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Chapter 4

Manganese Inhalation Induces Dopaminergic Cell Loss: Relevance to Parkinson's Disease

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Abstract

Parkinson's disease (PD) experimental models are crucial in the assessment of possible therapies. Nevertheless, even though PD was one of the first neurodegenerative conditions to be modeled, there are limitations such as spontaneous recovery; lack of bilateral damage, which is a PD characteristic; animal intensive care after neurotoxin administration; and ultrastructural and biochemical nonspecific alterations but mostly the neurodegenerative time course observed in humans. In this chapter, we investigated the effects of divalent and trivalent manganese inhalation on rats and mice to obtain a novel PD animal model inducing bilateral and progressive dopaminergic cell death. We found that after 5 or 6 months of inhalation, there was more than 70% decrease in the number of TH-immunopositive neurons, and these alterations are correlated with an evident motor performance deficits manifested as akinesia, postural instability, and action tremor. More interesting is the fact that these alterations were reverted with L-DOPA treatment, implying that the motor alterations are associated with nigrostriatal dopaminergic innervation, postulating new light for the understanding of manganese neurotoxicity as an appropriate PD experimental model. Our results are contributing to the development of a suitable PD animal model, reproducible, sensitive, time-efficient, and readily applicable behavioral tests.

Keywords: Parkinson's disease experimental model, rodents, manganese inhalation, dopaminergic cell loss

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1. Introduction

The typical motor symptoms of Parkinson's disease (PD) (akinesia, bradykinesia, rigidity, tremor, and postural abnormalities) are related to the loss of nigral dopaminergic cells and decay in caudate-putamen dopamine (DA) content that led to the introduction of DA replacement therapy [1]. Consequently, there has been a fundamental role for PD animal models in developing new approaches treating this disease, in innovative treatment strategies, and in understanding the nature of the pathogenic processes involved in the dopaminergic neuronal loss [1, 2].

Several models display many of the distinctive features of the disease; however, none resembles the complex chronic neurodegenerative features observed in human PD. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) are considered as neurotoxicants that rapidly and selectively kill dopaminergic neurons (in 1–3 days), while in PD patients, the disease is progressive [3].

Emborg [4] declares that a representative animal model must present pathology and behavioral manifestations that match the disease, involving its temporal path. The more the similarity of a model is to PD, the bigger the predictive strength for clinical efficacy will be.

The results regarding manganese (Mn) as an experimental PD model have been studied since its toxicity (commonly called manganism) shares neurological symptoms with numerous clinical disorders frequently described as "extrapyramidal motor system dysfunction," and, in particular, idiopathic PD [5–7]. Manganism is associated with high brain levels of Mn, primarily in those areas known to contain high concentrations of nonheme iron, particularly the striatum, globus pallidus (GP), substantia nigra compacta (SNc), and subthalamic nuclei [8].

There is some disagreement on the alterations induced by Mn; while some researchers reported that Mn alters nigrostriatal dopaminergic levels and produces a Parkinson-like disorder [9–12], other authors confirmed that Mn alterations are related to different aspects to those associated to PD in both etiology and pathology [13, 14] especially in the remarkable SNc dopaminergic cell conservation [15–19]. As stated by Calne et al. [5], Lu et al. [16], and others [20–22], the most important between these differences is the absence of clinical response to L-DOPA.

However, studies have reported ostensibly contradictory results on the dopaminergic effects of Mn (see Gwiazda et al. [23] and Guilarte [24] for review), including decrease [9, 25–28], increase [11, 29], both [30], or no modification [15, 31, 32] in SNc or striatum DA levels in Mn-exposed animals, probably indicating differences in exposure procedures on DA consequences. These inconsistencies might disclose changes in the route of exposure, magnitude, duration, Mn compound or concentration, experimental animals' species, age, etc., among investigations, revealing the complexity of Mn toxicity and suggesting that the features that cause the toxicity are not entirely recognized.

It appears that lesser dosages of Mn augmented DA and its metabolite concentrations, whereas the inverse was detected with more significant Mn concentrations [30, 32]. Similarly,

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it has been proposed that higher Mn dosages can drastically accelerate DA and other catecholamine oxidation, which concomitantly intensify reactive oxygen species formation of [33–35].

It seems that both trivalent and divalent Mn can be carried to the CNS through the brain barriers [36, 37]. Mn^{2+} is transferred into brain choroidal epithelial and capillary endothelial cells through nramp2 (DMT-1) or by divalent cation transporter DCT-1 [38]. On the other hand, trivalent Mn bound to transferrin is transported across the brain barriers via the receptor-mediated endocytosis [37]. Mn is then liberated into the endothelial cells by endosomal acidification [21], then is transported to the abluminal cell exterior for release into the extracellular fluid. Finally, it is delivered to the glial cells and neurons, for usage and storage [39]. It has been demonstrated that Mn inhibits complex I in the mitochondria altering the oxidative phosphorylation process. Also, it appears that trivalent Mn is more effective in inhibiting complex I than divalent Mn [40–43] and accelerating ferrous iron oxidation. Mn^{3+} increased facility to provoke oxidative stress has been established in rats treated with either Mn chloride [$MnCl_2$ (Mn^{2+})] or Mn acetate [$Mn(OAc)_3$ (Mn^{3+})] [41]; these researchers state that 1–1000 μM $MnCl_2$ induced increased reactive oxygen species in striatum, while $Mn(OAc)_3$ produced comparable results at significantly lower dosages (1–100 μM). Therefore, the Mn valence and metabolism appear to determine its toxicity.

Thus, since it has been suggested that trivalent Mn is more effective in producing oxidative stress and divalent Mn requires Mn^{3+} to induce oxidation and that there is an interaction between the two Mn compounds, this study examines Mn^{2+}/Mn^{3+} mixture inhalation effects on rats and mice to produce a unique PD experimental model provoking SNc dopaminergic cell death, progressive and bilateral, associating those changes with motor alterations. Moreover, we sought to determine if after Mn inhalation the motor alterations improve with L-DOPA treatment to ensure that the alteration's origin is dopaminergic.

2. Methods

Animals: 45 CD-1 male mice weighing 33 ± 2 g and 45 male Wistar rats weighing 180 ± 10 g were individually housed in hanging plastic cages under controlled light conditions (12 h light/dark regime) and fed with Purina Rodent Chow and water ad libitum (except the days of reaching task evaluation). The animals were weighed daily. The experiment was done according to the NIH Guide for the Care and Use of Laboratory Animals (No. 80-23 1996), Guide for Care and Use of Laboratory Animals certificated by SAGARPA-Mexico (NOM-062-ZOO-1999, Mexico) and approved by the Institutional Committee of Animal Care (UNAM). We made all attempts to reduce the number of rodents used and their distress.

2.1. Motor behavior

Before Mn exposure, all rodents were taught and trained for motor performance. Assessment and training were accomplished through the lighted part of the cycle, at the same hour every day. For the reaching task, the animals were kept without food to 90% of average body weight

for 24 h and received controlled quantities of food pellets once a day to sustain body weight and deprivation state. Behavior analyses were conducted the days the animals did not inhale. Each animal was tested once a week, a different day for each test.

2.2. The reaching task

The mouse reaching box was 19.5 cm × 8 cm and 20 cm high. A 1-cm vertical slot ran up the front of the box. A 0.2-cm-thick plastic shelf was displayed 1.1 cm from the floor on the box front. The rat-reaching box was 30 cm × 15 cm and 20 cm high. As for the mice box, this one has a 1-cm wide and narrow opening that ran up the front of the box. About 20-mg food pellets were positioned near the slot. Animals were habituated for 1 week by introducing them in the cages for 10 min. Pellets were initially reachable on the box floor and then within a short distance on the shelf. Food pellets were progressively raised from the box floor and positioned beyond the shelf (1 cm) until the rodents were obligated to retrieve the pellet with their preferred forelimb. According to Whishaw et al. [44], the pronation of the paw medially allows the mouse/rat to catch the food pellet with the forelimb and not with their tongue. The animals were independently trained and permitted to grasp with their preferred forelimb the pellets [44]. Each animal grasped for 20 food pellets each trial during the evaluating period. A successful reach was scored when the animal was able to retrieve with its forelimb and eat a pellet. When the pellet was knocked off the shelf or pulled into the chamber and dropped through the floor grating were scored as a failure [45]. The qualitative evaluation comprised the analysis of the "reaching performance": the posture, limb extension, aim, paw supination-pronation during grasping, and the pellet released into the snout.

2.3. The beam-walking task

This test evaluates the rodents' skills to traverse a narrow beam (3 mm) to reach an enclosed safety platform [46]. The mice apparatus is constructed by an elevating surface of a 10 × 100 cm × 3 mm wood beam 75 cm above the floor with two supports by 15° inclination. Rat's beam measured 2 m long and was elevated to a height of 1 m above the ground with wood supports with 15° inclination. A home box is situated near the end of the beam. On training days (4 days), each mouse/rat was positioned at the start of the beam with no inclination (four tests each day). When the animals traversed the apparatus in 20 s, they performed two more trials with the beam inclined. Mice were allowed up to 60 s and rats 120 s to traverse the wooden beam. The latency to cross the beam was recorded for each trial.

Video recording: the different trials were recorded with a Sony camcorder. The video camera was placed orthogonally to the reaching box to analyze the animal's behavior. Demonstrative motionless captures were taken from the video recordings with the Final Cut Pro X for Mac.

Neurological evaluation: Tremor and bradykinesia were assessed by inspection of Mn-exposed compared with control animals during the performance of the two tests.

2.4. Manganese inhalation

Afterward, two groups were formed: one group was exposed to deionized water (control groups; n = 20), while the second group (n = 20) was exposed to the mixture of chloride ($MnCl_2$)

0.04 M and acetate ($Mn(OAc)_3$) 0.02 M (Sigma-Aldrich, Co. Mexico). Inhalations were done as described by Avila-Costa et al. [47]. The animals were positioned in an acrylic chamber. Mn exposure was accomplished in locked acrylic boxes (35 cm × 44 cm and 20 cm high) attached to an ultra-nebulizer (Shinmed, Taiwan), with 10 l/min constant flux. The ultra-nebulizer produces 0.5–5-μm range droplets. A vapor was placed on the other side of the box with a sodium bicarbonate mixture to trap the residual metal. During inhalations, the rats/mice were examined continuously for respiration frequency, regularity, and depth. The inhalation chamber was monitored continuously for oxygen levels, temperature, and Mn concentration.

Based on the results found in the behavioral evaluations, we sacrifice the animals after being exposed to 40 (mice) and 72 (rats) inhalations (5/6 months of exposure) under deep anesthesia with sodium pentobarbital lethal dose IP (0.2 mg). Thus, when evident motor alterations were observed, twenty mice/rats were sacrificed (ten controls and ten Mn-exposed), anesthetized with sodium pentobarbital, and perfused via the aorta with phosphate buffer saline (0.1 M pH 7.4) containing 4% paraformaldehyde. The brain was removed and positioned in fixative solution for 2 h and processed for tyrosine hydroxylase (TH) and NeuN immunocytochemistry (five control and five Mn-exposed brains).

Later, the rest of the animals continued the Mn inhalation. Five were treated orally with 7.5 mg/kg L-DOPA (Sinemet [Carbidopa-L-DOPA 25/250]) every day during 2 months, five were reserved for the equivalent time but with no treatment, and five controls were kept for the same time and then sacrificed for further analysis; the motor behavior performance was assessed every week.

Additionally, the fresh tissue of other 10 control and 10 exposed animals, after 40 inhalations (mice) and after 72 inhalations (rats), was obtained to determine the concentrations of DA by HPLC in the striatum, SNc, and GP.

2.5. Sample preparation and immunohistochemistry

Tissue samples were serially sectioned at a thickness of 50 μm on a vibrating microtome (Pelco 101, Ted Pella Inc., Mexico) within 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 technique (Vector Lab, MI, USA) was performed for the cell analysis. All images were captured using an Optiphot 2 Nikon microscope. Images were analyzed using ImageJ software. The number of TH+ cells was calculated rostrocaudally through the SNc and ventral tegmental area (VTA) in nearby segments. The SNc was manually delineated to trace the region of interest (ROI) at low magnification (4×). The TH-positive cell number was calculated at the level of the third cranial nerve, within a 100-mm counting area at 40× only within this defined ROI [48, 49]. NeuN cell count of striatum and GP was done using 40× objective in seven sections per animal at 0.70 anterior, 0.48 mm posterior to bregma for dorsomedial striatum, and 0.80 anterior and 0.92 mm posterior to bregma for ventrocaudal GP according to [50] for rats and at rostrocaudal levels 0.86 anterior to 0.50 mm posterior to bregma for dorsomedial striatum and 0.62 anterior to 0.98 mm posterior to bregma for ventrocaudal GP according to [51] for mice, in a 11,550 and 3300 mm^2 counting area, respectively. It should be noted that both dorsomedial striatum and ventrocaudal GP receive the maximum dopaminergic innervation [52, 53].

2.6. Mn concentrations

The Mn concentration in the inhaling box was calculated by placing a filter at the gap of the inhaling chamber during the whole inhalation time; the flow rate was constant (10 l/min). After each exposure, the filter was detached and weighed; the metal concentration was calculated with a graphite furnace atomic absorption spectrometer (Perkin Elmer Mod. 3110, CT, USA). We analyzed six filters for each inhalation [54]. At the end of the experiment, rat/mice serum Mn levels were also estimated by graphite furnace atomic absorption spectrometry.

2.7. Dopamine concentrations

SNc, striatal, and GP DA contents were obtained after 5 months, for mice, and after 6 months for rats of Mn inhalation as described by [55]. Briefly, five controls and five Mn-exposed mice and five controls and five Mn-exposed rats were anesthetized and decapitated, and with a stereoscopic microscope, the tree structures were obtained. The tissue was homogenized in perchloric acid with 100 μl per brain. Then, the tissue was centrifuged (300 PSI, 2 min, Airfuge centrifuge, Beckman, Fullerton, CA, USA) and the supernatants filtered (0.22-μm membranes, Millipore, Bedford, MA, USA). The resulted tissue was resuspended, and by Bradford method, we performed the protein determination as reported elsewhere [56]. DA levels in 10 μl of supernatant were determined through HPLC reverse phase system attached to an electrochemical detector (BAS; West Lafayette, IN, USA). Results were analyzed using the Peak II integration software (SRI Instruments; Torrance, CA, USA). DA concentration is shown as pg./μg protein.

2.8. Statistical analysis

Unpaired t-test was used to analyze the number of TH and NeuN-positive cells. Repeated measures ANOVA analyzed motor behavior tests; post hoc comparisons were performed with Tukey's test. Group differences were established as statistically significant when $p < 0.05$. Statistical analysis was done using GraphPad 7 for Mac Software (San Diego, CA).

3. Results

After 5 (mice)/6 (rats) months of exposure, neither clinical alterations nor significant weight changes were detected in the exposed animals compared with controls.

3.1. Manganese concentrations

The average Mn concentration detected in the chamber filters was of 2676 μg/m^3 during the whole experiment. The average Mn concentration in serum of exposed mice was 30 ± 5 μg/l; control mice serum concentration of Mn was 0.05–0.12 μg/l. The average Mn concentration in serum of exposed rats was 45 ± 5 μg/l; control rat's serum Mn concentration was of 0.05 ± 0.12 μg/l.

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3.2. Single-pellet reaching task

The task includes the accomplishment of motor sequences, beginning with smelling a food pellet forward-facing the reaching slot, lifting the arm, adapting position to project the limb across the narrow slot to the food pellet, and taking the food (**Figure 1**).

Mice and rats were presented with 20 food pellets. **Figure 2** displays the success reaches throughout the experiment. Repeated measures ANOVA established a substantial effect of Mn-exposed groups since eight inhalations ($p < 0.001$). Mice/rats were similar in their skill to recover the pellets before Mn exposure, but Mn inhalation occasioned significant alterations in both number of successful recoveries ($p < 0.001$) and precision in both mice (**Figure 2A**) and rats (**Figure 2B**); however, with L-DOPA treatment, the animals recover their functioning when compared to the non-treated ones, like the control groups' performance ($p < 0.001$). Control animals were steady during the entire experiment and were notably better than Mn-exposed animals (**Figures 1** and **2AB**).

The qualitative assessment showed postural swing and deficiencies in limb extension (resulting in several shortened reaches), aim, and paw supination-pronation during grasping and release of the pellet into the slot (**Figure 3A–J**); both mice and rats exhibited unusual movements when recovering the food after Mn inhalation. The forelimb was frequently totally pronated and moves laterally over the food (**Figure 3F**, **G**, and **I**), or the animal hits at the pellet (**Figure 3I**); some mice/rats from Mn-exposed groups displayed such behavioral alterations that endured for the complete study.

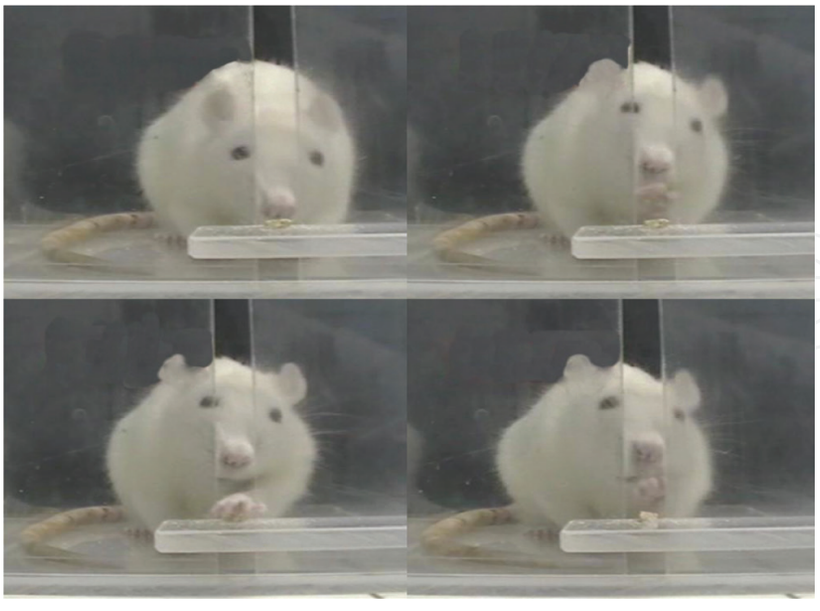


Figure 1. Characteristic pictures of a control animal taken during limb moving and withdrawal. The control animals moved their arm throughout the slot and opened their fingers; then, supinated their paw to take the food to the snout; and extended their digits to release the food into the mouth.

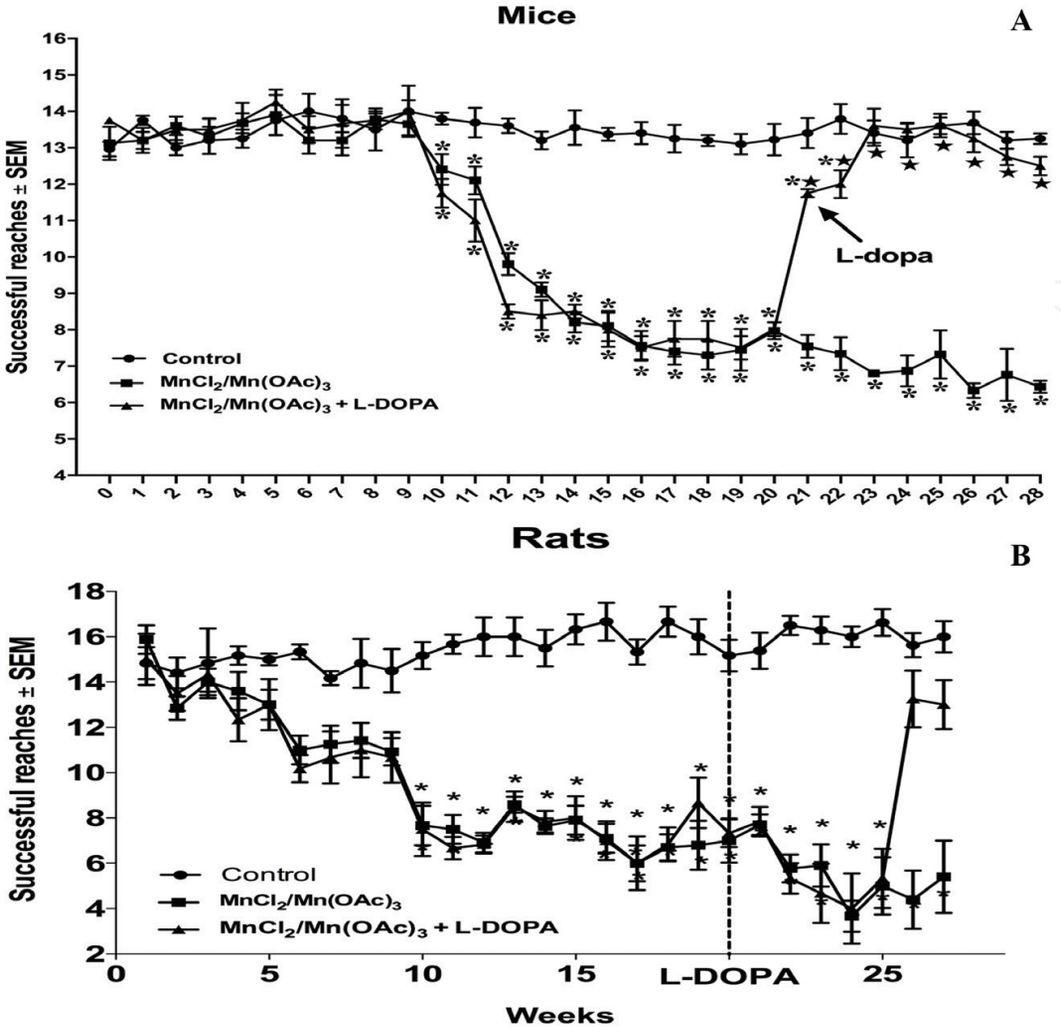


Figure 2. Reaching success scores (sum of food pellets taken out of 20; mean ± SEM) of control and Mn-exposed mice (A) and control and Mn-exposed rats (B) in the reaching task. The Mn-exposed group is impaired since week 12; note that L-DOPA treatment entirely reverses the alterations (*$p < 0.001$ vs. control group; repeated measures ANOVA).

The Mn-exposed groups are often incapable of accurately closing the digits around the pellet and dragging it to the slot without lifting the paw (**Figure 3F**, **G**, and **I**). These animals are also not capable of supinating the forelimb entirely and putting the mouth into the gap to recover the food with their tongue (**Figure 3J**). When the arm is withdrawn throughout the gap, Mn-exposed groups repeatedly turn their body and pursuit the food with the tongue instead of opening their fingers and introducing the food into the snout. The non-reaching forelimb is occasionally placed for support when recovering the food. Post-hoc analysis on the group's effect showed that at more Mn inhalations, success of retrievals was significantly lesser (**Figure 2**). These situations amazingly recover with L-DOPA treatment (**Figure 2A** and **B**). The treated animals adjust their posture and project the arm toward the food pellet, supinate and pronate the paw to obtain the food, close their digits, and drag the food to the snout; their motor performance with L-DOPA treatment was comparable to control groups (**Figures 1** and **2**).

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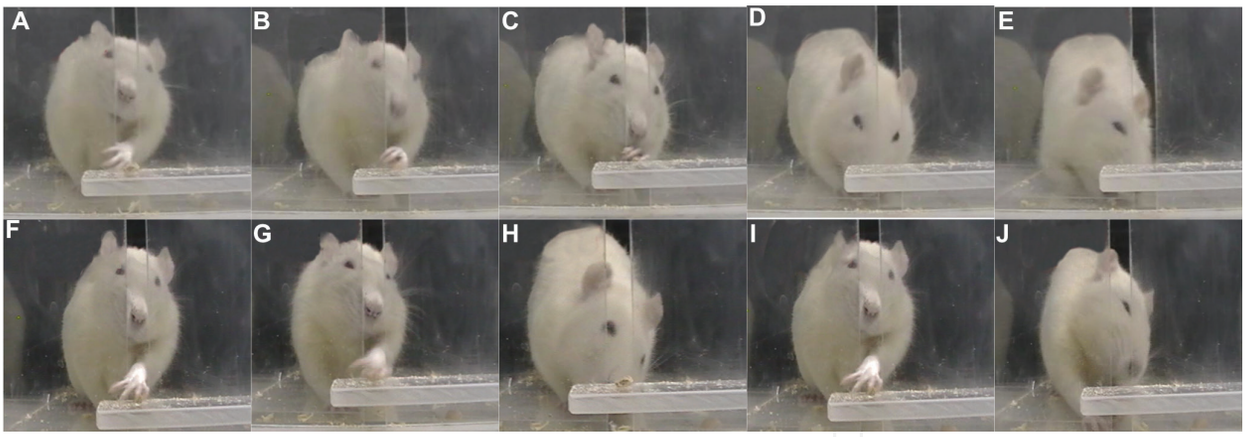


Figure 3. Illustrative still pictures of an exposed to Mn mouse (A–E) and a Mn-exposed rat (F–J). The animal approaches its forelimb by moving the elbow for the hand goes through the gap. As the arm moves closer to the food, the fingers open, and then the mouse pronates its forelimb by elbow adduction and rotates it around the wrist so that the hand is positioned on the top of the food. The pellet is grabbed by flexion of the fingers. The forelimb is withdrawn carrying the food. The animal lies on its hips to eat the pellet, which is secured by the hands. Frames (F–J) Mn-exposed rats displayed alterations characterized by severe postural modifications moving the forelimb obliquely throughout the gap making various small efforts without stretching the forelimb according to the midline of its body. The fingers are simultaneously adducted. The forelimb arises in front of the side or hits laterally, and the fingers do not take the food. The animal often pulls its forelimb across the gap and let fall the food to the floor cage chasing it with the tongue.

3.3. Beam-walking test

During the last day of evaluation before Mn exposure, we found no significant differences concerning the time in finishing the test for the controls and the Mn-exposed animals (ANOVA, $p > 0.05$). **Figure 4** depicts the mean of total time to traverse the beam. Mn-exposed mice (**Figure 4A**) and rats (**Figure 4B**) after 10 weeks of inhalation have a significant increase in the time to cross the beam compared with control groups; moreover, animals exhibit limb weakness, akinesia, postural instability, and action tremor. Mn-exposed mice have a significant reduction in the time taken to traverse the beam after two, four, six, and eight Mn inhalations (**Figure 4A**) proposing hyperactivity. Afterward there is a significant increase in the time to pass and a visible presence of freezing behavior time (data not shown), compared with control mice. As for the rats (**Figure 4B**) in the beam-walking test, Mn-exposed animals increased the execution at all-time points. While the control rats maintained an average of 20 s during the entire experiment, the Mn-exposed rats are slow and take more than 120 s to cross the beam after the tenth week (**Figure 4B**). This effect is completely reversed with L-DOPA treatment. Besides, all exposed animals also exhibited hind-limb weakness, delayed motor initiative (akinesia), postural instability, and action tremor. L-DOPA treatment reverted these motor alterations in both rats and mice.

3.4. Immunocytochemistry

3.4.1. TH immunocytochemistry

As for TH immunohistochemistry, mice (**Figure 5A**) exposed to 40 inhalations showed 67.58% decreased in the number of TH-immunopositive neurons in SNc compared to the control

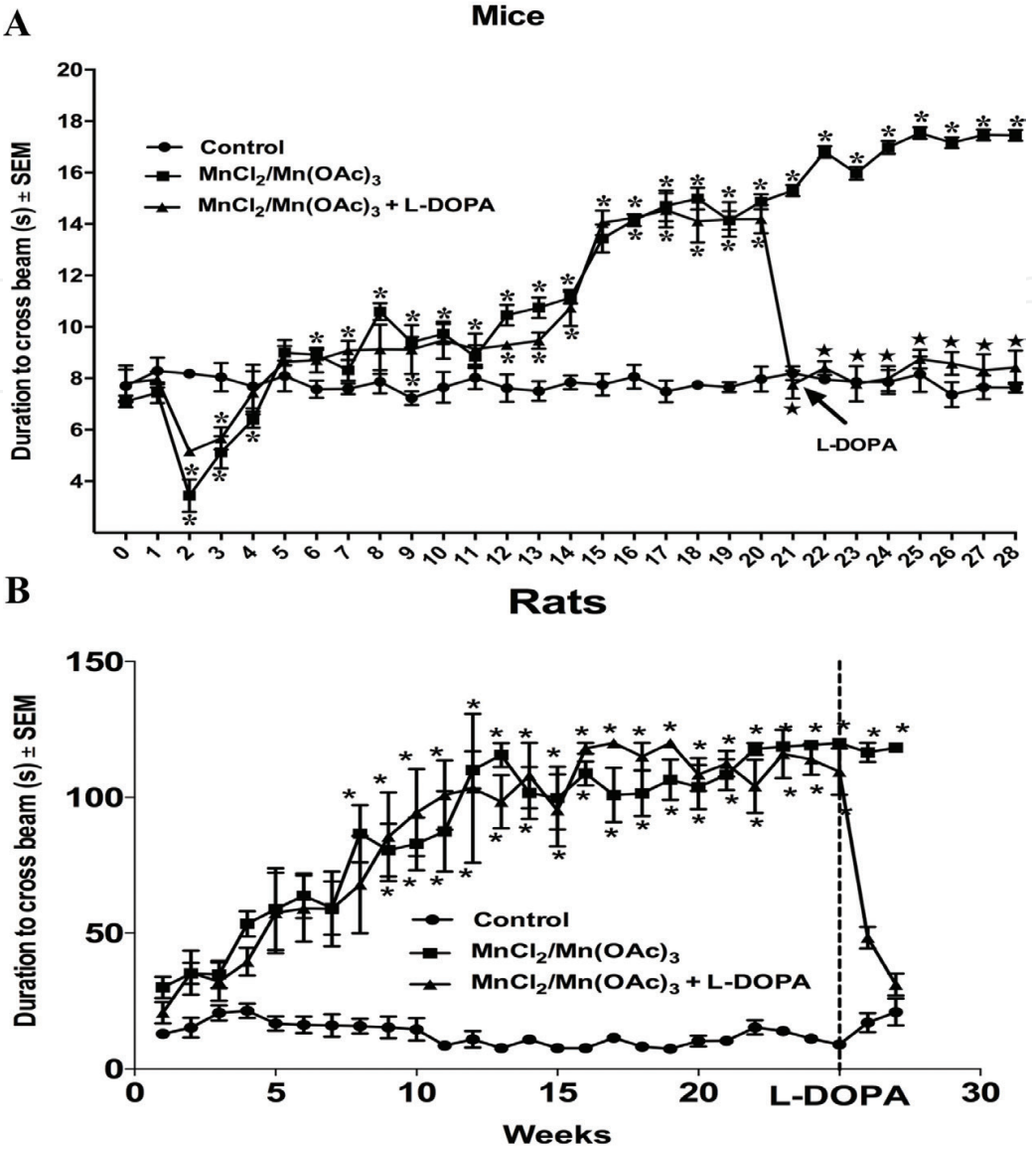


Figure 4. Mean latencies to traverse the beam (±SEM) before and after mice (A) and rats' (B) Mn inhalation and after L-DOPA treatment. It is notorious that after 2, 4, 6, and 8 of Mn inhalation, the mice significantly reduce the time to traverse the beam and afterward showed a significant increase in the time to cross the beam compared to controls. The Mn-exposed rats are significantly impaired since week 10. However, when the animals received the L-DOPA treatment, the time was reduced drastically resembling the values of the control group (*$p < 0.001$ vs. control group).

animals, while there was no loss of neurons in VTA of exposed animals compared to controls (**Figure 5A** and **6**). The rats showed a 75.9% loss in the number of TH immunoreactive neurons after 48 inhalations and, like mice, showed no neuronal loss in the VTA (**Figure 5B** and **6**).

3.4.2. NeuN immunocytochemistry

One of the required characteristics for animal models is the neuronal specificity for cerebral nuclei that are affected in humans, so to determine if the Mn mixture affects other brain structures, we performed anti-NeuN immunohistochemistry, a nuclear protein neuronal specific. In this respect, we found no significant loss in the number of neurons in any of the analyzed nuclei (data not shown).

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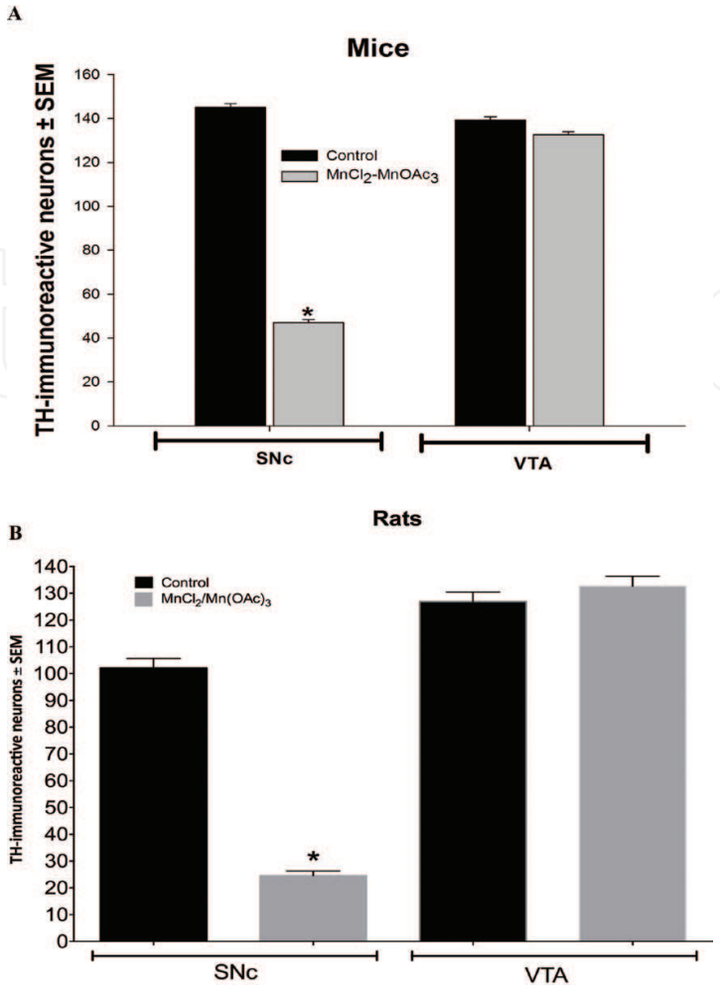


Figure 5. TH+ cell number from the SN) and VTA. The data are depicted as the mean ± standard error. A statistically significant diminution in TH+ cells was observed in the SNc (*$p < 0.05$ unpaired t-test) of Mn-exposed mice (A) and rats (B) compared to controls with no changes in the VTA.

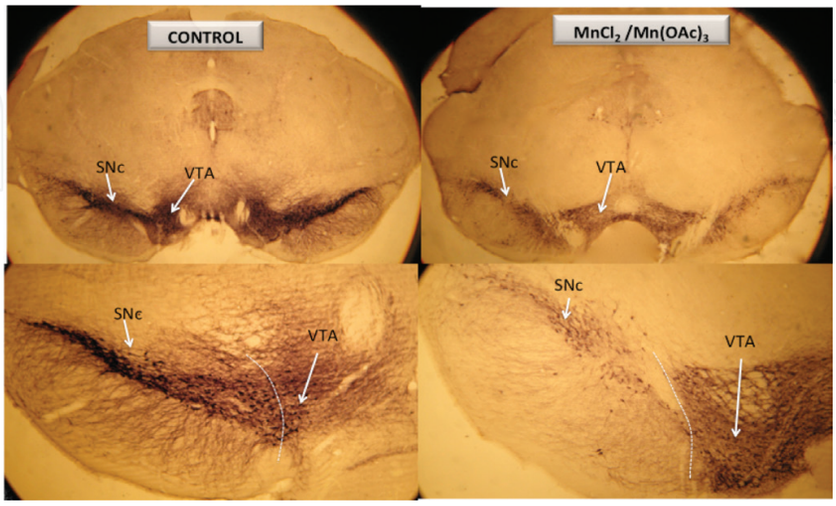


Figure 6. Characteristic TH+ immunostained from coronal sections comprising the SN and VTA of control and Mn-exposed animals showing the ROI which demonstrates the SNc area used for cell calculating. Note that the VTA contains many TH+ cells with no differences among groups and the SNc pronounced cell loss after Mn exposure (upper panel 4×, lower panel 10,000×).

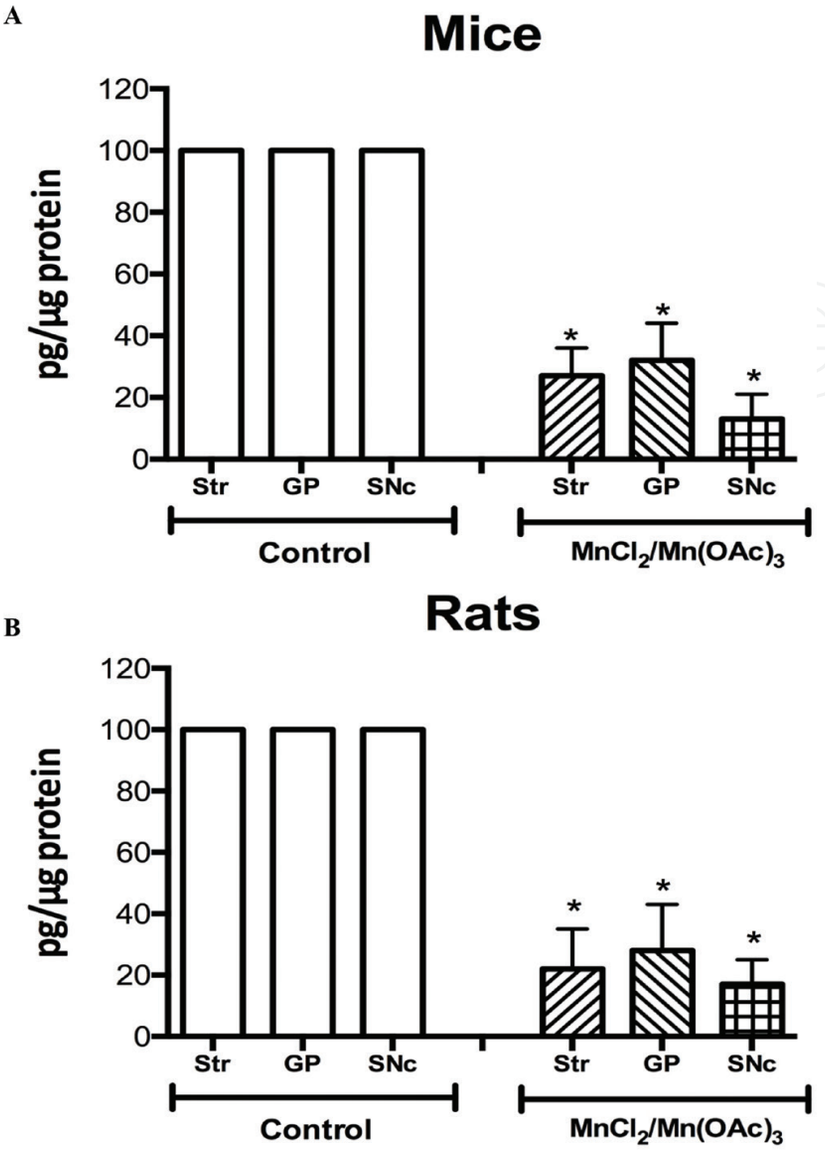


Figure 7. The decrease in dopamine concentrations in the striatum (str), GP, and SNc after 5 months (mice A) or 6 months (rats B) of Mn inhalation compared to controls. Contents are expressed as percentages, which were in pg/g of protein (*$p < 0.001$ vs. control group by one-way ANOVA with post hoc comparisons).

3.5. Dopamine concentrations

Figure 7 shows the change in DA content determined in the striatum (Str), GP, and SNc after 5 months (mice) or after 6 months (rats) of Mn inhalation compared to controls. The average content in the control mice was 96.545 ± 4.8820 and 28.008 ± 12.4500 pg/µg of protein for Mn-exposed mice; hence, DA content declines 71 and 76% for the rat's striatum.

4. Discussion

This research studied the fact that $MnCl_2$ mixed with $Mn(OAc)_3$ induces synergistic consequences by affecting the dopaminergic system reducing TH+ cell number in the SNc but not

in the VTA and reducing DA striatal, GP, and SNc levels, in both mice and rats. We found significant hyperactivity after the first weeks (2–8 inhalations) in mice and, afterward, evident reduction and alterations in locomotor activity; the motor changes improve drastically after L-DOPA treatment in both species. However, rats display different vulnerability to $MnCl_2$/$Mn(OAc)_3$ inhalation as they inhaled three times a week for 6 months. Nevertheless, regardless of the modified procedure, both species display notorious changes in motor behavior and a significant decrease in TH^+ cells in the SNc but not in VTA. Moreover, neither of the two species displayed neuronal death neither in the striatum nor the GP.

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 humans and rodents [57, 58]. PD patients are often described as having poor manual skills that worsen as the disease progresses [59, 60]. These patients experience difficulties performing tasks requiring unilateral and bilateral arm movements and sequential and alternating limb movements [58]. In our results, mice and rats took the food from the ledge without raising the forelimb and either place the mouth into the gap to recover the food pellet with the tongue or turn their body and chase the food with the mouth. Those changes could comprise impairments to basal ganglia structures responsible for grasping movements [61]. Our results thus demonstrate that Mn-exposed animals have impairment in their success in retrieving food pellets probably due to dopaminergic cell loss.

4.1.2. Beam-walking test

Both rats and mice showed extremity coordination disturbances, step length, and motor performance. With longer inhalation times, the Mn-exposed groups display more trouble for climbing the wooden beam. The motor alterations observed here are similar with published results in which C57-treated MPTP exhibited impairments in limb coordination, step length, and motor performance after 2 weeks [62].

Qualitative examination showed that the groups which inhaled Mn mixture displayed postural instability, akinesia, hind-limb weakness, prolonged freezing behavior, and action tremor. According to this, Autissier et al. [9] described that subchronically orally exposed to Mn mice exhibited akinesia; this alteration was related with low striatal DA levels; Eriksson and coworkers [25] reported that 5 months after Mn exposure the animals developed akinesia, action tremor, and unsteady gait. The exposed animals lacked strength in lower and upper limbs, and the limb movements were uncoordinated. Furthermore, the stereotaxic injection of Mn^{3+} into the rat SNc altered the rearing behavior and the spontaneous activity [63, 64].

4.2. Immunocytochemistry

Rats and mice exposed to Mn showed severe loss of SNc TH-immunopositive cells, but not in VTA, GP, or striatum. Our results disagree with other reports which found no loss of dopaminergic neurons [11, 18, 19, 32, 65, 66] and loss of striatal and GP cells [15, 17, 19]. The disagreements concerning our results and the conclusions that describe no TH+ SNc cell death and GP

and striatal cell loss after Mn exposure might be due to at least three causes; first, the mixture of two Mn compounds, which, by far, no report includes such mixture of Mn compounds. Agreeing to Aschner [67], it appears that the Mn toxicity degree is about its oxidation state. As we mentioned above, divalent Mn might be oxidized to trivalent Mn by the superoxide anion [40], and because the electron transport chain in the mitochondria is recognized as the major superoxide producer in the cells, it is understood that the alterations induced by Mn are linked to its oxidation state [40]. It has been proposed that Mn^{3+} is more effective in producing cell damage [68] and Mn^{2+} needs the presence of Mn^{3+} to reach oxidation. Thus it seems that there is synergy between the two Mn states [43]. It also has been said that the brain is an important target of attack for transition metal ions, such as Mn, due to its abundant catecholamine concentration and the rapid oxidative metabolism catalyzed by these metals [69]. In this regard, it has been hypothesized that Mn interacts with catechols specific to dopaminergic neurons to rapidly deplete them and render such cells no longer viable [33, 40]. Thus, it is conceivable that Mn-induced DA oxidation results in the generation of reactive oxygen species, oxidative stress, and secondary cytotoxicity to dopaminergic neurons [40, 70, 71]. Numerous explanations have been proposed to clarify the vulnerability of dopaminergic cells to Mn, such as the lack of cellular antioxidant defenses by the accumulation of the metal [72] and the disruption of mitochondrial oxidative energy metabolism [73]. Second, the concentration of Mn obtained in the inhalation box (2676 mg/m^3) and the time of exposure (5 or 6 mo) are sufficient to produce motor and cell alterations. It has been suggested that Mn toxicity results, most often, from the chronic exposure to very high Mn dosage (>1 mg/m^3) [7] and after long-term exposure [23]. Third, apparently the exposure method determines the delivery of Mn to the brain [74, 75]. Roels et al. [75] explored Mn levels in rat brains after exposing them to either to $MnCl_2$ or MnO_2. These compounds were given intratracheally (inhalation) or intragastrically (oral). This study proposed was to achieve comparable Mn concentrations in the blood and to reach for low oral absorption of Mn vs. the higher rate of absorption from the lung. When the exposition was 1.22 mg $MnCl_2$/kg intratracheally once a week for 4 weeks, there was an increase in blood Mn concentration (68%), which also results in augmented Mn concentrations in the striatum (205%) and cortex (48%) when compared to control group. Oral $MnCl_2$ administration (24.3 mg $MnCl_2$/kg once weekly for 4 weeks) produced about the same blood Mn concentration (68% increase comparing to controls) as intratracheal Mn administration in the same form, but they did not find significant Mn increase in the striatum or cerebral cortex (22% increase versus controls). Therefore, inhaled Mn delivery seems to be more efficient than oral administration in increasing brain Mn levels.

Moreover, it is relevant to indicate that, while Mn exposure provoked important SNc dopaminergic cell death, it appears that the VTA dopaminergic cells are not affected. We do not have the facts yet to demonstrate whether this indicates Mn selectivity for the SNc dopaminergic neurons and not for the VTA cells. Nevertheless, it has been proposed that Mn gets into the neurons via the dopamine transporter (DAT) [76, 77] as in the case of some neurotoxins such as MPTP [78], 6-OHDA [79], Maneb, and Paraquat [26], where SNc cells are more vulnerable than VTA cells. It appears that SNc neurons and VTA exhibit different biochemistry, topography, and susceptibility to pathological processes [81], VTA has lesser DAT levels than the SNc [78, 80, 81]. Therefore it is conceivable that Mn gets into SNc cells via the significant volumes of DAT located in these neurons. Nevertheless, further research is required to settle this fact.

4.3. Dopamine concentrations

Several studies have shown that Mn accumulates in the basal ganglia, particularly in the GP, the NE, and the SNc which cause neurodegeneration; Mn chronic exposure can induce similar changes to those observed in PD [82]. Patients with this disease present rigidity, tremor, akinesia, and postural changes. These signs reflect the SNc dopaminergic neuronal loss [83]. In this disease, there is a threshold; the motor symptoms appear when DA depletion in the striatum is about 80%, and about 60% of SNc dopaminergic neurons are lost [84]. These results are consistent with our data, which show that after $MnCl_2/Mn(OAc)_3$ mixture inhalation, the number of TH-positive SNc neurons decreases to 63% (in mice) and 75% (in rats) and DA content decreases in the studied nuclei, which could explain the motor disturbances observed in the behavioral assessments. Thus, the significant reduction in the quantity of SNc TH+ neurons after $MnCl_2/Mn(OAc)_3$ exposure and the decrease of striatal DA concentrations described here explains the evident DA reduction and the parkinsonian symptoms. Therefore, we assume that the motor alterations are exclusively due to dopaminergic changes because L-DOPA was able to reverse those motor disturbances.

Some authors described that Mn-induced damage includes the GP [17, 19]; nevertheless, with our data, we can guarantee that the $MnCl_2/Mn(OAc)_3$ mixture inhalation also compromises the dopaminergic nigrostriatal pathway. With our results, we prove that L-DOPA treatment significantly recovers the motor performance alterations observed after Mn inhalation, implying that this motor change origin is dopaminergic. Furthermore, the alterations produced by the inhalation of Mn mixture compounds were sufficiently extensive to cause motor deficits such as tremor, rigidity, postural instability, and akinesia. And unlike the complete DA denervation produced by some neurotoxins such as 6-OHDA, which is the most frequently used model, the inhalation of $MnCl_2/Mn(OAc)_3$ leaves a considerable portion of the nigrostriatal projection unharmed. As in early and middle stages of PD, the presence of an intact, functioning sub-portion of the nigrostriatal system could allow L-DOPA treatment to be effective.

4.4. Differences among species

It is well established that different vulnerability to neurotoxins occur among species. So, the best PD experimental model MPTP, in rats, is not actuality used, and the implications of the data obtained from this model are debatable [85, 86]. Rats injected with MPTP doses comparable to those employed in mice do not show any significant dopaminergic neurodegeneration [86, 87]. Only injections of much higher doses of MPTP (multiple applications of 30–60 mg/kg body weight) cause significant dopaminergic cell loss in rats [88]. Remarkably, these rats must be therapeutically pretreated, with guanethidine, to prevent peripheral catecholamine release and extensive mortality [86]. These findings indicate that rats are somewhat insensitive to MPTP. Consequently, rats are not recommended for MPTP research, since rats fail to develop parkinsonian characteristics, as those observed, e.g., for monkeys and mice [89]. The apparent insensitivity of rats to MPTP toxicity may be related to a species-specific metabolism of MPTP and sequestration of MPP+, which could be different in rats compared to mice and monkeys [89]. And despite that MPTP in nonhuman primates and mice provokes a well animal PD model, a spontaneous recovery of parkinsonian

symptoms has been described in both monkeys [90] and mice [91] 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 and closely imitates the progressive nature [92]. Nonetheless, rodents are most commonly used over nonhuman primates since rodent models have the advantage that rats and mice are widely available. They have high reproductive rates and require reduced living space, simple feeding, and drinking schedules and low costs [93]. Moreover, because of the economic, logistic, and ethical constraints that are related to experimental research in primates, primate models of PD are used in relatively few laboratories worldwide [94].

Furthermore, 6-OHDA model has been extensively used in rats; only scarce studies using mice with 6-OHDA lesions have been published. In these studies, 6-OHDA was injected mainly either intrastriatally [95, 96] or intraventricularly, and the mice were subjected to relatively slight behavioral assessment [97]. Furthermore, Cenci and Lundblad [98] performed the stereotactic unilateral 6-OHDA injection in rats and mice and then treated them with L-DOPA and reported abnormal involuntary movements (AIMs); these researchers indicated that while rat and mice AIMs can be evaluated with the same parameters, there are important differences among the two species. Mice motor behavior is less articulate and faster than rats. It is, therefore, more challenging to determine mice normal and abnormal movements with 6-OHDA model. Additionally, Iancu et al. [99] stereotactically lesioned mice SNc; they got 53 well-lesioned animals out of 110 lesioned. The small amount of well-lesioned mice is probably due to the SNc size since in mice it is extremely small. The slight variances in the inhalation procedure between species that we found here are likely because rat Mn absorption is a fast saturable process probably mediated by a high-affinity system [100]. Consequently, the rats, although with the same Mn concentrations, required more inhalations per week for 6 months instead of 5. However, both species, cytological and behavioral alterations, were very similar.

5. Conclusion

Contrasting to MPTP and 6-OHDA PD models, where the alterations occur in a range of days or weeks, while PD in humans develops over decades [90], our PD experimental model induced by Mn inhalation seems to be a suitable model because the dopaminergic cell degeneration is bilateral and progressive and the variances among species are minimum.

It has been established [88] that an acceptable PD experimental model must have these features: (1) an average number of SNc dopaminergic cells at birth followed by a gradual selective loss of these cells in adulthood; (2) merely demonstrable and measurable motor alterations; (3) the model should be established at reasonably short time course to replicate the PD pathogenesis (about 3–6 months), which would allow for therapeutic substances and strategies assessment; and (4) Lewy bodies must be present. Hence, with our Mn inhalation model, we produce three of those characteristics. Nevertheless, further studies are needed to clarify if Mn exposure generates Lewy bodies and determine if the animals recover after the inhalation period.

Finally, the results from this research provided essential contributions toward a better understanding of the mechanisms involved in nigrostriatal degeneration in PD because it is highly feasible and adequately simulates the neuroanatomical, neurochemical, and some of the PD behavioral characteristics.

In brief, the results of this research suggest that the motor alterations induced by the inhalation of the combination of $MnCl_2/Mn(OAc)_3$ are related to nigrostriatal dopaminergic function, providing new light for the understanding of Mn neurotoxicity as an adequate PD experimental model.

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Conflict of interest

Authors declare that there is no conflict of interest.

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