days. Since mild doses of MPTP was administered compared to the acute injection model, an incidence of death was lower in the subacute injection model. Loss of nigral dopaminergic neurons and striatal nerve terminal fibers was also less than acute injection model; ~50% loss of fibers and ~40% loss of nigral dopaminergic neurons were observed 3 weeks after the last day of MPTP administration. For the continuous administration model, MPTP was infused subcutaneously (s.c.) or intraperitoneally (i.p) using osmotic pumps. Our observation revealed that subcutaneous infusion of MPTP at 45 mg/kg/day for 28 days caused 50% loss of nigral dopaminergic neurons [19]. Continuous administration of MPTP subacutely or chronically caused less dopaminergic neuronal loss, which might reflect sprouting of residual fibers or *de novo* appearance of tyrosine hydroxylase-(TH-) positive dopaminergic neurons in DA-depleted striatum [22–25]. Therefore, chronic recovery and damage of TH fiber may occur simultaneously in nigrostriatal pathway.

The chronic administration model had several unique features which were regarded as better phenomena as PD model: (i) formation of inclusion bodies which were positive for alpha-synuclein and ubiquitin, (ii) loss of noradrenergic (NE) neurons in locus coeruleus, (iii) impairment of ubiquitin-proteasome system, and (iv) behavioral alteration. Especially, loss of NE neurons was observed as in human PD [26], and dopamine β-hydroxylase knockout ($Dbh^{-/-}$) mice which lack NE neurons showed more profound motor deficit compared to MPTP-treated mice [27]. Furthermore, bolus administration of MPTP did not induce inclusion bodies formation [21]. Therefore, chronic administration model using an osmotic pump could mimic human PD feature.

3. Oxidative Damage and Apoptotic Signals in MPTP Model

ROS, mostly a superoxide, is produced in mitochondria because of a leak of electrons from the respiratory chain inhibited by MPP^+ [28]. Energy metabolic inhibition and ROS overproduction have their peak several hours after MPTP administration, which trigger the downstream of cellular apoptosis and neurodegeneration days after MPTP treatment [29, 30]. In PD patients, iron level is increased selectively in SNpc, which leads to the greater accessibility of ferrous iron (Fe^{2+}) with hydrogen peroxide and thus generating hydroxyl radical (•OH) [31]. Moreover, lipid peroxidation, protein carbonyls, and 8-oxo-7,8-dihydroguanine (8-oxoG) are increased, which means that cellular lipids, proteins, and DNA are highly exposed to oxidative stress [32, 33]. Such oxidative damage occurs prior to the cellular apoptosis processes.

Sources of ROS are various, and ROS is produced not only in neurons but also in glial cells such as microglia when they become activated (reactive) and show morphological changes [34]. From dopaminergic neurons, superoxide is produced not only in mitochondria but also by auto-oxidation of DA [35]. It is known that auto-oxidation of DA leads to the production of DA (semi)quinones that are converted into aminochrome, which can generate superoxide [36, 37]. Increased ROS causes oxidative damage to DNA [38, 39], cellular lipid peroxidation [40, 41], and stress-related signaling activation such as MAPK and JNK activation [42–44].

Oxidative stress in DNA leads to cellular apoptosis which is mediated by p53 activation and p53-derived Bax translocation to mitochondria. Furthermore, Bax translocation and cytochrome c from mitochondria to the cytosol leads to caspase-dependent apoptosis [45]. Oxidative damage in DNA induces not only caspase-dependent apoptosis but also caspase-independent apoptosis. Among the five normal

Table 1: Comparison of representative MPTP-PD models. Each written model is representative and reproducible examples of MPTP-PD model because many researchers modify their own protocols in creating MPTP-PD model.

	Acute	Sub-acute	Chronic
Dose of MPTP	20 mg/kg	30 mg/kg	30 or 45 mg/kg/day (using osmotic pump)
Duration of MPTP	3 or 4 times at 2 h interval	Once a day for 5 consecutive days	28 days
Administration of MPTP	i.p.	i.p.	i.p. (30 mg) s.c. (45 mg)
Extirpation of brain	7 days after injection	21 days after injection	28 days after pump infusion
Anticipating nigral cell loss	70%	40%	50%
Anticipating striatal fiber loss	90%	50%	50%
Notable features	Undesirable death (~10%)	Less or no undesirable death Nitrated α-synuclein accumulation	Behavioral alteration (open-field test) Formation of inclusion bodies (stained for α-synuclein, ubiquitin) Loss of noradrenergic neurons
References	[18, 19]	[18, 20]	[18, 19, 21]

nucleobases, guanine is the most susceptible to oxidation, and the C8 position of free deoxyguanosine (dG) or dGTP is the most effectively oxidized by •OH in comparison to those in DNA. In fact, eight- to nine-times more 8-oxoG is formed in nucleotide dGTP than in DNA [46, 47]. Under the oxidative stress condition, 8-oxoG accumulates in mitochondrial and nuclear DNA, which can be selectively visualized by immunohistological technique [39, 48]. Systemic MPTP administration promoted the accumulation of 8-oxoG both in mitochondria DNA and in nuclear DNA [39]. Mitochondrial 8-oxoG (mt8oxoG) accumulated in nerve terminal in the striatum, prior to nuclear 8-oxoG (nu8oxoG) accumulation in nigral dopaminergic neurons. Oka et al. [49] demonstrated that accumulation of mt8oxoG causes mitochondrial dysfunction resulting in ATP depletion, which can open the mitochondrial membrane permeability transition (MMPT) pore. During replication of mitochondrial DNA (mtDNA) with an increased level of 8-oxoG, adenine is frequently inserted opposite 8-oxoG in mtDNA, and such adenine paired with 8-oxoG is selectively excised by adenine DNA glycosylase encoded by *Mutyh* gene. During the base excision repair (BER) process, apurinic/apyrimidinic (AP) endonuclease or AP lyases convert abasic sites to single-strand breaks (SSBs) [50–53]. It has been demonstrated that the MUTYH-initiated BER causes mtDNA degradation resulting in its depletion under oxidative stress [49]. This depletion may induce a decreased supply of mitochondria-encoded proteins, transfer RNAs, and ribosomal RNAs, leading to dysfunction of mitochondrial respiration. Therefore, accumulation of mt8oxoG results in the depletion of ATP. Furthermore, MMPT opening enables Ca^{2+} to leave mitochondria, and cytoplasmic Ca^{2+} increase activates calpain, a ubiquitous calcium-sensitive protease, thus inducing cell death [49, 54]. It has been well documented that calpain activation causes the cleavage of neuronal substrates that negatively affect neuronal structure and function, leading to inhibition of essential mechanisms for neuronal survival [55]. Moreover, inhibition of calpain is known to reduce the dopaminergic neuronal loss in the MPTP model [56]. Taken together, we propose that oxidative stress in dopamine neurons initiated by MPTP administration increases accumulation of mt8oxoG, and thereby causes mitochondrial dysfunction resulting in dopaminergic neuronal loss which is dependent on the calpain pathway (Figure 1).

On the other hands, SSBs are accumulated in nuclear DNA as a result of excision of adenine opposite nu8oxoG by MUTYH, and activate poly (ADP-ribose) polymerase (PARP) with the increase of poly-ADP ribosylation, leading to nuclear translocation of apoptosis inducing factor (AIF) and NAD/ATP depletion [49]. PARP, known as a molecular nick-sensor, binds SSBs specifically and utilizes β-NAD^+ as a substrates to catalyze the synthesis of (ADP-ribose) polymers (poly-ADP ribosylation) on nuclear proteins, including PARP itself with the increase of PARP activity [57, 58]. PARP activation signal induces AIF release from mitochondria and translocation to the nucleus, which results in a caspase-independent pathway of programmed cell death [59]. Activation of PARP leads to its autoconsumption, and depletes ATP content. Therefore, a loss of energy supply also contributes to cell death [49]. Several reports indicate that PARP activation is associated with MPTP-derived neurotoxicity [60, 61]. It is, however, noteworthy that MUTYH-dependent PARP activation requires replication of nuclear DNA [49], indicating that mitotic cells in brain such as glial cells other than neurons may be affected by the PARP-AIF pathway with increased level of nu8oxoG. Among glial cells, oligodendrocytes and astrocytes show PARP-AIF pathway mediated apoptotic cell death [62, 63]. Therefore,

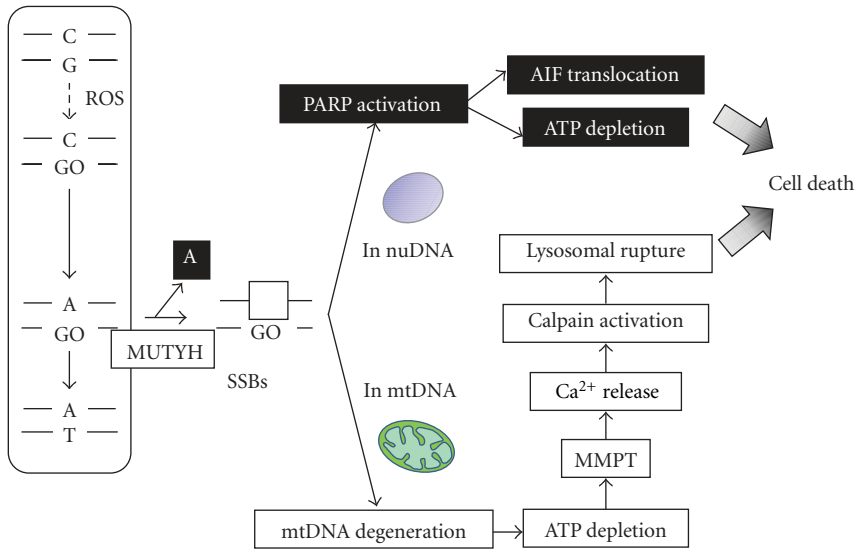

Figure 1: Scheme of apoptotic death signaling by accumulation of 8-oxoguanine (8-oxoG; GO) and single-strand-breaks (SSBs) in DNA. ROS, especially hydroxyl radical, increase the 8-oxoG accumulation and SSBs by MUTYH. In the case of SSBs in nucleus, activation of poly (ADP-ribose) polymerase (PARP), apoptosis inducing factor (AIF) translocation from mitochondria to nucleus, and ATP depletion followed by NAD^+ depletion leads to cellular apoptosis. On the other hands, in mitochondria, accumulation of SSBs induces mitochondrial DNA (mtDNA) degeneration. Loss of function of energy supply leads to ATP depletion, and mitochondrial membrane permeability transition (MMPT), and calpain activation results in lysosomal rupture, which potentiates cell death (modified from Figure 8, Oka et al., 2008 [49]).

accumulation of 8-oxoG in nuDNA in glial cells may thus cause caspase-independent cellular apoptosis, which might play critical roles in neurodegeneration (Figure 1).

4. 6-OHDA Model and Oxidative Damage in Nigrostriatal Neurons

For PD model animal, 6-OHDA is also used for deletion of catecholamine in the brain and in periphery [64]. 6-OHDA serves as a neurotoxin; which is readily auto-oxidized and deaminated by monoamine oxidase (MAO) [65]. Because 6-OHDA cannot penetrate blood-brain barrier, direct administration into the brain is required for the neurodegeneration in 6-OHDA model [66]. This neurotoxin can be generated within the brain by nonenzymatic reaction of dopamine, hydrogen peroxide, and free iron [67–69]. Auto-oxidation of dopamine by nitrite ions or manganese can also generate 6-OHDA [70, 71]. Oxidative damage via hydrogen peroxide and derived •OH are associated with the neurotoxic mechanism by 6-OHDA [64]. The steps to generate ROS are several varied processes: (1) in physiological condition, 6-OHDA is subjected to non-enzymatic auto-oxidation and generates several toxic products such as quinones, superoxide anion radicals, hydrogen peroxides, and •OH [65]; (2) Fenton reaction initiates and/or amplifies ROS generation. The deamination by MAO, or auto-oxidization increases the hydrogen peroxide [72, 73]. Both neurotoxins, MPTP and 6-OHDA, can potentiate the cellular apoptosis with the increase of oxidative damage in DNA, but SSBs-derived PARP activation does not affect 6-OHDA-derived cell death in embryonic nigral grafts [74]. This might be because of less formation of NO in grafted nigral neurons [75]. The apoptotic mechanism by 6-OHDA is explained by the role of p53 and Bax translocation, and caspase activation [66].

5. Hydrogen as a Therapeutic Antioxidant for Experimental Animal Models of PD

Since the first striking evidence indicating that molecular hydrogen acts as an antioxidant and inhalation of hydrogen-containing gas reduces ischemic injury in brain [76], there have been increasing reports which support therapeutic properties of hydrogen against oxidative stress-related diseases and damages in brain [77, 78], liver [79], intestinal graft [80], myocardial injury [81, 82], and atherosclerosis [83]. Hydrogen can be taken up by inhalation of hydrogen-containing gas (hydrogen gas) or drinking hydrogen-containing water (hydrogen water). One hour after the start of inhalation of hydrogen gas, hydrogen can be detectable in blood, at levels of 10 μM in arterial blood [76]. The content of hydrogen can be measured even after intake of hydrogen water by a catheter, which shows 5 μM in artery calculated after 3 min of hydrogen water incorporation [77]. Taking into account its continuous intake, it is easier and safer to drink hydrogen water than inhaling hydrogen gas.

We have previously reported that hydrogen in drinking water reduced the loss of dopaminergic neurons in MPTP-treated mice [19]. The therapeutic effects of hydrogen water against PD model have also been confirmed in another animal model, 6-OHDA-treated rats [84]. It is reported that 6-OHDA also causes 8-oxoG accumulation and mitochondrial dysfunction through oxidative stress [85], and thus our model shown in Figure 1 can be applied to the PD model.

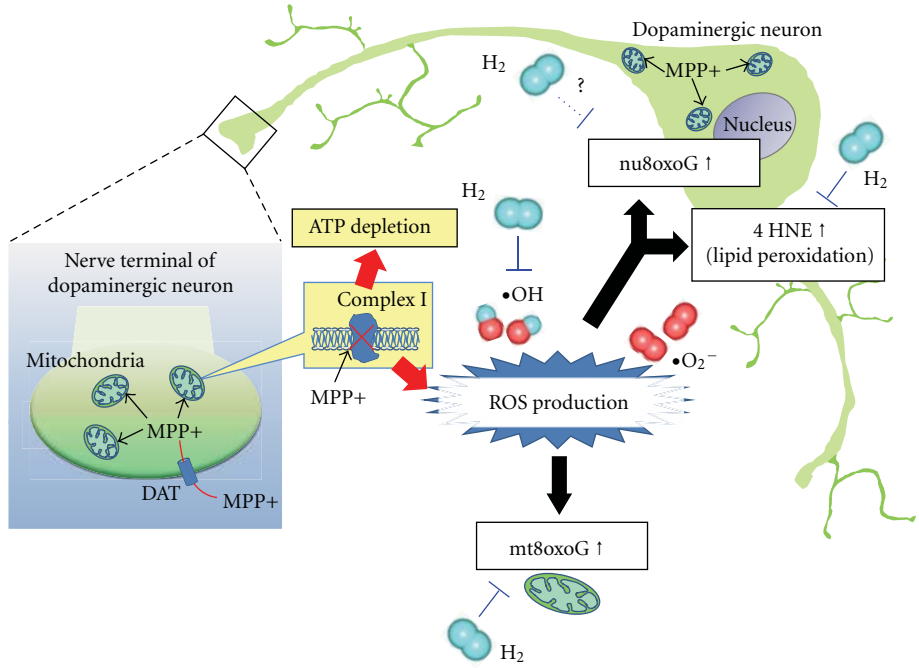

FIGURE 2: The effects of hydrogen in oxidative stress-derived neural apoptosis in dopaminergic cells. Hydrogen (H_2) selectively reduces hydroxyl radical (•OH) by direct reaction, and decreased oxidative damage such as mitochondrial/nuclear 8-oxoG (mt8oxoG/nu8oxoG) accumulation, and 4-hydroxynonenal (4-HNE) production in dopaminergic neurons. Each oxidative damage is involved in different neuronal apoptosis. Abbreviation; MPP^+: 1-methyl-4-phenylpyridinium ion, DAT: dopamine transporter, ROS: reactive oxygen species, ATP: adenosine 5′-triphosphate, •OH: hydroxyl radical, $•O_2^-$: superoxide, 4-HNE: 4-hydroxynonenal.

In these animal models, a number of dopaminergic neurons in SNpc, as well as nerve terminal fibers in striatum, were decreased by administration of the neurotoxin. However, hydrogen water significantly reduces the loss of both neuronal cell bodies and fibers compared with normal water. In MPTP-treated mice, chronic administration using an osmotic minipump results in neuronal loss as well as behavioral impairments observed by the open-field test [21]. Rats administered with 6-OHDA also show behavioral impairments assessed by the rotarod test. Hydrogen improved behavioral impairment in both MPTP and 6-OHDA model. From these observations, hydrogen water even prevents behavioral alteration which is regarded as a major symptom in PD.

It would provide us with useful information for the design of a therapeutic strategy to investigate how long the neuroprotection acquired by hydrogen water lasts. Continuous intake of hydrogen water before and during MPTP administration showed significant neuroprotection. However, intake of hydrogen water even after MPTP administration also reduced neurotoxic damage [19]. PD is regarded as a progressive neurodegenerative disease, so daily intake of hydrogen water might prevent the disease progression as well as the onset of neurodegeneration.

It has been reported that hydrogen reduced cytotoxic •OH selectively whereas the production of other radicals such as superoxide, hydrogen peroxide and nitric oxide was not altered by hydrogen [76]. This selectivity was proved by cell-free system, and in particular, the preference of scavenging of •OH rather than superoxide was confirmed in PC12 cell culture system [76]. According to Setsukinai et al. [86], both •OH and peroxynitrite ($ONOO^-$) were much more reactive than other ROS. This would be an answer why hydrogen shows selective reaction with only the strongest radicals both in the cell-free system and in PC12 cells.

Especially, •OH overproduction in oxidative and neurotoxic reaction by MPTP leads to lipid peroxidation observed by 4-hydroxynonenal (4-HNE) immunostaining in nigral dopaminergic neurons prior to cellular death. 4-HNE immunoreactivity in MPTP-treated mice is increased by three-times as much as in saline-treated mice [19], which was similar to the previous report of 4-HNE protein levels in substantia nigra observed at the same periods after MPTP administration using HPLC [41]. Hydrogen water significantly reduces the formation of 4-HNE in dopaminergic neurons in the substantia nigra to the level of control [19] (Figure 2). On the other hand, the increase of superoxide, which is detectable by administration of dihydroethidine (DHE) intravenously, was not significantly reduced by hydrogen water [19]. Although hydrogen reduces the production of superoxide in brain slices in hypoxia/reperfusion injury [87], hydrogen water might show a preferential reduction of •OH during the protection of dopaminergic neurons.

Hydrogen water significantly reduces the accumulation of 8-oxoG in striatum after MPTP administration [19] (Figure 2). As mentioned above, 8-oxoG, an oxidized form of guanine, accumulates both in mitochondria and in

nucleus; their nomenclature are mt8oxoG and nu8oxoG, respectively. Mt8oxoG accumulates in striatum which are rich in mitochondria in nerve terminal of dopaminergic neurons projected from the substantia nigra. Although nu8oxoG was not detected in nigral cell nucleus [19], hydrogen water might prevent the mt8oxoG-induced cellular apoptotic signals, not just reduce •OH in dopaminergic nerve terminals.

Hydrogen was effective when it was inhaled during reperfusion; when hydrogen was inhaled just during ischemia (not in reperfusion), infarct volume was not significantly decreased [76]. It was shown that hydrogen in the brain decreased immediately after stopping inhalation and completely disappeared within 10 min [19], indicating that the effect of hydrogen can be observed only during the period when the oxidative insults occur. Hydrogen could be detected in the blood 3 min after administration of hydrogen water into the stomach [77]. However, unpublished data showed that the half-life of hydrogen in the muscle in rats was approximately 20 min after the administration of hydrogen gas. Taking these reports into consideration, hydrogen in the brain and other tissues does not stay long enough to exert its ability as an antioxidant to ROS directly. Therefore, it is unlikely that direct reaction of hydrogen itself with ROS plays a major role in the neuroprotection, *especially by hydrogen in drinking water*, although hydrogen itself has the ability to reduce •OH preferentially. In accordance with this hypothesis, previous reports from Nakao et al. [88] has demonstrated that drinking hydrogen water increases urinary antioxidant enzyme, superoxide dismutase (SOD), an endogenous defensive system against ROS- (especially superoxide-) mediated cellular damage. Although it takes eight weeks for significant increase of SOD in humans, hydrogen has the ability to alter the expression level of urinary antioxidant enzyme. It was also reported that hydrogen water increased total bilirubin for four to eight weeks compared to baseline. Bilirubin is produced by the catalytic reaction of heme oxygenase 1 (HO-1), and degradation of heme generates bilirubin as well as carbon monoxide and free iron. The increase of HO-1 expression is likely due to the response to oxidative stress, and this response is also characterized as a phase II antioxidant which is positively regulated by several stress-responsive transcriptional factors [89]. Therefore, taking these observations into account, we might better have another aspect for protective effect of hydrogen in drinking water apart from inhalation. It is possible that drinking of hydrogen water has not only the ability to reduce cytotoxic radicals, but also novel mechanisms which are related to anti-oxidative defense system.

6. Conclusion

Oxidative stress is a key factor to induce cellular apoptosis in MPTP- and 6-OHDA-derived neurotoxicity. From studies using postmortem human brain of PD patients, increased iron, oxidation of proteins and DNA, lipid peroxidation in the SN appear to be important findings of oxidative stress [90–93]. Thought there are effective antioxidants or therapeutic strategies for PD, reduction of oxidative stress would be more desirable to attenuate neurotoxic damage in PD. Here, we would like to address that one of the most efficient ways to attenuate oxidative stress is taking low concentration of hydrogen in drinking water, a safer and easier way of hydrogen intake. Although the precise mechanism how hydrogen works is still under investigation, it will be possible to reveal the mechanisms using conventional PD models such as MPTP and 6-OHDA models. Not only that it is of great interest to know the neuroprotective mechanism of hydrogen but also hydrogen will bring great beneficial effects to reduce a risk of lifestyle-related oxidative damage and related neurodegenerative diseases including PD.

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Review Article

Limitations of Animal Models of Parkinson's Disease

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Most cases of Parkinson's disease (PD) are sporadic. When choosing an animal model for idiopathic PD, one must consider the extent of similarity or divergence between the physiology, anatomy, behavior, and regulation of gene expression between humans and the animal. Rodents and nonhuman primates are used most frequently in PD research because when a Parkinsonian state is induced, they mimic many aspects of idiopathic PD. These models have been useful in our understanding of the etiology of the disease and provide a means for testing new treatments. However, the current animal models often fall short in replicating the true pathophysiology occurring in idiopathic PD, and thus results from animal models often do not translate to the clinic. In this paper we will explain the limitations of animal models of PD and why their use is inappropriate for the study of some aspects of PD.

1. Introduction

The goal of most studies focused on understanding idiopathic PD is to identify the triggers and the mechanisms involved in the progressive neurodegeneration associated with the disease, to design treatments for the symptoms and to develop strategies to slow or stop neurodegeneration. Ideally, a model of idiopathic PD would be progressive in nature allowing the characterization of mechanistic changes in the brain and the onset of symptoms with time. Such a model would provide an opportunity to intervene as the disease progressed. Toxin-based models fall short in this regard since their acute nature, a single or a few injections given over a short period of time followed by rapid or immediate onset of symptoms, limits their usefulness. In addition, the best animal models should mimic the pathophysiology of the disease including the formation of alpha, synuclein, containing inclusions (Lewy bodies), the loss of neurons in the substantia nigra pars compacta (SNpc), and behavioral symptoms that arise during the course of the disease [1]. Taking these important issues into consideration, the best animal models for PD would provide a gradual onset of pathophysiological symptoms and only after manifestation of symptoms would a drug or neuroprotective agent be administered to test for effectiveness [2]. When a genetic model is used to study PD, treatment could be administered prior to the onset of the symptoms. This clinically driven approach that mimics the development of the disease in patients is rarely used in animal studies although there are a few exceptions [3, 4].

A widerange of models have been used to study PD from the small evolutionarily remote single cell yeast to the large evolutionarily similar nonhuman primate. Yeast [5], worms [6], and fruit flies [7] are useful for studying fundamental cellular processes involved with PD, such as apoptosis, autophagy, oxidative stress, protein misfolding and degradation, vesicle-mediated transport, and determining the function of proteins. Some of the factors known to be involved with PD have no known homologs in the smaller eukaryotes, nevertheless expression of human genes in these organisms has been useful in partially elucidating the role of the proteins. Whether it is possible to entirely determine the function of proteins using heterologous expression remains unclear particularly because important protein-protein interactions may not be evolutionarily conserved. In addition, these small animal models cannot be used to study many of the clinical manifestations of the disease [8], nor can yeast, worms, or fruit flies replicate the loss of neurons in the brain [7].

Throughout the years of PD research, rodents have been widely used to study the disease because they are readily

available, genetically malleable, and relatively low cost as compared to larger animals. There are several excellent studies that have used dogs, cats and nonhuman primates for PD studies, but the ethical concerns and costs of such studies have limited their utility. Because of the widespread use of rodent models and their similarities to humans, they will be the focus of this paper.

2. Modeling PD in Rodents Using Environmental Toxins

To the best of our knowledge, PD does not appear to develop naturally in any animals except humans. The standard models for PD are designed to produce nigrostriatal dopaminergic lesions usually with 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat or, rotenone [9–12]. Most of these models inhibit mitochondrial function and/or create reactive oxygen species, but none of them completely reproduces the clinical symptoms and pathology of PD seen in humans [12]. Although these models are used extensively to study the mechanism of disease onset and progression and the efficacy of therapeutic treatments, the results obtained using these models rarely translate to the clinic successfully [13, 14]. Part of the problem with most of the toxin models is their acute nature, which is completely different from the insidious progression of PD observed in patients. Compensatory changes may arise in patients over the course of the disease that would not have an opportunity to occur in the acute animal models. In addition, PD occurs most frequently in elderly patients, usually around the age of 60 or older. Unfortunately, most rodent models do not use older animals because of the inconvenience and cost of housing the animals for an extended period of time. In addition, a closer look at the differences in behavior, physiology, and gene expression between rodents and humans as described below partially reveals why the animal studies do not translate well to clinical studies.

3. Can Genetic Models Be Used to Study Idiopathic PD?

Some recently developed models for studying idiopathic PD have taken advantage of either genes known to play a role in PD from familial studies or genes whose expression is significantly altered in PD patients compared to controls. Models using inherited mutated familial genes are designed to create null mutations of recessive genes or to express additional copies of dominant genes in mice. The genetic models have recently been reviewed elsewhere and therefore are not described in detail in this paper except for a few of the most promising recently developed models [15, 16]. One of the mouse models expresses the human α-synuclein gene with two mutations (A30P/A53T) that produce dominantly inherited forms of PD under the control of the tyrosine hydroxylase (TH) promoter that restricts expression to catecholaminergic neurons [17]. The benefit of this genetic model is that an age-dependent loss of TH-positive neurons in the SNpc is observed along with a decline in motor activity. No Lewy bodies are observed in this model however. In addition, there are no known familial cases of PD in which both mutations have arisen in the α-synuclein gene, thus the relevance of the model has been questioned [15]. In another approach to developing genetic models, transgenic mice were created that used the TH promoter to overexpress truncated forms of α-synuclein [18, 19] that had been shown to be pathologically relevant to PD [20–23]. One of these models showed selective nigral DA neuron degeneration and impaired locomotive function that was reversed by L-DOPA treatment similar to PD in humans [19]. Unfortunately, the loss of neurons in this model was not progressive and occurred during embryogenesis thus substantially reducing the value of this model for collecting information pertinent to PD in humans. An alternative approach for overexpressing α-synuclein is stereotactic injection of the gene carried on viral vectors into the SN which produced rodents with DA neuron degeneration [24–26]. Despite the availability of numerous α-synuclein- based genetic models of PD, only the mouse prion promoter A53T α-synuclein transgenic mouse shows the same α-synuclein pathology and age-dependent neurodegeneration that is observed in humans [27–30]. One of the most recent additions to the selection of α-synuclein models of PD is a transgenic mouse that expresses the wild type gene with the regulated tetracycline (tet) system [31]. In this model, loss of neurons in the SN, progressive motor decline, hippocampal pathology and cognitive impairment were observed, but there were no fibrillary inclusions. This model has provided one very important piece of information in understanding PD, however. The ability to terminate expression allowed the investigators to conclude that continual expression of α-synuclein was required for disease progression [31].

There has been much more limited success in producing a genetic model of PD using several autosomal recessive genes including Parkin, PINK1 and DJ-1 (reviewed in [15]). Recently more attention has been directed at LRRK2 since mutations in this gene account for 5%–6% of patients with familial PD and 1%–3% of sporadic PD patients [32, 33]. Unfortunately, most of the transgenic mice that express wild type or mutated versions of LRRK2 exhibit minimal or no neurodegeneration [16]. This is also true of the wild type and mutant LRRK2 bacterial artificial chromosome (BAC) transgenic mice [34]. Despite this caveat, an advantage of the LRRK2 BAC transgenic mice is that they exhibit a progressive age-dependent motor deficit that responds to L-Dopa and apomorpine treatment [35].

Promising alternatives to the strict genetic models are genetic models that are additionally exposed to toxins such as MPTP. Since the development of PD may be caused by exposure to environmental toxins or heavy metals combined with a genetic vulnerability, these newer combination models could prove to be extremely beneficial for studying PD. In addition, some of the more refined genetic models of PD alter the expression of genes of interest in specific regions of the brain or specifically in neurons. One of the most promising models in this category is the MitoPark mouse [36]. In this model the mitochondrial transcription factor TFAM may

be conditionally inhibited in dopamine neurons. MitoPark mice exhibit motor impairment, reduced dopamine in the striatum and loss of dopamine neurons particularly in the SNpc. Intracellular aggregates form in the brain of MitoPark mice, but unfortunately they are not similar to the Lewy bodies that form in PD patients.

4. Behavioral Tests

Part of the problem with studying PD in animals is not simply the model, that is chosen, but in addition the assays used to assess changes between the healthy and diseased state. PD patients experience many motor symptoms including akinesia, bradykinesia, muscular rigidity, dystonia, resting tremors, gait abnormalities and postural instability due to progressive dopamine neuron loss and dysregulation of dopamine-modulated pathways in the basal ganglia [37, 38]. When assessing behavioral changes in rodent models, it is important to keep in mind that although the neuroanatomical components underlying motor control may be similar for humans and rodents, the manifestation of these motor deficits may be expressed differently between species.

There are various behavioral tests for rodents that are used to measure dopamine-induced motor deficits in animal models of PD. For example, there are exploratory tests such as the open field test and swim test, and then there are learned and/or innate skill tests. The latter tests include the rotarod, grid test, adjusting steps, inclined beam traversal, climbing down a pole, forelimb placing test, reaction-time test, staircase test, paw retraction test, adhesive removal and nesting behavior (for a full description of the tests see [37, 39]). These behavioral tests were largely designed to assess the innate motor skills/abilities of animals that are dopamine dependent, in order to relate the changes observed to the motor deficits seen in PD patients. However, many of these behavioral tests (with the exception of the stepping test) require the animal to learn the task first as most of these measures are complex tasks. Complex tasks can still measure innate motor skills though one does not know if the failure to perform a task is from a motor deficit or from a learning deficit. It is important to note that not all animals learn these complex tasks even prior to receiving the dopamine lesion and often are excluded from the results. In the animals that do learn the behavioral tasks one must keep in mind that the tests are reflective of akinesia and bradykinesia, and not necessarily tremor and rigidity. Although there are behavioral models that measure tremor and rigidity [39] the latter two symptoms are subtler and would probably be easier to characterize if rodents were less dependent on all four limbs for balance (for more information see Timothy Schallert's lab website: http://homepage.psy.utexas.edu/homepage/group/SchallertLAB/). To date, there are no behavioral models that can reproduce all of the motor deficits that are commonly seen to be in PD patients.

Another key point to consider is that the design of the paradigm influences the behavioral outcome. For example, the degree of dopamine loss, the timing and dose of the toxin injections, the time between injections and the behavioral testing and genetic manipulations will all impact the results of the behavioral study. When comparing the MPTP and 6-OHDA lesion models, the MPTP model would seem more favorable as it produces a bilateral dopamine lesion that can be delivered using a chronic regime [40, 41], similar to the slow onset of idiopathic PD, whereas the 6-OHDA model is classically a unilateral lesion [42], although bilateral lesions have been established [43–45]. In the classic unilateral 6-OHDA model only a single injection into the medial forebrain bundle is required to induce a full dopamine lesion approximately 2 weeks after injection. This is similar to what is seen in the bilateral 6-OHDA lesion models. The bilateral lesion models may be considered more relevant to PD since both hemispheres are dopamine depleted and they can have more specificity towards behavioral impairments depending on the dose and location of the injections [46]. Although both 6-OHDA models reproduce the major behavioral deficits seen in PD, the effect of the 6-OHDA toxin does not mimic the progressive loss seen in PD. The MPTP model also has its own caveats in that the extent of neuropathology observed is dependent on the age, sex, and strain of mouse used in the study [47]. In addition, the MPTP mouse models (as with the other toxin models of PD) fail to encompass the wide assortment of motor impairments seen in PD patients [37, 48]. Perhaps the current rodent models of PD would be more predictive of what will translate into human studies if the time course of dopamine neuron degeneration could be mimicked and behavioral tests were designed to assess the more subtle symptoms of tremor and rigidity.

Beyond the paradigm chosen for a particular study, there is a concern that applies to all animal research that is often neglected when interpreting results. There are factors introduced to the everyday laboratory environment by the experimenter that can cause undue stress to the animals. For example, rodents by nature are social creatures, and follow a social dominance hierarchy. Often a dominant male will suppress his subordinate cage mates by fighting and/or guarding the food and water to establish the hierarchy. Social interactions of this nature can lead to changes in dietary intake and overall behavior, an unwanted situation when conducting a behavioral experiment. The animals can also identify with the experimenter's smell (e.g., perfumes/colognes and scents from shampoos, deodorant, laundry soaps and lotions), including that of their lab coat. By using one specific lab coat only for behavioral testing throughout the entire experiment, animals can identify with the experimenter's smell and may be less stressed by their presence. Overall, it is important that investigators consider these subtle, though potentially important, confounds to their work.

5. Physiological Concerns

Although there is a great deal of similarity between the physiology of rodents and humans, it is clear that significant differences exist. Perhaps one of the most relevant examples of this difference with regards to PD research is the distinction between how humans and rodents metabolize MPTP. Rats

and mice are relatively resistant to MPTP, whereas humans are quite sensitive to this toxin. The sensitivity of humans to MPTP became apparent in 1983 when several drug addicts unfortunately injected themselves with MPTP thinking it was synthetic heroin. These young drug addicts very quickly developed symptoms similar to PD [49]. In contrast to this, MPTP is more effective when administered with the adjuvant probenecid (which blocks the rapid clearance of MPTP and its metabolites from the kidney) in rodents in order to produce some of the pathophysiological and behavioral symptoms seen in humans [40, 41]. There are likely to be additional differences in the metabolism of environmental toxins between rodents and humans that have not yet been identified and, therefore investigators must remain cautious in interpreting the results from studies of rodent models.

Differences between the blood brain barrier in humans and rodents must also be considered in this regard. There is evidence that the neuroinflammation associated with PD may make the blood brain barrier more leaky than in a healthy individual [50]. The function of the blood brain barrier is to act as a physical and metabolic barrier between the blood and central nervous system. If this barrier becomes leaky, immune mediators of the blood may enter the brain and contribute to the neurodegenerative process. Similar to humans, the blood brain barrier also becomes leaky in rodent models of PD [50]. The brain endothelial cells from rodents do not express the same enzymes as humans, however, and therefore the influx of nutrients that nourish the brain and efflux of toxic metabolites may be different between the species [50]. The transporter differences in the blood brain barrier between species again suggest that caution is required when applying data from animal studies to humans.

6. Regulation of Gene Expression

In the past, it was thought that transcription factors were conserved in sequence and function, allowing regulation of the same target genes across species. Recent studies, however, have now shown that although transcription factors may be conserved across species, the sites which they bind are different [51]. The divergence in the cis-regulatory networks between humans and mice was demonstrated in hepatocytes [52]. When the transcription factor binding sites in human chromosome 21 are compared to the orthologous regions in mice, only one-third to a half are conserved [53]. When mouse transcription factors were placed in a mouse nuclear environment, a human-like binding signature was observed on a human-derived chromosome indicating that the human chromosomal sequence is responsible for the placement of the transcription factors [53]. Studies similar to this have not yet been done in the brain, but the existence of cis-regulatory species-specific networks suggest that we cannot assume that the regulation of gene expression will be the same between humans and the animal models used for PD research. In this regard, major differences in the expression of transcription factors were observed between human and chimpanzees brains, which most likely results in coordinated differences in the expression of downstream genes [54]. Of particular interest to PD research, differences between the transcription regulation of human and mouse tyrosine hydroxylase have already been noted [55]. This is of interest because tyrosine hydroxylase is the enzyme that catalyzes the hydroxylation of tyrosine to produce L-dopa [56], which is the rate-limiting step in the synthesis of catecholamine neurotransmitters [57].

Species differences in posttranscriptional regulation of gene expression are just as important to consider as transcriptional changes when evaluating animal models. The regulation of alternative splicing plays an essential role in the diversity of proteins produced from a single gene. To determine the extent of alternative splicing in different species, Brett and colleagues studied expressed sequence tags and determined that the extent of alternative splicing is similar among species including humans and rodents [58]. Recently, however, it was shown that humans have more regulated alternative splicing than rodents using a similar approach [59]. The different results obtained in these two studies are most likely due to the fact that the newer study used only bona fide alternative splicing events, along with a few additional differences in the methodology [59]. Although some alternative splicing events have been evolutionarily conserved, the majority of these events have not been conserved between humans and mice [60]. With regard to the most prevalent form of alternative splicing, exon skipping, it has been estimated that >11% of the events are species-specific [61]. The results from all these studies combined suggest that species-specific alternative splicing has the potential to produce large differences in phenotypic complexity. These findings suggest that we must use caution when interpreting results from studies of animal models of PD because subtle molecular changes at the level of gene expression may result in large changes in signaling pathways and behavioral and physiological responses.

In addition to splicing changes in gene expression, non-coding microRNAs (miRNAs) fine-tune gene expression by binding to RNA sequences within the 3′-untranslated region and usually downregulate gene expression by destabilizing the RNA or inhibiting translation. Many miRNAs have been evolutionarily conserved, and there are many highly conserved motifs in the 3′ untranslated region of mRNAs in vertebrates, some of which most likely bind miRNAs [62]. Unfortunately, very few of the putative miRNA binding sites that have been identified through bioinformatics studies have been experimentally tested. Because of the importance of using animal models for studying diseases, further studies designed to assess the degree of evolutionary conservation of miRNA regulation of gene expression between species would be extremely helpful.

7. Conclusions

Rodents and nonhuman primates are an important resource for the study of PD, but the limitations of these models must be kept in mind when interpreting results. Nonhuman primate models are anatomically, physiologically, and behaviorally more similar to humans, but they are rarely

used because of cost and ethical concerns. Rats and mice are widely used for modeling PD, but no toxin or genetic model completely reproduces the pathophysiology seen in humans. Because it is currently thought that environmental factors and genetic susceptibility play a role in the onset and progression of PD, perhaps the most promising models are those that combine genetic models with exposure to toxins.

Because of the current limitations with PD models, some studies are best done in the clinic. An example of this type of study would be the search for noninvasive biomarkers of PD. If one is attempting to identify blood biomarkers of PD, the investigation could be done directly in humans and therefore the results obtained from the study would be directly applicable to patients.

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Research Article

Manganese Inhalation as a Parkinson Disease Model

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The present study examines the effects of divalent and trivalent Manganese (Mn^{2+}/Mn^{3+}) mixture inhalation on mice to obtain a novel animal model of Parkinson disease (PD) inducing bilateral and progressive dopaminergic cell death, correlate those alterations with motor disturbances, and determine whether L-DOPA treatment improves the behavior, to ensure that the alterations are of dopaminergic origin. CD-1 male mice inhaled a mixture of Manganese chloride and Manganese acetate, one hour twice a week for five months. Before Mn exposure, animals were trained to perform motor function tests and were evaluated each week after the exposure. By the end of Mn exposure, 10 mice were orally treated with 7.5 mg/kg L-DOPA. After 5 months of Mn mixture inhalation, striatal dopamine content decreased 71%, the SNc showed important reduction in the number of TH-immunopositive neurons, mice developed akinesia, postural instability, and action tremor; these motor alterations were reverted with L-DOPA treatment. Our data provide evidence that Mn^{2+}/Mn^{3+} mixture inhalation produces similar morphological, neurochemical, and behavioral alterations to those observed in PD providing a useful experimental model for the study of this neurodegenerative disease.

1. Introduction

Parkinson disease (PD) is a progressive neurodegenerative disorder that affects 1% of the population over 55 years of age. The pathologic hallmark of the disease is the loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNc) and the presence of intracytoplasmic inclusions named Lewy bodies, formed mainly by α-synuclein and ubiquitin. In the striatum, there is a loss of dopamine (DA) and its metabolites homovanillic acid and 3,4-dihydroxyphenylacetate [1–3]. The dopaminergic loss in the striatal spiny neurons is followed by a cascade of events that ultimately changes its structure and the activity of basal ganglia circuits, resulting in the development of PD symptomatology. The main symptoms of the disease are tremor, bradykinesia, hypokinesia, balance, and gait disturbances. The basic process behind the nigrostriatal degeneration still remains unsolved. However, among many other hypothetical degenerative mechanisms, oxidative stress has become an important candidate in producing the neuropathological alterations in PD [3].

Although the etiology of PD is still not fully understood, animal models have provided important clues. On the basis of experimental and clinical findings, PD was the first neurological disease to be modeled and, subsequently, to be treated by neurotransmitter replacement therapy [4]. All PD models are based on the concept that parkinsonian signs are related to dopaminergic nigral cell loss. Several models exhibit many of the characteristic features of the disease; however, none mimics the complex chronic neurodegenerative features of human PD. The 6-hydroxydopamine (6-OHDA) and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are neurotoxins which selectively and rapidly destroy catecholaminergic neurons (within 1–3 days), whereas in humans the PD pathogenesis follows a progressive course over decades.

According to Emborg [5], an ideal animal model can be described by presenting behavioral signs and pathology that

resemble the disease, including its time course. The closer the similarity of a model is to PD, the higher the predictive validity for clinical efficacy is.

It has been investigated the effects of Manganese (Mn) as a PD model, due to its toxicity (referred to as manganism) shares neurological symptoms with several clinical disorders commonly described as "extrapyramidal motor system dysfunction," and, in particular, idiopathic PD [6–8].

Postmortem studies in humans [9–13] and chronic studies in nonhuman primates [14–18] and rodents [19–22] revealed that Mn intoxication produces neuropathological changes in the basal ganglia, structures that include the globus pallidus (GP), caudate nucleus and putamen (striatum), and less frequently the substantia nigra (SN) [12, 21, 23].

The human central nervous system is an important target for Mn intoxication [6–8, 24–27]; its toxicity is targeted to DA-rich brain regions neurons possibly via the dopamine transporter (DAT) [28–31].

In vitro studies indicate that Mn produces an inhibition of oxidative phosphorylation [32], increasing of reactive oxygen species in synaptosomes [33], and enhances the rate of DA auto-oxidation [34–36], while intrastriatal administration of Mn leads to impaired energy metabolism, excitotoxic lesions, decreased DA, GABA, and substance P levels [37, 38] and accelerate the oxidation of DA [36]. Moreover, it has been reported that unilateral intranigral Mn administration induces ipsilateral turning, while bilateral infusion resulted in akinesia and dystonic posturing of the hind limbs, assuming that those alterations are due to decreasing DA levels [38–40].

The cellular, intracellular, and molecular mechanisms underlying neurotoxicity of Mn compounds are numerous, as it impacts many biological activities depending on levels and routes of exposure, dosage, age of the exposed individual, and duration of exposure [41].

Great discrepancy exists about Mn-inducing PD, including the specificity of Mn-damaging GP or SN [7, 17, 42]. Olanow [43], Perl and Olanow [44], Lucchini et al. [45], Guilarte [46], and others suggest that PD preferentially damages DA neurons in the SN, while Mn preferentially accumulates within and damages GP and striatum, while sparing the nigrostriatal system. According to Calne et al. [7], Lu et al. [47], Cersosimo and Koller [48], Aschner et al. [49, 50], and others, the most important among these differences is the lack of clinical response to L-DOPA.

However, studies have reported seemingly conflicting results on the dopaminergic effects of Mn (see Gwiazda et al. [51] and Guilarte [46] for review), including decrease [14, 21, 27, 40, 52–56], increase [19, 57], both, increase and decrease [20], or no change [25, 42, 58] in nigral or striatal DA concentrations in Mn-treated animals, possibly reflecting effects of the different exposure regimens on DA outcomes. These discrepancies may well reflect differences in exposure route, magnitude, duration, Mn concentration or compound, age of the experimental animals, and so forth between studies, though they also demonstrate the complexity of Mn toxicity and suggest that the factors contributing to its toxicity are not well understood.

It seems that at lower doses, Mn increased DA and its metabolite levels, while the opposite effect was seen at higher doses [20, 59]. Likewise, it has been suggested that higher concentrations of Mn may significantly accelerate the oxidation of DA and other catecholamines, which concurrently amplify the formation of reactive oxygen species [34, 36, 60].

It has been reported that divalent and trivalent manganese may be transported into the brain across the blood-brain and the blood-CSF barriers [61, 62]. Divalent Mn can be transported into brain capillary endothelial cells and choroidal epithelial cells via undefined divalent metal transporter DMT- 1, DCT-1, or nramp-2 [63]. In the brain, it is known that SN and striatum are regions rich in DMT-1 [64]. On the other hand, trivalent Mn bound to transferrin is transported across the brain barriers via the receptor-mediated endocytosis [62]. Mn is then released from the complex into the endothelial cell by endosomal acidification [50]. Mn released within the endothelial cells is subsequently transferred to the abluminal cell surface for release into the extracellular fluid. Mn delivered to brain-derived transferrin for extracellular transport, subsequently is taken up by neurons, oligodendrocytes, and astrocytes for usage and storage [65]. In the mitochondria, it has been demonstrated that Mn inhibits complex I thereby leading to altered oxidative phosphorylation, and it seems that Mn^{3+} is more potent at inhibiting complex I than Mn^{2+} [66–68] and accelerates the oxidation of ferrous iron. Low micromolar concentrations of Mn^{3+} are sufficient to trigger an immediate oxidation of ferrous iron, whereas divalent Mn at concentrations of 100-fold higher did not promote the conversion of ferrous to ferric [69].

The enhanced ability of trivalent Mn to induce oxidative stress has been confirmed in rats given either manganese chloride [$MnCl_2$ (Mn^{2+})] or manganese acetate [$Mn(OAc)_3$ (Mn^{3+})] [67]; these authors report that $MnCl_2$ (1–1000 μM) produced dose-dependent increases of reactive oxygen species in striatum whereas MnOAc produced similar increases at much lower concentrations (1–100 μM). Thus, the valence of Mn and its metabolism seem to influence its toxicity.

Therefore, the pro-oxidant activity of Mn^{2+} is dependent on trace amounts of Mn^{3+}, which may facilitate a small portion of Mn^{2+} to oxidize to Mn^{3+}. This synergistic relationship between Mn^{2+} and Mn^{3+}, results in continuous redox cycling [69]. These findings lead us to hypothesize that if the animals are exposed to the mixture of Mn^{2+}/Mn^{3+}, it would be possible to find cell and behavioral alterations resembling those found in PD.

Since it has been postulated that Mn^{3+} is more potent in producing oxidative stress and Mn^{2+} needs the presence of Mn^{3+} to reach oxidation and that there is a synergy between the two Mn states, the current study investigates the effects of Mn^{2+}/Mn^{3+} mixture inhalation on mice to obtain a novel animal model of PD inducing bilateral and progressive cell death in the SNc and correlating those alterations with motor disturbances. As a next step, we sought to determine if after Mn inhalation the movement alterations improve with L-DOPA treatment in order to ensure that the alterations origin is dopaminergic.

2. Experimental Procedures

Fourty-five CD-1 male mice weighing 33 ± 2 g were individually housed in hanging plastic cages under controlled light conditions (12 h light/h dark regime) and fed with Purina Rodent Chow and water *ad libitum*. Body weight was recorded daily. The experimental protocol was conduced in accordance with the Animal Act of 1986 for Scientific Procedures. All efforts were made to minimize the number of animals used and their suffering.

2.1. Motor Behavior. Prior to Mn inhalation, all the animals were trained in the reaching task and beam-walking test to evaluate motor performance. Training and testing were performed during the lighted portion of the cycle, at the same hour every time. For the reaching task mice were food deprived to 90% of normal body weight and received measured amounts of food once a day to maintain body weight and deprivation state. The motor behavior tests were performed during the days the animals did not inhale. Each mouse was tested once a week, a different day for each test. Two observers blind to the mice exposed or control status perform all behavioral assessments.

2.2. Single-Pellet Reaching Task. The plexiglas reaching box was 19.5 cm long, 8 cm wide, and 20 cm high. A 1-cm wide vertical slit ran up the front of the box. A 0.2 cm thick plastic shelf (8.3 cm long and 3.8 cm wide) was mounted 1.1 cm from the floor on the front of the box. Before training, animals were food deprived for 24 hr. Afterward, they received a restricted diet of ~10 gm/kg body weight adjusted to keep their weight constant. Twenty milligram food pellets were placed in indentations spaced 1 cm away from the slit and centered on its edges. Animals were habituated for 1 week by placing them in the cages for 10 minutes. Pellets were initially available on the cage floor and within tongue distance on the shelf. Pellets were gradually removed from the floor and placed farther away on the shelf (1 cm) until the mouse were forced to reach to retrieve the food. As the animal pronates the paw medially, this placement allows the mouse to obtain the pellet with a paw and not with the tongue. Mice were individually trained and allowed to reach with their preferred forelimb for food pellets [70]. Each animal reached for 20 pellets each day during the testing period. If an animal reached through the slot and obtained a food pellet, the reach was scored as a success. If an animal knocked the food away or dropped the food after grasping it, the reach was scored as a miss [71]. Qualitative assessment consisted in analyzing the "reaching performance," the postural shift and impairments in limb extension, aim, and supination-pronation of the paw during grasping, and release of the pellet into the mouth.

2.3. Beam Walking Test. The additional test to measure motor coordination of mice was assessed by measuring the ability of the animals to traverse a narrow beam (3 mm) to reach an enclosed safety platform [72]. The apparatus is constructed by elevating surface of a 10 × 100 cm × 3 mm wooden beam 75 cm above the floor with wooden supports with 15° inclination. A goal box is located at one end of the beam. During training, animals were placed at the beginning of the beam with no inclination and they were trained over 4 days (4 trials per day). Once the animals crossed the beam in a 20 seconds interval, they received two more consecutive trials with the inclined beam. Animals were allowed up to 60 sec to traverse the beam. The latency to traverse beam was recorded for each trial.

2.4. Video Recording. Performance during single pellet reaching and beam walking tests was video recorded using a Sony camcorder (1000th of a second shutter speed). The camera was positioned orthogonally to the reaching box such that the animal behavior was filmed from the front. Representative still frames were captured from digital video recordings with the video editing software Final Cut Pro. Pictures were cropped and adjusted for color and brightness contrast in Adobe Photoshop V.11.0.2 but were not altered in any other way.

Neurological Evaluation. Tremor and bradykinesia (slowed ability to start and continue movements and impaired ability to adjust body's position) were evaluated by inspection of Mn-exposed compared with control mice during the performance of the two tests.

2.5. Manganese Inhalation

2.5.1. Pilot Study. A pilot study was performed (5 control and 5 Mn exposure mice) with 0.02 and 0.03 M Manganese chloride ($MnCl_2$) and 0.01 and 0.02 M Manganese acetate [$Mn(OAc)_3$] (Sigma Chemical Co Mexico), and after 6, 8, 10, and 12 inhalations by light microscopy, some changes were observed in SNc tyroxine hidroxylase (TH) immunoreactive neurons. However, the loss of TH-immunostained cells were not enough to observe behavioral alterations (data not shown). Thus, higher doses were used; the mixture of 0.04 M $MnCl_2$ and 0.02 M $Mn(OAc)_3$, and knowing that the half-life of Mn is about 30–48 h and scarce information is available about inhalation, we planned a twice a week exposure protocol.

Inhalations were performed as described by Avila-Costa et al. [73]. Twenty animals were placed in an acrylic chamber inhaling 0.04 M $MnCl_2$ and 0.02 M $Mn(OAc)_3$ (Sigma Aldrich, Co. Mexico) 1 h twice a week for five months. Fifteen control mice inhaled only the vehicle—deionized water—for the same period. Inhalations were performed in closed acrylic boxes (35 cm wide × 44 cm long and 20 cm high) connected to an ultranebulizer (Shinmed, Taiwan), with 10 l/min continuous flux. The ultranebulizer is designed to produce droplets in a 0.5–5 μm range. A trap for the vapor was located in the opposite side with a solution of sodium bicarbonate to precipitate the remaining metal. During exposures, animals were continuously visually monitored for respiration rate, depth, and regularity. The exposure system was continuously monitored for temperature, oxygen level, and Mn concentration.

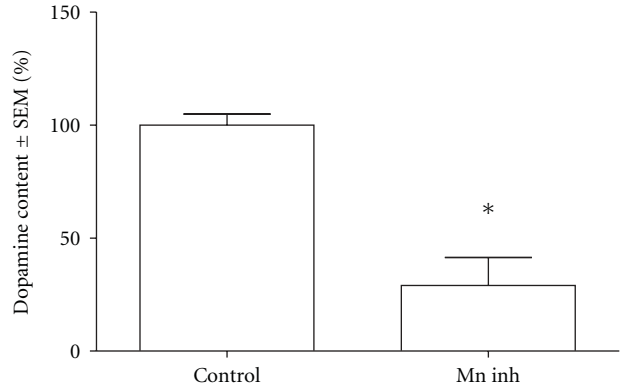

Figure 1: Decrease of dopamine content in the striatum after 5 months of Mn inhalation compared to controls. Contents are expressed as percentages, which were in pg/micro gram of protein (*$P < .001$ versus control group by one-way ANOVA with *post-hoc* comparisons).

After 5 months (40 inhalations), when important motor alterations were observed, 20 mice were sacrificed (10 control and 10 Mn-exposed), anesthetized with sodium pentobarbital lethal dose, and perfused via aorta with phosphate buffer saline (0.1 M p.H. 7.4) containing 2% glutaraldehyde and 2% paraformaldehyde. The brain was removed and placed in fixative solution for 2 hr and processed for TH inmunocytochemistry (5 control and 5 Mn-exposed brains).

Afterwards, the remaining mice continued inhaling Mn. Five were orally treated with 7.5 mg/kg L-DOPA (Sinemet [Carbidopa-L-DOPA 25/250]) daily for two months, 5 were kept for the same time without treatment, and 5 control were kept for the same time and then sacrificed for further analysis; the motor performance was evaluated weekly.

2.6. Immunocytochemistry. Coronal sections (50 μm) were obtained on a vibrating microtome through the mesencephalon for immunocytochemistry. Tyrosine hydroxilase (Chemicon International, Inc. CA, USA, 1 : 1000) immunostaining with the ABC detection method (Vector Lab MI, USA) was performed for light microscopic analysis. The analysis was conducted with a computer-assisted system (Image-Pro Plus, Media Cybernetics, L.P. Del Mar, CA, USA) connected by a CCD camera to Optiphot 2 microscope (Nikon, Japan). The number of TH-positive neurons was counted in 1500 μm^2 from 14 mesencephalic sections of each animal, the cell count included SNc and ventral tegmental area (VTA) [73].

2.7. Dopamine Concentrations. Striatal dopamine contents were obtained after 5 months of Mn inhalation as described elsewhere [74]. Briefly, 5 control and 5 Mn-exposed mice were anesthetized and decapitated, and using a stereoscopic microscope the striatum was dissected. The tissue was homogenized in perchloric acid utilizing 100 μl per brain. Homogenates were centrifuged (300 PSI, 2 min, airfuge centrifuge, Beckman; Fullerton, CA, USA), and the supernatants were filtered (0.22-μm membranes, Millipore; Bedford, MA, USA). The pellets were resuspended (120 μl of 0.1 M NaOH) and used for protein determination as reported by Bradford [75]. Dopamine content in 10 μl of supernatant was determined using a reverse phase HPLC system coupled to an electrochemical detector (BAS; West Lafayette, IN, USA). Chromatograms were analyzed using the Peak II integration software (SRI Instruments; Torrance, CA, USA). The DA content was expressed as pg/μg protein.

2.8. Mn Concentrations. The concentrations of Mn in the chamber were quantified as follows. A filter was positioned at the outlet of the ultranebulizer during the whole inhalation time at a flow rate of 10 l/min. After each exposure, the filter was removed and weighed; the element was quantified using a graphite furnace atomic absorption spectrometer (Perkin Elmer Mod. 3110, CT, USA). Six filters for each inhalation were evaluated [76]. Mn content in serum was also measured by graphite furnace atomic absorption spectrometry at the end of the experiment.

2.9. Statistical Analysis. One-way ANOVA was used to analyze the number of TH-immunopositive cells and behavioral data. Group differences were considered statistically significant at $P < .05$. When appropriate, *post-hoc* comparisons were made with the Tukey test. All analyses were conducted with SigmaPlot 11 (SYSTAT Software).

3. Results

After 7 months of exposure neither clinical alterations nor significant weight changes were detected in the exposed animals compared with controls.

3.1. Manganese Concentrations. The average Mn concentration measured in the filters of the chamber was of 2676 $\mu g/m^3$ during the whole experiment. The average Mn concentration in serum of exposed animals was of 30 ± 5 μg/l; control mice serum concentration of Mn was of 0.05–0.12 μg/l.

Figure 1 shows the change in dopamine content determined in the striatum after 5 months of Mn inhalation compared to controls. The average content in the control mice was 96.545 ± 4.8820 and 28.008 ± 12.4500 pg/μg of protein for Mn-exposed mice; hence, dopamine content declines 71%.

3.2. Single-Pellet Reaching Task. The task involves execution of a complex motor sequence, starting with sniffing a food pellet at the front of the reaching chamber, lifting the arm, adjusting posture to project the arm through a narrow slot toward the pellet, and grasping the target (Figure 2).

Animals were presented with 20 food pellets. Figure 3 shows the results of successful reaches over the course of the experiment. Repeated-measures ANOVA confirmed a significant effect of Mn-exposed group since 8 Mn-inhalations ($P < .001$). All animals were comparable in their ability to retrieve pellets before Mn inhalation, but the Mn exposure

Figure 2: Representative still frames of a control mouse captured during limb transport and limb withdrawal. The control animals advanced their forelimb through the slot and extended their digits, and they also supinated their paw to present the food to the mouth and extended their digits to release the food into the mouth (see text for detailed description.)

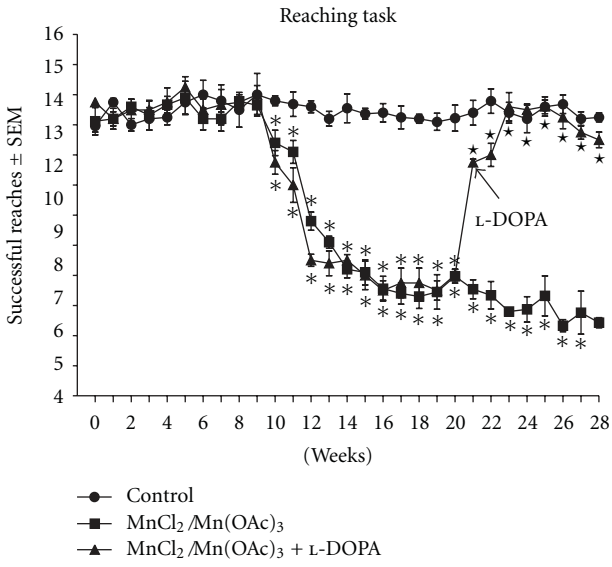

Figure 3: Reaching success (number of pellets obtained out of 20; mean ± SEM) by control, Mn exposed and Mn exposed + L-DOPA treatment mice in the single-pellet task before and after inhalation and after L-DOPA treatment. Note that the Mn-exposed group is impaired since 8 week and the L-DOPA treatment fully reverses the alterations ($^*P < .001$ versus control group; $^\star P < .001$ between L-DOPA treatment group versus Mn exposed group).

resulted in a marked impairment in both number of successful retrievals ($P < .001$) and accuracy; however, when L-DOPA treatment starts the mice improve their performance when comparing to the nontreated ones, resembling the control mice execution ($P < .001$). Control animals remained consistent throughout the duration of the experiment and performed significantly better than Mn-exposed animals at all time points (Figures 2 and 3).

Qualitative assessment resulted in a postural shift and impairments in limb extension (resulting in many shortened reaches), aim, and supination-pronation of the paw during grasping and release of the pellet into the mouth (Figures 4(a)–4(d)). Mice displayed abnormal movements when retrieving the pellet after Mn exposure. The paw is often fully pronated and moves either laterally (from the side) over the pellet (Figures 4(b) and 4(c)), or the mouse slaps at the pellet from above. Several animals from Mn-exposed group exhibited such motor abnormalities that persisted for the duration of the experiment.

The Mn-exposed mice are often unable to properly close the digits around the pellet and drag it to the slot without lifting the paw. Mice also fail to supinate the paw completely and place the snout into the slot to retrieve the pellet with the tongue. When the paw is withdrawn through the slot, Mn mice frequently rotate the body and "chase" the pellet with the snout instead of opening the digits and placing the pellet into the mouth. The nonreaching limb is seldom raised for support when retrieving the pellet. Post-hoc tests on the group effect indicated that at more Mn exposure success scores were significantly poorer (Figure 3). These conditions remarkably improve with L-DOPA treatment (Figures 4(e)–4(h)); the treated mice adjust their posture and project the arm toward the pellet, supinate and pronate the paw to obtain the pellet, close their digits, and drag the food to the snout; their motor performance was comparable to control mice (Figure 3).

3.3. Beam-Walking Test. We further tested Mn-exposed mice for possible alterations in motor activities using a beam traversal task. On the last day of testing before Mn inhalation, there was no significant difference between the latencies in completing the test for the controls (7.2 ± 6.9 sec) and the Mn-treated subjects (7.8 ± 3.1 sec) (ANOVA test; $P > .001$). Throughout the course of the experiment, none of the subjects fell from the beam.

Figure 5 illustrates the mean numbers of total time to cross the beam. Mn-exposed mice were observed to have a significant decrease in the duration to cross the beam after 2,4,6, and 8 Mn-inhalations suggesting hyperactivity; afterwards have a significant increase in the time to cross and a significant potentiation of freeze time (data not shown), compared with control mice. In addition, animals were also noted to exhibit hind-limb weakness, delayed motor initiative (akinesia), postural instability, and action tremor. L-DOPA treatment reverted these motor alterations.

3.4. TH-Immunocytochemistry

3.4.1. Pilot Study. The mean number of TH-positive neurons on the control SNc was 145 neurons (Figures 6 and 7(a)). In the 0.02 M $MnCl_2$-inhaled animals, TH-positive neurons in the SNc were reduced by 4.8–33% (138 and 98 neurons

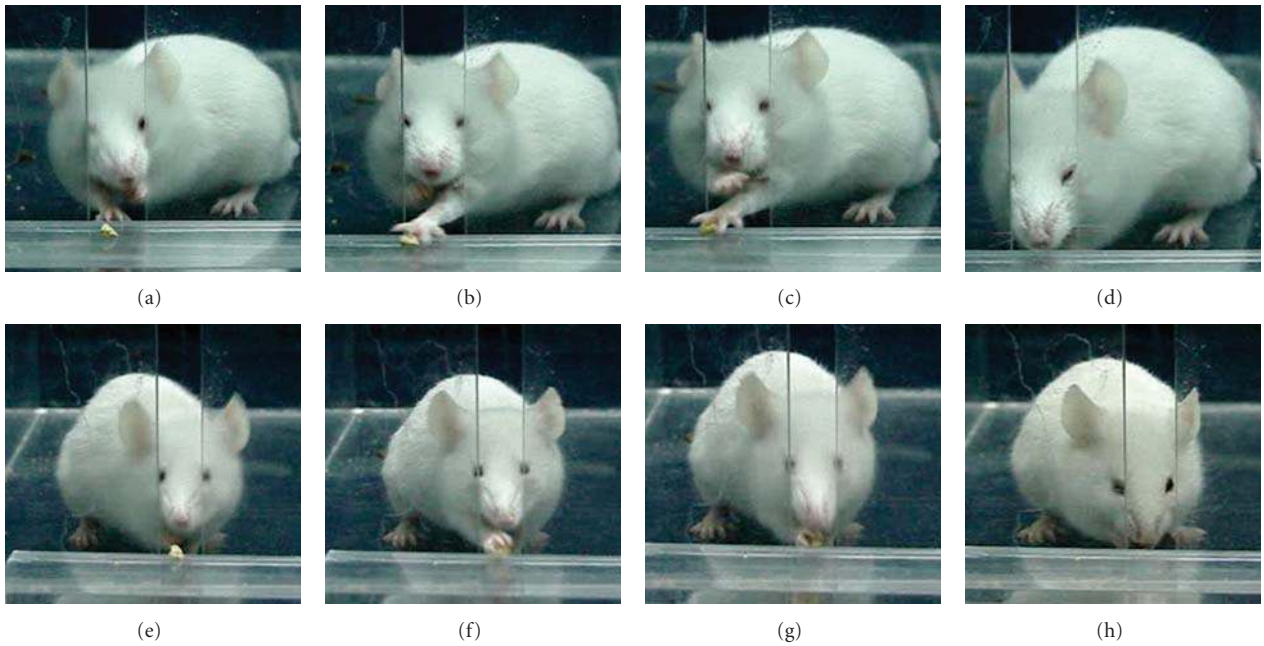

Figure 4: Representative still frames of a Mn-inhaled mouse (a–d) and Mn-inhaled mouse + L-DOPA treatment (e–h). In frames (a)–(d), the mouse showed impairments using extreme postural adjustments advancing the limb diagonally through the slot making many short attempts rather than aligning the limb with the midline of the body. The digits are concurrently adducted. The paw comes in from the side or slaps laterally, and digits do not contact the food pellet. The mouse is dragging its limb through the slot and dropping the pellet to the floor cage chasing the food with the tongue rather than fully pronating the paw and supinating it to present the food to the mouth. In contrast, in frames (e)–(h), the effect of L-DOPA treatment is evident, and the mouse adjusts its posture, directs the arm to the food pellet, and closes its digits to obtain it correctly.

resp. after 6 and 12 inhalations (Figures 6(a) and 7(b)); in the 0.03 M $MnCl_2$-inhaled mice, TH-positive neurons in the SNc were reduced by 11.03–38.6% (129 and 87 neurons resp. after 6 and 12 inhalations (Figures 6(c) and 7(c)); in the 0.01 M $Mn(OAc)_3$-inhaled mice the reduction was from 20 to 44.8% (116 neurons after 6 inhalations and 80 neurons after 12 inhalations (Figures 6(b) and 7(d)), and in the 0.02 M $Mn(OAc)_3$-inhaled mice the reduction was from 37.9 to 55.1% (90 neurons after 6 inhalations and 65 neurons after 12 inhalations (Figures 6(d) and 7(e))). Despite these reduction, after 10 inhalations in both cases the neuronal loss reached a plateau and there were no evident behavioral alterations. Hence, we decided to use higher doses and mix both compounds; afterwards we found glaring cell reduction (Figure 7(f)) and motor alterations described above.

3.5. $MnCl_2$/$Mn(OAc)_3$ Mixture. After 40 $MnCl_2$/$Mn(OAc)_3$-inhalations, a significant loss of the TH-positive neurons in the SNc was observed (67.58%) compared with the control group. However, the number and integrity of the TH-positive neurons in the VTA were not significantly affected by Mn-inhalation (7.6%) (Figures 8 and 9).

4. Discussion

This study examined the premise that exposure to $MnCl_2$ /$Mn(OAc)_3$, when combined, produces additive or even synergistic effects by impacting the DA nigrostriatal system by reducing TH cell counts in the SNc but not in the VTA and decreasing dopamine striatal concentrations. We found considerable hyperactivity immediately after the first inhalations (2–8 inhalations) and afterwards, evident reduction and alterations in locomotor activity, and the motor alterations improve drastically after L-DOPA treatment.

4.1. Motor Behavior Alterations

4.1.1. Single-Pellet Reaching Task. The single-pellet task examined both gross ability to retrieve pellets and reaching accuracy, which is more sensitive to subtle impairments and compensatory reaching strategies that may not be detected by other motor tests [77].

Detailed analyses of skilled limb movements, such as the reach-to-grasp movement, show very similar motor components in humans and in rodents [78, 79]. An analysis of the movements used by the rodents indicates that a reach consists of postural adjustments that result in the body being supported by the diagonal couplet of the hind limb ipsilateral to the reaching forelimb and its opposite forelimb. This postural strategy allows the body to shift forward and backward and so aid limb advancement and withdrawal. The reaching movement itself consists of a number of movement subcomponents that include aiming the limb, pronating the paw over the food in order to grasp, and supinating

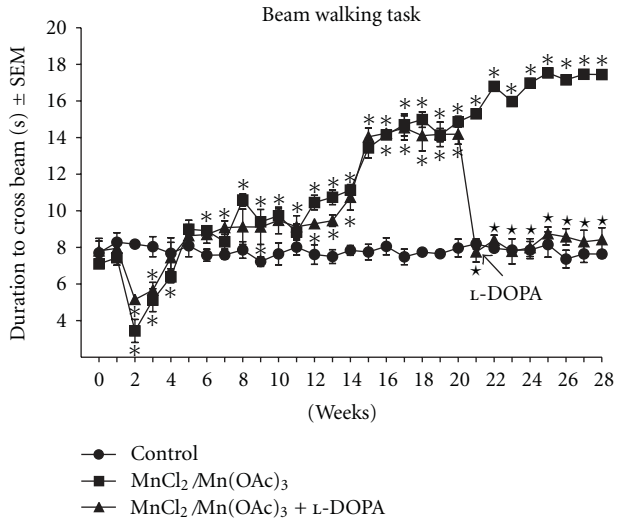

Figure 5: Mean latencies to cross the beam (±SEM) before and after Mn-inhalation and after L-DOPA treatment. Note that after 2, 4, 6, and 8 Mn-inhalations the mice significantly decrease the duration to cross the beam, and afterwards showed a significant increase in duration to transverse the beam compared to controls. However, when the mice received the L-DOPA treatment the time was reduced drastically resembling the values of the control group ($^*P < .001$ versus control group; $^\star P < .001$ between L-DOPA treatment group versus Mn exposed group).

the paw as it is withdrawn so that the food can be presented to the mouth. Humans with PD are often described as having poor manual dexterity that worsens as the disease progresses [80, 81]. They experience difficulties executing tasks requiring unilateral arm movements, bilateral arm movements, and sequential and alternating limb movements [79]. Movements by more distal body segments are more affected than movements by more proximal body segments.

After Mn exposure, mice commonly drag the pellet across the ledge without lifting the paw and either place the snout into the slot to retrieve the pellet with the tongue or rotate the body and "chase" the pellet with the snout when the pellet is withdrawn through the slot into the box. Those alterations could include damage to regions of the basal ganglia responsible for grasping movements [82].

With the results presented here, we confirm that bilateral DA-deficient mice have impairment in their success in retrieving food pellets. The video analysis of the reaching movements indicated that the Mn-exposed mice displayed impairment in supinating the paw to bring food to the snout. Rather than supinating, the paw was adducted across the snout so that the mouth contacted the upper surface of the paw. Food was lost because the paw is often fully pronated and moves either laterally over the pellet or the mouse slaps at the pellet from above. On the other hand, mice retained the ability to align and aim their limb to initiate a reach and to advance the limb to the food. Thus, the sensory and motor mechanisms underlying these movements must involve some motor cortical areas, which we assume intact; thus, in order to confirm that the motor alterations are due to basal ganglia damage, we utilized the beam walking test which is sensitive to impairments in the nigrostriatal pathway [83].

4.1.2. Beam Walking Test. The motor function impairments observed on the beam walking task are comparable with published findings in which C57 BL6/J mice treated with acute and subchronic dosing regimens of MPTP and were reported to display impairments in limb coordination, stride length, and motor function, at 1-2 weeks post-MPTP administration [84, 85]. In addition, the MPTP-induced increase in duration to traverse the beam also concords with published studies in which transgenic mouse models of PD were significantly slower in traversing a narrow, raised beam than wild-type control animals [86]. Qualitative analysis showed that Mn-exposed animals exhibit hind-limb weakness, delayed motor initiative (akinesia), postural instability, freezing behavior, and action tremor. Regarding these alterations, Autissier et al. [21] reported that mice subchronically exposed to Mn by intragastric gavage showed hypoactivity, this change was associated with a drop in striatal DA of 50%; Eriksson et al. [14] found that about 5 months after the start of the Mn exposure the animals became hypoactive with an unsteady gait and subsequently an action tremor. The animals lost power in both upper and lower limbs, and the movements of the paws were very clumsy. Moreover, Mn^{3+} injected into the rat SNc decreased spontaneous motor activity, rearing behavior, and acquisition of an avoidance response [38–40].

Regarding the hyperactivity observed after 2–8 Mn-inhalations (Figure 5), it has been reported that in early stages of Mn exposure the subjects manifest psychomotor excitement, irritability, and compulsive behavior [14, 42]. Nachtman et al. [87] indicate that acute exposure to Mn is associated with an increase in DA neurotransmission, which is also manifested as hyperactivity. Nevertheless, long-term exposure results in a loss of DA in the brain, and the concomitant neuronal cell damage could be expressed as a decrease in motor activity. Shukla and Singhal [88] reported that acute exposure to Mn^{2+} causes hyperactivity accompanied by elevated brain levels of catecholamines and their metabolites. Moreover, Tomas-Camardiel et al. [57] reported that experimental rats were significantly more active than control animals in the empty open field after Mn exposure.

It has been mentioned that rats with bilateral 6-OHDA lesions have postural abnormalities at rest and a reduced capacity to maintain balance after challenges with destabilizing forces. Likewise, spontaneous movements are greatly reduced [89].

Reports of parkinsonian-like tremor have been scarce in studies of 6-OHDA-lesioned rats [90, 91]; however, Schallert et al. [92] have observed occasional resting tremor in the wrist and the paw of rats with severe DA depletion (either bilateral or unilateral). This tremor can be seen only when the forelimb is positioned off the floor in a nonweight-bearing posture [92]. As it has been reported, rats with bilateral 6-OHDA lesions show all of the essential elements of parkinsonian motor syndromes. However, animals lesioned bilaterally with 6-OHDA is not a common model, as they

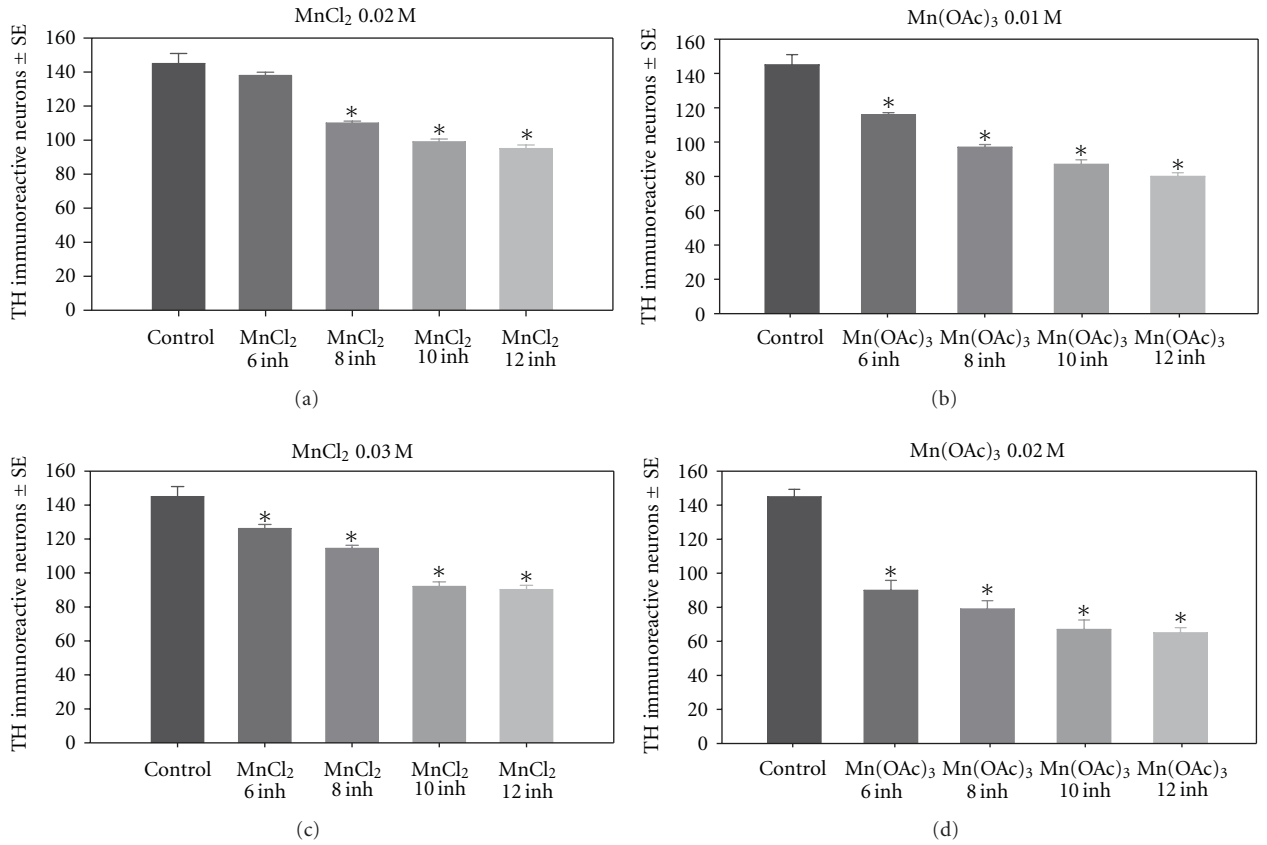

Figure 6: Pilot study. Number of SNc TH^+-immunostained neurons from control and exposed mice after different times of 0.02 M $MnCl_2$ (a) and 0.03 M $MnCl_2$ (c) inhalations and after different times of 0.01 M $Mn(OAc)_3$ (b) and 0.02 M $Mn(OAc)_3$ (d) inhalations. The data are presented as the mean ± standard error (SE) ($^*P < .05$ one-way ANOVA).

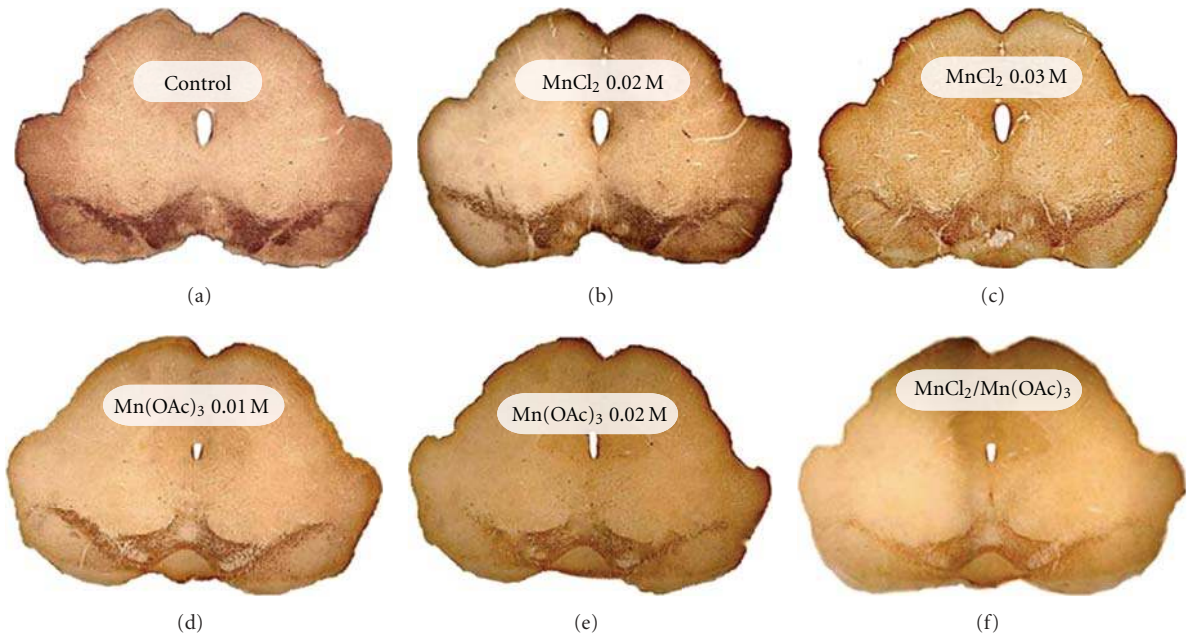

Figure 7: Pilot study. Representative coronal TH-immunostained sections through the SN and VTA of control and exposed mice to different Mn concentrations and compounds (4x).

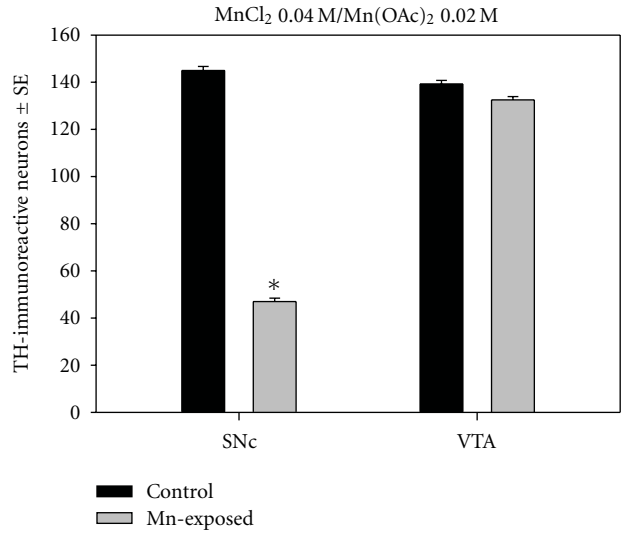

Figure 8: TH-immunoreactive cell counts from the Substantia Nigra compacta (SNc) and Ventral Tegmental Area (VTA). The data are presented as the mean ± SE. A statistically significant decrease in TH-immunoreactive cells was detected in the SNc ($*P < .05$ ANOVA test) of Mn-exposed mice compared to controls with no difference in the VTA.

require intensive nursing care [93]. So, rats with unilateral 6-OHDA lesion of the nigrostriatal dopamine pathway are the most widely used animal model of PD. However, this model does not mimic all the clinical and pathological features characteristic of PD. Furthermore, the acute nature of the experimental model differs from the progressive degeneration of the dopaminergic nigral neurons in PD.

4.1.3. TH-Immunocytochemistry. Contrary to previous reports [12, 14, 18, 23, 42–44, 57, 58], we found an important loss of TH-positive neurons as shown in Figures 8 and 9, exhibiting a pattern very similar to that observed in PD patients; according to our findings, some authors have been reported neurochemical changes in human and animal Mn intoxication including the reduction in DA levels and TH^+ immunoreactivity in the caudate nucleus, putamen, and SN [7, 8, 21, 27, 54–56]. In this way, it has been hypothesized that Mn interacts with catechols specific to dopaminergic neurons so as to rapidly deplete them and render such cells no longer viable [34, 66].

The controversy found here about the loss of TH cell count, decreased DA striatal concentrations, and the behavioral alterations, may be due to the fact that we included the mixture of $MnCl_2/Mn(OAc)_3$. According to some authors, the pro-oxidant activity of Mn^{2+} is dependent on trace amounts of Mn^{3+}, which may facilitate a small portion of Mn^{2+} to oxidize to Mn^{3+}. This synergistic relationship between Mn^{2+} and Mn^{3+} results in continuous redox cycling [69]. It seems that Mn^{2+} fails to induce oxidative effects; however, transition of Mn^{2+} to the trivalent state leads to an increased oxidant capacity of the metal which may result in the production of reactive oxygen species, lipid peroxidation, and cell membrane damage [59], and may in turn attack catecholamine neurotransmitters [40, 66]; thus, the inherent convertion of Mn^{2+} to Mn^{3+} and the presence of more Mn^{3+} could induce more reactive oxygen species and mitochondrial disfunction [94, 95] manifested as the evident DA cell loss and the motor disturbances found here.

Several explanations have been proposed to elucidate the vulnerability of dopamine to Mn, such as the impairment of cellular antioxidant defenses by the accumulation of the metal, and the disruption of mitochondrial oxidative energy metabolism [94]. This has led to the conclusion that excessive levels of brain Mn induce oxidative stress leading to neurodegeneration [69].

It has been mentioned that the brain is an important target of attack for transition metal ions, such as Mn, due to its great catecholamine concentration and the high speed of oxidative metabolism catalyzed by these metals [96]. DA is oxidized to aminochrome by reducing Mn^{3+} to Mn^{2+} [60], which may react with $O2^{-\bullet}$ radicals to generate hydrogen peroxide and more Mn^{3+} [66].

According to HaMai and Bondy [69], loss of the dopaminergic neurons in the nigrostriatal pathway of the basal ganglia, which are inhibitory, leads to heightened activity of neurons in the GP. Since GP efferences are also inhibitory, the sum of increased suppression of motor functions produces the symptoms characteristic of Mn-related parkinsonism. More specifically, rigidity and bradykinesia arise from the degeneration of neurons in the SNc, which project to the striatum. The Mn-induced alterations are focal to both pre- and postsynaptic terminals of the dopaminergic nigrostriatal pathway [52]. The subcellular localization of Mn occurs in the mitochondria, specifically inhibiting complex I, since Mn has a high affinity for the inner mitochondrial membrane [32, 54, 68, 95]. Salient features of the brain regions susceptible to Mn-provoked injury include their intense oxidative metabolism, major DA content, and high content of nonheme iron [32, 59, 69]. This raises the possibility that the mechanisms of Mn neurotoxicity relate to its potential for oxidative injury and promotion of DA auto-oxidation [36, 66]. The mechanisms by which the common neurotoxins kill dopaminergic neurons also involve mitochondrial dysfunction and oxidative damage. 6-OHDA is taken up by DAT, and it then generates free radicals [97]. MPTP is converted by monoamine oxidase B to 1-methyl-4-phenylpyridinium (MPP+). MPP+ is taken up by DAT and can then be accumulated by mitochondria, leading to complex I inhibition and the generation of free radicals [98]. In this way, Hirata et al. [99] suggest that the mechanisms by which Mn produces dysfunction of the nervous system are similar to those of MPTP.

It is also worth noting that, in this study, although Mn-inhalation caused significant damage to dopaminergic neurons in the SNc, the dopaminergic neurons in VTA did not appear to be affected. It is not clear whether this suggests any selectivity in Mn-induced toxicity between dopaminergic neurons in the SNc and those in the VTA; however, it has been mentioned that Mn enters the neurons possibly via DAT [29–31]; DAT has been shown to be involved in the selective neurotoxicity of MPTP [98], 6-OHDA [97], and that of Thiruchelvam et al. [27], where SNc is more

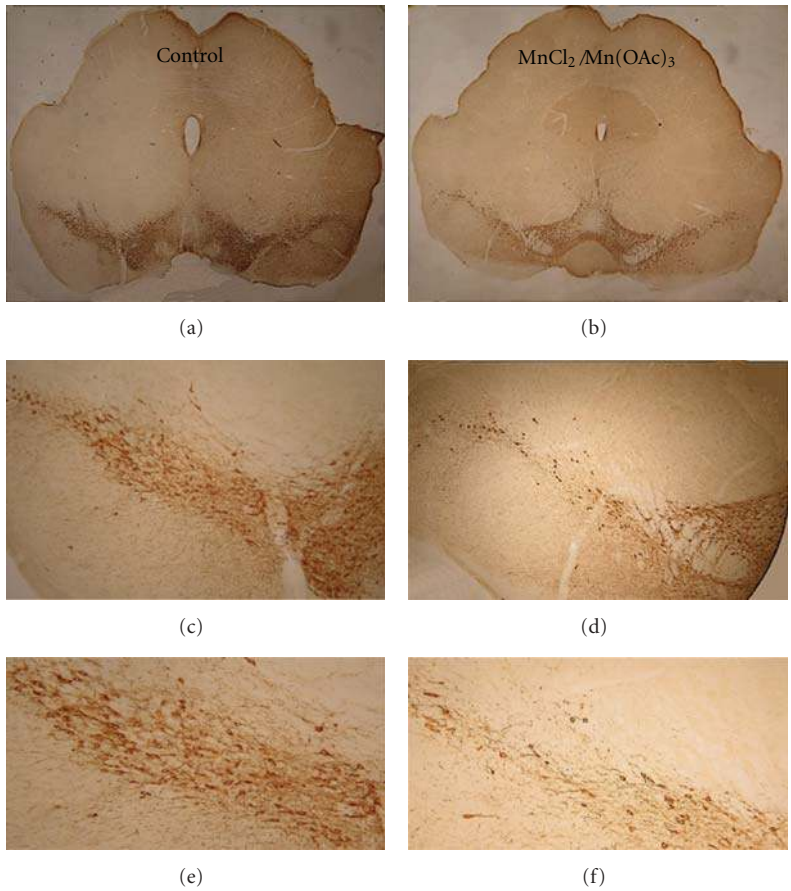

Figure 9: Representative TH-immunostained from coronal section containing the SN and VTA of control and Mn-exposed mice. Note the relative sparing in the ventral tegmental area and profound cell loss at all levels of SNc in the Mn-exposed group (upper panel 4x, middle panel 10,000x, and lower panel 40,000x).

susceptible than VTA. It seems that dopaminergic cells of the SNc and the VTA display differences in their topography, biochemistry, and susceptibility to pathological processes [100], VTA express lower levels of DAT than the middle and medial SNc [98, 101]; thus, it is possible that Mn reaches SNc dopaminergic cells via the large amounts of DAT found on those neurons; however, additional studies are needed.

Currently available animal models of PD have contributed greatly to our understanding of both the pathophysiology and potential neuroprotective therapeutics for PD, but as yet we do not have the optimal model. At present, MPTP neurotoxicity is the best available animal model from several standpoints, and it has been extremely valuable in testing neuroprotective and neurorestorative strategies. Nevertheless, the disadvantages of the MPTP model are: acute damage of the dopaminergic system and nonprogressive and rare generation of inclusion bodies [102]. Both, 6-OHDA and MPTP models differ significantly from the slowly progressive pathology of human PD [4]. In addition, genetic mouse models of PD have previously been observed to repeat some aspects of the disease in the absence of substantial neuronal loss in the affected brain subregions [103]. Transgenic mice overexpressing wild-type and FPD-linked mutant human alfa-synuclein exhibit motor deficits in the absence of loss of DA neurons [4, 104].

The significant decrease (67.58%) in the number of SNc TH-immunopositive neurons after $MnCl_2$/$Mn(OAc)_3$ inhalation and the evident reduction of striatal dopamine concentrations reported here demonstrates a glaring reduction of this chatecolamine content (71%). Hence, we assume that the alterations are due to dopaminergic loss since L-DOPA-treated mice almost completely improved their motor performance.

It has been reported that Mn effects involve the GP [43, 44, 105]; however, with these results we can assure that the $MnCl_2$/$Mn(OAc)_3$ mixture also jeopardizes the nigrostriatal pathway. In this study, we have demonstrated that L-DOPA treatment significantly improves the motor alterations found after Mn exposure, suggesting that these motor disturbances are of dopaminergic origin. Moreover, Mn mixture inhalation was extensive enough to induce substantial and stable deficits in spontaneous sensorimotor behaviors including tremor, posture instability, slowed movement, and rigidity; and in contrast to the complete nigrostriatal bundle lesion produced by other PD models such as 6-OHDA, which is the most commonly used model in functional experimental

studies, the Mn mixture inhalation leaves a considerable portion of the nigrostriatal projection intact. As in early stages of PD, the presence of an intact, functioning subportion of the nigrostriatal system could allow L-DOPA treatment to be efficient.

In summary, the results from this study suggest that the motor alterations induced by the inhalation of the combination of $MnCl_2/Mn(OAc)_3$ are related to nigrostriatal dopaminergic function, providing new light on the understanding of Mn neurotoxicity as a suitable PD experimental model.

In conclusion the data described in the present study provides further evidence that functional deficits following Mn exposure in mice can be quantified and are related to nigrostriatal DA function. The motor and immunocytochemical discrepancies reported here are probably due to the combination of $MnCl_2/Mn(OAc)_3$, since it has been reported that Mn^{3+} is more potent in producing oxidative stress and cell damage and Mn^{2+} needs the presence of Mn^{3+} to reach oxidation and that there is a synergy between the two Mn states, and so far, there is no research that has included this mixture. Therefore, we consider that the inhalation of $MnCl_2/Mn(OAc)_3$ mixture could be an appropriate PD model.

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Research Article

MPTP Neurotoxicity and Testosterone Induce Dendritic Remodeling of Striatal Medium Spiny Neurons in the C57Bl/6 Mouse

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Nigrostriatal damage is increased in males relative to females. While estrogen is neuroprotective in females, less is known about potential protective effects of testosterone in males. We determined if castration enhances neuronal injury to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Castrates or sham-castrated mice were sacrificed 1 week following injection of MPTP (4×20 mg/kg) or saline ($n = 11$-12/group). The right striatum was immunostained for tyrosine hydroxylase (TH). The left hemisphere was stained by Golgi Cox to quantify neuronal morphology in medium spiny neurons (MSNs) of the dorsolateral striatum. MPTP reduced TH, but there was no effect of castration and no interaction. For MSN dendritic morphology, MPTP decreased the highest branch order and increased spine density on 2nd-order dendrites. Castrated males had shorter 5th-order dendrites. However, there was no interaction between gonadal status and MPTP. Thus, castration and MPTP exert nonoverlapping effects on MSN morphology with castration acting on distal dendrites and MPTP acting proximally.

1. Introduction

Gonadal steroid hormones are potent modulators of neuronal survival and neuronal morphology [1]. In the adult, steroid hormones exert activational effects in steroid-responsive brain regions which include protective effects against neurodegeneration [2, 3]. However, sex differences exist in many neurodegenerative disorders, suggesting that the male and female brains are not equally responsive to gonadal steroids.

In this regard, Parkinson's disease (PD) is a common neurological disorder that demonstrates a substantial sex difference, with a one- to twofold higher incidence in men [4]. PD results from the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNC; [5]). Dopaminergic efferents from SNC project rostrally as part of the nigrostriatal pathway to the dorsolateral striatum, where they synapse onto medium spiny neurons (MSNs). These efferents are reduced in PD, leading to a depletion of striatal dopamine. Gonadal steroids modulate the function of the nigrostriatal system and are thought to contribute, in part, to this sex difference.

In females, estrogen promotes the function of the nigrostriatal system by enhancing striatal dopamine release, increasing dopamine metabolism and altering both dopamine receptors and uptake sites [6, 7]. In response to neurotoxic insult, estrogen is also neuroprotective in the nigrostriatal system [7]. This has been well demonstrated using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), neurotoxins which selectively deplete dopaminergic SNC neurons [8]. In both models, estrogen attenuates the loss of striatal dopamine and reduces the loss of SNC dopaminergic neurons [9–15].

In parallel to its effects in the female, estrogen also modulates nigrostriatal function in males [16, 17]. Estrogen in males is derived by the local aromatization of testosterone, although testosterone can also act as an androgen in the male brain. Whether testosterone has neuroprotective effects in the male nigrostriatal system, similar to the effects of estrogen in the female, is not well-established. After MPTP in castrated mice, striatal dopamine loss is attenuated by estrogen but not by testosterone [18, 19].

Previous studies investigating the effects of testosterone on nigrostriatal function in male rats and mice after 6-OHDA or MPTP have measured dopamine content and release [18–21], dopaminergic striatal input [21], and dopamine transporter binding [19, 21]. It is unknown whether testosterone reduces the loss of dopaminergic innervation to the striatum after MPTP in mice. Ultimately, the effects of MPTP lesion may also extend beyond the SNC dopamine neurons themselves. Loss of dopaminergic input may remodel MSN morphology, and testosterone has potential to attenuate this MPTP-induced deafferentation. The current study used castrated and gonad-intact adult male mice to determine whether testosterone reduces MPTP-induced deficits in striatal dopamine neurochemistry and MSN neuronal architecture.

2. Materials and Methods

2.1. Animals. Forty-five C57BL/6 adult male mice (8–10 weeks old) were obtained from Charles River Laboratories (Wilmington, MA). Mice were group-housed on a 12:12 LD photoperiod with access to food and water *ad libitum*. Experimental procedures were approved by USC's Institutional Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (DHEW Publication 80-23, revised 1985, Office of Science and Health reports, DRR/NIH, Bethesda, MD).

Initially, half of the mice ($n = 23$) were castrated (OrchX) via a midline scrotal incision. The other half ($n = 22$) received sham castrations. As measured by androgen-sensitive seminal vesicle weight, castration was effective (187.5 ± 14.2 mg in sham males versus 5.6 ± 1.4 mg in castrated males, $P < .05$). Two weeks later, half of the mice in each group received MPTP and half were given saline. MPTP (Sigma, St. Louis, MO) was dissolved in 0.9% saline and was administered in 4 injections of 20 mg/kg (free-base).ip. with an interinjection interval of 2 hours. Control mice received equivalent injections of 0.1 mL saline. This lesioning paradigm is a well-established method that leads to ca. 67% loss of nigrostriatal neurons and 90–95% depletion of striatal DA, as reported in previous studies from our laboratories [22, 23].

One week following MPTP, animals were sacrificed via intracardiac perfusion. This duration is sufficient for MPTP-induced cell death to occur [22]. Mice were deeply anesthetized with sodium pentobarbital (150 mg/kg BW) and perfused intracardially with 150 mL of 0.1 M sodium phosphate buffer (PB, pH = 7.4) containing 0.9% NaCl and 0.1% $NaNO_3$. The brains were removed and hemisected. To allow us to obtain TH and neuronal morphologic measures in the same animals, the right hemisphere for each brain was processed for TH immunocytochemistry and the left hemisphere was processed for Golgi-Cox staining. We are unaware of any evidence of laterality in striatal damage after.ip. MPTP injections in the mouse.

2.2. Tyrosine Hydroxylase Immunocytochemistry. The right hemispheres from each brain were postfixed in 4% paraformaldehyde in PB overnight at 4°C, then cryoprotected for 5 days at 4°C with 20% sucrose in PB. Hemispheres were rapidly frozen and sectioned coronally at 25 μm thickness through the rostrocaudal extent of the striatum. Sections were stored in PB with 0.01% sodium azide at 4°C until processed for TH immunocytochemistry.

Sections through the striatum at or rostral to the anterior commissure corresponding to Plates 18–28 of Paxinos and Franklin [24] were stained for TH. Tissue from mice in different groups was stained at the same time. Sections were incubated overnight at room temperature (RT) in polyclonal rabbit anti-TH antibody (1 : 5000; Chemicon, Temecula, CA) with 4% normal donkey serum and 0.3% Triton X-100 in PB. The following day, sections were incubated in biotinylated donkey antirabbit secondary antibody (1 : 200; Jackson Immunoresearch, West Grove, PA) and the avidin-biotin-horseradish peroxidase complex (Vector Elite Kit; Vector Laboratories, Burlingame, CA), each for 1 hour at RT with extensive washes in between. TH-labeled cells were visualized using NiCl-enhanced 3′,3-diaminobenzidine tetrahydrochloride with 0.25% hydrogen peroxide. Sections were mounted onto gelatin-coated slides, dehydrated in alcohols, cleared in xylenes, and coverslipped with Permount.

The relative expression of TH immunoreactivity was measured in dorsolateral striatum on coded slides by an observer blind to the treatment group. To ensure that differences in staining intensity were due to differences in antigen expression, multiple sections from each of the different treatment groups were handled concurrently in identical staining conditions. Control experiments excluding either primary or secondary antibody were also carried out to verify staining specificity. Three striatal sections rostral to the anterior commissure (Bregma 0.25-1.25 in [24]) were sampled per animal ($n = 8$-9 animals/group) using methods previously described by our laboratory [25, 26]. Briefly, striatal sections were digitally photographed at low magnification. The dorsolateral quadrant of each striatal section was outlined, and TH immunostaining was measured in a 1.6 mm^2 circular region of interest at the dorsolateral boundary of this quadrant (Figure 1). In previous studies [25, 26], this region shows the largest decrease in TH immunostaining after MPTP. The relative optical density (expressed as arbitrary units within the linear range of detection) was determined by subtracting the relative optical density of the corpus callosum as background. This measurement reflects both the area and intensity of TH immunostaining within the striatum. To ensure that the gray values represented an optical density within the nonsaturated

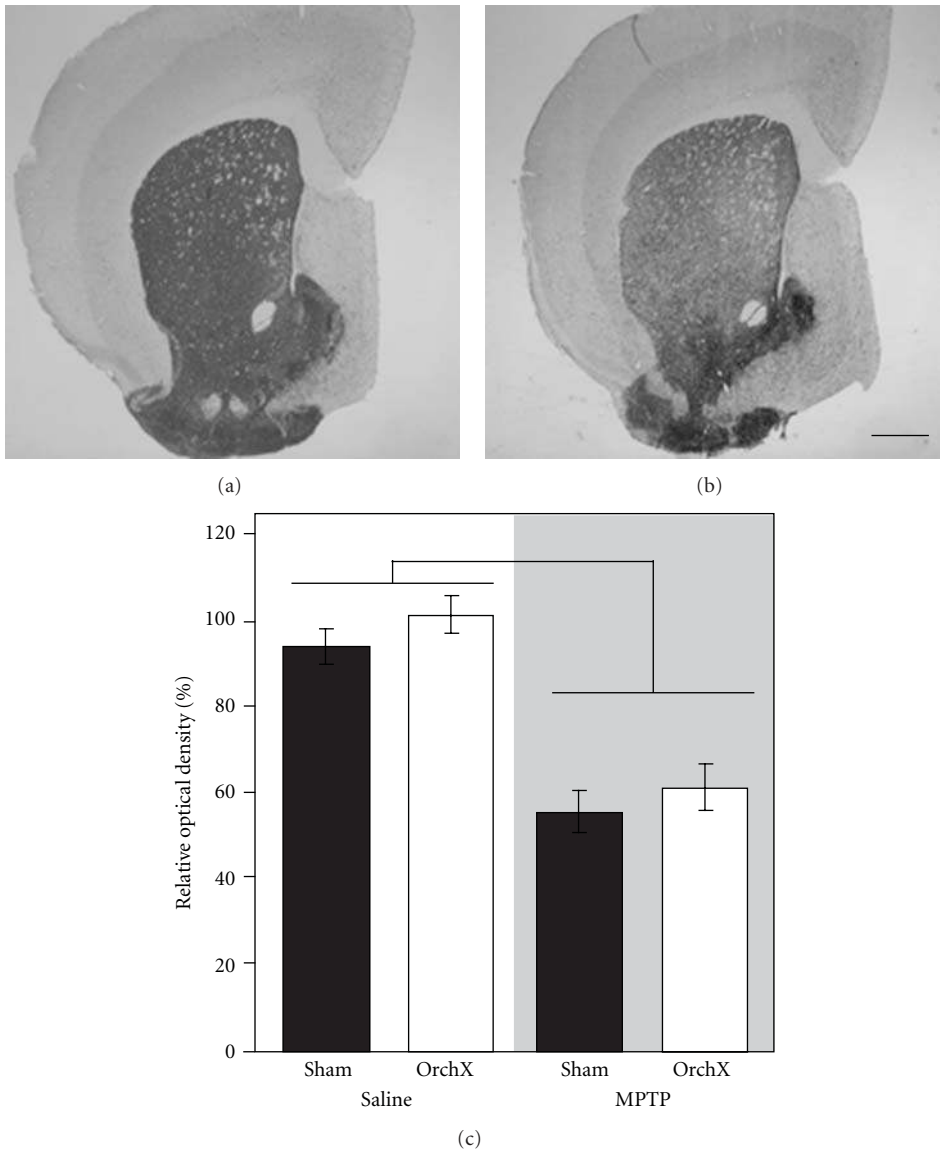


Figure 1: TH staining in the dorsolateral striatum. Photomicrographs of TH staining in gonad-intact saline-injected (a) and MPTP-treated (b) mice. Scale bar = 500 μm. (c) The density of TH staining in the dorsolateral striatum of saline-injected (clear) and MPTP-treated (shaded) gonad-intact (black bars) and orchidectomized (white bars) male mice (n = 8–10 mice/group). The bar represents an effect of MPTP ($P < .05$). OrchX: orchidectomized; Sham: sham-orchidectomized.

range of the image analysis, a Kodak photographic step tablet (density range to 255 OD units) captured by the CCD camera was used. Maximal tissue immunostaining relative OD units did not exceed the relative OD units of the tablet.

2.3. Golgi-Cox Staining. Golgi-Cox staining was performed on the left hemisphere of each brain using the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Ellicott City, MD). Pilot studies using Golgi staining (according to Gomez and Newman [27]), Golgi-cox staining (according to Gibb and Kolb [28]), and the rapid Golgi kit (FD NeuroTechnologies) were conducted to determine an optimal way to visualize neuronal morphology in our striatal tissue. The rapid Golgi kit provided the most complete staining of medium spiny neurons.

The hemispheres were placed in Golgi-Cox solution containing mercuric chloride, potassium dichromate, and potassium chromate for 2 weeks, and the solution was replaced after the first 24 hours. The brains were moved to a cryoprotection solution (GolgiStain Kit) for 48 hours and then sectioned coronally at 200 μm on a vibratome (Vibratome Series 1000). Sections through the rostral-caudal extent of the striatum were mounted on gelatin-coated slides. Slides were stored in a humidity chamber overnight and developed the following day according to the Rapid GolgiStain Kit protocol. Briefly, slides were rinsed in distilled water and placed in a developing solution for 10 minutes.

Immediately afterwards, the slides were rinsed, dehydrated in alcohols, cleared in xylenes, and coverslipped with Cytoseal-60 mounting medium (Richard-Allan Scientific). Slides were stored in the dark at RT until morphological analysis.

MSN morphology was analyzed on coded slides by an observer blinded to the treatment groups using a Nikon Eclipse 80i microscope (Nikon Instruments, Inc., Melville, NY) with motorized stage and MicroFire camera (Olympus America, Inc., Center Valley, PA). To compare dendritic morphology after castration and MPTP lesion, the entire dendritic tree from one primary dendrite was traced under a 100x oil immersion lens using the Neuron Tracing function in NeuroLucida (MicroBrightField,Inc., Williston, VT). Brains with well-differentiated Golgi-Cox labeling from 5 mice in each experimental group were selected for morphologic analysis; 5 neurons from each mouse were analyzed. Morphologic data from the 5 neurons/mouse were averaged to provide a single data point for each animal used in statistical comparison ($n = 5$/group). MSNs selected for analysis were located in the dorsolateral quadrant of the striatum at or rostral to the level of the anterior commissure (Plates 18–28 of [24]). Selected MSNs were fully impregnated with Golgi stain and had clearly visible spines with minimal or absent obstruction by neighboring Golgi-stained cells or blood vessels. Morphometric analysis was conducted using NeuroExplorer software (MicroBrightField, Inc.). Briefly, each dendritic segment was assigned a branch order with the dendritic segment proximal to the soma identified as the first branch order. Dendritic lengths, number of spines, and spine density were computed for each branch order. All dendrites subject to morphologic analysis had at least 3 branch orders. However, because not all dendrites had 4th- and 5th-order branches, the variability in dendritic length increased at higher branch orders. In addition, total spine density and total dendrite length were calculated for the entire dendritic tree. Due to the relative lack of spines on primary dendrites (typically, 1 or 2 spines/primary dendrite), branch order analysis was not performed on first-order dendrites.

2.4. Statistics. For comparison of both TH and Golgi-Cox labeling, morphologic data from each mouse were averaged to provide a single data point used in statistical comparison. Group differences for the 5 animals in each group were analyzed by two-factor (gonadal status and lesion) analysis of variance (ANOVA). Post hoc comparisons using the Fisher's LSD test were conducted when statistically significant differences ($P < .05$) were found.

3. Results

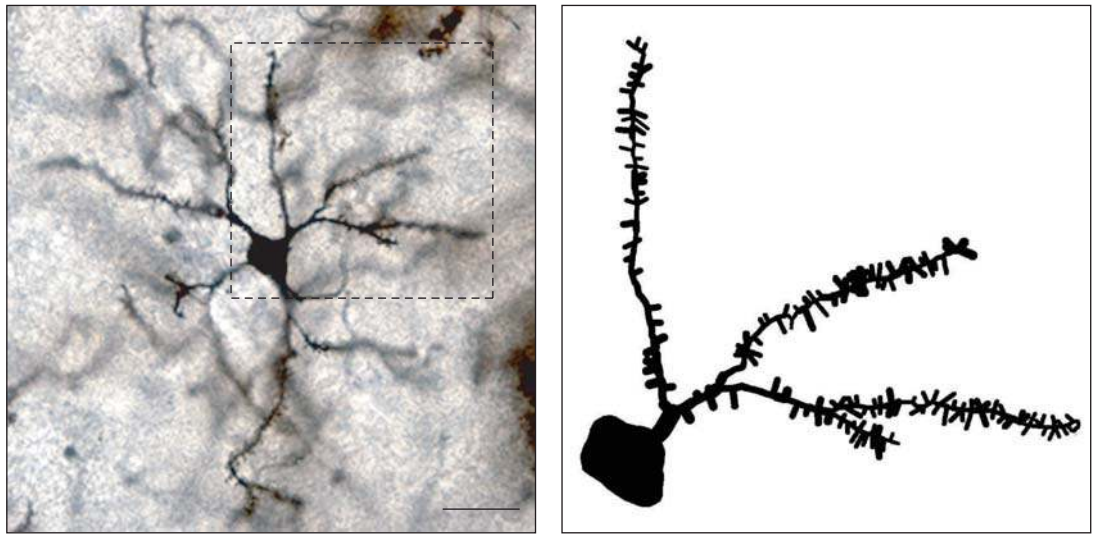
3.1. Striatal TH. Striatal TH immunocytochemistry was used here as a measure of lesion damage in gonad-intact and castrated males. Damage to midbrain DA neurons causes a loss of striatal DA terminals, which produces equivalent changes in striatal levels of presynaptic dopamine transporter and TH [23, 29, 30]. Similar to previous reports by our lab and others [25, 26, 31, 32], MPTP decreased TH immunoreactivity in the striatum (Figures 1(a) and 1(b), $n =$ 8–10/group). Striatal TH was reduced by 40% after MPTP (mean gray level 59.4 ± 3.7 versus 98.5 ± 3.1 in unlesioned mice, $F(1, 31) = 64.883$, $P < .05$, Figure 1(c)). However, there was no effect of castration on TH immunoreactivity and no interaction between MPTP and castration ($P > .05$, Figure 1(c)). This finding parallels previous studies which have found no effect of castration on the number of TH-positive neurons in SNC [33].

3.2. Medium Spiny Neuron Morphology

3.2.1. Spine Density. MSNs have elaborate dendritic arbors with a high density of dendritic spines (Figure 2). In saline-treated sham-castrate controls, spines were largely absent from primary dendrites (1.2 ± 0.2 spines/10 μm), but increased on more distal dendrites (5th-order dendrites 6.8 ± 0.4 spines/10 μm). The density of dendritic spines in the present study ($n = 5$/group) is comparable to that reported previously in mice [34–36]. When combining castrated and gonad-intact mice, MPTP produced a modest but significant increase in total spine density (spines/10 μm) on MSNs (6.9 ± 0.1 versus 6.3 ± 0.1 in saline-injected castrate and intact mice, $F(1, 16) = 10.22$, $P < .05$, Figure 3(a)). When analyzed according to branch order, the increase in spine density was restricted to proximal dendrites (Figure 4(a)). Specifically, MPTP-treated mice had a higher spine density on 2nd-order (5.3 ± 0.3) and 3rd-order (7.1 ± 0.2) dendrites, compared with 4.2 ± 0.2 and 6.5 ± 0.2 in saline-injected mice, respectively ($F(1, 16) = 9.659$ and 5.700, $P < .05$, Figure 4(a)). However, there was no effect of castration on spine density and no interaction ($P > .05$, Figures 3(a) and 4(a)).

3.2.2. Branch Order. MPTP significantly decreased the average highest branch order (3.9 ± 0.1) compared to saline-injected males (4.3 ± 0.1, $F(1, 16) = 4.595$, $P < .05$, Figure 3(b)). As with other measures of overall neuronal morphology, castration was without effect and there was no interaction (Figure 3(b)).

3.2.3. Dendritic Length. There was no effect of MPTP on total dendrite length (402.0 ± 17.4 μm versus 415.5 ± 20.2 μm in saline-injected mice, $P > .05$, Figure 3(c)). However, in parallel to the increase in spine density, we observed a selective increase in dendritic length after MPTP on 2nd-order dendrites (80.2 ± 6.8 μm versus 61.6 ± 4.6 μm in saline-injected mice, $F(1, 16) = 4.796$, $P < .05$, Figure 4(b)). Castration had no effect on total dendritic length. However, castrated mice had a prominent reduction in dendritic length of distal dendrites. Specifically, 5th-order dendritic length was significantly reduced in castrated mice (78.9 ± 14.8 μm) compared with gonad-intact males (138.3 ± 13.2 μm, $F(1, 16) = 6.659$, $P < .05$, Figure 4(b)). There was no interaction between MPTP treatment and gonadectomy (Figures 3(c) and 4(b)).



(a) (b)

Figure 2: Golgi stained MSNs. Photomicrograph of a representative Golgi-impregnated medium spiny neuron with high-magnification inserts of a primary dendritic branch and fifth-order dendritic branch (a) and corresponding neurolucida tracing (b). Scale bar = 10 μm.

4. Discussion

The current study used the MPTP mouse model of PD to investigate the effects of castration on dopamine-depleting lesions of the nigrostriatal system. MPTP decreased striatal TH immunoreactivity, reduced the average highest branch order on MSNs, and increased proximal spine density. Separately, castration reduced dendritic length of distal dendrites. We predicted that testosterone would act as a neuroprotectant to attenuate the effects of MPTP and that castrated mice would have increased nigrostriatal damage after MPTP compared with gonad-intact mice. However, there was no interaction between gonadal hormone status and MPTP, suggesting that testosterone does not attenuate the neurotoxic effects of MPTP in the nigrostriatal system of males.

The current study used a well-established lesioning protocol [22, 23] that produced a moderate lesion, as measured by TH immunostaining. This is relevant to the study of how gonadal steroid hormones act on the nigrostriatal system because the neuroprotective effects of gonadal hormones are likely to be evident earlier in PD. For example, PD symptom severity is sexually dimorphic in early stages of the disease, with women experiencing less severe motor impairments [4, 37, 38]. This has been attributed, in part, to the neuroprotective effects of estrogen on the nigrostriatal system. In later stages of PD, sex differences are not reported, presumably because severe nigrostriatal degeneration obscures the effects of neuroprotective factors, including estrogen. This is paralleled in animal studies, where moderate lesions do not overwhelm the potential for gonadal hormones to attenuate the nigrostriatal response to MPTP. In 6-OHDA- lesioned rats, Gillies et al. [21] have demonstrated sex differences with small doses of 6-OHDA that disappear with larger doses. Using MPTP, our laboratory has demonstrated sex differences in motor impairments after relatively small lesions [39]. Even so, in the current study, castration did not alter the morphologic response to MPTP. Larger lesions should produce a more dramatic depletion of TH, but seem unlikely to reveal an interaction with castration.

Nonetheless, our results do demonstrate a broad impact of MPTP on basal ganglia circuitry. Although MPTP selectively kills dopamine-producing neurons of SNC, its effects are not limited to dopaminergic neurons themselves. In fact, morphological effects of MPTP were observed on efferent targets in the striatum. Specifically, MPTP increased dendritic spine density on proximal dendrites of striatal MSNs. This result was initially surprising because loss of dopaminergic input to MSNs is expected to reduce spine density, at least as demonstrated in vitro [34, 40]. Importantly, the increased spine density observed in our study was restricted to proximal dendrites. Proximal MSN dendrites receive synaptic inputs from within the striatum, while distal dendrites receive extrinsic inputs from the cortex and SNC [41]. This suggests that the increase in proximal dendritic spine density after MPTP is driven by intrinsic striatal neurons, rather than dopaminergic neurons of SNC. This may reflect a compensatory reaction to dopamine depletion and demonstrates the dynamics of spine morphology with degree of lesion, model, and time postlesion.

Striatal MSNs receive a variety of afferent inputs which include glutamatergic inputs from cortex as well as dopaminergic projections from SNC. Interestingly, MSN spine density is increased after methamphetamine, which also depletes striatal dopamine [42]. However, the methamphetamine-induced increase in spine density is selective to distal dendrites [43–45], while the effect that we observed occurred proximally. Given the importance of dopaminergic projections to the striatum, the absence of MPTP-induced

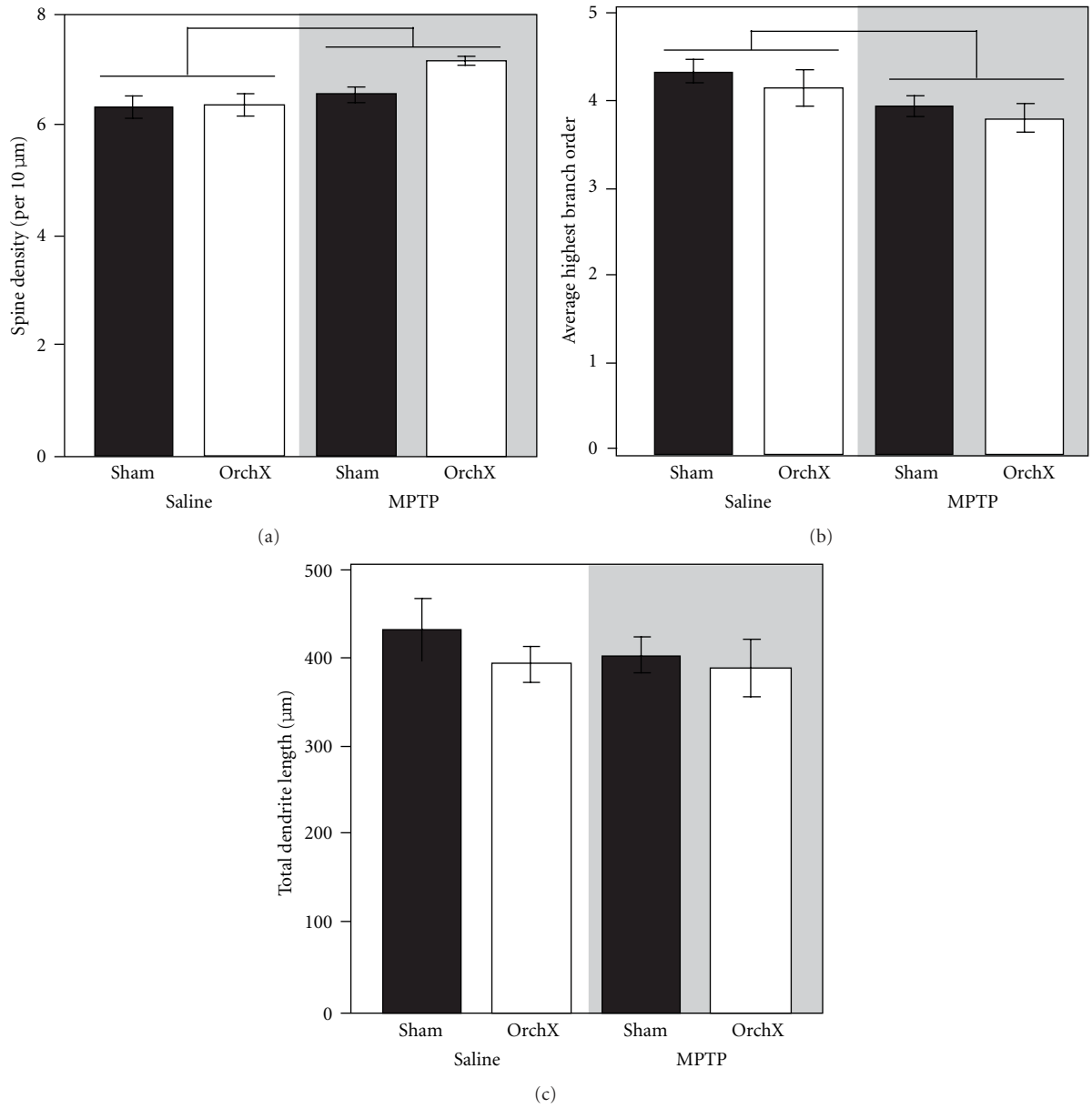


FIGURE 3: Neuronal morphology totals. Total spine density (a), average highest branch order (b), and total dendrite length (c) in saline-injected (unshaded panel) and MPTP-treated (shaded panel) gonad-intact (black bars) and orchidectomized (white bars) male mice (n = 5 mice/group). Bars represent an effect of MPTP ($P < .05$). OrchX: orchidectomized; Sham: sham-orchidectomized.

structural changes to MSN distal dendritic spines is indeed unexpected.

The absence of MPTP-induced structural changes to distal dendrites may relate to the distribution of striatal MSNs and the heterogeneity of striatal structure. MSNs represent a heterogeneous population comprised of both D1 receptor-containing neurons of the direct, striatonigral pathway and D2 receptor-containing neurons of the indirect, striatopallidal pathway [41]. The indirect pathway has also been implicated behaviorally, with D2 receptor knockout mice exhibiting PD-like akinesia and bradykinesia [46]. Behavioral deficits in D1 receptor knockout mice are minimal [47] or absent [48]. Recently, Day et al. [34] demonstrated a selective effect of dopamine-depleting lesions on D2 receptor containing MSNs using 6-OHDA. Therefore, it is possible that MPTP-induced spine changes are also confined to the D2 receptor-containing subpopulation of MSNs.

The other key observation from our study was that castrated and gonad-intact males had the same response to MPTP, suggesting that testosterone fails to protect against MPTP-induced neuronal damage. Initially, we postulated that testosterone would be neuroprotective in males, similar to the effects of estrogen in females. This is because testosterone can be converted to estrogen and because PD occurs most often in older men whose endogenous androgens are in decline. Although our hypothesis was not supported,

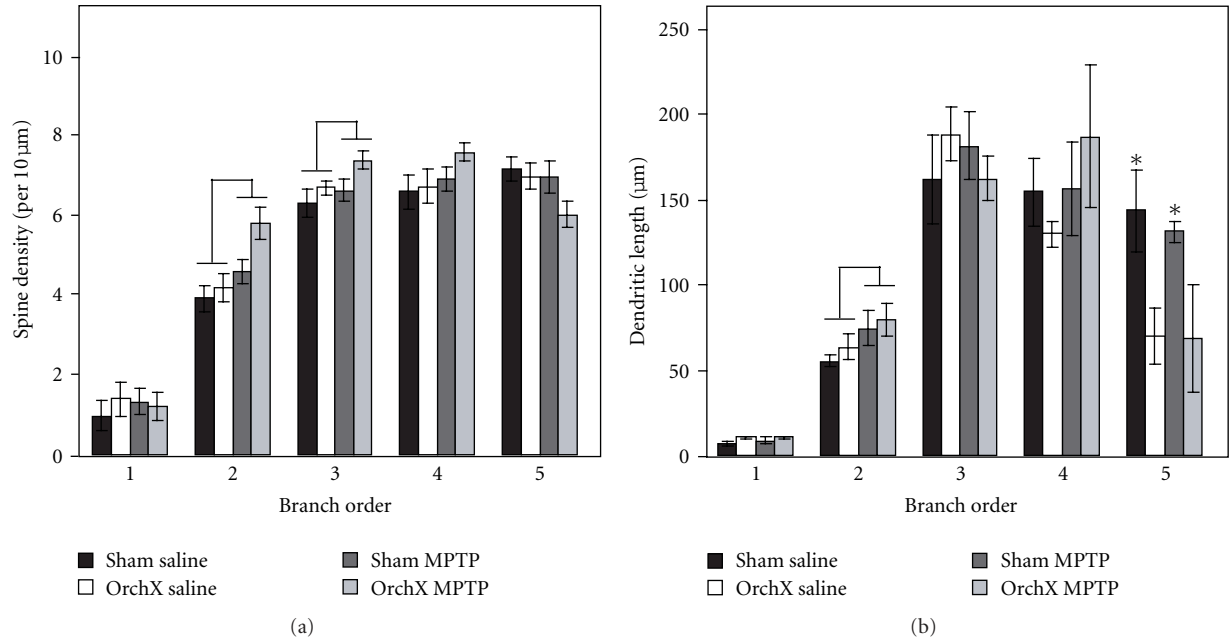


Figure 4: Branch order totals. Spine density (a) and dendritic length (b) for first- to fifth-order branches in striatal medium spiny neurons ($n = 5$ mice/group). Saline-injected mice include gonad-intact (black bars) and orchidectomized (white bars) males. MPTP-treated mice include gonad-intact (dark gray bars) and orchidectomized (light gray bars) males. Bars represent an effect of MPTP ($P < .05$), and asterisks represent an effect of gonadectomy ($P < .05$). Abbreviations: OrchX, orchidectomized; Sham, sham-orchidectomized.

our findings are in agreement with previous studies which found no effect of castration on striatal dopamine loss after MPTP [18] or on TH neurons in SNC after 6-OHDA [33]. In addition, studies using methamphetamine, which also depletes striatal dopamine, have shown that dopamine depletion after methamphetamine as well as amphetamine-induced stereotyped behaviors are similar in gonad-intact and castrated animals [49–52]. Interestingly, other studies have reported that testosterone increases neurotoxicity after dopamine-depleting lesions [13, 21, 53]. Due to the tremendous variability among published studies, these results are difficult to interpret. However, testosterone does not appear to have neuroprotective effects in the male nigrostriatal system.

Despite having no effect on MPTP-induced morphologic changes, castration decreased MSN dendritic length, but this effect was restricted to the distal branches of the dendritic tree. These effects of castration suggest that testosterone promotes dendritic growth in striatal MSNs. This raises an important question. How does testosterone promote growth of distal dendrites, but fail to attenuate MPTP-induced structural remodeling? To understand this issue, it is important to understand how hormones modulate neuronal plasticity, where hormones act in relation to the striatum and, in the case of testosterone, whether they act via androgenic or estrogenic mechanisms.

In hormone-sensitive areas of the brain, testosterone-driven changes in structural morphology are driven by classical hormone receptors for androgen or estrogen. For example, castration decreases dendritic branching in the posterior medial amygdala (MeP) and medial preoptic area (MPOA), and reduces spine density on hippocampal CA1 neurons in adult rodents [31, 54, 55]. The findings in the current study are similar. However, while MeP, MPOA and CA1 each contains an abundance of classical receptors for both androgens and estrogen, the male mouse striatum is largely devoid of classical hormone receptors [56]. Moreover, classical hormone receptors are also sparse in the major dopaminergic input to MSN dendrites from SNC [56]. Although previous studies in rats and mice have found some evidence of AR and ER in SNC [56–58], it appears that few steroid-sensitive neurons are TH-positive and project to the striatum [56, 58]. This relative absence of hormone receptors severely limits the ability of testosterone to exert direct or indirect effects on MSN dendrites via classical mechanisms. It is more likely that testosterone acts through nonclassical mechanisms to drive changes in the striatum, as suggested previously [56, 59]. The present results extend this potential mechanism to include morphologic changes as well.

In the brain, testosterone can act as an androgen, but it can also act via estrogenic mechanisms after aromatization. The ability of gonadal hormones to attenuate nigrostriatal neurotoxicity is largely attributed to estrogen, which is neuroprotective in the female. In animal models, estrogen attenuates striatal dopamine depletion [9, 60, 61] and partially prevents the loss of striatal TH immunoreactivity [14, 62] after MPTP. Indeed, experimental evidence has shown that estrogen also protects against nigrostriatal degeneration in the male striatum. However, while circulating estrogen is abundant in females, striatal estrogen available in males is minimal due to the low levels of aromatase in the striatum [63–66]. Therefore, the inability of testosterone

to act through estrogenic mechanisms effectively prevents hormone-driven neuroprotection in the striatum. However, it does not influence the neurotrophic actions of testosterone on MSN distal dendrites, which likely occur via androgenic mechanisms.

Clinically, the actions of gonadal steroid hormones in the nigrostriatal system are important because estrogen is thought to be neuroprotective in several neurological disorders, including Parkinson's disease. In fact, women are more likely to develop PD after hysterectomy or menopause, when endogenous estrogen is eliminated [67, 68]. One of the potential benefits of hormone replacement therapy on postmenopausal women is the potential to delay the onset and/or decrease the severity of neurodegenerative disease. Men also experience a loss of testosterone with age, albeit less severe than the complete loss of gonadal steroids in the female. Although androgen replacement therapy is available for men with hypoandrogenism, the results of the current study suggest that androgen replacement will not attenuate nigrostriatal neurodegeneration in the male.

Abbreviations

6-OHDA:	6-hydroxydopamine
CA1:	CA1 region of the hippocampus
D1:	Dopamine receptor D1
D2:	Dopamine receptor D2
MeP:	Posterior medial amygdala
MPOA:	Medial preoptic area of the hypothalamus
MPTP:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSN:	Medium spiny neuron
PD:	Parkinson's disease
SNC:	Substantia nigra pars compacta
TH:	Tyrosine hydroxylase.

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Research Article

Protective Role of rAAV-NDI1, Serotype 5, in an Acute MPTP Mouse Parkinson's Model

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Defects in mitochondrial proton-translocating NADH-quinone oxidoreductase (complex I) have been implicated in a number of acquired and hereditary diseases including Leigh's syndrome and more recently Parkinson's disease. A limited number of strategies have been attempted to repair the damaged complex I with little or no success. We have recently shown that the non-proton-pumping, internal NADH-ubiquinone oxidoreductase (Ndi1) from *Saccharomyces cerevisiae* (baker's yeast) can be successfully inserted into the mitochondria of mice and rats, and the enzyme was found to be fully active. Using recombinant adenoassociated virus vectors (serotype 5) carrying our *NDI1* gene, we were able to express the Ndi1 protein in the substantia nigra (SN) of C57BL/6 mice with an expression period of two months. The results show that the AAV serotype 5 was highly efficient in expressing Ndi1 in the SN, when compared to a previous model using serotype 2, which led to nearly 100% protection when using an acute MPTP model. It is conceivable that the AAV-serotype5 carrying the *NDI1* gene is a powerful tool for proof-of-concept study to demonstrate complex I defects as the causable factor in diseases of the brain.

1. Introduction

Parkinson's disease (PD), the second most common neurodegenerative disorder, is characterized by a loss of dopaminergic neurons in the substantia nigra (SN) which leads to a decrease in dopamine levels and a loss of motor control. The challenge in treating PD stems from a lack of understanding with regard to what triggers the onset of the disease. Studies of the disease through human pathology or from toxin-induced models, specifically 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), have identified three possible sources; oxidative stress, mitochondrial defects, and abnormal aggregation of proteins [1–4], and in some rare cases, inherited genetic defects. The discovery of MPTP as a neurotoxin has given neuroscientists the ability to develop animal models, mostly in mice and nonhuman primates, to investigate the mechanism(s) that may lead to PD in humans [5, 6]. Current evaluation strategies involve the testing of animal models through neurochemical analysis (HPLC, western blots, and immunohistochemistry) and behavioral assessment [7–9].

The use of recombinant-associated adeno virus (rAAV) has been widely explored as a gene therapy tool for the past 20 years [10]. Through extensive research, a number of serotypes have been isolated (AAV1-11) and engineered, with each showing differing selectivity and efficiency at infecting tissues ranging from the CNS (neurons) to skeletal muscles [11]. The most common serotypes that are used for gene delivery in the CNS include serotypes 2 and 5 with serotype 2 being the most widely used thus far. The goals of current gene therapy models include promoting cell survival or modification of activity in the damaged region [12]. Some of the gene therapies attempted to date include glial cell line-derived neurotrophic factor (GDNF) and enzymes

involved in dopamine synthesis (tyrosine hydroxylase (TH) and aromatic acid decarboxylase (AADC)) with limited success [12, 13]. More recently, a preliminary report on a clinical trial in humans was published indicating the safety of using AAV as a vehicle to introduce genes into the brain [14]. In addition, they were able to demonstrate an improvement in the patients with the use of a gene that regulates the level of GABA in the basal ganglia [14]. This initial study provides great potential for further studies and the use of other genes to modify signaling in the brain as a treatment for PD and other neurodegenerative diseases.

As mentioned above, one of the possible triggers of Parkinson's disease may involve defects in the mitochondrial respiratory chain. Therefore, our approach involves a gene therapy to complement the damaged mitochondria using the internal NADH-ubiquinone oxidoreductase derived from *Saccharomyces cerevisiae* (baker's yeast), NDI1 [15–20]. The *NDI1* gene will be expressed in the SN of mice using rAAV serotype 5, as a comparison to a previous study with this gene using rAAV serotype 2. In addition to immunohistochemical data, behavioral testing will be used to evaluate the ability of NDI1 to protect against the toxic effects of MPTP.

2. Materials and Methods

2.1. Animals. Twelve-week-old male (25–30 g) C57Bl/6 mice (obtained from our in-house breeding colony) were housed four per cage in a temperature-controlled environment under 12-hr light/dark cycle with free access to food and water. The housing and treatment of the animals was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Institutional Animal Care and Use Committee at The Scripps Research Institute approved all procedures.

2.2. Injection of AAV-NDI1. Recombinant AAV serotype 5 (rAAV5) carrying the *NDI1* gene (designated rAAV5-NDI1) was produced by and purchased from Applied Viromics (Fremont, CA). The final viral particle concentration, estimated by dot blot assay, was determined to be 3.1×10^{12} viral particles/ml. Surgical procedures were performed as previously described by Seo et al. [19]. Briefly, anesthesia was induced with 3% of isoflurane in O_2, and mice were secured in a stereotaxic frame (Kopf Instruments, Tujunga, CA). Anesthesia was maintained for the duration of the surgical procedure with 1.5–2% of isoflurane in O_2 through a nose tip fixed to the stereotaxic frame. All rAAV5-NDI1 injections were made using a 5-μL Hamilton microsyringe with a 30-gauge beveled needle. A single injection of 2 μL rAAV5-NDI1 (suspended in PBS containing 0.1% of fluorescent beads) was made in the right hemisphere at the following coordinates (measured from bregma/dura): AP: −3.3 mm, ML: 1.5 mm, and DL: −3.9 mm, at a rate of 0.2 μL/min. Expression levels were verified two months after injection, and prior to any drug treatment.

2.3. MPTP Treatment. MPTP handling and safety measures were in accordance with the chemical hygiene plan developed at The Scripps Research Institute. Approximately 2 months after the rAAV5-NDI1 injection, mice were subjected to acute MPTP treatment, as reported by Seo et al. [19]. Briefly, MPTP (in sterile saline) was administered intraperitoneally at a dose of 15 mg/kg of body weight. A total of 4 injections were performed at 2-hour intervals with a 100% survival rate. The MPTP-treated groups were divided into either NDI1+MPTP ($n = 14$) or MPTP only ($n = 8$). Control animals (NDI1+Saline, $n = 10$) were injected with the vehicle (sterile saline) in place of the MPTP solution.

2.4. Behavioral Testing. Behavioral testing was performed as described previously by Baber-Singh et al. [7]. One week prior to and 1-week post-MPTP treatment animals were tested using the elevated body swing (EBS) [7, 21, 22] and methamphetamine- (MA-) induced rotation tests. EBS and MA-induced rotation trials were videotaped and analyzed at a later time by an unbiased observer. For the EBS test, each animal was held 1 cm from the base of the tail and suspended approximately 1 cm above the table for 60 sec. Movements greater than 30° from vertical were counted as a swing, and the next swing was counted only after the animal returned to or passed through the neutral position.

Prior to the administration of MA (1.5 mg/kg), animals were allowed 5 min to acclimate to the bowl environment. Evaluation of activity was initiated 15 min after the administration of MA, to allow for the drug to take effect, and then continuously for 40 min. The number of quarter turns around the bowl was used to evaluate any bias created by the protection of NDI1 when challenged with MPTP or saline.

2.5. HPLC Analysis. One-half of the mice from each treatment group were euthanized for HPLC analysis of striatal DA and its metabolite levels [19, 23]. The mice were perfused with saline, after which brains were quickly removed and frozen on dry ice, and maintained at −80°C until chemical analysis was performed. The method used was similar to that outlined by Seo et al. [19]. Brains were dissected with a razor blade to approximately 2 mm thick sections. Striatal regions from each side of the brain were isolated separately and weighed. Each sample was homogenized by sonication in 5 volumes of ice-cold 0.2 M perchloric acid and deproteinized by centrifugation at 14,000 rpm for 15 min at 4°C. Aromatic amines and their metabolites were separated using ion-paired reversed phase HPLC coupled with electrochemical detection (Eicom ECD-300, Kyoto, Japan). Samples (6 μL) kept on ice were injected into the HPLC system equipped with an SC-3ODS column (3 μm, 3 × 100 mm; Eicom) with a flow rate of 4 ml/min, at room temperature. The mobile phase was composed of 0.1 M citrate-acetate buffer, 1 mM sodium octane sulfate, and 13 μM EDTA·2Na with a final pH adjusted to 3.5 prior to adding 20% (v/v) of methanol. The analytes were detected on a graphite-working electrode set at +750 mV versus Ag/AgCl reference electrode. The data were collected using an EPC-500 processor (Eicom); peak

areas were calculated using the PowerChrom software and quantified from a calibration curve of standards.

2.6. Immunohistochemistry. The remaining mice from each group were perfused with saline followed by cold 4% (w/v) paraformaldehyde solution. The brains were removed and postfixed in the paraformaldehyde solution for 1 hr at 4°C. Brains were frozen in OCT compound (Sakura, Torrance, CA) and stored at −20°C until further processing. 30-μm sections were collected using a cryostat (Microm, Germany), directly mounted onto slides, and stored at −20°C. Immunohistochemistry using antibodies against NDI1 (1 : 250, prepared in our laboratory), tyrosine hydroxylase (TH, 1 : 500, EMD Bioscience/Calbiochem, La Jolla, CA), and glial fibrillary acidic protein (GFAP, 1 : 250, Sigma-Aldrich, St. Louis, MO) was carried out on slide sections as previously described by Seo et al. [19]. Briefly, each section was first rinsed in PBS, followed by incubation in a 3% hydrogen peroxide solution for 30 min to quench native peroxidases, followed by permeabilization and blocking for nonspecific binding with 10% goat serum, 5% horse serum, and 0.1% Triton X-100/PBS at room temperature for 1 hr. Sections were then incubated with primary antibody overnight at 4°C. For TH and GFAP, sections were subsequently incubated with biotinylated secondary antibody for 1 hr at room temperature followed by revelation with the ABC elite kit (Vector Laboratories, Burlingame, CA) and DAB (3,3′-diaminobenzidine tetrachloride, Sigma-Aldrich, St. Louis, MO). NDI1 protein staining was done using the tyramide signal amplification following the manufacturer's procedure (PerkinElmer, Boston, MA). The sections were blocked using Image-iT FX (Molecular Probes, Eugene, OR), followed by primary antibody overnight at 4°C, and horseradish peroxidase-conjugated goat antirabbit IgG (1 : 1000, EMD Bioscience/Calbiochem) at room temperature for 2 hrs. The sections were then rinsed 3 times in PBS for 10 min and then incubated with the fluorophore tyramide amplification solution (1 : 75, dilution with amplification buffer) for 7 min at room temperature followed by rinsing in PBS.

2.7. NADH Activity Staining. Histochemical staining for NADH dehydrogenase activity was based on the NADH-tetrazolium reductase reaction [19]. Brain sections were incubated with an NADH-tetrazolium reductase solution (0.2 M Tris-Cl, pH 7.4, 1.5 mM NADH, and 1.5 mM nitro blue tetrazolium) at room temperature until sections were overstained ($t > 10$ min, bright purple), followed by removal of excess color in a series of acetone solutions (30%, 60%, and 90%) for 1 min each, and rinsed 3 times 10 min in deionized water.

2.8. Western Blotting. Samples used for HPLC analysis were further processed for Western blotting using a protocol outlined in Barber-Singh et al. [7]. Samples were thoroughly mixed and neutralized with 1 M Tris (pH 11), after which 2 μL of DNase (50 mg/ml, Roche, Indianapolis, IN) was added, along with a protease inhibitor cocktail (Complete Mini, Roche) containing 1 mM EDTA. To this, SDS at a final concentration of 5% was added, and the samples were incubated at room temperature for 1 hr prior to protein evaluation in each sample using the Bradford method. Samples were diluted in 2 × sample buffer to a final concentration of 4 μg/μL. Sixty micrograms of total protein was loaded and separated on a 10% SDS-polyacrylamide gel then transferred to a 0.22-μm nitrocellulose membrane (Scleicher and Schueller, Germany). Detection was performed using the following antibodies: monoclonal mouse anti-TH 1 : 1000 (EMD Bioscience/Calbiochem), polyclonal rabbit anti-VMAT2 1 : 1000 (Chemicon, Temecula, CA), monoclonal rat-anti-DAT 1 : 5000 (Chemicon), monoclonal mouse anti-GAPDH 1 : 2000 (Chemicon), and rat-anti-NDI1 1 : 5000 (prepared in our laboratory) [24]. Visualization of the protein bands was accomplished using the appropriate secondary, either goat antimouse horseradish peroxidase (HRP) (1 : 1000, Pierce), antirabbit HRP (1 : 5000, GE Healthcare, United Kingdom), or antirat HRP (1 : 10,000, Chemicon) followed by revelation with SuperSignal West Pico chemiluminescence kit (Pierce, Rockford, IL). Chemiluminescence signals were collected on autoradiography film and the density of each band was measured using the ImageJ software [25].

2.9. Statistical Analysis. Statistical analysis was performed using the Student's t-test. Results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis of staining density was collected and evaluated using the ImageJ software. Threshold values were set for the entire group of subjects, for example, GFAP or TH staining and applied to all sections prior to evaluating density values. Statistical significance was set as follows, unless indicated elsewhere: ** if $P < .01$, *** if $P < .001$.

3. Results

3.1. Expression of the NDI1 Protein in the Mouse SN. An important aspect of gene therapy is the ability to target the appropriate structure *in vivo* and to have widespread expression of protein in the desired region without interfering in the native function of surrounding regions. It is therefore crucial to verify the location and extent of NDI1 protein expression in the substantia nigra (Figures 1(a) and 1(b)). The AAV-NDI1 serotype 5 that was chosen for use in this experiment shows high levels of expression throughout the SN (Figure 1(a)), when compared to TH levels (Figure 1(b)). In addition, NADH activity staining reveals widespread NDI1 expression throughout much of the SN in serial sections, averaging 900 μm in both the saline and MPTP-treated groups (Figures 2(a)–2(d)). In contrast, previous trials using AAV-NDI1 serotype 2 showed that the expression of NDI1 was predominantly limited to regions near the injection point (Figure 1(c)) when compared to TH levels (Figure 1(d)). Also, the spread (anterior-posterior) of virus throughout the SN was limited, typically 300–450 μm measured by the number of NDI1-positive sections. This ranges from 10 to 15 sections depending on the placement of the injection, as the time for expression was equal

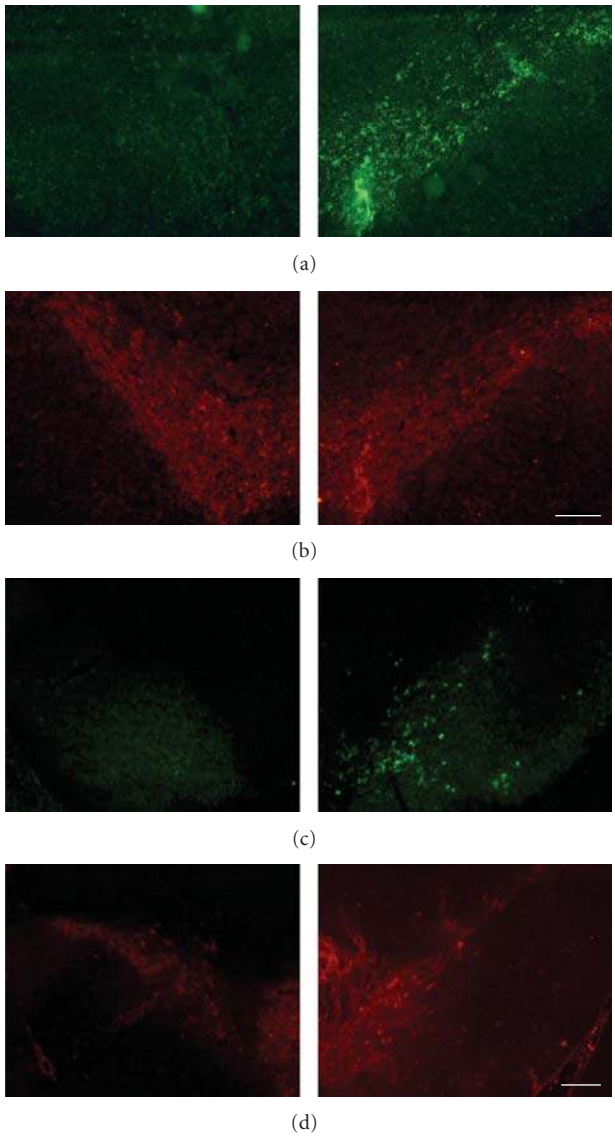

Figure 1: Comparison of NDI1 expression levels in the SN when using AAV serotype 5 versus serotype 2. All images were obtained approximately 2 months after injection of AAV-NDI1 in control mice. (a) *NDI1* expression after injection of rAAV5-NDI1, (b) TH levels in SN corresponding to (a). (c) *NDI1* expression after injection of AAV-NDI1 serotype 2, (d) TH levels in SN corresponding to (c). Scale bar = 200 μm for (a) and (b), and 200 μm for (c) and (d).

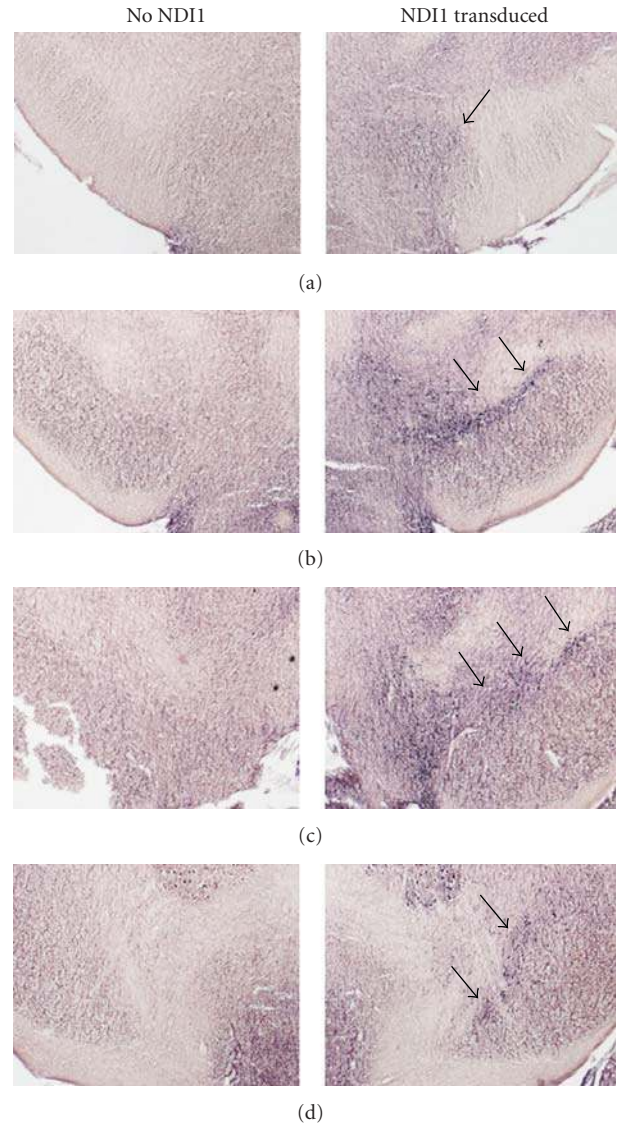

Figure 2: Representative SN images from the NDI1 + Saline group demonstrating the spread of functional NDI1 2 months after injection. Expression of NDI1 was revealed by NADH activity staining. Image position is given relative to the injection point, which was determined by the presence of fluorescent beads added to rAAV solution prior to injection. (a) ~150 μm anterior of injection point, (b) point of injection, (c) ~180 μm posterior of (b), and (d) ~720 μm posterior of (b). Arrows indicate the SN.

for both serotypes. The increase in NDI1 expression, for serotype 5, is further established with the behavioral and immunochemical data represented below.

3.2. Effect of NDI1 Expression on Behavior Following either Saline or MPTP Treatment. The elevated body swing test (Figure 3(a)) was chosen due to the fact that it is a nondrug-based test for lateralized activity in unilateral Parkinson's models. It has been reported that the effect of administering amphetamines multiple times can lead to a sensitization resulting in poor correlation between neuronal loss and behavioral effects. Each group of animals was first tested 1 week prior to and again approximately 1 week after MPTP treatment to evaluate the protective effect of NDI1 expression in the nigrostriatal pathway. In the pre-MPTP trials, the results confirmed no bias towards one side or the other (data not shown). The % left and % right swings were as follows (mean ± SEM): MPTP Only (MO) = 52.5% (± 2.6, L) and 47.5% (± 2.6, R); NDI1 + Saline (NS) = 47.5% (± 2.8, L) and 52.5% (± 2.8, R); NDI1 + MPTP (NM) = 48.4% (± 2.1, L) and 51.6% (± 2.1, R). Analysis of the post-MPTP treatment

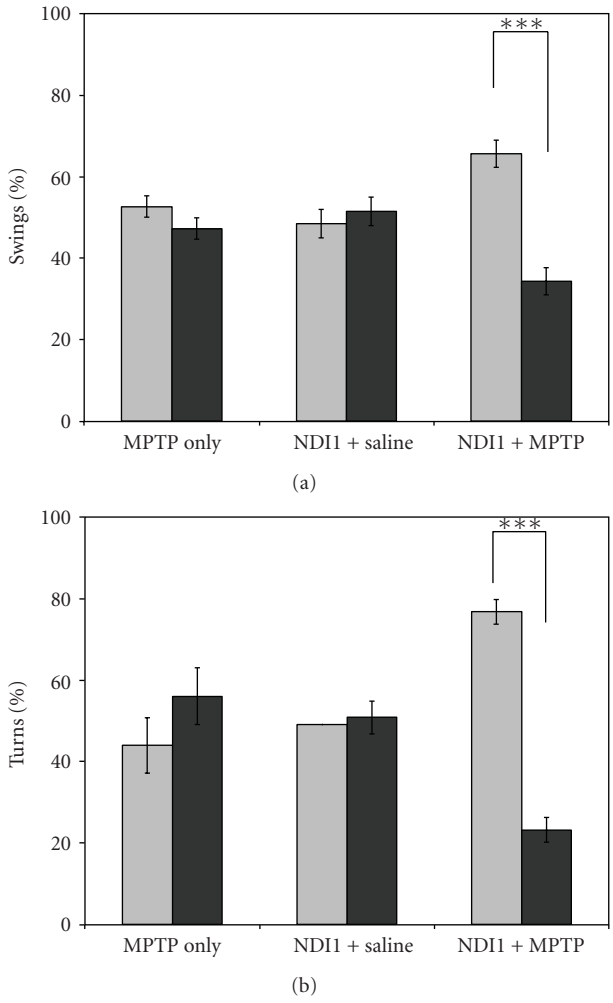

Figure 3: Analysis of mouse behavior tests 1 week after MPTP treatment. All trials were videotaped and analyzed at a later time by an observer blind to the identity of the animal. (a) For the EBS test, each animal was held 1 cm from the base of its tail and suspended approximately 1 cm above the table for 60 sec. The number of swings greater than 30° from the vertical position was counted, and the next swing was counted only after the animal passed through vertical. (b) For the drug-induced rotation test, the number of quarter turns around the bowl and the direction were used to evaluate any bias created by the protection of NDI1 when challenged with either MPTP or saline. Data are represented as mean ± SEM (MPTP Only, $n = 8$; NDI1 + Saline, $n = 10$; NDI1 + MPTP, $n = 14$) *** = $P < .0005$. Light grey column, movement left; dark grey column, movement right.

revealed a significant change in behavior for the NM group only (Figure 3(a)), to 67.9% (± 3.4, L) and 32.1% (± 3.4, R).

Methamphetamine- (MA-) induced rotation provides a robust method for evaluating unilateral damage in the Parkinson's mouse model. One week prior to and 1 week after MPTP treatment, animals were injected with 1.5 mg/kg MA. Rotational behavior (Figure 3(b)) was monitored for 40 min, with no significant bias for the pre-MPTP treatment test (results not shown). The % turns were as follows (mean ± SEM): MO = 47.4% (± 7.1, L) and 52.6% (± 7.1, R), NS = 52.4% (± 5.1, L) and 46.6% (± 5.1, R), and NM = 55.0% (± 4.0, L) and 45.0% (± 4.0, R). One week after MPTP treatment, the test was repeated (total time between trials was 3 weeks), which resulted in a significant bias for the NM group only (Figure 3(b)), 76.8% (± 3.1, L) and 23.2% (± 3.1, R).

3.3. Neurochemical Analysis of the Dopaminergic System. To evaluate the level of protection that expression of *NDI1* provided to the nigrostriatal system, HPLC analysis of the striatal region was used to evaluate the levels of DA, DOPAC, and serotonin (Figures 4(a)–4(c)). Significant decreases were observed for the MPTP-Only (MO) group, to approximately 30% of control (NS) for DA (Figure 4(a)) and 75% of control for DOPAC (Figure 4(b)), as well as the nontransduced hemisphere for the *NDI1*-MPTP (NM), 32% of control for DA, (Figure 4(a)) and 73% of control for DOPAC (Figure 4(b)). For the *NDI1*-transduced hemisphere 100% protection was observed for both DA (Figure 4(a)) and the major DA metabolite DOPAC (Figure 4(b)). As expected, there were no significant changes in the serotonin levels with the administration of MPTP (Figure 4(c)).

Following HPLC measurements, samples were further prepared for Western blotting analysis of the two dopaminergic transporters, VMAT2 and DAT, as well as TH and NDI1 levels after MPTP treatment (Figure 5(a)). When compared to the control group (NS), both the MO group and the nontransduced side of the NM group showed significant decreases in all proteins, with TH levels falling below the limit of detection. Statistical analysis of the Western blots for each protein further shows that the *NDI1*-transduced hemisphere provided protection against MPTP, with levels similar to that of the control group (NS) (Figure 5(b)).

3.4. Immunohistochemical Assessment of Neurodegeneration following MPTP Treatment. In order to determine functionality of the NDI1 protein, sections were incubated with NADH and tetrazolium as the substrates (Figure 6(a)). Darker blue staining in the SN and CPu (right hemisphere, white arrow) corresponds to the regions of functional NDI1 expression, and lighter blue/purple staining in the opposite hemisphere is presumably due to native complex I activity. Both the MO and the NM groups clearly have weaker NADH activity in the left hemisphere in both the SN and CPu when compared to the saline-treated group (Figure 6(a)).

In addition, to assess the degree of protection provided to the nigrostriatal system by NDI1 expression, serial striatal sections were analyzed using two immunohistochemical markers, GFAP and TH (Figure 6(b)). The GFAP staining clearly shows increased damage in the MO group, in both hemispheres, and in the nontransduced hemisphere of the NM group. Comparable results were observed for the TH staining, with a significant difference in staining between the nontransduced and *NDI1*-transduced hemispheres, as well as a significant decrease in TH staining for the MO group when compared to control. Statistical analysis of all tissue stained for GFAP and TH (Figures 7(a) and 7(b)) further confirms the significant differences between MO

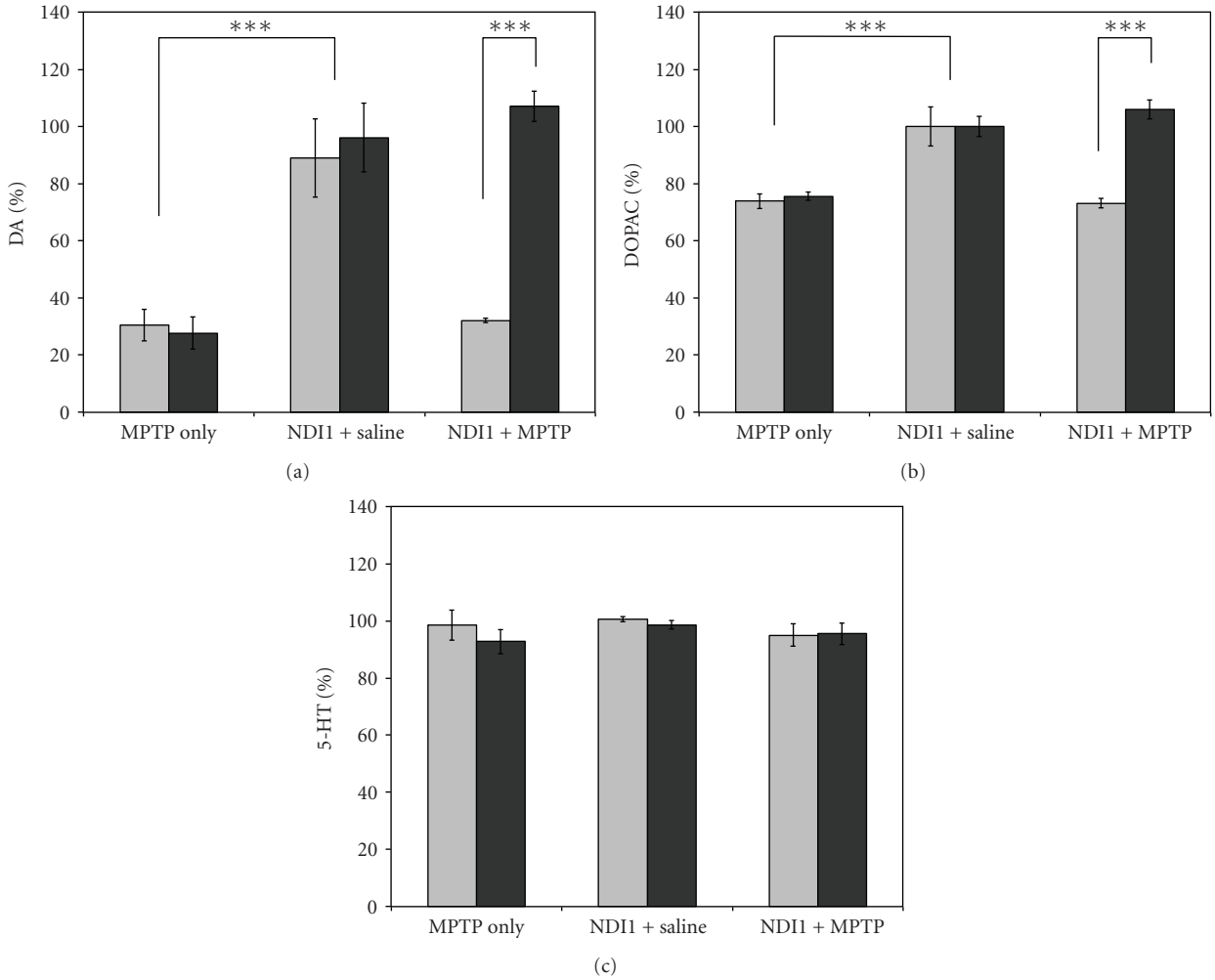

Figure 4: Measurement of aromatic amine levels in the mouse striatum 2 weeks after MPTP treatment. One-half of the animals from each group were euthanized, and brains were immediately frozen on dry ice prior to HPLC analysis. Striatal sections from each hemisphere were isolated separately and processed as described in the materials and methods section. (a) Dopamine (DA), (b) DOPAC, the major metabolite of DA, and (c) Serotonin (5-HT). Results are expressed as mean ± SEM (MPTP Only, $n = 5$; NDI1 + Saline, $n = 5$; NDI1 + MPTP, $n = 7$) *** = $P < .0001$. Light grey column, left hemisphere; dark gray column, right hemisphere.

and NS as well as the significant difference between the hemispheres of the NM hemispheres. For the MO group, the GFAP (Figure 7(a)) staining was more than 150% of NS levels, and the nontransduced hemisphere of the NM group was also 150% of saline-treated animals. For TH staining (Figure 7(b)), MO animals had a 60% reduction in TH levels compared to saline-treated animals. In addition, the NM group had nearly a 90% reduction in TH staining in the nontransduced hemisphere and a slight decrease (not significant) in the TH staining in the *NDI1*-transduced hemisphere.

4. Discussion

The use of gene therapy for the treatment of Parkinson's disease has become more widespread in recent years with a focus on introducing neurotrophic factors and enzymes responsible for the production of neurotransmitters (e.g., DA and GABA) as a means to prevent further loss of neurons [12, 13, 26]. These remedies are most commonly introduced into the appropriate brain region using recombinant adenoassociated viruses (rAAVs). The most commonly used serotype in gene therapy applications, using rAAV, has been type 2 which has been shown to transduce neurons in a number of brain regions, including the substantia nigra (SN) [10, 11, 26]. However, further research has produced additional recombinant serotypes that demonstrate higher transduction efficiency for specific brain regions. For example, serotype 5 was shown to be highly specific for the SN [10]. This has been confirmed with the difference in NDI1 expression levels found in the SN 2 months after injection, for serotype 2 (low) versus 5 (high). The level of expression strongly correlates with the protection provided, as evidenced through neurochemical analysis. Previous trials using rAAV2-NDI1 found 60% retention of DA levels in the striatum after acute MPTP treatment [19]. In contrast, the results here revealed

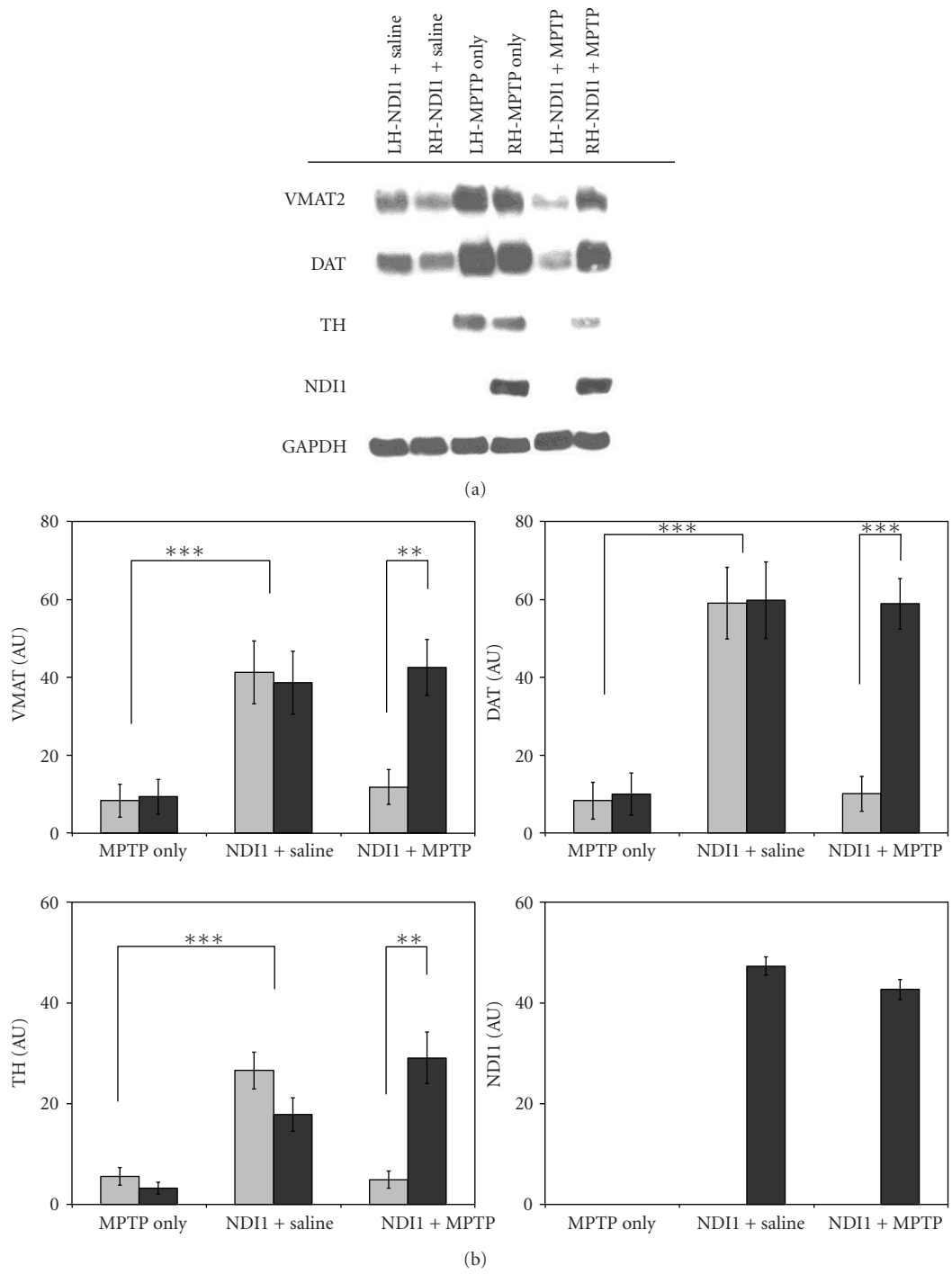

Figure 5: Evaluation of MPTP effects on the DAergic neurons of the striatum using mouse brain homogenates. (a) Representative Western blots, 60 μg of protein per lane was loaded on a 10% SDS-PAGE gel, (b) statistical analysis of western blots for each treatment group MPTP Only (MO), NDI1 + Saline (NS), and NDI1 + MPTP (NM), VMAT2 ($n = 7, 7, 10$), DAT ($n = 8, 8, 11$), TH ($n = 5,5,7$), and NDI1 ($n = 0, 9, 13$). ** = $P < .005$, *** = $P < .00001$. Light grey column, left hemisphere; dark grey column, right hemisphere.

that stronger and more widespread expression can result in 100% retention of DA levels in an acute MPTP model, when compared to controls.

Behavioral analysis of our MPTP model revealed lateralization of movement in the NM group only, for both the elevated body swing (EBST) and drug-induced rotation tests. For the methamphetamine-induced rotation, as expected, the animals rotated towards the lesioned side. The source of ipsilateral rotation has been described in a number of papers regarding the unilateral depletion of dopamine in the

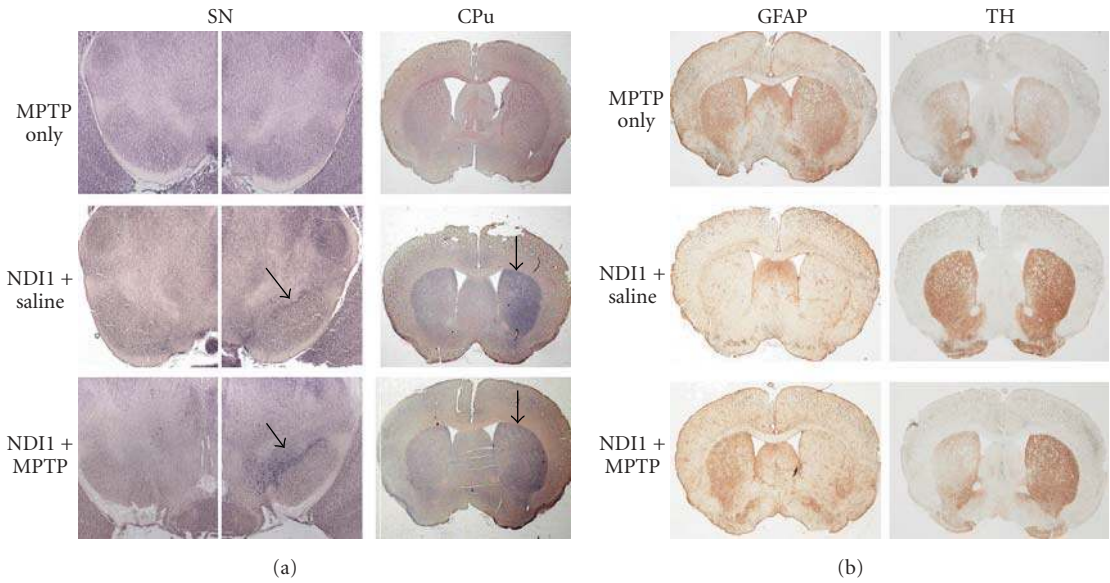

Figure 6: Functional expression of NDI1 in the SN and CPu and protective effects following treatment with either saline or MPTP. Two weeks after the last injection, one-half of the mice were sacrificed for immunohistochemical analysis. (a) Representative NADH activity staining in the SN and CPu for each treatment group. Arrows in SN indicate the substantia nigra. Arrows in CPu indicate the injection side. (b) GFAP and TH levels in CPu.

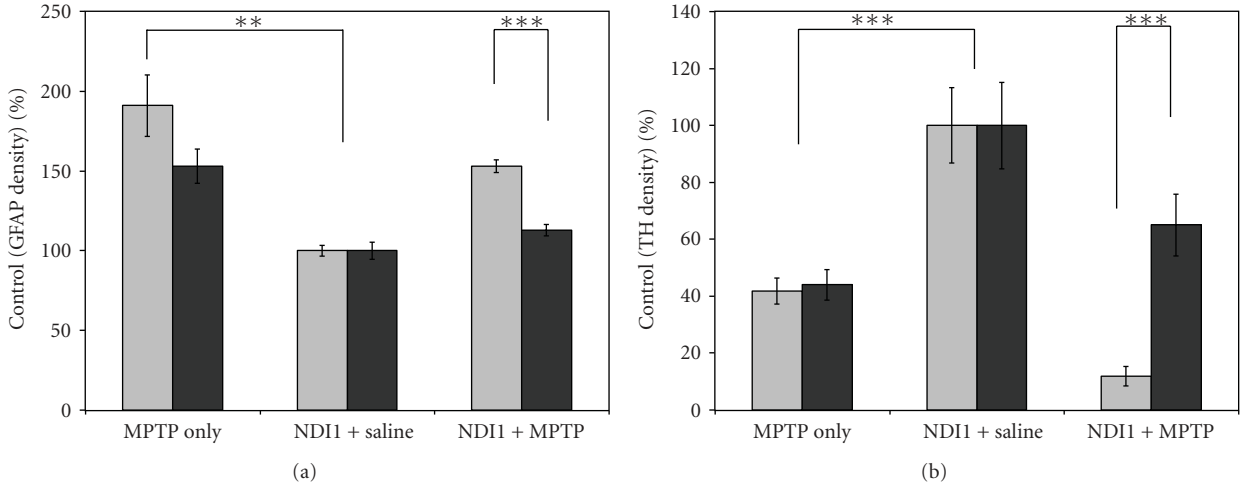

Figure 7: Statistical analysis of GFAP and TH staining density in the mouse striatum: a comparison between left and right hemisphere in MO, NS, and NM groups. Images with GFAP ($n = 28$) and TH ($n = 35$) immunostaining were collected and processed using the ImageJ software. (a) Density measurement of GFAP staining was evaluated for each hemisphere and compared to control (*NDI1* + Saline, $n = 10$), results given as % control ± SEM (MPTP Only, $n = 6$; *NDI1* + MPTP, $n = 12$). (b) The same procedure was used to evaluate TH density of each hemisphere, reported as % control ± SEM. (MPTP Only, $n = 8$; *NDI1* + Saline, $n = 12$; *NDI1* + MPTP, $n = 15$) $^{**} = P < .005$,$^{***} = P < .0001$. Light grey column, left hemisphere; dark grey column, right hemisphere.

nigrostriatal system [22, 27, 28]. The elevated body swing test (EBS) has been used extensively with the unilateral 6-OHDA rat and mouse models [21, 22, 27, 29] and previously described for MPTP-treated mice by our group in a chronic MPTP study [7]. The results for the EBS test exhibited good correlation with the drug-induced rotation test, movement towards the lesioned side for the NM group (ipsilateral), and with a previous test in a chronic MPTP mouse model [7]. Similar tests in a 6-OHDA Parkinson model showed either a contralateral movement or no effect [21, 22, 29]. However, an interesting result published by Abrous et al. [27] demonstrated that this test may be dependent on a few factors. First, being the extent of the lesion, and second, the length of time after treatment that the test is administered; both of which may affect the changes in activity over long periods of time (i.e., months). However, in our chronic MPTP Parkinson model, we achieved the same outcome as in this experiment when testing animals more than 3 week

after treatment [7]. And as demonstrated both in the acute and chronic models through immunohistochemical analysis, the MPTP-treated animals have extensive loss of nigrostriatal neurons, resulting in ipsilateral movement in the *NDI1*-transduced + MPTP-treated group.

An immunohistochemical hallmark of MPTP treatment is the loss of TH-positive neurons in both the SN and CPu [8, 30]. This result was clearly observed in the MO group, as well as significant loss on the non-*NDI1* side of the NM group. In addition, a significant increase in GFAP staining corresponded well with the loss of TH in both the SN and CPu. Again, the presence of NDI1 in both the SN and CPu shows a protective effect when challenged with an acute MPTP treatment, and all immunohistochemical results were supported by the Western blot analysis. As was expected, the amount of both monoamine transporter proteins, in non-*NDI1* samples, in the CPu was decreased when compared to controls as well as the levels of TH after 2 weeks. This marked decrease in monoamine transporters is expected as they are the primary route of MPP^+ sequestration and consequent toxicity in the brain [6, 31–33]. In addition, once MPP^+ has entered the neuron, there is a passive transport into the mitochondria resulting in inhibition of complex I activity and possibly the overproduction of reactive oxygen species (ROS) [3, 34–37]. The presence of NDI1 in the NM group has prevented the deleterious effects of MPP^+ in the neuron which resulted in transporter levels that were not significantly altered compared to controls and only a slight decrease in TH levels.

5. Conclusion

In conclusion, all results obtained demonstrate a clear protective effect of NDI1 in the dopaminergic system. The use of serotype 5 in the dopaminergic neurons resulted in greater expression efficiency and consequently better protection when challenged with MPTP in an acute PD mouse model. The use of behavioral testing in conjunction with neurochemical analysis provided a more complete evaluation of the unilateral MPTP PD model. These results provide further support for the use of NDI1 as a gene therapy for the treatment of PD and the possibility for use in other mitochondrial complex I-deficient diseases.

Acknowledgments

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Research Article

Effects of Human Alpha-Synuclein A53T-A30P Mutations on SVZ and Local Olfactory Bulb Cell Proliferation in a Transgenic Rat Model of Parkinson Disease

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A transgenic Sprague Dawley rat bearing the A30P and A53T α-synuclein (α-syn) human mutations under the control of the tyrosine hydroxylase promoter was generated in order to get a better understanding of the role of the human α-syn mutations on the neuropathological events involved in the progression of the Parkinson's disease (PD). This rat displayed olfactory deficits in the absence of motor impairments as observed in most early PD cases. In order to investigate the role of the mutated α-syn on cell proliferation, we focused on the subventricular zone (SVZ) and the olfactory bulbs (OB) as a change of the proliferation could affect OB function. The effect on OB dopaminergic innervation was investigated. The human α-syn co-localized in TH-positive OB neurons. No human α-syn was visualized in the SVZ. A significant increase in resident cell proliferation in the glomerular but not in the granular layers of the OB and in the SVZ was observed. TH innervation was significantly increased within the glomerular layer without an increase in the size of the glomeruli. Our rat could be a good model to investigate the role of human mutated α-syn on the development of olfactory deficits.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder. It is mainly characterized by a progressive and massive loss of dopaminergic (DA) neurons in the substantia nigra *pars compacta* (SNpc), which leads to several clinical motor symptoms such as akinesia, rigidity and resting tremor [1, 2]. The molecular pathways leading to these concomitant clinical alterations remain obscure, but it is believed that it may result from environmental factors, genetic causes, or a combination of the two [3]. The first gene discovered involved in the disease was the α-synuclein (α-syn) gene. Mutations of this gene are responsible for autosomal dominants forms of PD [4, 5]. Indeed, three missense mutations in the α-syn gene have been found in patient families: A30P, A53T, and E46K [4, 6, 7]. Alpha-syn has an increased propensity to aggregate due to its hydrophobic nonamyloid-β component domain and the presence of fibrillar α-syn as a major structural component of Lewy body, a pathological hallmark of Parkinson's disease and suggests a role of aggregated α-syn in disease pathogenesis [8]. Alpha-syn is a natively unfolded presynaptic protein which has a role in compartmentalization, storage, and recycling of neurotransmitters. It is involved in physiological regulation of certain enzymes such as tyrosine hydroxylase (TH) and increases the number of dopamine transporter molecules [1]. On the contrary, it is implicated in downregulation of the activity of the vesicular monoamine transporter-2 (VMAT-2).

In order to understand better the effects of the α-syn mutation on the neuropathology and progression of PD, transgenic mouse models were generated. However, mostly due to the choice of the promoter, the expression of mutated human α-syn was often located in non-DA brain structures [9–11]. In addition, when α-syn aggregates were visualized in the SNpc, no significant DA neuronal cell loss was noticed although motor deficits were observed [1, 11–15].

These transgenic mice models were more suitable to study whole brain α-synucleinopathy than to investigate the precise role of α-syn on DA structures, therefore a search for better animal models of PD continued. Thus, about a decade ago, Lo Bianco et al. [16] and Yamada et al. [17] showed that lentiviral vectors expressing wild-type or mutant human A30P and A53T forms of α-syn injected into the rat SNpc induced, in contrast to transgenic mouse models, a selective loss of nigral DA neurons, DA denervation of the striatum as well as significant motor impairments [18]. These studies demonstrated that the rat has specific sensitivity of SNpc DA neurons to human α-syn, but this new model of PD lacked the progressive nature of the disease observed in humans. In the light of these previous studies, we generated a transgenic rat bearing both the A30P and A53T α-syn human mutations [19, 20] in order to get a better understanding of the human α-syn role on the neuropathological events involved in the progression of the disease. In the mouse, the A30P mutant form [21] or the A53T mutant form [22] of α-syn showed a decrease in the neurogenesis in the glomerular and granular layers of the olfactory bulbs (OBs). This is the reason why the present study focused on the subventricular zone (SVZ) proliferation and the OB local proliferation. Alteration of SVZ and local OB proliferation could also affect OB function, as most of the time, hyposmia precede clinical motor symptoms in Parkinson's disease [23].

In the adult brain, neural stem cells from the anterior portion of the SVZ give rise to neuroblasts that migrate along the rostral migratory stream to the OB [24]. Within the granule cell and glomerular layers of the OB, a persistent proliferative activity of progenitor cells is observed [24]. Then, the cells differentiate into functional granular GABAergic and periglomerular DA olfactory interneurons.

To summarize, we investigated the effects of the human double A30P and A53T α-syn mutations on SVZ and local OB proliferation with an additional focus on OB DA innervations [25].

2. Materials and Methods

2.1. Generation of the Transgenic Human A53T and A30P α-synuclein Rat. The transgene construct pUTHTV $hm^2\alpha$-SYN (Figure 1) was created by Richfield et al. [14] and kindly given by H.J Federoff (University of Rochester, New York). Briefly, the transgene was composed of the A30P and A53T double mutated form of human α-syn under the control of the rat tyrosine hydroxylase (TH) promoter. The current method of gene transfer, microinjection, which is widely used in transgenic mouse production, was successful in obtaining transgenic rats. The microinjection of Sprague Dawley rat ovocytes into male pronucleus were generated by the INSERM UMR643 transgenic rat common facility (Nantes, France) and by genOway company (Lyon, France). The present study was performed with one of the 3 transgenic rat lines that were generated, the MA3 transgenic rat line.

All experiments were carried out in accordance with the regulations of the University of Nantes Animal Health Committee.

2.1.1. Analysis of the Olfaction, Modified from Lemasson et al. [26] and Gross Motor Locomotion. After habituation, the animal was placed one time per month in the middle of an open field apparatus (600 × 600 × 400 mm) equipped with infrared beams and connected to a computer to analyze locomotion and time spent in the four quadrants of the maze for a 2-minute period (Imetronic, Pessac, France). In one corner, a filter paper (70 × 30 mm) located at a height of 10 cm, was soaked in fresh coconut milk (half diluted in distilled water; Tables du Monde, Leclerc Company France). Coconut milk is known to be a very attractive odor for the rat. In the opposite corner, the same size paper filter was soaked in distilled water, considered as a neutral odor. The time spent by the rat in both corners is recorded. The results are expressed as the ratio of time spent in the corner with the coconut milk filter paper/the time spent in the corner with the distilled water filter paper. A higher ratio (i.e., more time spent in the corner with the coconut milk filter paper) demonstrates that the animal was able to smell the odor of the coconut milk. During habituation and olfaction testing, the animals were studied for locomotor impairments by a hidden observer. Five animals were used in both groups. The day before the first olfaction testing, the rat is placed in the apparatus (without filter paper) for 30 min. During olfaction testing, the rat is recorded to examine if there was any gross impairments in the motor pattern.

2.1.2. Lateral Stepping Test and Movement Initiation. Each animal was systematically handled on a regular basis for several days before the first assessment. Briefly, in this test evaluating the initiation of the movement, the experimenter firmly suspended the rat's hindquarters and restricted one of its forelimbs, while the rat supported its weight on the other forelimb. Then, the experimenter moved the rat along the table (0.9 m in 5 seconds) on the right limb, three times consecutively per session. Then, the rat undergoes the same test for its left paw. All the sessions (left and right) were recorded to allow the number of adjusting steps to be counted by an investigator blinded to the state of the rat (i.e., transgenic or wild type). For each session (left and right), the total score calculated was the mean of the number of adjusting steps observed in the three tests (for the right and the left paw). Then data from left and right were averaged giving one value per animal. Data are presented as mean values per group.

2.2. BrdU Injection. Groups of 5 wild-type (WT) and 4 transgenic female Sprague Dawley rats were sacrificed at

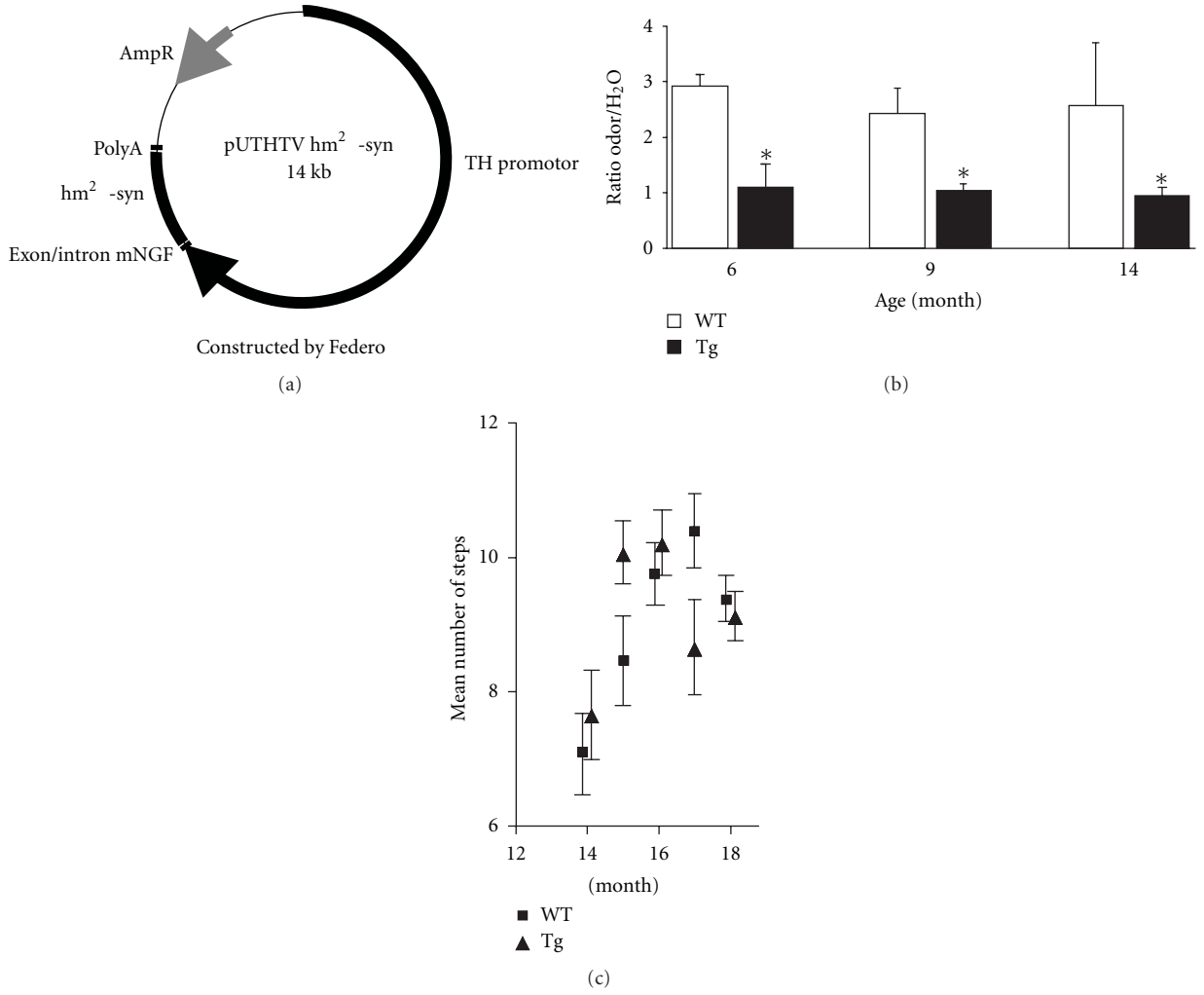

Figure 1: (a) Transgene construction. The transgene construct pUTHTV hm$^2\alpha$-Syn is composed of double mutated form of human α-syn (hm$^2\alpha$-SYN) with A30P and A53T mutations under the control of the 9-kb rat tyrosine hydroxylase promoter. (b) There is a significant difference in the ratio odor/H20 between the 2 groups of rats ($^*P < .05$, with the odor being from the coconut milk) in the olfaction test. The WT rats spent more time in the corner with the coconut milk filter paper as compared to the corner with the distilled water (ratio superior to 2). Transgenic animals spent less time in the corner with the coconut milk as a ratio of 1 corresponds to the same period of time spent in both corners. No significant ratio difference was observed between 6, 9, and 14 months for both groups of animals. (c) There is no significant difference in lateral stepping performance between WT and Tg animals indicating that no motor deficits in 18-month old Tg animals.

25 months of age. At that age, transgenic rats displayed a severe olfactory deficit. In order to label proliferative cells, BrdU (100 mg/Kg) was injected intraperitoneally once a day during 5 consecutive days and the animals were sacrificed 5 hours after the last injection. This protocol was aimed to detect local proliferation rather than neurogenesis as changes in local OB proliferation could take part in the olfactory alterations observed in our transgenic rat.

2.3. Tissue Preparation. All animals were deeply anesthetized with Rompun/Ketamine (1 mL/Kg i.m.) and transcardially perfused with ice-cold 4% paraformaldehyde in phosphate buffered (PB). Brains were rapidly removed, immersed in the same fixative for 24 h at 4°C and stored in 15% sucrose in PB for 48 hours and then in 30% sucrose for an additional 24 hours. Brains were then frozen at −40°C in isopentane (Prolabo, Fontenay-sous-Bois, France). Serial sixteen-micrometer-thick coronal sections through the whole brain were cut on a cryostat (Leica, CM 3050) and then collected on gelatin-coated slides.

2.4. Immunohistochemistry. The brain sections were thoroughly washed with PBS prior to immunohistochemical labeling. They were then labeled with antibodies against TH to identify catecholaminergic neurons (1 : 1000; Pel-Freeze, Brown Deer,WI), against the human-α-syn to characterized neurons expressing the human α-syn (1 : 500; Invitrogen, Cergy Pontoise, France) and against BrdU to quantify proliferative cells (1 : 200; BD, USA). One section out of 6 serial sections was stained for each immunohistochemistry labeling.

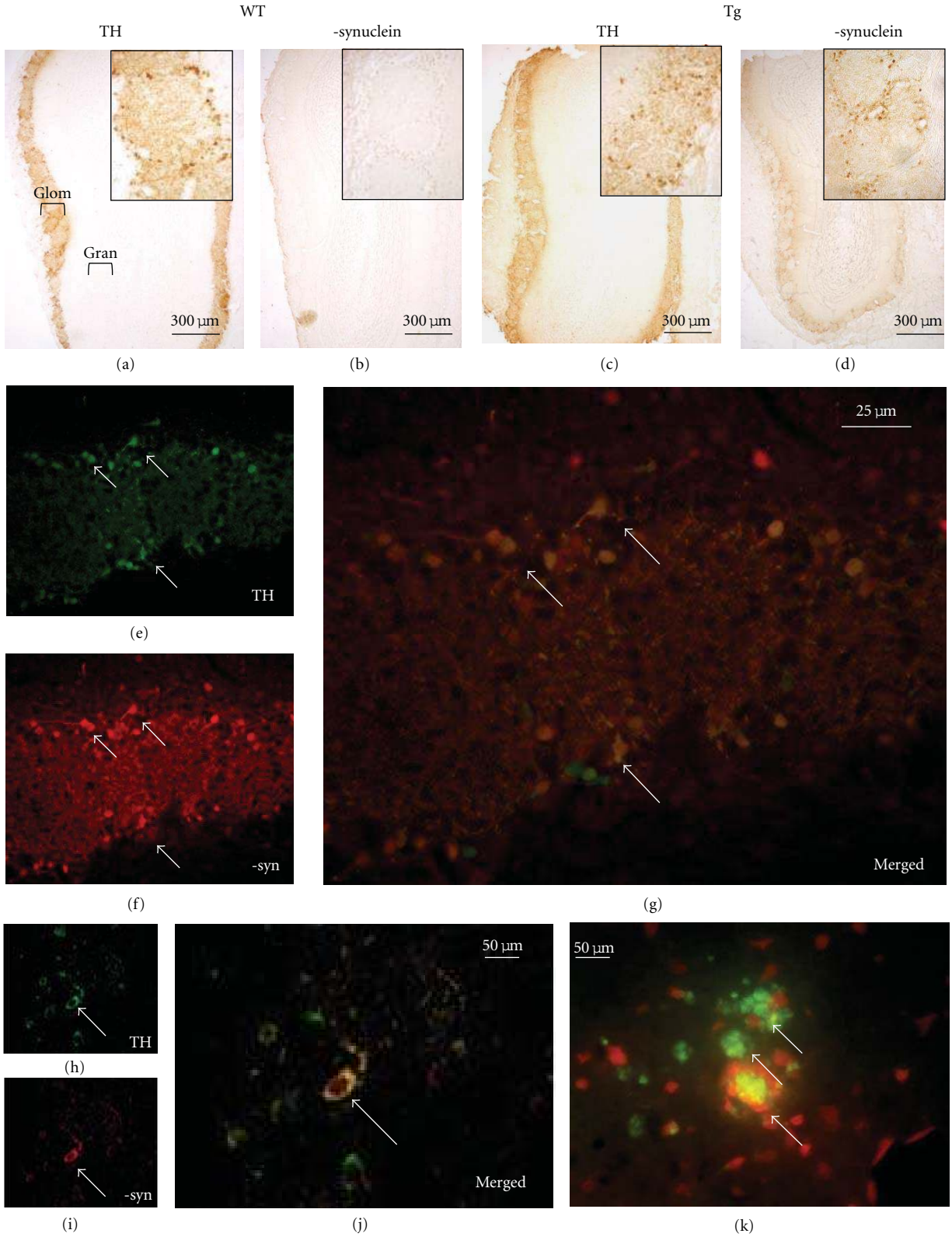

Figure 2: Alpha-synuclein, tyrosine hydroxylase (TH), thioflavin T, and BrdU stainings in wild-type (WT) and transgenic (Tg) rats. Overview of TH expression in wild-type (WT, a) and transgenic (Tg) olfactory bulbs (c). Alpha-syn immunostaining on a section of olfactory bulb of WT (b) and transgenic rat (d). Higher magnification of a Tg glomerular layer stained for TH in green (e), for α-syn in red (f), and with merged stainings (g). Arrows point out some of the many neurons expressing both TH and α-syn (stained in yellow in g). Confocal visualizations in transgenic rat of TH (h), human α-syn (i), and merged TH and α-syn (j). Arrows in h, i, and j point out a TH positive neuron (in green) expressing the human α-syn (in red). (k) Visualization of protein aggregates (arrows) in a Tg glomerular layer using thioflavin T, cell bodies are in red.

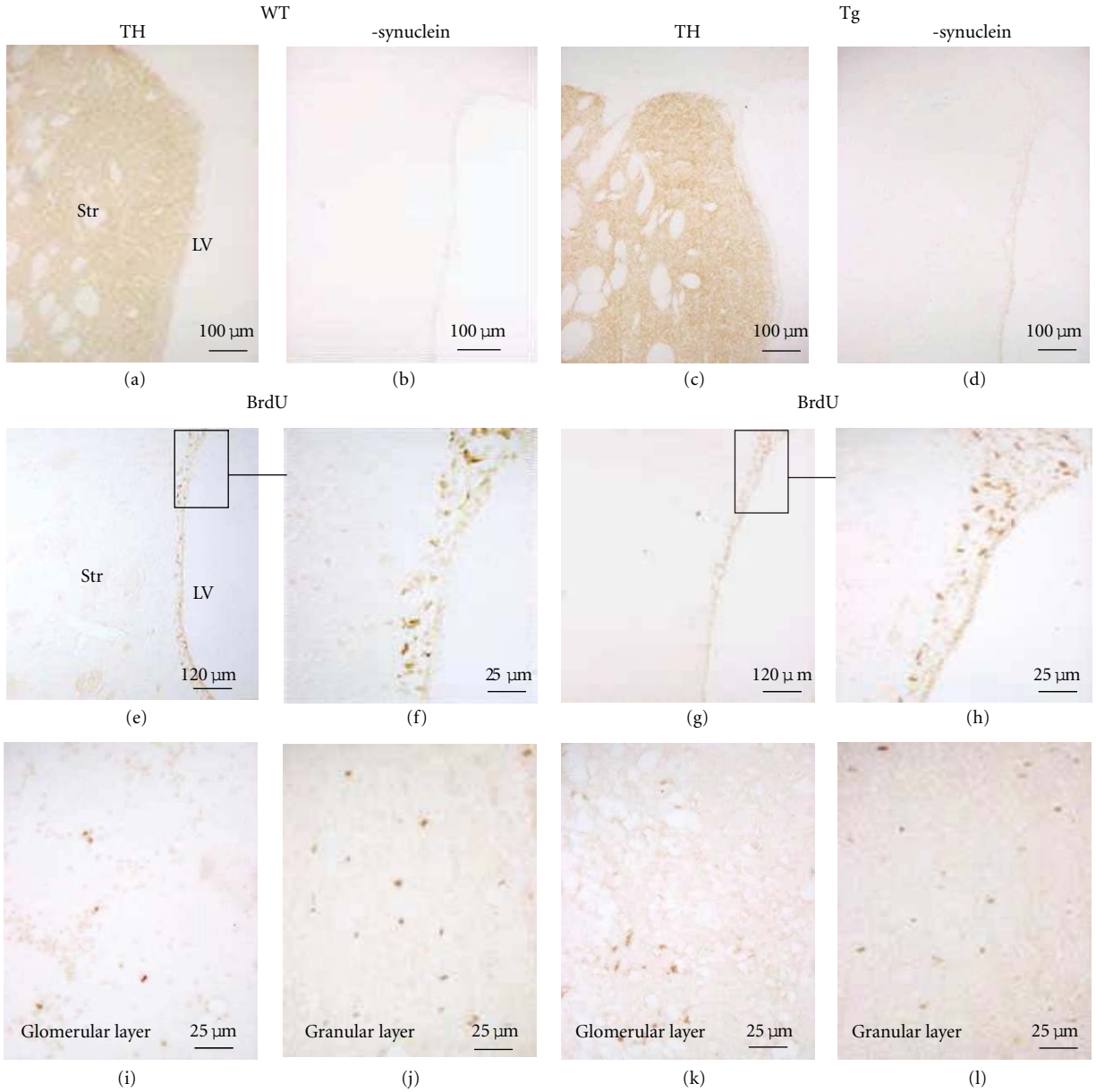

Figure 3: Alpha-synuclein, tyrosine hydroxylase (TH), and BrdU immunoreactivity in wild-type (WT) and transgenic (Tg) rats. TH and α-syn immunostainings in SVZ in a WT rat (a, b) and in a transgenic rat (c, d). BrdU immunostaining in SVZ in a WT rat (e, f) and in a transgenic rat (g, h). No difference in the number of BrdU positive cells is observed between both groups. F and H are a higher magnification of E and G, respectively. LV: lateral ventricle; Str: striatum. BrdU immunostaining in glomerular layer (I: WT; K: Tg) and granular cell layer (J: WT; L: Tg) of a section of an olfactory bulb. We can observe an increase of the number of BrdU positive cells in the glomerular layer of transgenic rats as compared to WT animals.

Briefly, after treatment with H_2O_2 3% in PBS, sections were incubated overnight in a dilution of primary antibodies. Then, sections were immersed in a 1 : 500 dilution of secondary biotinylated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Then sections were transferred to a Vectastain ABC Kit/PBS for 1 hour (Vector Laboratories, Burkingame, CA); 3,3 diaminobenzidine served as chromogen in the subsequent visualization reaction.

For double immunochemistry labeling and confocal visualization of TH and human α-syn, immunofluorescent secondary antibodies were used (anti-mouse IgG alexa568; Invitrogen, Cergy Pontoise, France and anti-rabbit IgG FITC; JacksonimmunoResearch Laboratories, West Grove, PA).

For BrdU immunohistochemistry, we used a DNA denaturation method consisting in first 30 min incubation at 37°C with 2 N HCl in PBS followed by a second incubation in 0.1 M Borax, pH : 8.6 for 30 min.

Thioflavin T (Sigma, St Louis, USA) was used to detect amyloid structure in proteins. Thioflavin T is a reagent known to become strongly fluorescent upon binding to amyloid fibrils. After 3 washes in PBS, brain sections were immersed in a 1 : 500 dilution of TO-PRO-3 (Invitrogen,

Cergy Pontoise, France) to label the cell body in red. Then sections were incubated for 1 hour with thioflavin T (0.04%) in a glycine solution (50 mM).

2.5. Quantification Procedure

2.5.1. Proliferative Cells. For BrdU quantification of positive cells within the SVZ, a rectangle (2500 × 1000 mm) was drawn around the structure and all stained cells (with a clearly visible positive nucleus) were counted on each section for a total of 10 equally spaced sections from bregma levels 1.70 to −0.40 mm. The mean of these 10 values (one value per section) was calculated giving one final value per animal.

To get an unbiased estimate of the density of BrdU positive cells within the OB granular and glomerular layers, we used the dissector principle and random systematic sampling [27]. The Mercator stereology analysis software (Explora Nova, La Rochelle, France) was utilized to perform unbiased stereological counts of BrdU positive cell. For the unbiased quantification, a line was drawn around the granular or glomerular layers of each section (12 sections from the 12 different rostrocaudal levels in right and left side of the brain were used). The observer was blinded to the rat group. Cells were counted with a 40X objective (NA, 0.85) using a Nikon Eclipse E600 microscope (Tokyo, Japan) with a motorized stage (x, y, and z). Random and systematic counting frames (50 × 50 μm squares, regularly spaced by 200 μm) were used [28]. Only BrdU positive cells within the frame were counted on sections (16-um serial sections, one every six sections). A BrdU positive neuron was defined as a clearly visible BrdU-immunoreactive nucleus.

The total number of BrdU positive neurons in both layers was also calculated using the formula $N_t = V_t/Vu \times Nu$, where N_t is the total number of BrdU positive neurons in the layer, V_t is the total volume of the layer, Vu is the unit volume in which the number of neurons was counted, and Nu is the number of neurons counted in the unit volume. The average of the total amount of cells for each group within the granular and the glomerular layers was then statistically analyzed.

The global volume of the glomerular layer and granular cell layer was estimated without bias (i.e., without systematic error) from the profile areas of the cut sections of the glomerular and granular cell layers. An unbiased estimate of each layer's volume was done using Cavalieri's principle. Accordingly, we multiply the sum of the profile areas of each layer on all sections (regularly spaced) with the distance between the sections [29]. With V_t = sum of profile areas × spacing between sections, spacing between sections = 16 μm (sections thickness) × 6 (one every six sections). The average of the total volume for all animals was then calculated.

2.5.2. TH Immunoreactivity in the OB. A measure of the width of TH immunoreactivity within the glomerular layer in the OB was performed. First, the OB was divided in 3 equal parts, from the anterior to the posterior axis (giving 3 values: one anterior (at bregma 6.7 mm), one medial (at bregma 6.2 mm), and one posterior (at bregma 5.7 mm)), each part containing 4 TH-stained sections spaced by 16 microns (for a total of 12 sections for the entire OB). Then, for each animal, these 12 coronal sections were visualized using the X4 magnification of an optical microscope (Nikon Eclipse E600, Tokyo, Japan). The width of TH staining corresponding to TH innervation was measured at 3 dorso-ventral levels per section (dorsal, median, and ventral levels; see Figure 4) in both left and right OB using the ImageJ software (National Institutes of Health, Bethesda, MD). Then the left and right measures from these 3 dorsoventral levels obtained for each section were added together and the mean of the 4 values from each 3 anterior-posterior OB levels was calculated giving one final value per animal for the anterior part of the OB, one final value per animal for the medial part of the OB and one final value per animal for the posterior part of the OB.

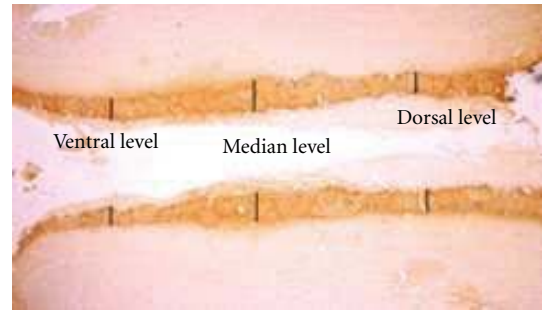

Figure 4: Representation of the 3 dorso-ventral levels used to quantify the width of TH innervation in the olfactory bulb glomerular layer in wild-type and transgenic rats.

2.5.3. Area of the Glomeruli. A measure of the area of the glomeruli in the OB was performed on 12 TH stained coronal sections (from bregma 6.7 mm to 5.7 mm) in 3 transgenic and 3 wild-type animals using the ImageJ software (National Institutes of Health, Bethesda, MD). This quantification was performed in order to investigate if a variation in glomeruli area could interact with a difference in glomerular layer TH innervation pattern between the 2 groups of animals. A total of 45 to 50 areas of glomeruli were measured per animal. Seven area intervals were arbitrary used to rank the glomeruli per size: 0–4330 μm^2; 4331–8660 μm^2; 8661–12990 μm^2; 12991–17320 μm^2; 17321–21650 μm^2; 21651–25980 μm^2; 25981–30310 μm^2. Data are represented as the percentage of glomeruli for each area interval for each animal.

2.6. Statistical Analysis of the Data. Results from the behavior were expressed as mean± SEM and analyzed using an ANOVA and Mann-Whitney. Data from BrdU labeling in the SVZ and in the OB, glomerular TH innervation in the OB and glomeruli area were expressed as mean ± SEM. The density and total number of BrdU-positive cells and the width of glomerular TH innervation were then analyzed using the Mann-Whitney statistical test (one-tailed; PRISM; Graph Pad 4.0 software, CA, USA).

3. Results

3.1. Generation of the A30P and A53T α-syn Transgenic Rat. Using the transgene construction from Richfield et al. ([14], see Figure 1), 3 lines of transgenic rats expressing both the A30P and A53T mutated forms of the human α-syn gene under the control of the TH promoter have been generated. However, only 2 founders were able to transmit the transgene to several generations of offsprings as characterized by PCR (not shown). The MA3 line characterized by 1 or 2 transgene copies was used in our present investigation.

3.2. Investigation of the Olfaction. In the open field, the habituation pattern was identical for both groups of rats (no corner preference). During the olfaction test, WT rats were attracted by the odor of the coconut milk and spent significantly more time in the corner with the coconut milk filter paper as compared to the corner with the distilled water. A ratio of 2 indicates that the rat spent twice as much time visiting the corner with the coconut milk as compared to the corner with the distilled water. On the contrary, transgenic animals significantly spent less time in the corner with the coconut milk as a ratio of 1 corresponds to the same period of time spent in both corners. Therefore, transgenic animals presented olfactory impairments when compared to WT animals (Figure 1(b)). This impairment remained stable from 6 months of age.

No gross alteration in locomotion pattern was observed during the habituation and the olfaction test between both groups of animals.

3.3. Lateral Stepping Test. No significant difference in movement initiation was observed between WT and Tg rats from 14 to 18 months (Figure 1(c)). Results from gross locomotor observations during the investigation of olfaction were confirmed in older rats with the lateral stepping test.

3.4. Immunohistochemistry. Distribution of human α-syn protein and DA-labeled cells/processes in the OB and the SVZ was evaluated by immunohistochemical investigation. All transgenic MA3 brains expressed human α-syn protein labeling in the OB (Figure 2(d)). Tyrosine hydroxylase and human α-syn immunostainings were only detected in the OB glomerular layer (Figures 2(a), 2(b), 2(c) and 2(d)) of transgenic rats (the same location where the TH staining was observed in WT animals, Figure 2(a)). Higher magnification using merged fluorescence of TH and α-syn stainings showed than most of TH positive neurons in the OB glomerular layer also expressed the human α-syn (Figures 2(e), 2(f), and 2(d)). The confocal analysis confirmed than both human α-syn and TH molecules were colocalized in the same cell bodies and processes with a diffuse cellular α-syn staining pattern. The cell body contained dense patches positive for human α-syn staining and a prominent immunoreactivity in the processes (Figures 2(h), 2(i) and 2(g)). In addition, using the Thioflavin T staining, numerous aggregates of protein were noticed within the cell body (Figure 2(k)). The antibody used to detect the human α-syn was validated by the absence of any human α-syn staining in the OB of WT rats (Figure 2(b)). Neither human α-syn positive cells nor TH-labeled cells were visualized within the SVZ from WT or transgenic animals (Figures 3(a), 3(b), 3(c), 3(d)).

BrdU-positive nuclei were observed in the SVZ area in both WT and transgenic rats (Figures 3(e), 3(f), 3(g), and 3(h)). Proliferative-BrdU-labeled cells were also noticed in the OB glomerular (Figures 3(i), 3(k)) and granular cell layers (Figures 3(j), 3(l)). Fewer cells were noted in the WT glomerular layer than in the Tg glomerular layer.

3.5. Quantification of SVZ Proliferation. We performed counts of BrdU immunoreactive cell bodies to measure the level of SVZ proliferation. No significant difference in BrdU positive cells within the SVZ was noted between the 2 groups of rats (Figures 3(e), 3(f), 3(g), 3(h), and Table 1).

3.6. Quantification of OB New Generated Cells Within Glomerular and the Granular Cell Layers. Following BrdU injections, proliferation in the OB was assessed using unbiased stereology. When comparing WT and transgenic rats, no significant difference was observed in the density of resident proliferated cells in the granular cell layer (mean density of BrdU positive cells in transgenic group: 6.32×10^3 versus 5.78×10^3 in the WT group: (Figures 3(j), 3(l), and Table 1). In contrast, we observed a statistically significant increase in the density of BrdU positive cells in the glomerular layer of transgenic rats as compared to WT animals (+68%; $P < .05$; Figures 3(i) and 3(k) and Table 1). The analysis of the total number of local proliferative cells also showed a significant increase only in the glomerular layer of the transgenic animals as compared to the WT rats (Table 1).

No significant variation in the volume of the glomerular and granular layers was observed between the 2 groups of animals (Table 1).

3.7. Quantification of DA Innervation in Glomerular Layer. To analyze DA innervations in the OB (Figure 4), the width of the TH positive area within the glomerular layer evidenced by TH immunolabeling was measured at 3 different bregma levels per section (anterior (6.7 mm), medial (6.2 mm) and posterior (5.7 mm)). An overall 7.9% increase in the width of TH innervation was observed in the transgenic rat as compared to WT animals. However, when the OBs were divided along the anterior-posterior axis, only the width calculated at Bregma 6.2 mm level was significantly increased by 12.4% in transgenic rat as compared to WT rats ($P < .01$; Table 2).

3.8. Area of the Glomeruli. Glomeruli were distributed from areas inferior to 4330 μm^2 to a maximum area of 30310 μm^2 with most of them having an area comprised between 8660 μm^2 and 12990 μm^2. No significant difference was observed concerning the percentage of glomeruli in each area interval, except for the smaller interval where significantly more transgenic glomeruli (3.47%) were contained in the "0 to 4330 μm^2" area interval as compared to 1.40% for the WT glomeruli ($P < .05$; Table 3).

Table 1: Quantification of BrdU positive cells and layer's volume in wild-type and transgenic rats from the olfactory bulb glomerular and granular cell layers and in the SVZ. Mean ± SEM. $^{*}P < .05$; ns: no statistical difference between the 2 groups. Only the number of BrdU positive cells in the glomerular layer of transgenic rats was significantly increased by 68% as compared to WT animals. Values are expressed as density per mm^3 and total number of BrdU positive neurons.

	Wild-type group	Transgenic group	$P < .05$
Glomerular layer density in mm^3	$1.72 \times 10^3 \pm 0.38 \times 10^3$	$2.90 \times 10^3 \pm 0.51 \times 10^3$	*
Glomerular layer total number	$2.35 \times 10^3 \pm 0.52 \times 10^3$	$4.13 \times 10^3 \pm 0.72 \times 10^3$	*
Granular cell layer density in mm^3	$5.78 \times 10^3 \pm 0.79 \times 10^3$	$6.32 \times 10^3 \pm 1.14 \times 10^3$	ns
Granular cell layer total number	$16.6 \times 10^3 \pm 2.28 \times 10^3$	$19.27 \times 10^3 \pm 3.48 \times 10^3$	ns
Subventricular zone total number	$85.1 \times 10^3 \pm 21.4 \times 10^3$	$121 \times 10^3 \pm 16.1 \times 10^3$	ns
Glomerular layer volume in mm^3	1.364 ± 0.21	1.423 ± 0.06	ns
Granular layer volume in mm^3	2.872 ± 0.41	3.050 ± 0.202	ns

Table 2: Measure of the width of TH innervation in the olfactory bulb glomerular layer in wild-type and transgenic rats. The width was measured in the anterior level (bregma 6.7 mm), in the median level (bregma 6.2 mm), and in the posterior level (bregma 5.7 mm) of the olfactory bulb. Mean ± SEM. $^{**}P < .01$; ns: no statistical difference between the 2 groups. A significant increase of the TH innervation was observed for bregma 6.2 mm level in transgenic as compared to wild type rats.

	Wild-type group	Transgenic group	$P < .01$
Bregma: 6.7 mm	434.2 ± 16.46	480.9 ± 34.23	ns
Bregma: 6.2 mm	572.5 ± 17.63	643.6 ± 8.18	**
Bregma: 5.7 mm	684.4 ± 51.34	701.0 ± 48.79	ns

4. Discussion

Our study used the first α-syn transgenic rat bearing the human A30P and A53T mutations under the control of the TH promoter. As previously stated [19, 20] transgenic animals displayed some long-lasting olfactory deficits and the human-mutated α-syn protein was observed in the OB, the SNpc, and the LC. It was colocalized with TH immunostaining (as shown for the OB in the present paper) which is consistent with the fact that TH was the transgene promoter. Olfactory deficits appeared long before the motor alterations as 18-month old animal did not present yet any deficit in movement initiation. Deficits in motor coordination appeared at 19 months of age (not shown). Twenty-five-month-old transgenic rats were used in this study as clinical and pathological manifestations of the α-syn mutations appear in advanced age in PD, generally [22]. Tyrosine hydroxylase was used as the promoter in order to obtain the human α-syn synthesis only in catecholaminergic structures. Indeed, we were able to observe transgene expression in the 3 main catecholaminergic brain areas involved in the course of PD: the OB, the SNpc, and the locus coeruleus. To date, there is a growing evidence of a prion-like transmission of α-syn contained in aggregates from donor cell to recipient cell [30]. However, it did not seem to be the case in our transgenic rat as non-TH positive brain structures did not contain any human α-syn molecule. However, we cannot rule out that this mechanism did not happen within catecholaminergic structures, thus potentiating the effect of the transgene.

The OB is a brain region of particular interest because Lewy neurites and bodies are present in this area in the very early stages of the PD [31]. These inclusions consist of aggregated form of α-syn with other components such as phosphorylated neurofilaments and ubiquitin [31]. As in PD patients, we have shown that our mutant human A53T and A30P α-syn expressing rat presented protein aggregates in the glomerular layer suggesting an implication of the human-mutated α-syn in the cellular processing of aggregates, which could in turn alter local OB proliferation.

Our data showed an increased number of proliferative cells in the glomerular layer but not in the granular cell layer. It is worth mentioning here that the BrdU protocol used in the present study rather revealed local OB proliferation than migrated cells from the SVZ to the OB as the animals were given BrdU for 5 days and sacrificed 5 hours after the last injection.

Interestingly, Winner et al. [32] showed in 2-month-old female Wistar rats that the local dividing cells represented less than 5% of the total number of new cells. Their total number of BrdU positive cells in the granular (8,200 cells) and glomerular layer (250) are lower than ours (16,600 cells and 2,350 cells, resp.). This important difference in numbers can be related to the concentration of BrdU used in Winner's study being half of the one we used, to the time of the sacrifice after the last injection (2 h versus 5 h in our study), to the age of the animal (2 months versus 25 months) and could point out for a few cells an increase in granular and glomerular layer local proliferation due to aging.

No variation in the SVZ proliferation was induced by the double α-syn mutation. This later result can be explained by the absence of any transgene expression within the SVZ. Our observation in the SVZ is in agreement with the findings of Maxreiter et al. [21] using a mouse expressing the human A30P mutant form of the α-syn, who did not find any change in SVZ proliferation. Using mice expressing the A30P mutant form of α-syn under the control of the calcium/calmodulin-dependent protein kinase II alpha (CaMK) promoter [21]

Table 3: Distribution of glomeruli (in %) by area from TH-stained sections in wild-type and transgenic groups. Seven intervals of area were used: 0–4330 μm^2; 4330–8660 μm^2; 8660–12990 μm^2; 12990–17320 μm^2; 17320–21650 μm^2; 21650–25980 μm^2; 25980–30310 μm^2. Mean ± SEM. $^*P < .05$; ns: no statistical difference between the 2 groups. There is no significant difference between both groups except for the smaller interval.

Interval	Wild-type group	Transgenic group	$P < .05$
0–4330 μm^2	1.403 ± 0.701	3.471 ± 0.486	*
4331–8660 μm^2	17.06 ± 3.556	19.11 ± 1.780	ns
8661–12990 μm^2	37.67 ± 3.353	31.26 ± 0.077	ns
12991–17320 μm^2	31.72 ± 4.339	22.56 ± 3.162	ns
17321–21650 μm^2	7.423 ± 2.246	17.63 ± 3.261	ns
21651–25980 μm^2	3.405 ± 0.642	3.217 ± 1.725	ns
25981–30310 μm^2	1.307 ± 1.307	2.731 ± 0.254	ns

or expressing the A53T mutant form of α-syn under the control of the PDGF-promoter [22], two groups studying OB neurogenesis found a decrease in newly generated neurons in the glomerular and granular layers. Taken together, the data on OB neurogenesis and from our own investigation suggest that human α-syn A30P/A53T mutations impacts newly generated neuroblasts during OB integration/differentiation as well as local OB proliferation. In contrast to our observations and certainly due to the promoter they used, Winner et al. [22] and Maxreiter et al. [21] also observed some transgene expression in noncatecholaminergic structures. Some data suggest that the mutated α-syn could spread using a prion-like transmission from cell to cell [30]. As a result, it is possible that a more "α-syn toxic brain environment" was created in the A30P and the A53T transgenic mouse brains than in our rat brain. The increased local proliferation that we noticed in the glomerular layer is in agreement with data from the glomerular layer of PD patients [23]. This later finding suggests that our rat model is a suitable tool concerning the effects of the α-syn mutations in the OB.

As proliferative cells within the glomerular layer are known to differentiate in DA neurons [24], we investigated the TH innervation within the glomerular layer. We observed an increase in width of the TH positive area in the glomerular layer without an increase in the size of the glomeruli (except for the smaller interval in transgenic animals) suggesting that this increased TH innervation was not induced by an increased glomerular layer areas (as an increased glomeruli size would have increased the size of the glomerular layer which in turn could have enlarged the pattern of TH innervation). This result is agreement with the 100% increase in DA cell number in the glomerular layer from PD patients [23]. Although the mechanisms underlying the enhanced DA innervation in the OB glomerular layer remains to be determined, various growth factors which play an important role in OB proliferation and DA differentiation could be involved such as BDNF, GDNF and CNTF [33]. Interestingly, this increase in DA innervation observed in our transgenic rat and in PD patients could explain, at least in part, the olfactory deficit observed both in our rat and in patients as DA in the OB has an inhibitory action. Hyposmia can be detected in PD patients in early stage of disease. Our transgenic rats have been tested for olfactory function at different ages (from one week to 25 months of age) and at 6 months of age they presented an alteration of olfaction. Dopamine has an important role in mediating olfactory information into the brain [34]. TH innervation is found exclusively in glomerular layer of the OB [25]. In our rat, the increase in TH innervation in this area might suggest an increase in DA release. DA is known to induce an inhibition between olfactory receptor cells and mitral cells within glomerular layer [23]. D2 receptors are the most abundant subtype of DA receptors in the glomerular layer [35] and are involved in the decrease in synaptic transmission [34]. The increase of DA neurons caused by the A30P and the A53T mutant forms of α-syn could induce a depression in synaptic transmission and therefore compromises the threshold for olfaction. This circuit is the first step in the process of final consciousness of smell and therefore is essential for the proper function of olfactory circuits. Data from biopsies of patients diagnosed with PD support the idea that olfactory impairment in PD do not result from damage to the olfactory epithelium but is the consequence of central-nervous alterations [36]. Thus our rat could be a good model to investigate the role of human mutated α-syn in the development of olfactory deficits.

In conclusion, we generated a human double mutated α-syn (A30P and A53T) transgenic rat presenting an alteration of the local proliferation in the glomerular layer but neither in the granular cell layer of the OB nor in the SVZ. In addition, an increase in DA glomerular layer innervation was noticed, which might be related to the increased proliferation observed in this layer. Further investigation should examine the time course of the changes in the olfactory function in regards to alterations in OB local proliferation as well as elucidate the role of the increased DA function in the olfactory deficits we observed in our transgenic rat.

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