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Research report

Cognitive, motor and tyrosine hydroxylase temporal impairment in a model of parkinsonism induced by reserpine

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HIGHLIGHTS

- Repeated reserpine treatment induces a progressive rat model of Parkinson's disease.
- Cognitive and motor signs are detected in the same animal.
- Memory impairment precedes motor alterations in the course of the treatment.
- Alterations were accompanied by decreased tyrosine hydroxylase levels.

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ABSTRACT

Studies have suggested that cognitive deficits can precede motor alterations in Parkinson's disease (PD). However, in general, classic animal models are based on severe motor impairment after one single administration of neurotoxins, and thereby do not express the progressive nature of the pathology. A previous study showed that the repeated administration with a low dose (0.1 mg/kg) of the monoamine depleting agent reserpine induces a gradual appearance of motor signs of pharmacological parkinsonism in rats. Here, we showed this repeated treatment with reserpine induced a memory impairment (evaluated by the novel object recognition task) before the gradual appearance of the motor signs. Additionally, these alterations were accompanied by decreased tyrosine hydroxylase (TH) striatal levels and reduced number of TH+ cells in substantia nigra *pars compacta* (SNpc). After 30 days without treatment, reserpine-treated animals showed normal levels of striatal TH, partial recovery of TH+ cells in SNpc, recovery of motor function, but not reversal of the memory impairment. Furthermore, the motor alterations were statistically correlated with decreased TH levels (GD, CA1, PFC and DS) and number of TH+ cells (SNpc and VTA) in the brain. Thus, we extended previous results showing that the gradual appearance of motor impairment induced by repeated treatment with a low dose of reserpine is preceded by short-term memory impairment, as well as accompanied by neurochemical alterations compatible with the pathology of PD.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder. It affects approximately 1–2% of the population over the age of 65, with increasing incidence at older ages [1]. PD is a chronic, progressive, neurodegenerative disorder associated with a massive death of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) accompanied by an expressive reduction of striatal dopamine levels [2]. This disease is characterized by bradykinesia, tremor, postural instability and rigidity [3–5]. In addition, cognitive impairments can be present in PD patients [6–8]. Recent studies have shown that cognitive impairments can occur at the premature stages of PD [7,9,10]. These cognitive alterations include deficits in recognition learning and memory [11] and executive function, such as planning, working memory [12] and attentional deficits [13], and dementia [14].

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Several phenotypical features of human PD can be mimicked in pharmacological models using rodents [15] such as the administration of 6-hydroxydopamine (6-OHDA) [16,17], 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [18,19], rotenone [20], and paraquat [21]. Although those models induce irreversible deficits in motor function, the acute administration of the monoamine depleting agent reserpine induces temporary pharmacological parkinsonism (hypomobility, catalepsy, tremor, and muscular rigidity) and is also useful for the study of PD in animal models [22–26]. The doses used in those studies (1.0–5.0 mg/g) usually promote severe motor impairment, precluding the possibility of other behavioral analysis, including cognitive tasks. However, some studies demonstrated memory impairment in the absence of motor disorders after the administration of lower single doses of reserpine [27,28]. These results are in accordance with clinical observations that cognitive impairment could precede motor disorders in PD [7,9,10].

Reserpine irreversibly blocks the vesicular monoamine transporters 1 and 2 (VMAT-1 and VMAT-2). This blockage interferes with the storage of monoamines into the vesicle, which results in the depletion of catecholamines in nerve terminals [29]. The accumulation of neurotransmitters in the synaptic terminal leads to an increase in the metabolism of these substances by monoaminoxidase, generating free radicals and cellular damage by increased oxidative stress [30–33] demonstrated that repeated treatment with reserpine induces an increase in striatal oxidative stress, measured by an increase in lipid peroxidation and the ratio of oxidized/reduced glutathione (GSSG/GSH). In accordance, we have recently demonstrated the increase in striatal oxidative stress after repeated administration of a low dose (0.1 mg/kg, every other day) of reserpine in rats [34]. Interestingly, that treatment induced gradual alterations in motor function, indicating a possible application of this gradually induced parkinsonism to study the progressive nature the motor signs in PD. However, the motor deficits in that study were not accompanied by changes in long-term memory. In addition, the previous study did not evaluate possible neuronal correlates of the motor alterations.

The aim of the present study was to extend the characterization of repeated administration of a low dose of reserpine as a model of gradual cognitive and motor features of pharmacological parkinsonism. Specifically, along with the motor tests across the treatment, short-term recognition memory evaluation was included in the protocol. We also investigated if those alterations were accompanied by neuronal changes compatible with parkinsonism, by measuring tyrosine hydroxylase (TH) brain levels. Finally, we investigated the possible persistence of behavioral and neuronal alterations after the termination of pharmacological treatment.

2. Materials and methods

2.1. Animals

Twenty-two 7-month-old male Wistar rats were used. All animals were housed in groups of 4–5 per cage (30 cm × 37 cm × 16 cm) under conditions of acoustic isolation and controlled airflow and temperature (25 ± 1° C), with a 12 h light/12 h dark cycle (lights on 6:30 a.m.). Food and water were available *ad libitum*. Animals used in this study were handled in accordance with the guidelines of the Brazilian law for the use of animals in research (Law Number 11.794) and all procedures were approved by the local ethics committee. All efforts were made to minimize animal pain, suffering or discomfort.

2.2. Drug treatment, general procedures and experimental design

Reserpine (Sigma Chemical Co., St. Louis, MO) was dissolved in glacial acetic acid and diluted to the correct concentration in distilled water. Vehicle consisted of the same amount of acetic acid and water as in the reserpine solution. These solutions were injected subcutaneously (s.c.).

The animals were handled daily for 10 min during 5 days before the beginning of the experimental procedures. Afterwards, the rats were randomly assigned to one of three groups: control (CTR: $n = 7$), reserpine-treated (RESt: $n = 8$) and reserpine-withdrawn (RESw: $n = 7$) groups. The animals received 10 subcutaneous injections of vehicle (CTR) or 0.1 mg/kg of reserpine (RESt and RESw) at a volume of 1 ml/kg body weight, every other day. The rats of the RESt group were sacrificed 48 h after the 10th injection, while CTR and RESw animals were sacrificed 30 days after the 10th injection, for immunohistochemmical analysis. Therefore, until the 20th day of treatment all the groups went through the behavioral procedures, while evaluations from the 21st to the 50th days were conducted with CTR and RESw groups.

Rats were submitted to the following behavioral procedures (from 8:00 a.m. to 4:00 p.m.): (1) catalepsy test before the 1st injection and every other day throughout the treatment and for 32 days after the last injection; (2) evaluation of open field behavior on the 8th (48 h after the 4th injection), 12th (48 h after the 6th injection) and 50th (30 days after the 10th injection) days; (3) novel object recognition task on the 8th (48 h after the 4th injection) and 50th (30 days after the 10th injection) days. All behavioral tests performed 48 h of a given injection were conducted before the following injection. The behavioral tests listed were performed at least 48 h after the referred injection, before the animals received another injection. Experimental design is shown in Fig. 1.

In some tests (open field behavior and novel object recognition task), data from the groups RESt and RESw were considered together for analysis (RES, $n = 15$).

2.3. Behavioral testing

2.3.1. Catalepsy test

The catalepsy behavior was assessed by placing the animal's forepaws on a horizontal bar positioned at 9 cm above the bench surface. The duration of catalepsy, which was defined as an immobile posture, keeping both forepaws on the bar, was measured up to a maximum of 180 s. Three trials were carried out for each animal in each observation day and the results were analyzed considering the mean value of the three trials.

2.3.2. Open field

The apparatus was a circular open field arena (84 cm in diameter) with 32 cm high walls, made of wood and painted in black. Animals were placed in the center of the apparatus for free exploration during 10 min. The sessions were recorded by a digital camera above the apparatus and the behavioral parameters were registered by an animal tracking software (Any-maze, Stoelting, USA). We quantified the distance traveled in the whole arena and in the center (in meters), the average speed (in meters/second) and the time in the center of the open field (in seconds).

2.3.3. Novel object recognition task

The task was carried out in the same arena used in the open field test. Three sets of objects with four copies each were used randomly among animals. The objects used were a sugar bowl, a mug and a goblet, all made of plastic material and filled with cement to ensure that animals could not displace them. The objects differed in height, width, color and shape. A previous experiment demonstrated that rats do not show spontaneous preference for any of these objects.

In the training session, rats were exposed to two copies of an object for 5 min. The same procedure was carried out 1 h later (test session), except that one of the objects was replaced for a new one. Different objects were used on the 50th day to avoid a possible recognition of objects used on the 8th day. The apparatus and objects were cleaned with a 5% alcohol solution after each behavioral session. The sessions were recorded by a digital camera and the behavioral parameters were registered by Any-maze®. The time rats spent exploring each object was measured in both sessions. Exploration behavior included touching with forepaws or nose, sniffing and biting the objects. The percent time exploring each object (time exploring old or new object/time exploring both objects) was calculated.

2.4. Tyrosine hydroxylase (TH) immunohistochemistry

Upon completion of the behavioral procedures, rats ($n = 6$ per group) were deeply anesthetized with intraperitoneal injection of the sodium thiopental (40 mg/kg) and perfused transcardially with 200 ml phosphate-buffered saline (PBS), pH 7.4, containing 500 IU heparin (Liquemin, Roche, Brazil), followed by 300 ml 4.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed from the skull, postfixed in the same fixative solution for 2–4 h, and transferred to a solution containing sucrose 30% in 0.1 M PBS, pH 7.4. Each brain was serially cut in the coronal plane into 30-μm thick sections with a cryostat microtome (Leica, Germany) at a temperature of −20 °C. The sections were placed sequentially in five compartments (one section per compartment), with the distance between one section and the next in the same compartment being approximately 150 μm. All sections in the five compartments were stored in antifreeze solution. For the detection of TH, free-floating sections were incubated for 18–24 h with a monoclonal anti-TH primary antibody raised in rabbits (cat # AB152 Chemicon, USA, 1:10,000, as described by Bezin et al. [35]), containing 2% goat normal serum diluted in 0.3% Triton X-100 and 0.1 M phosphate buffer, pH 7.4. Afterwards, the sections were incubated with the biotinylated secondary antibody goat anti-rabbit (1:1000; cat # S-1000 Vector Labs, USA) for 2 h at room temperature, washed, and incubated with avidin–biotin-peroxidase solution (ABC Elite kit, Vector Labs, Burlingame, USA) for 90 min. The reaction was developed by the addition of diaminobenzidine

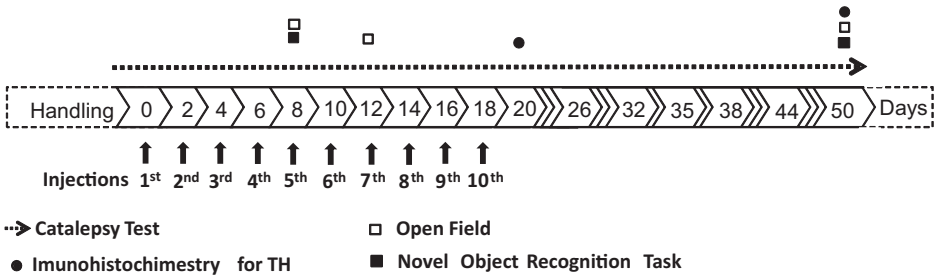

Fig. 1. Schematic representation of the experimental design.

tetrahydrochloride (Sigma, USA) and 0.01% H_2O_2 in 0.1 M phosphate buffer, pH 7.4. The sections were washed (4×, 5 min) with 0.1 M phosphate buffer, pH 7.4, between each step and at the end of the procedure. Then, the sections were dried, dehydrated in a graded alcohol series, cleared in xylene, and coverslipped with Entellan (Merck). All the immunostainings were performed concomitantly, minimizing possible differences in background between the animals. An adjacent series was stained with tionine to serve as a reference series for cytoarchitetural purposes. Sections were examined under brightfield illumination (Olympus Microscope, BX-41), images were captured using a CCD camera (Nikon, DXM-1200) and the locations of areas were determined using the atlas of [36].

In order to estimate the number of dopaminergic cells in the SNpc, ventral tegmental area (VTA) and *Locus coeruleus* (LC), four sections of each animal were selected for each region evaluated (SNpc, VTA and LC): one at the rostral level, two at medium level and one at caudal level, representative of the rostrocaudal extension of each area of interest. The exact location of the regions was determined on the basis of the Paxinos and Watson [36] rat brain atlas. The TH± cell count was performed for the whole extension of the evaluated regions within each section.

Additionally, TH+ levels were assessed by analysis of relative optical densitometry (ROD) in the following areas: hippocampus (subregions GD, CA3 and CA1); prefrontal cortex (PFC), and dorsal striatum (DS) using the software Image J (Version 1.46i, NIH). Four representative sections of the rostrocaudal extension of each region were chosen. In each section, 4 fields evenly distributed throughout the areas of interest were analyzed. The medium pixels in the target area were subtracted from de medium values of a control region (areas that should not have specific TH staining) of the same tissue (cortex or *corpus calosum*). Finally, all values were normalized considering the control group, in order to evaluate proportional alterations.

2.5. Data analysis

Catalepsy behavior was compared between groups across the treatment using ANOVA with repeated measures followed by Tukey's test. The independent samples *t* test was used to analyze differences between the groups RESt or RESw and CTR in all parameters of the open field behavior. In the novel object recognition task, within-subject comparisons for percentage of time to explore old × new objects were conducted with paired-samples *t* tests. The number of cell counts and ROD data underwent analysis of variance (one way ANOVA) followed by Tukey's test. Correlation tests between behavioral parameters and neurochemical values were conducted by Pearson's test (r). Results are expressed as mean ± S.E.M. and $p < 0.05$ was considered to reflect significant differences.

3. Results

3.1. Catalepsy behavior

ANOVA with repeated measures revealed time (days of treatment) [$F(10,190) = 20.71$, $p < 0.001$], treatment (CTR, RESt or RESw) [$F(2,190) = 4.36$, $p = 0.027$] and time × treatment interaction effects [$F(20,190) = 4.28$, $p < 0.001$], for values until the 20th day (48 h after the 10th injection). Moreover, ANOVA with repeated measures revealed time (days of treatment) [$F(14,168) = 8.99$, $p < 0.001$], treatment (CTR or RESw) [$F(1,168) = 4.36$, $p = 0.006$] and time × treatment interaction effects [$F(14,168) = 9.12$, $p < 0.001$] for values obtained from the 22nd day (96 h after the 10th injection) until the end treatment (30 days after the 10th injection). Repeated treatment with reserpine showed progressive increases in the duration of catalepsy behavior (RESt and RESw), which were significantly different from CTR from the 14th (48 h after the 7th injection) until the 38th (20 days after the last injection) days of treatment (see Fig. 2).

3.2. Open field

Open field data are displayed in Table 1. On the 8th day (48 h after the 4th injection) animals repeatedly treated with reserpine showed a decrease in the distance traveled in the inner zone [$t(20) = 2.32$, $p = 0.030$] and in the time in the inner zone [$t(20) = 2.17$, $p = 0.042$], but not in the total distance traveled [$t(20) = 0.86$, $p = 0.395$] and average speed [$t(20) = 1.02$, $p = 0.317$].

On the 12th day (48 h after the 6th injection) we found significant effects of treatment in the total distance traveled [$t(12) = 2.42$, $p = 0.032$], but not in average speed [$t(12) = 0.87$, $p = 0.400$], time in the inner zone [$t(12) = 4.94$, $p = 0.629$] and distance traveled in the inner zone [$t(12) = 0.93$, $p = 0.366$] (Table 1). The decrease in total distance traveled on the 12th day shows a possible change in motor behavior caused by repeated administration of reserpine.

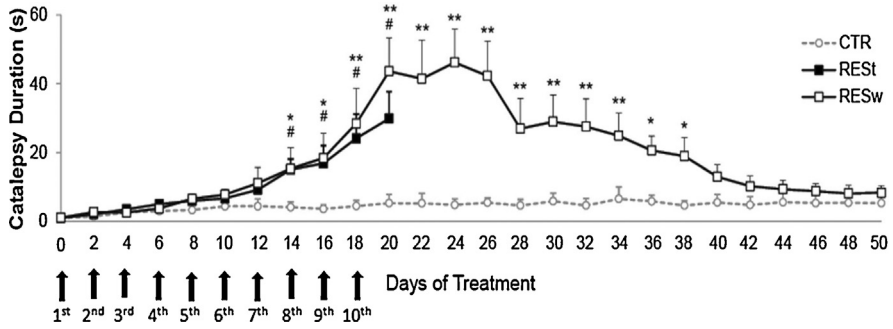

Fig. 2. Effects of repeated administration of 0.1 mg/kg reserpine (reserpine treated – RESt, $n = 8$; reserpine withdrawn – RESw, $n = 7$) or vehicle (control group – CTR, $n = 7$) on catalepsy behavior. Arrows indicate reserpine or vehicle injections. Data are expressed as mean ± S.E.M. ANOVA with repeated measures revealed time, treatment and time × treatment interaction effects. $^{*}p < 0.05$ and $^{**}p < 0.01$ comparing RESw to CRT; $^{\#}p < 0.05$ comparing RESt to CTR (*post hoc* with Tukey's test).

Table 1
Effects of repeated administration of 0.1 mg/kg reserpine (RES) or vehicle (CTR) on open field behavior. Data are expressed as the mean ± S.E.M. On the 8th day: RES, n = 15 and CTR, n = 7 and on the 12th and 50th days: RES, n = 7 and CTR, n = 7.

Day/treatment	Distance traveled (m)	d	Average speed (m/s × 10^{-2})	Time (inner zone) (s)	Distance (inner zone) (m)
8	CTR	23.26 ± 4.97	4.02 ± 0.81	40.14 ± 8.20	4.33 ± 1.01
	RES	18.64 ± 2.96	3.39 ± 0.64	22.84 ± 3.95*	2.13 ± 0.43*
12	CTR	20.51 ± 3.30	3.88 ± 0.82	38.68 ± 6.47	4.57 ± 0.93
	RES	10.90 ± 2.19*	2.94 ± 0.96	34.33 ± 5.63	3.25 ± 1.04
50	CTR	16.73 ± 3.14	3.21 ± 0.52	28.52 ± 5.54	2.98 ± 0.92
	RES	27.51 ± 3.45*	4.19 ± 0.63	60.35 ± 1.32*	5.82 ± 1.30

* $p < 0.05$ compared to CTR (independent samples t test).

On the 50th day (30 days after 10th injection) we found significant effects of treatment to total distance traveled [$t(12) = 2.35$, $p = 0.036$] and time in the inner zone [$t(12) = 2.23$, $p = 0.045$], but not to average speed [$t(12) = 1.36$, $p = 0.199$] and distance traveled in the inner zone [$t(12) = 1.83$, $p = 0.091$] (Table 1). In this case, however, animals that have been previously treated with reserpine have shown an increase in motor activity.

3.3. Novel object recognition task

The CTR group showed an increased percentage of novel object exploration compared to the old object on the 8th (48 h after the 4th injection) [$t(12) = 2.63$, $p = 0.021$] and 50th (30 days after the 10th injection) [$t(12) = 2.39$, $p = 0.034$] days, indicating adequate performance of the task. The RES group did not show differences in the percentage of novel object exploration compared to the old object on the 8th (48 h after the 4th injection) [$t(24) = 1.39$, $p = 0.175$] and 50th (30 days after the 10th injection) [$t(12) = 0.56$, $p = 0.583$] days (see Fig. 3A and B).

3.4. TH immunohistochemistry

For the number of TH+ cells, one way ANOVA revealed significant differences between groups for SNpc [$F(2,15) = 4.89$, $p = 0.023$], VTA [$F(2,15) = 18.84$, $p < 0.001$] and LC [$F(2,15) = 16.00$, $p < 0.001$]. *Post hoc* analysis revealed a decrease in the number of TH+ cells in the SNpc, VTA and LC in RESt when compared to CTR. Additionally, a partial recovery was verified in RESw, which showed a significant increase in the number of TH+ cells in the SNpc, VTA and LC when compared to RESt, although remaining significantly reduced when compared to CTR (see Figs. 4 and 6).

Regarding the TH levels by the application of ROD in brain areas (hippocampal areas: GD, CA3 and CA1; PFC and DS), one-way ANOVA revealed statistically significant difference between groups (CTR, RESt and RESw) in the GD [$F(2,15) = 11.51$, $p < 0.001$], CA1 [$F(2,15) = 6.98$, $p = 0.007$], PFC [$F(2,15) = 32.97$, $p < 0.001$] and DS [$F(2,15) = 11.70$, $p < 0.001$], but not in CA3 area [$F(2,15) = 2.01$, $p = 0.168$] (Fig. 6A–F). *Post hoc* analysis revealed that repeated treatment with reserpine (RESt) induced decreases in the TH levels, when compared to CTR. Moreover, withdrawn from the repeated treatment with reserpine (RESw) resulted in recovery of TH levels in CA1, PFC and DS, because ROD in these areas 30 days after the end of the treatment was increased when compared to RESt, and not different when compared to CTR. However, ROD in GD for RESw group was still different from CTR (see Figs. 5 and 6).

3.5. Correlations

Correlation tests were applied between the number of TH+ neurons (SNpc, LC and VTA) or relative optical density (DS and PFC) and motor behavior (time of catalepsy and distance traveled in the open field). Correlations were detected between catalepsy behavior (20th day) and the values of ROD (DS and PFC) and the number of

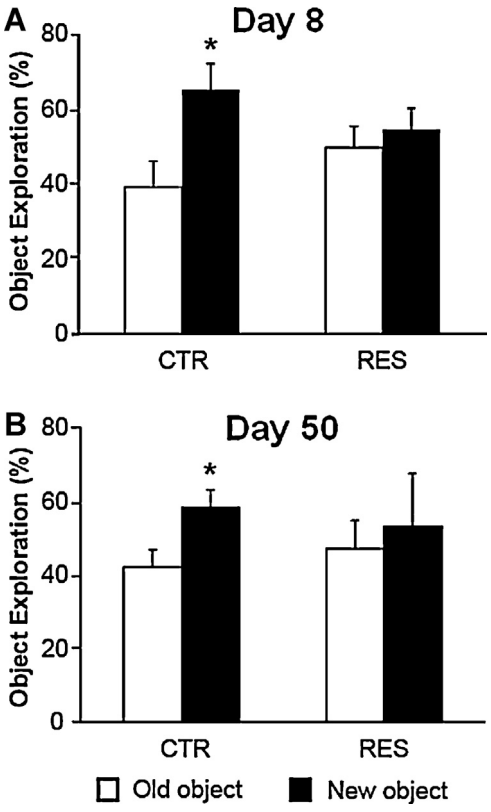

Fig. 3. Effects of repeated administration of 0.1 mg/kg reserpine (RES) or vehicle (CTR) on novel object recognition task. In (A), novel object recognition task performed on the 8th day (48 h after the 4th injection) – RES ($n = 15$) and CTR ($n = 7$). In (B), novel object recognition task performed on the 50th day (30 days after the 10th injection) – RES ($n = 7$) and CTR ($n = 7$). Data are expressed as mean ± S.E.M. (exploration rate). *$p < 0.01$ compared to old object (paired-samples t test).

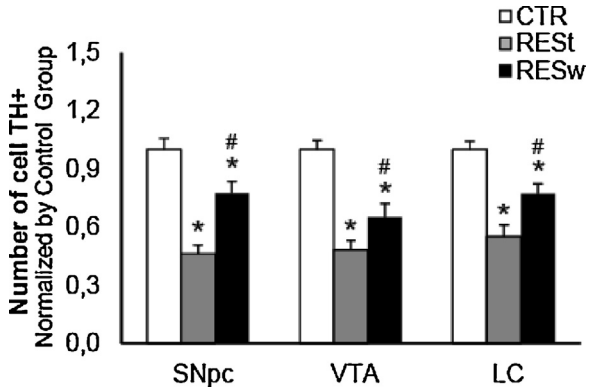

Fig. 4. Effects of repeated administration of 0.1 mg/kg reserpine (reserpine treated – RESt, $n = 6$; reserpina withdrawn – RESw, $n = 6$) or vehicle (control group – CTR, $n = 6$) on TH+ neurons in SNpc, VTA and LC normalized by CTR values. One way ANOVA revealed statistically significant difference between groups for all analyzed areas. *$p < 0.05$ comparing to CRT; #$p < 0.05$ comparing to RESt (*post hoc* with Tukey's test).

TH+ neurons in SNpc: DS [$r = -0.587$, $p = 0.021$] and PFC [$r = -0.584$, $p = 0.022$].

We also ran correlations tests between the exploration rate of the new object in the test session of the novel object recognition task and the values of ROD (hippocampal areas, DS and PFC) and number of TH+ cells (SNpc and VTA). Correlations were detected between the exploration rate of the new object (50th day) and the number of TH+ neurons in VTA [$r = 0.534$, $p = 0.040$].

Values were omitted for non-significant correlations. All correlation tests performed are summarized in Table 2.

4. Discussion

In this study, we investigated the motor, cognitive and neurochemical effects during and after repeated administration with a low dose of reserpine. Corroborating the previous study [34], we observed that this treatment induced gradual motor impairments. In addition, these alterations were reversed 22 days after withdrawal of the treatment. These results can be seen in the evaluation of catalepsy behavior performed 48 h after each injection (Fig. 2). Furthermore, we observed a deficit in short-term memory (evaluated by novel object recognition task) before the motor impairment that remained until 30 days after the end of the treatment (Fig. 3). Locomotor parameters evaluated in the open field were not altered in the RES group 48 h after the 4th injection, but the treatment induced decreased exploration of the inner zone of the arena, suggesting an anxiogenic effect. The locomotor deficit was detected by diminished distance traveled in the open field on the 12th day, 48 h after the 6th injection. In addition, increased locomotor activity was observed in the RESw group on the 50th day (30 days after 10th injection) (Table 1). In relation to neuronal analyses, repeated reserpine treatment induced a decrease in the TH staining (GD, CA1, PFC and DS) and in the number of TH+ cells (SNpc, VTA and LC), with different levels of reversal of this effect after treatment withdrawal (30 days after 10th injection) (Figs. 4–6). Finally, some motor and cognitive alterations induced by repeated reserpine treatment correlated with the decrease in TH levels and the number of TH+ cell (Table 2).

Motor symptoms (bradykinesia, tremor, postural abnormalities and rigidity) are the cardinal signs used in the diagnosis of PD [3–5,37,38]. The motor impairments in PD manifest only when there is a reduction of 60–80% of dopaminergic neurons [2,39]. In animal models of PD, the motor impairments are visible after an irreversible lesion of dopaminergic neurons with MPTP [40], 6-OHDA [41,42], and rotenone [43]. Notwithstanding, the same motor impairments can also be detectable after acute haloperidol and reserpine injections [44]. However, at least when given acutely, these drugs do not cause the death of neurons and their effects are reversible any time after the end of treatment [45,46].

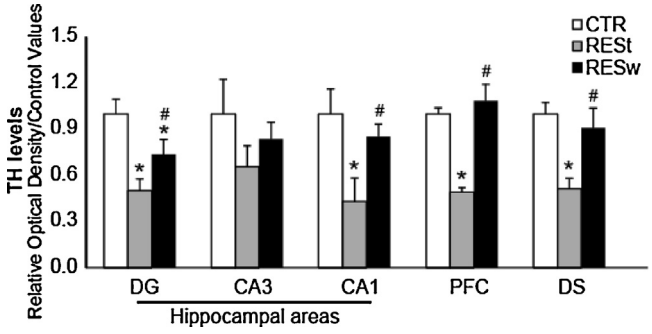

Fig. 5. Effects of repeated administration of 0.1 mg/kg reserpine (reserpine treated – RESt, $n = 6$; reserpine withdrawn – RESw, $n = 6$) or vehicle (control group – CTR, $n = 6$) on TH levels analyzed by relative optical density (ROD) normalized by CTR values (hippocampal areas DG, CA3 and CA1, pre-frontal cortex – PFC – and dorsal striatum – DS). One-way ANOVA revealed statistically significant difference between groups for all analyzed areas, except CA3. $^{*}p < 0.05$ comparing to CRT; $^{\#}p < 0.05$ comparing to RESt (*post hoc* with Tukey's test).

Table 2
Correlation between the number of TH+ neurons (SNpc, LC and VTA) or relative optical density (DS and PFC) and motor behavior (time of catalepsy, duration of oral twitching, frequency number of tongue protrusions, number of vacuous chewing movements and distance traveled) or % exploration of the new object in the novel object recognition task.

Area	Behavior		
	Catalepsy	Distance traveled	%Exploration (new object)
SNpc	(−)**		
DS	(−)*		
LC			
VTA			(+)*
PFC	(−)*		

Significant correlations (Pearson correlation – r) when $^{*}p < 0.05$; $^{**}p < 0.01$. (−) and (+) for negative and positive correlations, respectively.

Catalepsy includes the inability to initiate a movement and it has been widely used to assess motor alterations in animal models of PD [47]. This difficulty in initiating the movement is also common in patients with PD [48]. Acute reserpine administrated in doses ranging between 1.0 and 5.0 mg/kg promotes catalepsy in rodents [49,50]. Nevertheless, high doses can cause hypolocomotor effect [49–51], and as a consequence, difficulty to assess other behaviors. Our research group recently demonstrated that repeated administration with a low dose (0.1 mg/Kg) of reserpine in middle-aged rats induces a gradual appearance of typical motor signs, evaluated by catalepsy behavior, that appeared only 48 h after a 7th injection [34]. In the present study, we used the same chronic treatment with reserpine and catalepsy behavior was also observed 48 h after the 7th injection, lasting until day 40 (20 days after the 10th injection) (Fig. 2).

Regarding a methodological issue, although the present study was conducted following the same treatment schedule from the previous work [34], there were differences in the scale of results when comparing previous and present catalepsy evaluation. Differences in behavioral analysis among studies could reflect natural behavioral variation, but other factors could be related to this difference. For example, there was a difference in the age of the subjects (7 months against 6 months in the previous study). Regardless, in both studies, a gradual increase in latency to step down the bar was observed when compared to respective controls.

Interestingly, Neisewander et al. [52] observed that rats injected daily with reserpine (1.0 mg/kg) for 6 weeks showed progressive alterations of motor behavior accompanied by increased D2 receptor density in the caudate-putamen (CPu) and nucleus accumbens. However, this progressive motor alteration was assessed by measuring the frequency of tongue protrusion, and not catalepsy (possibly because this amount of reserpine induced severe immobility since the first injection).

Further, catalepsy durations have shown negative correlations with number of TH+ cells in the SNpc and TH levels in the DS (Table 2), suggesting a relationship between the hypofunction of the nigrostriatal pathway and the presence of motor alterations. In this sense, the present results reveal that a chronic treatment with a low dose of reserpine induces an increase in catalepsy behavior, reinforcing the face validity for the cardinal features of PD. Therefore, this model can contribute to the understanding of the neuronal mechanisms involved in the adaptation during chronic depletion of neurotransmitters concomitantly to a progressive motor impairment in PD.

Interestingly, in the present study, motor alterations were detectable up to 22 days after the 10th injection (Fig. 2), but not on the 50th day (30 days after the 10th injection), indicating a

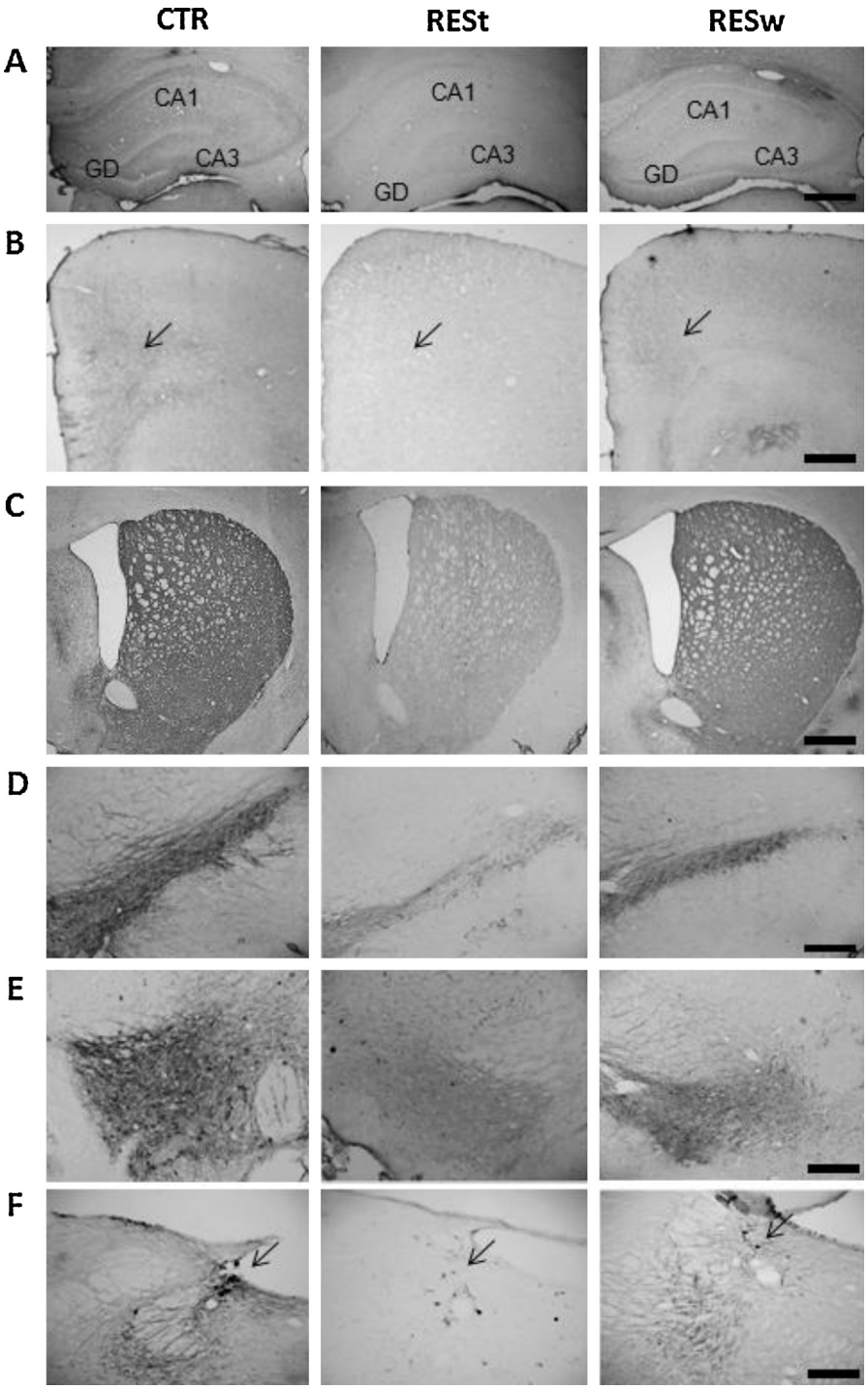

Fig. 6. Representative photomicrographs of brain coronal sections of (A) hippocampal areas (GD, CA3 and CA1), (B) pre frontal cortex (PFC), (C) dorsal striatum (DS), (D) substantia nigra pars compacta (SNpc), (E) ventral tegmental area (VTA) and (F) locus coeruleus (LC) of rats repeatedly treated with vehicle (CTR) or 0,1 mg/kg reserpine sacrificed 48 h (RESt) or 30 days (RESw) after the last injection. Scale bar in (A), (D), (E) and (F): 200 μm; (B): 500 μm; (C): 1000 μm.

reversal of the motor effect induced by reserpine. These findings are consistent with previous studies (conducted with acute reserpine treatment) suggesting that reserpine does not induce neuronal death, and its effects are reversible [45,46]. However, although not discarding the possibility of an additive effect on dopaminergic function, *i.e.*, increasing dopamine depletion throughout the treatment being the responsible for the effects found here, there is also evidence in favor of neurotoxicity underlying the behavioral and neuronal damage (not necessarily neuronal death).

In this context, one of the goals of the present study was to verify if the protocol we used would induce reduction in the TH levels similarly to other pharmacological models of PD. Indeed, to our knowledge, this kind of alteration has not been demonstrated previously using reserpine repeated treatment. Further, a previous study has shown that the classical acute treatment (with a dose 10 times higher than the one we used) did not cause a reduction in TH, although causing an important motor impairment [53]. This is an indicative that the protocol used is not a cumulative effect of the drug; at least this is probably not the only factor leading

to the alterations found. In this respect, as mentioned, it has been shown not only in our previous study but also by others [31,54,55] that reserpine treatment increases brain oxidative stress (as a consequence of dopamine metabolism in the cell cytoplasm) which potentially leads to neuronal damage [56,57]. Additionally, endogenous and exogenous antioxidants seem to protect the organism from the effects of reserpine on motor function [30,31,54,58,59], suggesting that oxidative stress may be related to the changes observed alternatively or additionally to the changes caused by depletion of neurotransmitters *per se*. In addition, part of the alterations induced by the treatment were not recovered after 30 days of withdrawal (specifically, the short term memory deficit remained, and only a partial recovery of TH staining in various areas). Thus, these remaining alterations could be consequence of the neurotoxic effect of the treatment with reserpine.

In relation to the locomotor effects of reserpine evaluated in the open-field, an absence of general locomotor deficit was observed at the same time-point of no changes in catalepsy (48 h after the 4th injection of reserpine), reinforcing the gradual aspect of the motor impairments induced by repeated reserpine. However, the evaluation of time and distance traveled in the inner zone in this open field session showed a decreased exploration of this area of the arena by reserpine-treated animals. Relative decrease in exploration of this more aversive area of the arena (not protected by the circular wall) indicates a possible anxiogenic effect [60]. Since its development by Hall [61], the open field test has become a very widely used tool in behavioral tests, in particular to assess general activity and anxiety-like behaviors in rodents [62]. In this respect, studies have shown that depression and anxiety symptoms can be observed in patients with PD [63–68], and some researchers have suggested that these symptoms appear in early stages of this disease [69]. Previous studies in reserpine treated models of PD have shown anxiety-like [70] or depressive-like behaviors [71,72]. However, these studies were performed with a single administration of reserpine [71,72] or genetic VMAT2-deficient animals [69], which does not feature a chronic animal model of PD.

The evaluation of open-field behavior on the 12th day (48 h after the 6th injection) showed decreased distance traveled in the arena by reserpine-treated animals, which is consistent with the other motor alterations present around this time of treatment (Table 1). Conversely, the results also showed an increase in the total distance traveled and in the time in the inner zone on the 50th day (30 days after the 10th injection) by animals that have been treated with reserpine (Table 1). This effect could be related with compensatory mechanisms of increased neurotransmission activated as a consequence of prolonged depletion with reserpine. The prolonged treatment with low doses of reserpine could give rise to a dopaminergic hypersensitivity, which has been reported to be related to increases in motor activity [73,74]. Interestingly, this alleged compensatory response could be related not only to the hyperlocomotion but also to the reversal of the increase in catalepsy behavior after withdrawal of reserpine treatment.

In addition to the motor deficits symptoms, cognitive impairments are highly prevalent in PD patients [6–8]. PD seems to produce recognition memory deficit [11], executive dysfunction that involve working memory [12], and attentional deficits [13]. We evaluated short-term recognition memory of objects of the animals on the 8th day of reserpine treatment (48 h after the 4th injection) and observed that the RES group showed deficits in recognizing the new object (Fig. 3A). The novel object recognition task is based on the fact that rats recognize a previously presented object, and therefore would spend more time exploring the new object in the test session. This test involves recognition memory and executive functions. Interestingly, this cognitive impairment preceded the motor alterations induced by reserpine, in accordance with the premature manifestation of cognitive functioning in PD [7,9,10].

Furthermore, deficits in recognizing the new object were still present on the 50th day (30 days after 10th injection) in the RES (Fig. 3B), indicating that the compensatory mechanisms that might be involved to the motor recovery after withdrawal of reserpine were not developed in brain areas related to cognitive performance. Although possible neuronal damages by increased oxidative stress have been described by a similar reserpine treatment [34], neuronal death was not specifically evaluated in the present study. In addition, decreased TH levels and TH+ cells induced by reserpine treatment were, in general, recovered after termination of treatment. Nevertheless, it has been shown that small variations in the levels of monoamines induced by reserpine can promote cognitive deficits in the absence of the motor impairments [27,28]. In addition, previous studies have shown that the acute administration of low doses of neurotoxins may cause cognitive impairments similar to that occur in early stages of PD [18,72,75–77]. Moreover, other findings [72,78–81] demonstrated that nearly 40% depletion of striatal DA is capable of causing deficits in memory tasks, while the motor impairments are visible only when there is a reduction of 60–80% of dopaminergic neurons [2,39].

Regarding the cognitive impairment reported here, one of the main structures involved in object recognition memory is suggested to be the perirhinal cortex [82]. However, other areas such as the hippocampus, the mediodorsal thalamic nucleus, prefrontal cortex other rhinal cortices have been suggested to be involved in this kind of task as well [83]. In this study, we found memory impairment in novel object recognition task in early stages of treatment with a repeated low dose reserpine, which was possibly related with neurochemical changes in brains regions involved. Specifically, we observed a decrease in TH+ cell number in the VTA (Fig. 4), and this alteration was correlated with performance of RESw animals in the object recognition task (Table 2). In this respect, it has been suggested that not only nigrostriatal but also mesolimbic pathways are involved in the death of dopaminergic neurons in PD [84]. This association can be confirmed by a long-term follow-up of Parkinson disease patients and experimental animal models with lesion in the ventral DA pathway [85]. In addition, Chen et al. [86] observed, in an animal model of PD, a decrease in TH levels in VTA and cognitive and emotional changes that are possibly associated with early stages of parkinsonism. Interestingly, Kato [87] showed changes in the ventral midbrain area in humans with PD.

Despite the absence of statistical correlation between the exploration of the new objects and the reduction of TH levels in hippocampal areas (GD and CA1), the involvement of hippocampal damage in the memory impairments found in animal models of PD cannot be ruled out. As mentioned, the cognitive deficits remained after treatment withdrawal, but the neurochemical alterations were recovered. However, recovery of TH levels in GD was only partial, and this hippocampal subregion has been implicated in object recognition tasks [88].

In summary, the evaluation of immunohistochemistry for TH showed decreases in the TH levels in hippocampal areas (GD and CA1), PFC and DS (Fig. 5). This decrease was accompanied by a reduction in number of TH+ cell in the SNpc, VTA and LC (Fig. 4) corroborating studies in other animal models of PD [72,79,80,89]. Importantly, the reduction of TH levels in the DS and the number of TH+ cell in the SNpc correlated with the motor alteration (catalepsy behavior – Table 2).

The decrease in TH+ cell number in the LC found in the present study indicates a reduction in the production of norepinephrine. Similar results were presented by [90] after combined injections in SNpc and LC with 6-OHDA, resulting in a decrease of TH+ cell in both structures. These results are in accordance with the involvement of the noradrenergic neurotransmission in PD [91–95]. Considering that despite the dopamine neurotransmission, the noradrenergic,

serotonergic and adrenergic systems seem be involved in the pathophysiology of the disease [91–95], the fact that reserpine unspecifically blocks monoamines vesicular uptake might provide an advantage of this agent as a pharmacological model of PD.

As discussed above, the effects reported here could be related to neurotoxic effects consequent of repeated treatment with reserpine. However, there is the possibility that the repeated administration with the VMAT blocker is producing additive effects on the dopamine system. As a consequence, the gradual behavioral alterations found would be related to a dose response effect. Although our data do not allow rejection of this hypothesis, the maximum VMAT blockage occurs around 24 h after reserpine administration [96,97]. Because we performed all behavioral evaluations at least 48 h after the last injection across the treatment, the possibility that the behavioral outcome is a direct effect of the last administration (alone or added to the previous injections) seems unlikely. In addition, the relationship between VMAT functioning with the etiology of Parkinson's disease seems to be more direct than previously thought. Indeed, studies have shown diminished VMAT-2 expression in patients with familiar Parkinson's disease [98–100].

In conclusion, we found that repeated administration with a low dose of reserpine in rats induces a gradual appearance of motor impairments and these motor signs were reverted after interruption of treatment. The motor alterations are correlated with decrease of TH levels (GD, CA1, PFC and DS) and number of TH+ cells (SNpc and VTA) in the brain. Repeated administration with a low dose of reserpine was also able to induce memory impairment in novel object recognition task before the appearance of the motor impairments. In addition, an increase in anxiety seen in PD patient was also induced by the present treatment. In this sense, this animal model present an extend face validity when compared to other animal models of the disease. Importantly, this model also presents the advantage of mimicking the progressive nature of the PD symptoms. Furthermore, this model can be useful for studying compensatory mechanisms of production of neurotransmitters and plasticity during the progression of PD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2013.06.031.

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