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Review

Application of neurotoxin- and pesticide-induced animal models of Parkinson's disease in the evaluation of new drug delivery systems

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Parkinson's disease (PD) is the second most prevalent neurodegenerative disease after Alzheimer´s disease. It is characterized by motor symptoms such as akinesia, bradykinesia, tremor, rigidity, and postural abnormalities, due to the loss of nigral dopaminergic neurons and a decrease in the dopamine contents of the caudate-putamen structures. To this date, there is no cure for the disease and available treatments are aimed at controlling the symptoms. Therefore, there is an unmet need for new treatments for PD. In the past decades, animal models of PD have been proven to be valuable tools in elucidating the nature of the pathogenic processes involved in the disease, and in designing new pharmacological approaches. Here, we review the use of neurotoxin-induced and pesticide-induced animal models of PD, specifically those induced by rotenone, paraquat, maneb, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-OHDA (6-hydroxydopamine), and their application in the development of new drug delivery systems for PD.

Keywords: Parkinson's disease, rotenone, paraquat, maneb, MPTP, 6-OHDA, animal model, controlled drug delivery

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INTRODUCTION

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder after Alzheimer´s disease. It is a chronic and progressive disease that mainly involves dopamine depletion in the central nervous system (CNS). It is characterized by the early death of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) and the accumulation of Lewy bodies, which are abnormal intracellular aggregates containing proteins, such as α-synuclein and ubiquitin (1, 2). This deficiency of dopamine in the basal ganglia causes movement disorders resulting in the typical symptoms of the disease, such as akinesia, bradykinesia, rigidity, resting tremor and postural abnormalities. Moreover, PD is also associated with several non-motor symptoms, such as olfactory dysfunction, cognitive impairment, psychiatric symptoms, sleep disorders, neurodegenerative dysfunction, pain and fatigue, among others (3).

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Diagnosis of the disease is currently based on medical history, physical and neurological examination of the subject and the presence of certain symptoms. The latest advances in diagnosis (functional imaging, neuropsychological tests) can help the diagnosis based on a suspicion related to the clinical evaluation of the symptoms and signs present in the patient (4, 5). Currently, there are no biological markers that allow for a quick and accurate diagnosis of PD. For instance, the clinical criteria used by the UK Parkinson Disease Society Brain Bank, which are commonly used, have a diagnostic accuracy of only 80 % at the early stages of PD (6). Therefore, functional imaging is necessary to confirm the clinical diagnosis and understand the underlying pathophysiology. The definitive diagnosis is the presence of SNpc degeneration and Lewy pathology in the postmortem examination (7).

Treatment of PD is only symptomatic since there is no cure for the disease, with many of the drugs aiming to increase dopamine concentrations or directly stimulate dopamine receptors. The American Academy of Neurology recommends initiating one of the following available drug therapies once the patient has developed symptoms of functional disability (8). Since dopamine does not cross the blood-brain barrier BBB and direct infusion into the brain is not possible in human subjects, the clinical management of PD is usually performed by means of the administration of dopamine agonists, the dopamine precursor L-DOPA, catechol-*O*-methyltransferase (COMT) inhibitors and monoamine oxidase B (MAO-B) inhibitors, *N*-methyl-D-aspartate (NMDA) receptor inhibitors, anticholinergics, alone or in combination. Dopaminergic therapy is highly effective in bradykinesia and stiffness in which dopamine and levodopa agonists help reduce disease progression and disability. Tremor responds to anticholinergic drugs such as trihexyphenidyl, but with a poor and inconsistent response to dopamine replacement therapy (9, 10).

With the need for new drugs and more effective treatments, several models have been developed to mimic features resembling those of human PD in experimental animals, and in order to design and evaluate drug molecules that favor neuroprotection and decrease neuronal cell death as occurs in PD. In this work, we present a review of the main experimental models of PD based on the use of pesticides or neurotoxins, and their relevance for the study and knowledge of the disease, with a special focus on the application of these experimental models in the development of controlled drug delivery systems for the treatment of PD.

In recent years, new drug delivery systems are being developed to improve the efficacy of the pharmacological treatments of PD. In this regard, the design of controlled delivery systems is of great interest as they can maintain constant drug levels for prolonged periods of time, which allows reducing the frequency of administration and improve patient compliance, among other benefits. They can be either biodegradable or non-biodegradable depending upon the nature of the polymer or material used for their fabrication. Among controlled drug delivery systems, the hydrogels and polymeric or lipid microparticles (MPs) and nanoparticles (NPs) seem to be the most effective in providing neuroprotection and facilitating the delivery of drugs and small molecules to the brain. *E.g.*, lipid-based nanocarriers are very interesting for the development of nanosystems destined to reach the CNS after intranasal administration.

MODELS BASED ON PESTICIDES

Pesticides are substances of natural or synthetic origin used to exterminate, prevent, and control any unwanted pest or organism and can be classified as insecticides, herbicides and fungicides (11). In the late 1980s, several epidemiological studies found an increased

risk of developing PD in populations exposed to pesticides, herbicides and industrial compounds (12). A recent study performed by Belvisi *et al*. (13) indicated that in five out of eight studies reviewed (14–18) greater risk of developing PD was associated with such substances but in the other three studies (19–21) no significant association was found between occupational exposure to pesticides and PD. These opposite results could be explained by the cohort sizes and follow-up duration of the different studies which were not comparable. Therefore, correlations between agrochemical exposure in populations and increased risk of developing PD have been difficult to conclude due to a lack of details on exposure to particular agents. Nevertheless, the emphasis has been made on rotenone, paraquat and maneb, as possible environmental causes of PD.

Rotenone

Rotenone (RT) is an organic pesticide extracted from the roots of some tropical plants of the genera *Lonchocarpus* and *Derris.* Chemically it is the (2*R*,6a*S*,12a*S*)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-*b*]furo(2,3*H*)chromen-6-one (Table I). Due to its high liposolubility, it can pass through biological barriers and cell membranes (22). RT was firstly used as a causative agent of PD in 1985 when Heikkila *et al*. (23) injected this mitochondrial complex I inhibitor directly into the brain of rats. They demonstrated that at a concentration of 5 mmol L^{-1}, approximately five hundred thousand times greater than its maximal inhibitory concentration (CI_{50}), RT was able to eliminate dopaminergic neurons. This contribution opened a new field of research since PD was related to systemic mitochondrial defects. In this respect, various research groups began to work with the systemic administration of mitochondrial toxins such as rotenone to investigate PD. Ferrante *et al*. (24), reported that the administration of rotenone (10–18 mg kg^{-1} per day) produced non-specific brain lesions and peripheral toxicity in rats. Betarbet *et al*. (25) demonstrated that the administration of RT (2–3 mg kg^{-1} per day) to rats for 33 days caused selective nigrostriatal dopaminergic degeneration which could be associated behaviorally with hypokinesia and rigidity. They also found fibrillar cytoplasmic inclusions containing ubiquitin and α-synuclein proteins in nigral neurons of RT treated rats.

It was also reported that systemic administration of RT may reproduce other features of PD in experimental animal models such as the alterations of the retina, loss of testosterone and sleep disorders (26–28). Other studies performed in mice have demonstrated that the selective toxicity of RT can be related to inhibition of the NADH gene dehydrogenase ubiquinone proteins Fe-S4 (NDUFS4) which encode the mitochondrial complex I which is inhibited by RT. Other underlying mechanisms have also been reported for RT, such as depolymerization of microtubules and accumulation of reactive oxygen species (ROS) in mesencephalon cultures (29).

In summary, the RT model is one of the most frequently used models for inducing parkinsonian symptoms in experimental animals due to the following characteristics:

(*i*) RT model reproduces most of the motor disorders and histopathological features of PD, including Lewy bodies and some conditions associated with α-synuclein (α-Syn) accumulation (30, 22),

(*ii*) RT and other pesticides are potent mitochondrial inhibitors associated with a high incidence of sporadic PD in humans mainly in rural/agricultural areas,

(*iii*) RT is a lipophilic compound and, as such, can pass through biological barriers without relying on transporters (22).

However, RT has some drawbacks such as high photoreactivity and systemic (cardiovascular) toxicity which leads to significant mortality rates (~30 % of the animals), regardless of the administration route (25). This toxicity makes it necessary to use a large number of animals to assure the statistical significance of the results obtained when performing behavioral, biochemical and histological analysis. Moreover, due to its low water solubility selection of the appropriate vehicle and the administration route is more complex (31). Another drawback, as indicated previously, is that RT also causes non-PD-related symptoms such as cardiovascular effects (32).

RT is usually dissolved in polar vehicles with the incorporation of surfactant agents such as carboxymethyl cellulose (CMC) or naturally occurring oils (sunflower, castor oil). The incorporation of dimethylsulfoxide (DMSO) can minimize the use of surfactants or even prevent it (33). Regarding the route of administration, RT can be given either subcutaneously, intraperitoneally, orally or intracerebrally with the selection of the dose depending on the administration route and the type of treatment chosen (acute or chronic) (34).

Controlled-release systems using the rotenone model. – Some of the new controlled-release systems have been recently evaluated in RT-induced animal models of PD. For example, rasagiline mesylate (RM) is a potent, selective and non-reversible MAO-B inhibitor that exhibits neuroprotective effects and it's currently used as monotherapy in early PD. However, due to its short elimination half-life (1.5–3.5 h), RM must be given orally in daily doses. Kanwar *et al*. (35) encapsulated RM in polycaprolactone microspheres (MPs) in order to expand the dosing intervals, taking into consideration that patients with PD develop dysphagia, *i.e.* difficulty in swallowing. The new formulation was evaluated in Sprague-Dawley rats after stereotaxic administration of RT [6 mg in 2 mL DMSO:PEG (1:1)]. The administration of the RM-loaded formulation improved several behavioral (locomotor activity, grip strength) and biochemical (lipid peroxidation, reduced glutathione, *etc*.) parameters. Non-significant differences were found between the daily administration of RM in solution and the polymeric MPs given once a month, which makes this approach very interesting for treating PD patients with dysphagia. Fernandez *et al*. (36) also developed a new formulation consisting of RM-loaded MPs using poly(lactic-co-glycolic) acid (PLGA) as a biodegradable polymer. The system was assayed in the RT model of PD induced in Wistar rats. Daily *i.p.* doses of RT (2 mg kg^{-1}) induced neuronal and behavioral changes similar to those occurring in PD. Once an advanced stage of PD was achieved, animals received RM in saline (1 mg kg^{-1} per day) or encapsulated within PLGA MPs (amount of microspheres equivalent to 15 mg kg^{-1} RM given on days 15 and 30). After 45 days, RM showed a robust effect on all analytical outcomes (brain histology, immunochemistry, behavioral testing) with non-statistically significant differences found between the administration of RM in solution or encapsulated within polymeric MPs.

Many of the experimental applications of the RT model found in the literature are related to the evaluation of dopaminergic agonists, a therapeutic strategy to provide continuous dopamine (DA) receptor stimulation. Most DA agonists show low bioavailability due to extensive first-pass metabolisms after oral administration, thereby implying the need for the daily administration of various doses. This fact makes them good candidates for the development of controlled delivery systems. With this aim, Barcia *et al*. (37) developed ropinirole (RP) PLGA nanoparticles (NPs) to improve the access of RP to the CNS. The RT model was induced in Wistar rats at a dose of 2 mg kg^{-1} per day given *i.p.* Once

neurodegeneration was established (15 days), animals received RP in saline (1 mg kg^{-1} per day for 35 days) or RP-loaded PLGA NPs (amount of NPs equivalent to 1 mg kg^{-1} per day, every 3 days for 35 days). Brain histology and immunochemistry Nissl-staining, glial fibrillary acidic protein (GFAP) and tyrosine hydroxylase (TH) immunohistochemistry, and behavioral testing (catalepsy, akinesia, rotarod and swim test) showed that RP NPs were able to revert PD like symptoms of neurodegeneration in the RT-induced model.

Negro *et al*. (38) developed RP-loaded PLGA MPs and assayed the formulation in the RT model induced in Wistar rats. After 15 days of daily *i.p.* doses of RT (2 mg kg^{-1}), animals received RP in saline (1 mg kg^{-1} per day for 45 days) or RP-loaded PLGA MPs at two dose levels (amount of MPs equivalent to 7.5 or 15 mg kg^{-1} RP given on days 15 and 30). All the outcomes analyzed (brain histology, immunochemistry, and behavioral testing) showed that animals receiving RP either daily in solution or every two weeks encapsulated within the MPs reverted the PD symptoms, with the best results obtained in animals receiving the RP microspheres at the highest dose level.

Patel *et al*. (39) have developed nanocarriers (microemulsions, ME) of RP for transdermal application which are considered highly efficient as colloidal soft nanocarriers in dermal and transdermal drug delivery systems. In this study and in order to achieve sustained and controlled permeation of RP through the skin, ME was converted into a ME-based gel (MEG) using a biocompatible gelling agent. The system was assayed in Sprague-Dawley rats after *s.c.* administration of RT (2 mg kg^{-1} per day) for 11 days. Chronic administration of RT caused the destruction of dopaminergic neurons resulting in motor dysfunctions (rigidity, slower movement, and inability to move). Administration of the MEG developed for RP (2.16 mg kg^{-1} per day *via* transdermal route) improved the motor function by 76 %, whereas the marketed tablet suspension of RP (2.16 mg kg^{-1} per day *via* oral route) showed only 5 % restoration of the normal function. Moreover, the new transdermal delivery system successfully restored the catalase and superoxide dismutase levels which were significantly reduced by RT administration.

Resveratrol (RSV) has recently drawn attention since it is considered a red wine-derived polyphenol with cardioprotective and neuroprotective effects. In this regard, Palle and Neerati (40) developed RSV-loaded nanoparticles (NPs) by temperature-controlled antisolvent precipitation. The new therapeutic system was evaluated for its neuroprotective effects in the RT-induced model in Wistar rats. For this, the administration of RT (2 mg kg^{-1}, *s.c.*) for 35 days produced motor deficits, decreased rearing behavior, mitochondrial dysfunction, and oxidative stress in the animals. Oral administration for 35 days of RSV-loaded NPs (40 mg kg^{-1}) 30 min before the administration of RT showed better efficacy than the oral administration of RSV in solution (40 mg kg^{-1}) in attenuating the RT-induced behavioral, biochemical and histological alterations observed in the animals.

Accumulation of α-Syn protein, mitochondrial dysfunction, oxidative stress, and neuronal cell death are among the main pathological hallmarks of PD. In this regard, the combination of piperine and curcumin may have potential interest due to their beneficial cognitive and antioxidant properties. However, the access of drugs to the brain is limited by the BBB. To overcome this passage, Kundu *et al*. (41), designed a new delivery system consisting of dual drug curcumin and piperine co-loaded glyceryl monooleate NPs (CPNPs) coated with various surfactants to improve their passage to the brain. The new system was evaluated in the RT-induced mouse model of PD after oral administration of RT (30 mg kg^{-1}) for 28 days. CPNPs were given orally at an equivalent dose of 200 mg kg^{-1}

every alternate day, 30 min before the administration of RT. The results obtained showed that the new formulation was able to cross the BBB, rescue the RT-induced motor coordination impairment, and restrained dopaminergic neuronal degeneration in the PD animal model.

The sapogenin (camelliagenin) isolated from *Camellia oleifera* seeds has demonstrated antioxidative, anti-inflammatory and analgesic activities which could be related to its neuroprotective effects. In this regard, Yang *et al*. (42) synthesized iron-sapogenin NPs. The neuroprotective effects of the formulation were evaluated in RT-induced neurodegeneration in Kunming mice injected *s.c.* with RT (50 mg kg^{-1} per day) for 6 weeks and treated by *i.v.* injection of the iron-sapogenin complex at three dose levels (25, 50 and 100 mg kg^{-1} for 7 days). Behavioral disorders were attenuated by the delivery system and increased superoxide dismutase activity, tyrosine hydroxylase expression, dopamine and acetylcholine levels in the brain were increased in a dose-dependent manner. Iron-sapogenin NPs showed significantly better effects than the sapogenin alone.

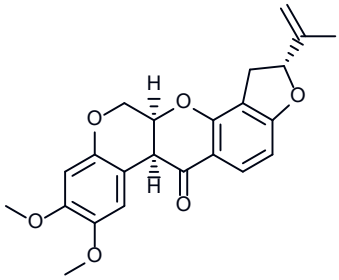
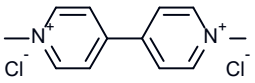
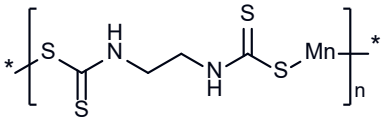
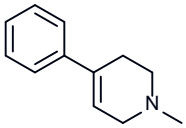
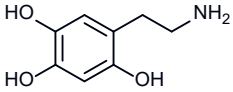
The occurrence of ocular manifestations in many neurodegenerative diseases, including PD, emphasizes the strong connection between the brain and the retina. PD patients often suffer from visual symptoms such as reduced visual acuity, low contrast sensitivity, and disturbed color vision. Normando *et al*. (43) used the RT-induced rodent model of PD to investigate retinal manifestations in PD. In this study, RT was injected *i.p.* to Dark Agouti rats at a dose of 2.5 mg kg^{-1} per day for 60 days. The retinal evaluation was assessed by optical coherence tomography (OCT), and by longitudinal *in vivo* imaging with detection of apoptosing retinal cells (DARC). The results obtained showed increased apoptosis of retinal ganglion cells with a transient swelling of the retinal layers after 20 days of initiating RT. By day 60, histological neurodegenerative changes in the *substantia nigra* and *striatum* were observed suggesting that retinal changes precede the "traditional" pathological manifestations of PD. The efficacy of a novel rosiglitazone (RG) liposomal formulation was evaluated in this PD model. For this, RT was administered for 10 days, followed by a single *i.p.* injection of liposome-encapsulated rosiglitazone (1 or 1.4 mL kg^{-1}). Administration of this new liposomal formulation resulted in greater neuroprotective effects both in the retina (day 20) and brain (day 60) than daily administration of the free drug. Moreover, DARC and OCT measures in the retina were good predictors of therapeutic efficacy in the brain.

Paraquat

Paraquat (PQ) (1,1′-dimethyl-4,4′-bipyridyl dichloride) is a quaternary nitrogen herbicide (Table I) frequently used in agriculture for broadleaf weed control due to its low cost, rapid action and a broad spectrum of activity (44). It was first introduced to the market in the '60s; however, its use is currently banned in many countries, including the 27 countries of the European Union, due to its toxicity on plants, animals and humans.

A study performed in 1985 by Barbeau *et al*. (45) showed that PQ caused a significant reduction in dopamine concentrations resulting in behavioral changes similar to those induced by MPP^+ (1-methyl-4-phenylpyridinium). The pathophysiological mechanism of PQ resembles that of the MPP^+ cation since PQ as a divalent cation (PQ^{++}) can undergo the redox pathway in microglia, resulting in a monovalent cation (PQ^+) accompanied by the production of superoxide that results in oxidative stress and cytotoxicity. PQ^+ is also

Table I. Chemical structure and mechanism of action of pesticides and neurotoxins used in animal models of Parkinson's disease

Model	Chemical structure	Mechanism of action
Rotenone		Inhibition of NADPH and mitochondrial complex I
Paraquat		ASK1 kinase activation triggers neuronal DA apoptosis
Maneb		Inhibition of glutamate transport and disruption of DA absorption and release
MPTP		Formation of free radicals and inhibition of NADPH dehydrogenase
6-OHDA		Formation of free radicals and H_2O_2

recognized by the dopamine transporter (DAT) causing accumulation in dopaminergic neurons which in turn generates superoxide and reactive dopamine species leading to dopaminergic neurotoxicity (46).

PQ can cross the BBB affecting the dopaminergic system as is transported into dopaminergic neurons *via* the dopamine transporter (47). Systemic administration of PQ in C57BL/6 mice resulted in a reduction of dopaminergic neurons, degeneration of the dopaminergic fibers of the *striatum* and a decrease in ambulatory activity, with the neurotoxicity exerted by PQ associated with its ability to induce the formation of free radicals, facilitate fibrillation of α-synuclein and cause cell death by apoptosis. Other studies also showed that PQ produces an increase in reactive oxygen species level, aggregations of α-synuclein, the formation of Lewy bodies and neuroselective lesions in the SNpc, features resembling those encountered in human PD (46, 48). PQ also activates other molecular signaling pathways including an increase in NADPH oxidase expression, depletion of oxidized glutathione, and Jun *N*-terminal kinase (JNK) activation (49).

Table II. Characteristics of the pesticide-/neurotoxin-induced models of Parkinson's disease

Model	Behavioral symptom	Nigrostriatal damage	Synuclein aggregation/Lewy body formation	Drawback
RT	Decreased motor activity in rodents.	Loss of DA neurons accompanied by reduced DA innervation in *striatum*.	Synuclein aggregation in DA neurons.	Substantial morbidity and mortality.
PQ/MB	Not clear motor déficit.	Moderate neuron loss and DA deficiency in the nigro-striatal projection; neurons in other regions may be affected.	No inclusions present, but increased synuclein immune-reactivity in DA neurons of the SNpc.	Not extensively tested. Effects in other neurotransmitter systems.
MPTP	Motor impairments in primates. Less obvious in acute rodent models.	Loss of DA neurons depending on dosing, reaching 95 % in acute high-dose conditions. Reduced DA levels in striatum with midbrain DA neuron loss.	Few cases of synuclein aggrega-tion in primates. Increased synuclein immunoreactivity in rodents.	Non-progressive model of cell death. Inclusions are rare.
6-OHDA	Rotational beha-vior after unilateral injection.	Loss of DA innervation at injection site (striatum).	No inclusions found.	Intracerebral injection, very little synuclein involvement.

MB – maneb, PQ – paraquat

Different studies have confirmed the ability of PQ to destroy nigral dopaminergic neurons in animal models (mice, rats) (50, 51). One important feature of PQ toxicity is that it exhibits high selectivity for SNpc dopaminergic neurons which in turn results in almost 50 % loss after multiple injections (52). It has also been demonstrated the relationship between age and PQ neurotoxicity as older animals (mice, rats) exhibit enhanced vulnerability to this pesticide (53). However, doubts have been raised in recent studies regarding the effects of systematic PQ administration since it does not produce dopamine depletion in the *striatum* or evident motor deficits (54).

Currently, PD models induced with PQ are mainly developed either with the divalent cation (PQ^{++}) or the stable form (PQ^{+}). PQ^{++} is not transported *via* DAT but is converted to PQ^{+} through redox cycling (55).

In most of the studies that evaluate the neurotoxicity of PQ, the agent is administered by *i.p.* once weekly for several weeks. One particular feature of the PQ model is that it can be used alone or in combination with other agents such as maneb, as concurrent exposure to both chemicals in adult mice has resulted in marked DA fiber loss, altered DA turnover and decreased locomotor activity (56, 57).

Controlled-release systems using paraquat model. – Srivastav *et al.* (58) have recently synthesized biocompatible piperine-coated gold nanoparticles (AuNPspiperine) to specifically target PQ-induced metabolic complications both in *Drosophila melanogaster* and SH-SY5Y (human neuroblastoma) cells. Piperine, a natural alkaloid found in *Piper longum* and *Piper nigrum*, exerts potential antiparkinsonian activity due to its anti-inflammatory and antioxidative properties (59) also being able to counter motor dysfunctions and dopamine depletion (60). The authors found that in the *in vitro* model tested AuNPspiperine were able to maintain the mitochondrial membrane potential thereby protecting the cells against PQ-induced toxicity. Moreover, in PQ-treated flies, the new controlled delivery system was able to suppress oxidative stress and mitochondrial dysfunction leading to inhibition of apoptotic cell death. AuNPspiperine also improved locomotor function and life span in PQ exposed flies.

Maneb

Maneb (MB) is a fungicide in the form of a polymeric complex of manganese with anionic ligand ethylene-bis(dithiocarbamate) (Table I). It is used either as a contact pesticide to treat seeds or for direct application to emerging soil crops. MB exacerbates the toxicity of other agents such as PQ in mouse models, just as other dithiocarbamates disrupt the function of the ubiquitin-proteasome system, a system that is believed to be involved in genetic and idiopathic forms of PD (11).

In vitro studies have shown that the neurotoxicity exerted by MB is related to inhibition of the enzymatic activity of mitochondrial complex III and oxidation of catecholamines. Moreover, systemic administration of both PQ and MB can induce a synergistic decrease of DA in the *striatum*, degeneration of SNpc and motor dysfunction (57).

Berry *et al*. (48), in a literature review, indicated that the study performed by Thiruchelvam *et al*. (57), in C57BL/6 male mice injected *i.p.* with PQ (0.3 mg kg^{-1}) or MB (1 mg kg^{-1}), showed that exposure to these pesticides during the postnatal period produced permanent

Table III. Controlled-release systems developed for the treatment of PD and evaluated with pesticide or neurotoxin-induced animal models of PD

Animal model	Controlled--release system	Drug	Results	Ref.
Rotenone	Microparticles	Rasagiline	Rasagiline-loaded PLGA microparticles showed a robust effect on all analytical outcomes (brain histology, immunochemistry, and behavioral testing) evaluated in rotenone-treated Wistar rats.	36
	Nanoparticles	Ropinirole	Brain histology and immunochemistry (Nissl-staining, glial fibrillary acidic protein (GFAP) and tyrosine hydroxylase (TH) immunohistochemistry), and behavioral testing (catalepsy, akinesia, rotarod and swim test) showed that ropinirole-loaded PLGA nanoparticles efficiently reverted PD-like symptoms of neurodegeneration in rotenone-treated Wistar rats.	37
	Microparticles	Ropinirole	Ropinirole-loaded PLGA microparticles given every two weeks were able to revert PD-like symptoms induced by rotenone in Wistar rats.	38
	Microemul-sions in transdermal system	Ropinirole	Ropinirole microemulsion via transdermal route improved the motor function by 76 % when compared to the oral administration of the drug to rotenone-treated Sprague-Dawley rats. The transdermal delivery system also successfully restored catalase and superoxide dismutase levels.	39
	Nanoparticles	Resveratrol	Resveratrol-loaded nanoparticles showed better efficacy than the drug given orally in solution in attenuating the rotenone-induced behavioral, biochemical and histological alterations observed in rotenone-treated Wistar rats.	40
	Nanoparticles	Curcumin and piperine	Curcumin-piperine co-loaded glyceryl mono-oleate nanoparticles were able to cross the BBB and rescue the rotenone-induced motor coordination impairment, and restrained dopaminergic neuronal degeneration in rotenone-treated mice.	41
	Nanoparticles	Iron-sapogenin	Iron-sapogenin nanoparticles showed better efficacy than sapogenin alone in reverting behavioral disorders induced by rotenone in rotenone-treated Kunming mice. Increased superoxide dismutase activity, tyrosine hydroxylase expression, and dopamine and acetylcholine levels in the brain were also obtained.	42
	Liposomes	Rosiglitazone	Rosiglitazone liposomes produced greater neuroprotective effects both in the retina and brain of rotenone-treated Dark Agouti rats than daily administration of the free drug. DARC (detection of apoptosing retinal cells) and OCT (optical coherence tomography) measures in the retina were good predictors of therapeutic efficacy in the brain.	43
MPTP	Microparticles	Glial cell line-derived neuro-trophic factor (GDNF)	GDNF-loaded PLGA 503H microparticles administered within the putamen achieved sustained levels of the neurotrophic factor in the brain, which were able to improve both motor and dopaminergic function in MPTP-treated *Macaca fascicularis* non-human primates.	75

Animal model	Controlled--release system	Drug	Results	Ref.
6-OHDA	Nanoparticles	Dopamine	Dopamine-loaded PLGA nanoparticles significantly recovered neurobehavioral changes and increased dopamine levels and its metabolites and a reduction of dopamine-D2 receptor supersensitivity in the *striatum* of parkinsonian rats.	93
	Microparticles	L-DOPA/ benserazide	L-DOPA/benserazide-loaded PLGA microparticles improved motor function and ameliorated the expression of L-DOPA induced dyskinesia in 6-OHDA-treated Sprague-Dawley rats.	95
	Nanoemulsion gel in a trans-dermal system	Ropinirole	Ropinirole nanoemulsion gel restored the biochemical markers of oxidative stress (glutathione antioxidant enzymes, thiobarbituric acid reactive substances, and catalase activity) in 6-OHDA-treated Wistar rats.	96
	Microparticles	Rotigotine	Rotigotine-loaded PLGA microparticles showed high and stable plasma and striatal drug levels for up to two weeks and exhibited steady efficacy in 6-OHDA-treated Sprague-Dawley rats.	99
	Solid lipid nanoparticles	Apomorphine	Apomorphine nanoparticles increased the bioavailability and distribution of the drug in the striatum, which resulted in a significant improvement of lesions induced by 6-OHDA in rats.	100
	Microparticles	Glial cell line-derived neurotrophic factor (GDNF)	GDNF-loaded PLGA microparticles produced a consistent improvement in the behavior of 6-OHDA-treated Sprague-Dawley rats. More than 50 % of the animals fully recovered from their rotational asymmetry with the response being accompanied by a higher fiber density in the GDNF treated *striatum*.	101
	Liposomes	Basic fibroblast growth factor (bFGF)	The bFGF liposomal formulation enhanced the neuroprotective effects of bFGF on dopaminergic neurons, improved the behavioral alterations induced by 6-OHDA in rats, increased the number of Nissl bodies, and ameliorated the loss of tyrosine hydroxylase (TH)-positive neurons.	102
	Nanoparticles	Basic fibroblast growth factor (bFGF)	bFGF in phospholipid-based gelatin nanoparticles stimulated dopaminergic function in surviving synapses and exerted neuroprotective effects after intranasal administration to 6-OHDA-treated rats.	103
	Microparticles	Vascular endothelial growth factor (VEGF) and GDNF	GDNF-loaded microspheres and VEGF and GDNF-coloaded microspheres improved the rotation behavioral test and resulted in higher levels of neurorepair and neuroregeneration in 6-OHDA-treated Sprague-Dawley rats.	104

and progressive alterations in the nigrostriatal DA system, that could be involved in the induction of neurodegenerative disorders.

MB is also able to potentiate both the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and the PQ effects (56, 61). In rodents, the combination of PQ and MB can elicit dopamine depletion in the *striatum*, selective dopaminergic cell loss, accumulation of α-synuclein and PD-like motor and non-motor symptoms. Moreover, a synergistic effect has been demonstrated after the administration of PQ (10 mg kg^{-1}) and MB (30 mg kg^{-1}) to mice twice a week for 6 weeks, a dosage regimen in which exacerbated parkinsonism was observed when compared to the administration of each compound alone (56).

However, with the use of MB as occurs with RT, the animal model has led to contradictory results, variable loss of striatal DA content and cell death (62). Therefore, research efforts are still needed to elicit the exact mechanisms by which PQ induces neurodegeneration.

To date, no articles have been found in the literature regarding the evaluation of controlled-release systems in MB-induced animal models of PD.

MODELS BASED ON NEUROTOXINS

Neurotoxin models remain the most popular models in PD animal studies with MPTP and 6-OHDA being the most frequently used. However, they lack the ability to mimic the pathological features of PD, as they only mimic the symptoms. These models only resemble the symptoms and motor dysfunctions occurring once severe dopamine depletion is reached (63).

MPTP

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Table I) was first described as an agent causing parkinsonism in 1982, in California, when a group of drug addicts developed a severe acute stiffness syndrome which improved upon receiving levodopa and dopamine agonists (64).

Due to its lipophilicity, MPTP readily crosses the BBB. When in contact with monoamine oxidase B it transforms into 1-methyl-4-phenylpyridinium (MPP^+), which is the active compound that enters the dopamine neurons of the SNPc. Once inside, it blocks complex I of the mitochondrial electron transport chain causing a decrease in adenosine triphosphate (ATP) and accumulation of ROS, which in turn results in cell death (65). MPTP causes damage to the dopaminergic pathway which is easily reproducible in animal models. Moreover, when infused over a period of 30 days, it can induce the formation of ubiquitin and α-synuclein inclusions resembling the features of human PD (66).

It has also been postulated that the toxicity of MPTP through the MPP^+ entity may be dependent on the neuromelanin content since the affinity of MPP^+ for neuromelanin is very high (24) and MPP^+ can remain in the cytosol interacting with enzymes (67).

Induction of parkinsonian syndromes with MPTP is usually performed in rodents, dogs, cats and non-human primates. The effects of MPTP in mice vary with age, dose, route of administration and number and frequency of injections. It has been demonstrated

that the damage caused by MPTP at the mitochondrial level increases with age as brains of older mice accumulate more MPP^+ than those of younger animals, which may contribute to greater sensitivity to MPTP toxicity.

Of the numerous studies performed with MPTP in mice, some have been based on the *s.c.* or *i.p.* administration of 10–30 mg kg^{-1} per day for several days, depending on whether an acute or chronic model is being developed. For instance, for the acute model doses of MPTP ranging between 20–30 mg kg^{-1} given the same day have been described, whereas, for the chronic model, two dosage regimens have been described: administration of daily doses of 30 mg kg^{-1} for five consecutive days or daily doses of 4 mg kg^{-1} for 20 days (68, 69).

The MPTP mouse model has some advantages over the one induced by 6-OHDA (6-hydroxydopamine) due to the fact that it does not require stereotaxic surgery for its administration, Moreover, it is also able to develop bilateral degeneration of the nigrostriatal tract resembling that found in human PD, and also mimic other known biochemical features such as decrease in striatal levels of DA and TH (tyrosine hydroxylase), increase of both striatal precursor protein preproenkephalin-A (PPE-A) and acetylcholine (ACh) levels, increase of extracellular glutamate levels and reduction of glutathione (GSH) in basal ganglia (70). The MPTP mouse model does, however, have some disadvantages over the 6-OHDA model, especially regarding reproducibility and the range of behavioral outcomes that can be obtained. Moreover, mice are less sensitive to MPTP than primates, therefore, requiring higher doses that can be lethal, due to the resulting peripheral neuro- or cardiotoxicity (71).

All primate species in which MPTP has been tested are sensitive to this neurotoxin, including squirrel, monkeys, baboons, macaques, marmosets and vervet monkeys. Repeated systemic administration of MPTP by *i.p.*, *s.c.*, *i.v.* or *i.m.* injections, with varying doses depending on species and route, are able to rapidly develop motor alterations resembling those found on human PD, such as bradykinesia, postural instability and rigidity (72), as well as non-motor symptoms such as cognitive deficits (73) and temporary autonomic disturbances (74).

Controlled-release systems using the MPTP model. – Glial cell line-derived neurotrophic factor (GDNF) is a potent neuroprotective agent currently considered as a promising candidate for the treatment of PD, due to its potent trophic effect on the dopaminergic system. However, GDNF is not able to cross the BBB and has a relatively short elimination half-life. For this, attempts have been made by Garbayo *et al.* (75) to design new controlled delivery systems to increase the stability and retention of neurotrophic factors such as GDNF aiming to improve the treatment of PD. In this study, GDNF-loaded microparticles were prepared by the solvent extraction-evaporation method using PLGA 503H as polymer. The new formulation was applied to *Macaca fascicularis* non-human primates that received weekly *i.v.* doses of MPTP (0.5 mg kg^{-1}) until the animals reached stable severe parkinsonian symptoms. Administration of the new drug delivery system was performed by stereotaxic surgery. The results obtained showed that a single administration of microencapsulated GDNF (25 mg) within the putamen achieved sustained levels of the neurotrophic factor in the brain, which were able to improve both motor and dopaminergic function. Moreover, in the SNpc GDNF was able to increase the number of dopaminergic neurons, regardless of the severity of neurodegeneration.

6-OHDA

6-Hydroxydopamine (6-OHDA) (Table I) is another neurotoxin widely used to induce parkinsonism in experimental animal models. It is a highly oxidizable DA analog that can be captured by the dopamine transporter, which in turn results in selective damage of dopaminergic neurons in the SNpc (76).

6-OHDA does not cross the BBB, therefore, making it necessary to perform its direct administration into the brain parenchyma. This represents one of the main drawbacks as specialized stereotaxic surgical instruments and training is required for its administration. Regarding the cytotoxicity exerted by 6-OHDA three mechanisms have been proposed: intra-extracellular self-oxidation of 6-OHDA resulting in the formation of hydroxyl, hydrogen peroxide and superoxide radicals, the appearance of hydrogen peroxide due to the monoamine oxidase, and inhibition of mitochondrial electron transport chain complexes I and IV [NADH dehydrogenase (complex I) and cytochrome c oxidase (complex IV)]. It is postulated that these events resemble those occurring in the PD brain, which supports the interest and validity of the 6-OHDA model. Moreover, as a result of these, ROS are produced, which in turn explains the oxidative stress leading to the cytotoxic effect of 6-OHDA. Recent studies have demonstrated that the administration of 6 μg of 6-OHDA in the dorsal *striatum* of rats is able to produce an increase in the oxidation level of proteins and lipoperoxidation products as a function of time (77), which could be due to mitochondrial oxidative stress leading to both neuroinflammation and cell death by apoptosis (78).

When trying to understand the *in vivo* effects and neurodegenerative mechanisms of 6-OHDA, injection of the neurotoxin can be performed into the medial forebrain bundle (79), the *substantia nigra* (80), or at the intrastriatal site (81–83). Bilateral lesions of the ascending forebrain dopaminergic system have been reported to induce severe aphagia, adipsia and akinesia, with the need for tube feeding of the animals (84). Intranigral lesions usually cause pain and massive degeneration of the injured nucleus, which hinders their use when evaluating the mechanisms involved in neurotoxicity and death, as a consequence of long-term oxidative stress.

On the other hand, the intrastriatal lesion model produces a progressive loss of dopaminergic neurons in the SNpc, resembling the nigrostriatal lesions found in PD after unilateral intracranial injection (85, 86) and bilateral injection (87). In the late '90s, Kirik *et al*. (88) established the optimal parameters when causing unilateral ventrolateral *striatum* lesions after injecting a 6-μg dose of 6-OHDA at three different *striatum* sites. The results obtained indicated that the effect of the intrastriatal injection depends on the site of injury and the dose given. In this study, 80 % reduction in striatal innervation and almost 90 % loss of the nigral dopaminergic neurons was observed.

The 6-OHDA model does not reproduce the presence of Lewy bodies (89). To overcome this drawback, α-Syn murine models have been developed, based on knockout models (90), gene expression models (91), or intracerebral injection of α-Syn (92). Although further research is needed, this is an interesting approach since this protein is a key component of Lewy bodies.

Since the 6-OHDA model reproduces several of the cellular alterations occurring in PD, it is a valuable model to study the cytotoxicity mechanisms involved and the cellular processes activated by oxidative stress (neuroinflammation and neuronal death).

Controlled-release systems using the 6-OHDA model. – When trying to increase the DA levels at the CNS, a novel approach is based on the use of DA encapsulated within polymeric nanoparticles (93). DA-loaded NPs were prepared with copolymer PLGA by means of the double emulsion solvent evaporation method. This formulation was evaluated in a 6-OHDA-induced rat model of PD, being able to successfully release the drug in the *striatum*. Systemic *i.v.* administration of DA-loaded NPs caused a significant increase of DA levels and its metabolites and a reduction of DA-D2 receptor supersensitivity in the *striatum* of parkinsonian rats. Moreover, the formulation significantly recovered neurobehavioral changes induced by 6-OHDA without causing any additional production of ROS, dopaminergic neuron degeneration, and structural changes in the *striatum* and SNpc when compared to 6-OHDA-lesioned rats.

Nowadays, L-DOPA is still considered the most effective drug in the treatment of PD. However, chronic administration of this agent usually results in L-DOPA-induced dyskinesia (LID), which has been related to its peak plasma concentration (94). To avoid this, Ren *et al*. (95) developed L-DOPA/benserazide-loaded PLGA microparticles which were administered to Sprague-Dawley rats in which PD was induced by stereotactic administration of 6-OHDA. Subcutaneous administration of the microparticulate formulation improved motor function and ameliorated the expression of L-DOPA-induced dyskinesia (LID).

As previously indicated, ropinirole (RP), a dopamine agonist, stimulates striatal DA receptors being effective both as monotherapy and in combination therapy with levodopa. However, RP has low oral bioavailability and short elimination half-life which results in frequent dosing. Continuous delivery of RP from a transdermal system may avoid these drawbacks and help prevent or delay the onset of L-DOPA-related motor complications due to continuous dopaminergic stimulation. In this regard, Azeem *et al*. (96) developed a nanoemulsion gel for RP transdermal delivery. The new formulation was evaluated in a PD animal model induced in Wistar rats after stereotaxic administration of 10 mg 6-OHDA in 2 μL of 0.1 % in ascorbic acid-saline. The efficacy of the nanoemulsion was tested by analyzing three biochemical markers of oxidative stress (glutathione antioxidant enzymes, thiobarbituric acid reactive substances and catalase activity) with the results demonstrating that the new formulation was able to effectively restore the biochemical changes induced by 6-OHDA.

Rotigotine (Ro) is a non-ergoline agonist of DA D3/D2/D1 receptors indicated for the treatment of both early and advanced PD. Giladi *et al*. (97), developed a transdermal patch for early PD that delivered the drug in patients at a constant rate for 24 h providing continuous plasma concentrations. However, there are some important side-effects related to the use of skin patches such as variability in the absorption rates from different skin areas and possible irritation after long-term application of the transdermal device (98). As PD patients require long-term treatments it would be interesting to develop a sustained--release preparation of Ro to achieve continuous dopaminergic stimulation for long periods of time. For this purpose, Wang *et al*. (99), encapsulated Ro within PLGA microparticles (RoMPs) by an oil-in-water emulsion solvent evaporation technique and evaluated the delivery system in 6-OHDA-lesioned Sprague-Dawley rats (4 μg μL^{-1} 6-OHDA in 0.9 % saline, containing 0.04 % ascorbic acid) in comparison with pulsatile L-DOPA administration. The pharmacokinetic study performed after intramuscular administration of RoMPs showed high and stable plasma and striatal drug levels for up to two weeks. Moreover, the micro-

particulate Ro formulation exhibited steady efficacy which lasted for 2 weeks without any significant differences found between RoMPs treatment and pulsatile L-DOPA.

Tsai *et al*. (100) encapsulated apomorphine, a DA receptor agonist, in solid lipid nanoparticles (SLNs) in order to improve its very low oral bioavailability (< 2 %). The brain regional distribution of the nanoparticles was also evaluated. For this, pharmacokinetic studies were carried out in rats. Drug bioavailability was increased with NPs in respect to a reference solution. Apomorphine distribution in the *striatum*, the predominant site of its therapeutic action, also increased when using the solid-lipid nanoparticles (SLN). The anti-parkinsonian activity of apomorphine was evaluated in rats with 6-OHDA induced lesions. Animals treated with orally administered SLNs presented a significant improvement when compared to those treated with the drug formulated in solution.

Glial cell line-derived neurotrophic factor (GDNF) is studied for the treatment of neurodegenerative disorders of basal ganglia origin such as PD. Garbayo *et al*. (101) investigated the neurorestorative effects of controlled GDNF delivery when given as GDNF-loaded PLGA microparticles, which were prepared by a solvent extraction/evaporation method. The new delivery system was assessed in Sprague-Dawley rats that previously were stereotaxically injected with a total dose of 20 μg 6-OHDA dissolved in 10 μL of saline with 0.1 % ascorbic acid. The results demonstrated the efficacy of GDNF-loaded PLGA microparticles which resulted in a consistent improvement in behavior 6 weeks after stereotaxic injection of the delivery system. Moreover, 60 % of the animals treated with new formulation fully recovered from their rotational asymmetry 8 weeks after treatment, a response that was accompanied by a higher fiber density in the GDNF treated *striatum*.

Regarding lipid-based nanocarriers, Yang *et al*. (102) developed basic fibroblast growth factor (bFGF)-loaded liposomes for intranasal administration due to the neuroprotective effects of bFGF. The liposomal formulation was evaluated in a PD model induced in rats by 6-OHDA administration. The authors demonstrated that the new liposomal formulation was able to deliver bFGF to the *striatum* and SNPc of rats and enhanced the neuroprotective effects of bFGF on dopaminergic neurons. Moreover, the bFGF liposomal formulation markedly improved the behavioral alterations induced by 6-OHDA, increased the number of Nissl bodies, and ameliorated the loss of tyrosine hydroxylase (TH)-positive neurons.

Zhao *et al*. (103) formulated bFGF in phospholipid-based gelatin nanoparticles destined to target the CNS also *via* intranasal administration. Hemi-parkinsonism was generated in rats by stereotaxic injection of 10 μL 6-OHDA solution in the right-side *striatum*. After administration, the integrity of the nasal mucosa was not affected. The results obtained showed that bFGF not only stimulated dopaminergic function in surviving synapses but also played a neuroprotective effect.

In another approach, vascular endothelial growth factor (VEGF), a potent angiogenic factor with survival effects in neuronal cultures, was microencapsulated either alone or in combination with GDNF (104), in order to evaluate the efficacy of the formulation in the advanced stages of PD. For this, Sprague-Dawley rats were injected with 6-OHDA (7.5 and 6 μg at two coordinates) to establish the PD model. The results showed that treatment with GDNF microspheres and with both VEGF and GDNF microspheres improved the rotation behavioral test, and resulted in higher levels of neurorepair and neuroregeneration.

Table II summarizes the characteristics of the pesticide and neurotoxin-induced animal models of PD. Finally, Table III summarizes different controlled-release systems developed for the treatment of PD and evaluated with pesticide or neurotoxin-induced animal models of PD.

CONCLUSIONS

Several animal models of PD are currently being used to help understanding the pathogenesis of this neurogenerative disorder as well as to evaluate the potential of new treatments developed for the disease. Some of these models are based on the use of neurotoxins in which substantial nigrostriatal degeneration is generally developed, as well as motor symptoms resembling those occurring in human PD. Regarding the development and evaluation of new drug delivery systems for PD, 6-OHDA is the one most frequently used. Although it needs to be injected intracerebrally, it exerts great selectivity of damage facilitating damage assessment. In addition, the scientific evidence on this neurotoxin is very extensive. MPTP is another neurotoxin frequently used. Unlike 6-OHDA, MPTP has the ability to cross the BBB, thereby facilitating its administration. Its mechanism of action has been extensively studied, with the MPTP-monkey model being considered the gold standard for preclinical testing of new therapeutic approaches for PD. However, the lack of facilities and expertise of researchers with monkey models is one of its main drawbacks.

Regarding pesticide-based animal models of PD, the agents usually employed are rotenone, paraquat and maneb. Systemic administration of rotenone mainly targets mitochondrial structures thereby triggering oxidative stress causing apoptosis of the dopaminergic neurons of the SNpc. Moreover, the rotenone rat model can cause the formation of α-synuclein inclusions as well as motor deficits resembling those occurring in PD. Paraquat is also thought to cause dopaminergic degeneration by inducing oxidative stress, however, after chronic administration, this agent is not able to produce dopamine depletion in the *striatum* or clear motor deficits.

From this review, it has been found that when evaluating new drug delivery systems developed for PD, 6-OHDA and rotenone are the most frequently used agents. However, there is still a need for further research since none of the animal models based either on the use of neurotoxins or pesticides can exactly mimic the neuropathological features occurring in human PD.

Abbreviations, acronyms, symbols. – 6-OHDA – 6-hydroxydopamine, bFGF – basic fibroblast growth factor, COMT – catechol-*O*-methyltransferase, CPNPs – curcumin and piperine co-loaded glyceryl monooleate NPs, DA – dopamine, DARC – detection apoptosing retinal cells, DAT – dopamine transporter, GDNF – glial cell line-derived neurotrophic factor, GFAP – glial fibrillary acidic protein, LID – L-DOPA-induced dyskinesia, JNK – Jun *N*-terminal kinase, MAO-B – monoamine oxidase B, MB – maneb, ME – microemulsion, MEG – ME-based gel, MPP^+ – 1-methyl-4-phenylpyridinium, MPs – microparticles, MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, NDUFS4 – NADH dehydrogenase ubiquinone proteins Fe-S4, NMDA – *N*-methyl-D-aspartate, NPs – nanoparticles, OCT – optical coherence tomography, PD – Parkinson's disease, PLGA – poly(lactic-co-glycolic) acid, PPE-A – protein preproenkephalin-A, PQ – paraquat (1,1'-dimethyl-4,4'-bipyridyl dichloride), RM – rasagiline mesylate, RG – rosiglitazone, Ro – rotigotine, RoMPs – rotigotine microparticles, RP – ropinirole, RSV – resveratrol, RT – rotenone, SLNs – solid lipid nanoparticles, SNpc – *substantia nigra pars compacta*, TH – tyrosine hydroxylase, VEGF – vascular endothelial growth factor, α-Syn – alpha-synuclein

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