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Manganese mixture inhalation is a reliable Parkinson disease model in rats

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ABSTRACT

Manganese (Mn) is an essential trace metal. Regardless of its essentiality, it has been reported that the overexposure causes neurotoxicity manifested as extrapyramidal symptoms similar to those observed in Parkinson disease (PD). Recently, our group reported that mice that inhaled for 5 months the mixture of manganese chloride ($MnCl_2$) and manganese acetate $Mn(OAc)_3$ developed movement abnormalities, significant loss of substantia nigra compacta (SNc) dopaminergic neurons, dopamine depletion and improved behavior with L-DOPA treatment. However, this model has only been characterized in mice. In order to have a well-supported and generalizable model in rodents, we used male Wistar rats that inhaled a mixture of 0.04 M $MnCl_2$ and 0.02 M $Mn(OAc)_3$, 1 h three times a week for 6 months. Before Mn exposure, animals were trained to perform motor tests (Beam-walking and Single-pellet reaching tasks) and were evaluated each week after the exposure. The mixture of $MnCl_2$/$Mn(OAc)_3$ caused alterations in the motor tests, 75.95% loss of SNc dopaminergic neurons, and no cell alterations in Globus Pallidus or striatum. With these results we conclude that the inhalation of the mixture of Mn compounds is a useful model in rodents for the study of PD.

1. Introduction

Parkinson disease (PD) is characterized by a progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNc); the neurochemical consequence of this loss is a marked decrease in the concentrations of dopamine (DA) in the caudate nucleus and putamen (striatum) (Dunnett and Björklund, 1999; Lang and Lozano, 1998; Olanow and Tatton, 1999). The main symptoms of the disease are tremor, bradykinesia, hypokinesia, balance and gait disturbances.

Although the etiology of PD is still not fully understood, animal models have provided important clues. On the basis of experimental and clinical discoveries, PD was the first neurological disease to be modeled and, subsequently, to be treated by neurotransmitter replacement therapy (Betarbet et al., 2002). Several models exhibit many of the characteristic features of the disease; however, none resembles the complex chronic neurodegenerative features of human PD. 6-Hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are neurotoxins which selectively and rapidly destroy dopaminergic neurons (within 1–3 days), whereas in humans PD pathogenesis follows a progressive course over decades.

When choosing an animal model for idiopathic PD, one must consider the degree of similarity or discrepancy between the physiology, anatomy and behavior between humans and animals. The existing models have been useful for understanding the etiology of the disease and offer resources for proving new treatments (Potashkin et al., 2010). However, the loss of the nigrostriatal dopaminergic pathway that has been replicated in animals, either unilaterally or bilaterally, using a variety of selective toxins or by genetic manipulations, is rapid and not progressive, and for those derived through genetic manipulations relevant to human PD, the loss, although more progressive, may be limited in extent or may not even occur at all (Emborg, 2004; Meredith and Kang, 2006).

It has been reported that diverse susceptibility to neurotoxins exists between species. Mice are more sensitive to MPTP than rats due to their higher cerebral levels of MAO-B and lower expression of the vesicular monoamine transporter 2 (VMAT2), which in rats seems to sequester MPP+ and diminishes its toxicity (Russo et al., 1994). Furthermore, different strains of mice (and even within a given strain obtained from different traders) can exhibit distinct sensitivity to MPTP (Emborg, 2004). This diversity acts in an

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autosomal dominant manner and two trait loci on chromosome 15 and 13 have been suggested to be linked to MPTP susceptibility (Sedelis et al., 2003). Gender, age and body weight also affect MPTP sensitivity and reproducibility of the lesion (Emborg, 2004). Older animals show increased sensitivity to dopaminergic neurotoxins (Ricaurte et al., 1987). In the case of stereotaxic 6-OHDA lesions, after adjusting the coordinates to weight and strand (Paxinos and Watson, 1986), strand but not age seems to affect the effectiveness of the lesioning (Collier and Sortwell, 1999). Lewis rats young and old, compared to Fischer or Sprague–Dawley rats, require dosing two-fold higher of 6-OHDA to induce a measurable lesion (Emborg, 2004).

The effects of manganese (Mn) as a PD model have been investigated due to the fact that 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 (Calne et al., 1994; Cook et al., 1974; Pal et al., 1999).

Manganism is associated with elevated brain levels of Mn, primarily in those areas known to contain high concentrations of nonheme iron, especially the striatum, Globus Pallidus (GP), substantia nigra (SN), and subthalamic nuclei (Aschner et al., 2005). Manganism is initially characterized by a psychiatric disorder (*locura manganica*) that resembles schizophrenia, may be due to Mn acute exposure causes hyperactivity accompanied by elevated brain levels of catecholamines and their metabolites (Shukla and Singhal, 1984; Tomás-Camardiel et al., 2002; Zhang et al., 2011). Symptoms include compulsive and violent behavior, emotional instability and hallucinations. As exposure continues and the disease progresses, patients may develop prolonged muscle contractions (dystonia), decreased muscle movement (hypokinesia), rigidity, and muscle tremors (Pal et al., 1999). These signs are associated with damage to dopaminergic neurons within brain structures that control muscle movement (Aschner et al., 2005).

There has been some controversy about the alterations produced by Mn; while some authors found that Mn alters dopaminergic functions specifically in the basal ganglia and produces Parkinson-like disorder (Autissier et al., 1982; Daniels and Abarca, 1991; Tomás-Camardiel et al., 2002; Zhang et al., 2009; Zhao et al., 2009), others indicated that Mn intoxication appears to be different from PD in both etiology and pathology (Liu et al., 2006; Yamada et al., 1986) particularly in the notable preservation of SNc dopaminergic somas (Calabresi et al., 2001; Lu et al., 2005; Olanow, 2004; Peneder et al., 2011; Perl and Olanow, 2007; Yong et al., 1986).

Recently, we developed a novel PD experimental model in mice by the inhalation of the mixture of Mn compounds, Manganese chloride ($MnCl_2$) and Manganese acetate ($Mn(OAc)_3$). After Mn mixture inhalation, the mice presented an important loss of SNc TH-positive neurons; the loss of these neurons was 67.58% (Ordoñez-Librado et al., 2008). Later on, we determine whether L-DOPA treatment improves the behavior to ensure that the alterations are of dopaminergic origin (Ordoñez-Librado et al., 2010). In summary, after 5 months of Mn mixture inhalation, striatal dopamine content decreased 71%, SNc showed significant 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 provided evidence that $MnCl_2/Mn(OAc)_3$ mixture inhalation produces similar morphological, neurochemical and behavioral alterations to those observed in PD, suggesting a useful experimental model for the study of this neurodegenerative disease (Ordoñez-Librado et al., 2011). Additionally, Mn inhalation is progressive and bilateral, which makes it more reliable. However, this model has only been characterized in mice, and since it has been postulated that there are different susceptibility between species to the most common used neurotoxins, our goal is to prove the inhalation of $MnCl_2/Mn(OAc)_3$ mixture in rats to determine if these animals are also susceptible, and if so, extrapolate our PD model to other species.

2. Experimental procedures

Thirty male Wistar rats weighing 180 ± 10 g were individually housed in hanging plastic cages under controlled light conditions (12/12 h light/dark regime) and fed with Purina Rat Chow and water ad libitum. Body weight was recorded daily. The experimental protocol was in accordance to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and to the Rules for Research in Health Matters (Mexico). 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, rats 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 rat was tested once a week, a different day for each test. Two observers blind to the rats exposed or control status perform all behavioral assessments.

2.2. Single-pellet reaching task

The plexiglas reaching box was 30 cm long, 15 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 (Ordoñez-Librado et al., 2008). Before training, animals were food deprived for 24 h. Afterward, they received a restricted diet of ~10 g/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 min. 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 rats were forced to reach to retrieve the food. As the animal pronates the paw medially, this placement allows the rat to obtain the pellet with a paw and not with the tongue. Rats were individually trained and allowed to reach with their preferred forelimb for food pellets (Whishaw et al., 1991). 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 a rat knocked the food away or dropped the food after grasping it, the reach was scored as a miss (Farr and Quantitative, 2002). Qualitative assessment consisted in analyze 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 (Ordoñez-Librado et al., 2008, 2011).

2.3. Beam-walking test

The additional test to measure motor coordination was assessed by evaluating the ability of the animals to traverse a narrow beam (12 mm wide) to reach an enclosed safety platform (Perry et al., 1995). The beam measured 2 m long and was elevated

to a height of 1 m above the floor with wooden supports with 15° inclination. Each test session consisted of four trials in which latency to cross the beam was recorded. Five trials were averaged to give a mean latency, and testing was done every week (Gutierrez-Valdez et al., 2012).

2.4. Video recording

Performance during single pellet reaching and beam walking tests were video recorded using a Sony camcorder (1000th of a second shutter speed). The camera was positioned orthogonally to the reaching box in such a way that the animal's 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 rats during the performance of the two tests.

2.5. Manganese inhalation

We previously carried out a pilot study in mice to obtain the optimal Mn concentrations with 0.02 (low) and 0.03 M (high) Manganese chloride ($MnCl_2$) and 0.01 (low) and 0.02 M (high) Manganese acetate [$Mn(OAc)_3$] alone, and after 6, 8, 10 and 12 inhalations by light microscopy some changes were observed in SNc Tyrosine hydroxylase-immunoreactive neurons (see Ordoñez-Librado et al., 2011 for details). However, the cell loss was not enough to observe behavioral alterations. Thus, higher doses were used and Mn compounds were mixed; we used 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 (Ordoñez-Librado et al., 2011). In the present study, we also perform a pilot study (5 control and 10 Mn-exposure rats) with the mixture of 0.04 M $MnCl_2$ and 0.02 M $Mn(OAc)_3$ (Sigma–Aldrich, Co. Mexico); the rats inhaled 1 h twice a week (the same protocol that we used in mice), and after 40 inhalations (5 months) by light microscopy some changes were observed in SNc tyrosine hydroxylase (TH) immunoreactive neurons. However, the loss of TH-immunostained cells were not enough to observe behavioral alterations. Thus, we decided to use the same Mn mixture concentrations but with three times a week inhalation protocol.

Inhalations were performed as described by Avila-Costa et al. (2004). 10 rats were placed in an acrylic chamber inhaling 0.04 M $MnCl_2$ and 0.02 M $Mn(OAc)_3$ 1 h three times a week for six months. 5 control rats inhaled only the vehicle—deionized water—for the same period. Inhalations were performed in closed acrylic boxes (40 cm wide × 70 cm long and 25 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.

After 6 months (72 inhalations), when significant motor alterations were observed, the rats were sacrificed, anesthetized with sodium pentobarbital lethal dose and perfused via aorta with phosphate buffer saline (0.1 M pH 7.4) containing 2% glutaraldehyde and 2% paraformaldehyde. The brain was removed and placed in fixative solution for 2 h and processed for TH and NeuN immunocytochemistry.

2.6. Immunocytochemistry

Coronal sections (50 μm) were obtained on a vibrating microtome (Pelco 101, Ted Pella Inc., Mexico) through the mesencephalon for TH, and GP and striatum for NeuN immunocytochemistry. TH (Chemicon International, Inc., CA, USA, 1:1000) and NeuN immunostaining (Chemicon International, International, Inc., CA, USA, 1:200,) 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 total number of TH-positive cells was counted manually rostro-caudally through the SNc and ventral tegmental area (VTA) in adjacent sections. The SNc was outlined using a manually traced region of interest (ROI) at low magnification (×4). The number of TH positive cells was counted in both hemispheres at the level of third cranial nerve, within a 100 μm × 100 μm counting area at high magnification (×40) only within this defined ROI. The level of the third cranial nerve provides a strong anatomical landmark where the SNc can be reliably delineated from the VTA as described elsewhere (Bukhatwa et al., 2009; Chan et al., 2010; Iravani et al., 2002). Although not a stereological technique, previous studies have shown that the 3rd nerve rootlets provide a reliable anatomical landmark at which the extent of cell loss is reflective of cell loss throughout the entire SN (Iravani et al., 2002). Striatal and GP NeuN cell count was performed using ×40 objective in seven coronal sections per animal at rostrocaudal levels 0.70 anterior to 0.48 mm posterior to bregma for dorsomedial striatum and −0.80 anterior to −0.92 mm posterior to bregma for ventrocaudal GP (Paxinos and Watson, 1986) in a 11,550 μm^2 and 3300 μm^2 counting area, respectively. It should be noted that both, dorsomedial striatum and ventrocaudal GP receive the greatest dopaminergic innervation (Jan et al., 2000; Lex and Hauber, 2010; Voorn et al., 1988).

2.7. 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 (Ordoñez-Librado et al., 2011). Mn content in serum was also measured by graphite-furnace atomic-absorption spectrometry at the end of the experiment.

2.8. Statistical analysis

Unpaired *t*-test was used to analyze the number of cells. Reaching task and Beam-Walking test were analyzed using repeated-measures ANOVA on mean values of motor activity per certain period during Mn exposure, post hoc comparisons were made with Tukey's test. Group differences were considered statistically significant at $p < 0.05$. All analyses were conducted with GraphPad Prism Software Inc., Version 5.

3. Results

After 6 months of exposure, neither clinical alterations nor significant weight changes were detected in the exposed animals compared with controls.

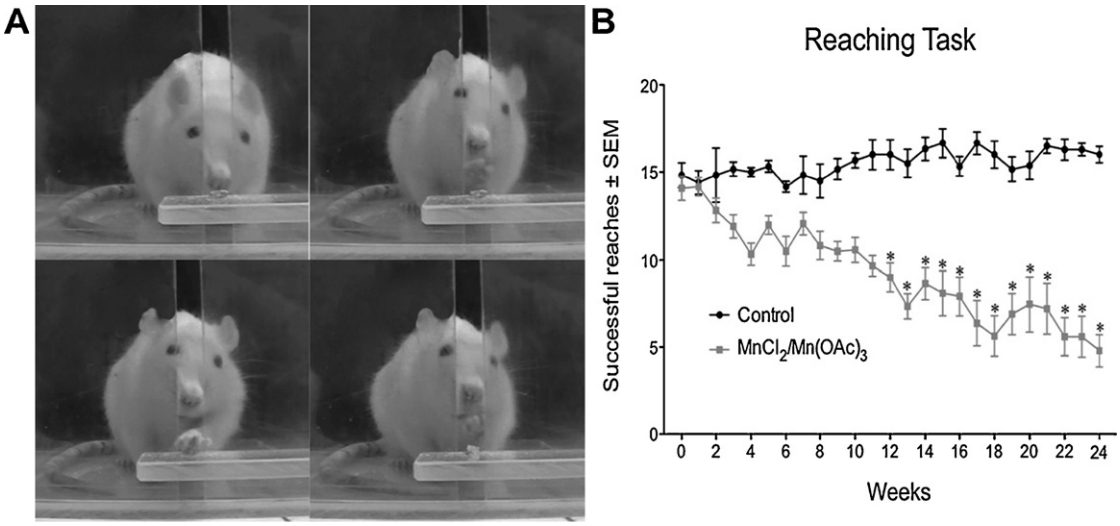

Fig. 1. (A) Representative still frames of a control rat captured during limb transport and limb withdrawal. The control animals advanced their forelimb through the slot and extended their digits, also supinated their paw to present the food to the mouth and extended their digits to release the food into the mouth. (B) Reaching success (number of pellets obtained out of 20; mean ± SEM) by control and Mn-Exposed rats in the Reaching task. Mn-exposed group is impaired since week 12 ($^*p < 0.001$ vs control group; repeated-measures ANOVA).

3.1. Manganese concentrations

The average Mn concentration measured in the filters of the chamber was of 2676 μg/m^3 during the whole experiment. The average Mn concentration in serum of exposed animals was of 45 ± 5 μg/l; control rats serum concentration of Mn was of 0.05 ± 0.12 μg/l.

3.2. Single-pellet reaching task

The task involves execution of a complex motor sequence, starting with sniffing the 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 (Figs. 1A and 2 sequence A).

Animals were presented with 20 food pellets. Fig. 1B shows the results of successful reaches over the course of the experiment. Repeated-measures ANOVA confirmed a significant effect of Mn-exposed group since week 12 ($p < 0.001$). All animals were comparable in their ability to retrieve pellets before Mn inhalation, but Mn exposure resulted in a marked impairment in both number of successful retrievals ($p < 0.001$) and accuracy. Control animals remained consistent throughout the duration of the experiment and performed significantly better than Mn-exposed animals at all time points (Figs. 1 and 2 sequence A).

Qualitative assessment resulted in a postural swing and impairments in limb extension (Fig. 2 sequences B and C), aim, and supination–pronation of the paw during grasping and release of the pellet into the mouth. Rats displayed unusual movements when retrieving the pellet after Mn-exposure. The paw is

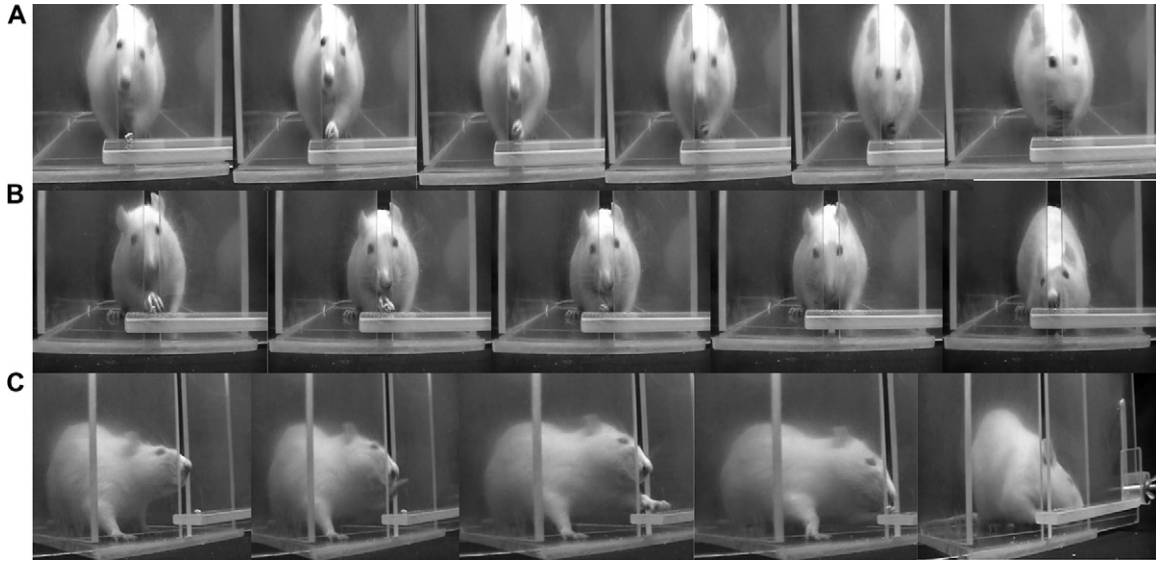

Fig. 2. Comparison in the motor execution between control rat (Sequence A) and Mn-exposed rats (Sequences B and C). Sequence (A) Control reaching behavior. The rat advances its limb by adducting the elbow so that the limb passes through the slot. As the limb advances the digits open. The rat pronates the paw by adduction of the elbow and a rotation of the paw around the wrist so that the palm of the paw is placed on the top of the pellet. The pellet is grasped by flexion of the digits. The limb is withdrawn carrying the pellet. The rat sits on its haunches to eat the food, which is held by the paws. Sequences (B) and (C) Mn-exposed rats 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 rat often drags its limb through the slot and drops 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.

frequently fully pronated and moves either laterally over the pellet (Fig. 2 sequences B and C), or the rat slaps the pellet from above. Several animals from Mn-exposed group displayed such motor abnormalities that persisted for the duration of the experiment. Mn-exposed rats are frequently unable to close the digits around the pellet and drag it to the slot without lifting the paw (Fig. 2 sequence C). Rats also fail to supinate the paw completely and place the snout into the slot to retrieve the pellet with the tongue (Fig. 2 sequences B and C). When the paw is withdrawn through the slot, Mn rats often rotate the body and "chase" the pellet with the snout instead of opening the digits and placing the pellet into the mouth (Fig. 2 sequences B and C). Post hoc tests on the group effect indicated that at more Mn-exposure success, scores were significantly poorer (Fig. 1B).

3.3. Beam-walking test

On the last day of testing before Mn inhalation, there was no significant difference between the latencies in completing the test for the controls and the Mn-treated rats (repeated-measures ANOVA, $p > 0.05$).

Fig. 3 demonstrates the mean of total time to cross the beam in seconds. Mn-exposed rats after 10 weeks of inhalation have a significant increase in the time to cross the beam compared with control rats. Moreover, rats exhibit limb weakness, akinesia, postural instability and action tremor.

3.4. Immunocytochemistry

3.4.1. TH-Immunocytochemistry

3.4.1.1. Pilot study. The mean number of TH-positive neurons on the control SNc was 102.13 ± 3.5 (Fig. 4). In the twice a week 0.04 M $MnCl_2$ and 0.02 M $Mn(OAc)_3$ inhalation protocol, the number of TH-positive neurons in the SNc were reduced 48.53% (52.57 ± 1.7 neurons after 5 months) (Fig. 4). We noticed that with the mice inhalation protocol (Ordoñez-Librado et al., 2011) the rats neuronal loss was not enough to produce evident behavioral alterations. Hence, we decided to use the same dose but with an inhalation protocol of three times a week for 6 months, afterwards we found pronounced cell reduction (Figs. 5 and 6) and motor alterations described above.

3.4.2. $MnCl_2/Mn(OAc)_3$ mixture

After 72 $MnCl_2/Mn(OAc)_3$-inhalations, a significant loss of the TH-positive neurons in the SNc was observed (75.95%) compared with the control group. The number and integrity of the TH-positive neurons in the VTA were not significantly affected by Mn-inhalation (Figs. 5 and 6).

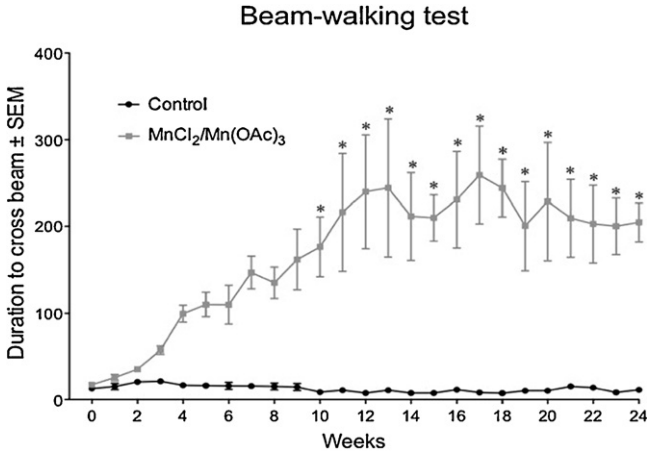

Fig. 3. Mean latencies to cross the beam (seconds ± SEM) before and after Mn-inhalation. Mn-exposed rats showed a significant increase in duration to transverse the beam compared to controls. The Mn-exposed rats are significantly impaired since week 10 (*$p < 0.001$ vs control group; repeated-measures ANOVA).

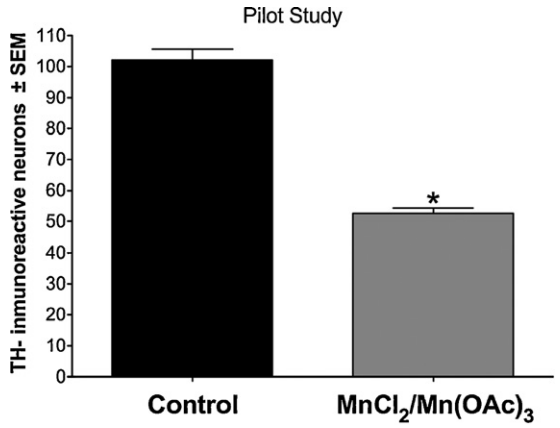

Fig. 4. Pilot study. Number of SNc TH^+-immunostained neurons from control and 0.04 M $MnCl_2$/0.02 M $Mn(OAc)_3$ twice a week exposed rats. The data are presented as the mean ± standard error (SEM) (*$p < 0.05$ unpaired *t*-test).

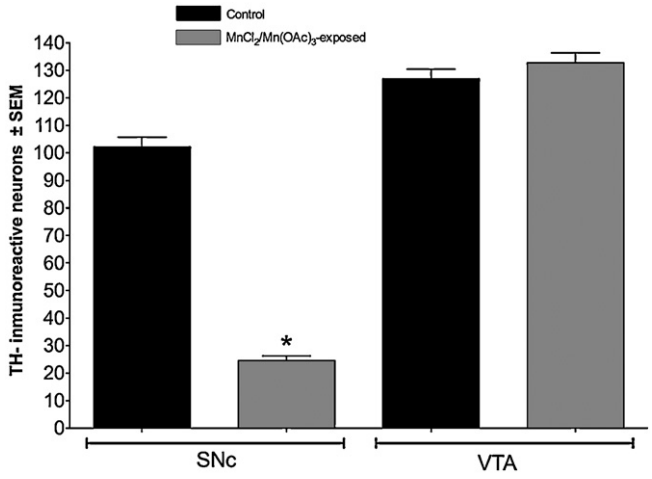

Fig. 5. TH-immunoreactive cell counts from the substantia nigra compacta (SNc) and ventral tegmental area (VTA). The data are presented as the mean ± standard error. A statistically significant decrease in TH-immunoreactive cells was detected in the SNc (*$p < 0.05$ unpaired *t*-test) of Mn-exposed rats compared to controls with no difference in VTA.

3.4.3. NeuN-immunocytochemistry

Globus Pallidus and Striatal cell count reveal no neuronal loss after 72 $MnCl_2/Mn(OAc)_3$ inhalations (Figs. 7 and 8).

4. Discussion

This study demonstrate that rats display different susceptibility to the inhalation of $MnCl_2/Mn(OAc)_3$ since they were exposed three times a week for six months instead of twice a week for five months. However, despite the different protocol, the rats, as the mice, display evident alterations in locomotor activity and a significant reduction in TH^+ cell counts in the SNc but not in VTA, nor in GP or striatum (see Figs. 5–8 and Ordoñez-Librado et al., 2008).

4.1. Motor performance alterations

4.1.1. Single-pellet reaching task

It has been demonstrated that skilled limb movements, such as the reach-to-grasp, display very similar motor components in

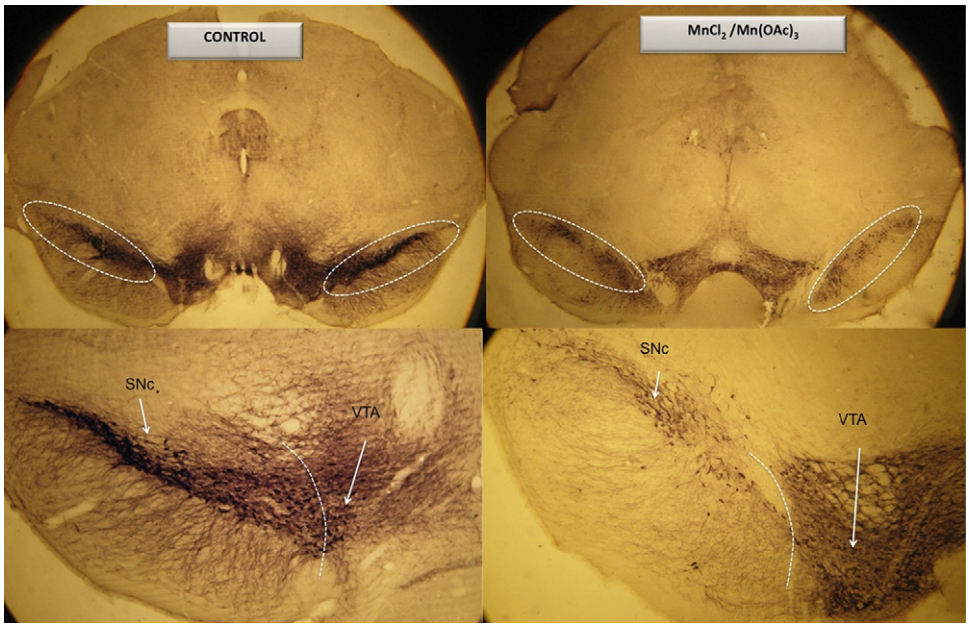

Fig. 6. Representative TH-immunostained from coronal section containing the SN and VTA of control and Mn-exposed rats showing the ROI including the SNc used for cell counting. Note the relative sparing in the VTA and profound cell loss of SNc in the Mn-exposed group (upper panel 4×, lower panel 10,000×).

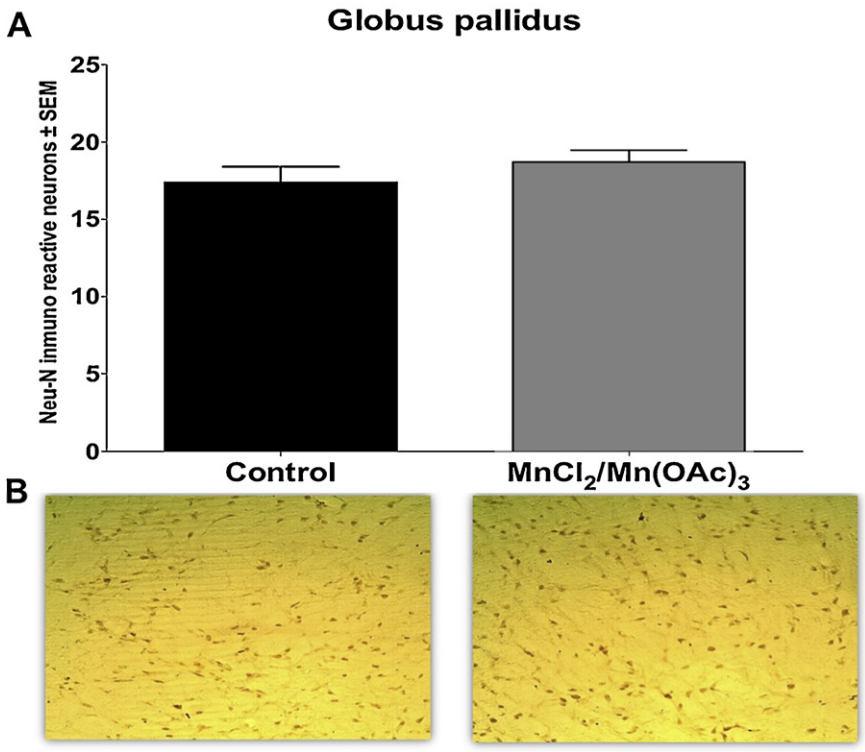

Fig. 7. (A) NeuN-immunostained cell counts from ventrocaudal GP. The data are presented as the mean ± standard error. There were no statistical differences between control and Mn exposed groups ($p > 0.05$ unpaired t-test). (B) Representative NeuN-immunostained from coronal section containing ventrocaudal GP of control and Mn-exposed rats (10,000×).

humans and in rodents (Metz et al., 2001; Whishaw et al., 2002). Humans with PD are often described as having poor manual skills that worsens as the disease progresses (Castiello et al., 1999; Jackson et al., 2000). These patients experience difficulties performing tasks requiring unilateral and bilateral arm movements and sequential and alternating limb movements (Whishaw et al., 2002).

Here, the rats drag the pellet across the shelf without raising the paw and either place the snout into the slot to retrieve the pellet with the tongue, or rotate the body and pursue 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 (MacLellan et al., 2006). Our results thus demonstrate, that rats have impairment in

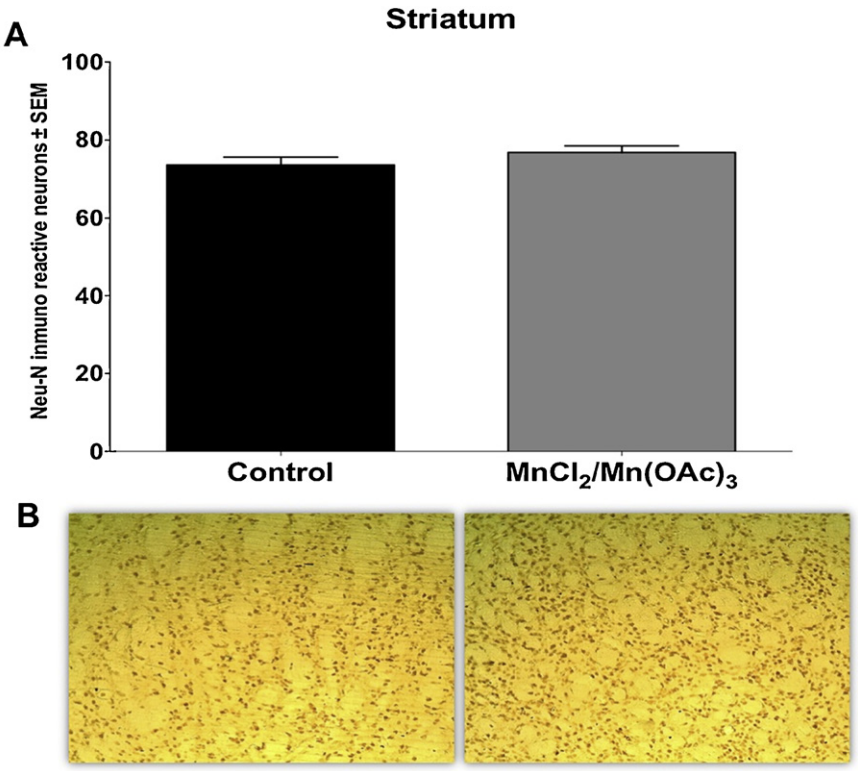

Fig. 8. (A) NeuN-immunostained cell counts from dorsomedial striatum. The data are presented as the mean ± standard error. There were no statistical differences between control and Mn exposed groups ($p > 0.05$ unpaired t-test). (B) Representative NeuN-immunostained from coronal section dorsomedial striatum of control and Mn-exposed rats (10,000×).

their success in retrieving food pellets probably due to dopaminergic cells loss.

4.1.2. Beam walking test

The motor function impairments observed on the beam-walking task are comparable with those found in mice exposed to Mn mixture (Ordoñez-Librado et al., 2008, 2011), that displayed impairments in limb coordination, stride length and motor performance. As time goes on, the rats show more difficulty ascending the beam (Fig. 3). The motor function impairments observed on the beam walking task are also comparable with published findings in which C57 BL6/J mice treated with acute and sub-chronic dosing regimens of MPTP, were reported to display impairments in limb coordination, stride length and motor function at 1–2 weeks post-MPTP administration (Fernagut et al., 2002; Ogawa et al., 1985).

Qualitative analysis showed that Mn-exposed rats exhibit hind-limb weakness, akinesia, postural instability, action tremor and freezing behavior. With regard to these alterations, Autissier et al. (1982) 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. (1987) found that about 5 months after Mn exposure the animals became hypoactive with an unsteady gait, and subsequently 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 (Brouillet et al., 1993; Díaz-Véliz et al., 2004).

4.2. Immunocytochemistry

The rats exposed to Mn showed severe loss of SNc TH-immunopositive cells comparable to what we observed in Mn exposed mice (Ordoñez-Librado et al., 2008, 2011), but not in VTA, GP or striatum. Our results disagree with other reports where found no loss of dopaminergic neurons (Guilarte et al., 2006; Gwiazda et al., 2002; Peneder et al., 2011; Perl and Olanow, 2007; Struve et al., 2007; Tomás-Camardiel et al., 2002) and loss of striatal and pallidal cells (Calabresi et al., 2001; Olanow, 2004; Perl and Olanow, 2007).

The contradictions between our findings and the studies that report no SNc cell loss and striatal and GP cell alterations after Mn exposure, could be due to at least three factors; first, Mn compounds and their combination, which as far as we know, there is no study that has included the mixture of such compounds. According to Aschner (2006) it seems that the extent of Mn neurotoxicity appears to be determined by its oxidation state. Mn^{2+} can be oxidized to the powerful oxidizing agent, Mn^{3+} by superoxide (Archibald and Tyree, 1987) and since the mitochondrial electron transport chain is recognized as the largest producer of superoxide in the cell, a common hypothesis of Mn-induced damage is related to the oxidation of important cellular components by Mn^{3+} (Archibald and Tyree, 1987). It has been proposed that Mn^{3+} is more potent in producing cell damage (Reaney et al., 2006) and Mn^{2+} needs the presence of Mn^{3+} to reach oxidation, thus it seems that there is synergy between the two Mn states (HaMai and Bondy, 2004). It also 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 rapid oxidative metabolism catalyzed by these metals (Stokes et al., 1999). In this regard, 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 (Archibald and Tyree, 1987; Donaldson et al., 1982). Thus, it is possible that Mn-induced DA oxidation results in the generation of reactive oxygen species, oxidative stress, and secondary cytotoxicity to dopaminergic neurons (Archibald and Tyree, 1987; Graham, 1984;

Hussain et al., 1997; Simonian and Coyle, 1996). Several explanations have been proposed to elucidate the vulnerability of dopaminergic cells to Mn, such as the deficiency of cellular antioxidant defenses by the accumulation of the metal (Desole et al., 1997), and the disruption of mitochondrial oxidative energy metabolism (Morello et al., 2008).

Second, Mn levels measured in the inhalation chamber (2676 $\mu g/m^3$) and the exposure time (6 months) are sufficient enough to produce behavioral and cytological disorders, since it has been proposed that overt Mn neurotoxicity most often results from the chronic inhalation of very high (>1 mg/m^3) Mn concentrations (Pal et al., 1999) and after long-term exposure (Gwiazda et al., 2007).

Third, it seems that the route of exposure can influence the delivery of Mn to the brain (Andersen and Gearhart, 1999; Roels et al., 1997). Roels et al. (1997) investigated brain Mn concentrations in rats following exposure to either a soluble ($MnCl_2$) or insoluble (MnO_2) forms. These chemicals were administered via intratracheal injection (as a surrogate for inhalation) or by gavage (oral administration). This experiment was designed in order to achieve similar blood Mn concentrations and was thus designed to account for low oral absorption of Mn versus the higher rate of absorption from the lung. When administered intratracheally once a week for 4 weeks, 1.22 mg $MnCl_2$/kg as resulted in a 68% steady-state increase in blood Mn concentration after the dosing period. This dose also resulted in significantly increased concentrations of Mn in the rat striatum (205% increase) and cortex (48% increase) when compared to control rats. $MnCl_2$ administration by gavage (administration of 24.3 mg $MnCl_2$/kg as once weekly for 4 weeks) caused roughly the same amount of increased Mn in the blood (68% increase vs. controls) as intratracheal administration of Mn in the same form, but it did not cause as significant increase of Mn in the cortex (22% increase vs. controls); striatum Mn concentrations were unaffected following gavage administration. Thus, pulmonary delivery of Mn appears to be more efficient than ingestion in increasing Mn concentration in the brain. Moreover, Calabresi et al. (2001) found no behavioral alterations after give the rats drinking solutions of $MnCl_2$ (20 mg/ml of water).

On the other hand, it is important to indicate that, although Mn-inhalation induced significant damage to dopaminergic neurons in the SNc, the VTA dopaminergic neurons did not seem 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 suggested that Mn enters the neurons via DAT (Anderson et al., 2007; Erikson et al., 2005; Hastings et al., 1996; Ingersoll et al., 1999) as in the case of MPTP (Haber et al., 1995), 6-OHDA (Decker et al., 1993; Permual et al., 1992) and Paraquat and Maneb (Thiruchelvam et al., 2000), where SNc is more susceptible than VTA. It seems that SNc cells and VTA display differences in their topography, biochemistry and susceptibility to pathological processes (Blanchard et al., 1994; Uhl, 1998), VTA express lower DAT levels than the middle and medial SNc (Blanchard et al., 1994; Ciliax et al., 1999; Haber et al., 1995), thus it is possible that Mn reaches SNc dopaminergic cells via the great amounts of DAT found in these neurons, however additional studies are needed to confirm this matter.

4.3. Differences between rats and mice

It is known that diverse susceptibility to neurotoxins exists between species. In this way, the best PD model MPTP, in rats is not being widely used, and the significance of data obtained from MPTP-treated rats are controversial (Kopin and Markey, 1988). Rats injected with MPTP doses comparable to those used in mice do not show any significant dopaminergic neurodegeneration (Giovanni et al., 1994a,b). Only injections of much higher doses of MPTP (multiple applications of 30–60 mg/kg body weight) cause significant dopaminergic neurodegeneration in rats (Schober, 2004).

Remarkably, these rats have to be therapeutically pretreated, with guanethidine, to prevent peripheral catecholamine release and extensive mortality (Giovanni et al., 1994a). These findings indicate that rats are relatively insensitive to MPTP. Thus, rats are not recommended for MPTP studies, because rats fail to develop parkinsonian features, as shown, e.g. for monkeys and mice (Schmidt and Ferger, 2001). The evident insensitivity of rats to MPTP toxicity may be related to a species specific metabolism of MPTP and/or sequestration of MPP^+, which could be different in rats compared to mice and monkeys (Russo et al., 1994; Schmidt and Ferger, 2001). And despite that MPTP in non-human primates and mice provokes a well animal model, a spontaneous recovery of parkinsonian symptoms has been described in both, monkeys (Eidelberg et al., 1986; Taylor et al., 1997) and mice (Sedelis et al., 2000, 2001) after MPTP administration, which causes concern to use this model for an assessment of long-term therapeutic effects. However, it has been reported that chronic administration of low doses of MPTP to macaques, reproduces all the signs of PD (tremor, bradykinesia, rigidity, hypokinesia, and postural impairment) and closely mimics the progressive nature of PD (Brownell et al., 1998, 2003). Nevertheless, rodents are most commonly used over non-human primates since rodent models have the advantage that rats and mice are widely available, they have high reproductive rates, require reduced living space, simple feeding and drinking schedules and low costs (Fox et al., 1984). Moreover, because of the economical, logistic and ethical constraints that are related to experimental research in primates, primate models of PD are used in relatively few laboratories worldwide (Cenci et al., 2002).

On the other hand, 6-OHDA model has been widely used in rats; only few studies concerning mice with 6-OHDA-lesions have been published. In these studies, 6-OHDA was injected mainly either intrastriatally (Bensadoun et al., 2000; Brundin et al., 1986; Cunningham and Su, 2002; Lundblad et al., 2004) or intraventricularly, and the mice were subjected to relatively little behavioral assessment (Archer et al., 2003; Asanuma et al., 1995; Bensadoun et al., 1998). Moreover, Cenci and Lundblad (2007) unilaterally lesioned rats and mice with 6-OHDA and determined abnormal involuntary movements (AIMs) after L-DOPA treatment, these authors stated that although rat and mouse AIMs can be rated according to the same principles, there are differences between the two species. Compared to rats, movements in mice are much faster and less articulate. It is therefore more difficult to discriminate between normal and abnormal movements in mice with this model. Iancu et al. (2005) lesioned mice in the medial forebrain bundle (MFB), selected 53 mice out of 110 mice subjected to the lesion. They selected mice showed a wide variability regarding loss of dopaminergic SN neurons probably due to the small size of the MFB that makes it difficult to target in mice.

The slight differences in the inhalation protocol between mice and rats that we found here, are probably due that in the rat, Mn absorption is thought to be a rapidly saturable process probably mediated by a high-affinity low capacity transport system (Garcia-Aranda et al., 1983), thus the rats, although with the same Mn concentrations, needed more inhalations per week during 6 months instead of 5. However, both, in behavioral and cytological alterations, the rats behaved in a way similar to mice.

5. Conclusion

Unlike the models of MPTP and 6-OHDA, where all alterations induced appear in a range of days or weeks, whereas PD in humans develops over decades (Schmidt and Ferger, 2001), our Mn-inhaled model seems to be an adequate model since the degeneration is

progressive and bilateral, and the differences between species are minimal.

According to Schober (2004) an adequate PD animal model should have the following features: (i) a normal set of nigral dopaminergic neurons at birth followed by a selective gradual loss of these cells beginning in adulthood; (ii) easily detectable and quantifiable motor deficits; (iii) Lewy bodies should be generated; (iv) the model should have a relatively short time course to mimic the pathogenesis of PD (about 3–6 months), which would allow a rapid screening of therapeutic substances and strategies. Hence, with our model, we reproduce at least three of those features. However, further analyses are needed to elucidate whether Mn inhalation decreases DA striatal concentrations, generates Lewy bodies and determine if the animals after the inhalation period show recovery.

Finally, the results of this work and the findings of the Mn-model in mice provided important contributions toward a better understanding of the mechanisms involved in nigrostriatal degeneration in PD because it is highly viable and adequately mimics the neurochemical, neuroanatomical and some of the behavioral characteristics of PD.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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