Behavioral Analysis of the Reserpine Induced Motor Changes in a Parkinsonian Mouse Model

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Summary

In mice, the systematic administration of reserpine at a pre-determined dose leads to severe brain and peripheral monoamines depletion. This results in the appearance of heterogeneous symptoms regarding motor control resembling those present in human Parkinson's disease; such as tremor, rigidity, akinesia, gait alterations and posture deformities. Here we analyze some of the standard tests, which have been exposed in previous studies to typify behavioral changes in mice. For that purpose, mice were treated intraperitoneally with reserpine for five consecutive days at a concentration of 5 mg/kg to then apply several motor tests. Posterior to seventy-two hours of evolution, animals showed a 78% decrease in normal motor function and at the fifth day we observed 95% of parkinsonian motor symptoms. The topic presented here will be useful not only to validate the reserpine mouse model for behavioral characterization, but more importantly, for the usage of the animal model if it is intended to be used to test new treatments.

Keywords: Reserpine; behavioral analysis, motor activity, Parkinson's disease, animal model, mice, dopamine.

Análisis Comportamental de los Cambios Motores Inducidos por Reserpina en un Modelo de Parkinson en Ratones

Resumen

La administración sistémica de reserpina a dosis predeterminadas en ratones produce una deficiencia severa de monoaminas periféricas y cerebrales. Esto da como resultado la aparición de síntomas heterogéneos, dentro de los cuales

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destacan los síntomas motores que asemejan a los que se presentan en la enfermedad de Parkinson en humanos; como el temblor, rigidez, acinesia y alteraciones posturales y de la marcha. Aquí, analizamos pruebas conductuales estandarizadas, las cuales han sido demostradas en estudios previos para tipificar los cambios conductuales en ratones. Para este propósito, los ratones fueron tratados intraperitonealmente con reserpina (5 mg/kg) por cinco días consecutivos, después de este régimen fueron aplicados las diferentes pruebas. Setenta y dos horas después de la inyección de reserpina, los animales mostraron un 78% de disminución en la función motora, y al quinto día fueron observados el 95% de los síntomas motores parkinsónicos. El tópico presentado aquí, será muy útil no solo para la caracterización del modelo conductual de reserpina, si de manera más importante para considerar su uso, cuando se intente probar nuevos tratamientos para la enfermedad de Parkinson.

Palabras clave: Reserpina, análisis conductual, actividad motora, enfermedad de Parkinson, modelo animal, ratones, dopamina.

Introduction

Parkinson's disease (PD) is characterized by the progressive degeneration of numerous neurons inhabiting different regions of the brainstem, most remarkably of dopaminergic neurons located in the *substantia nigra pars compacta* (SNpc) (Hirsch, Graybiel, & Agid, 1988); which consequently generates a drawback of their axon terminals input to the *striatum* (caudate, putamen and *fundus striati*). A distinctly diminished concentration of dopamine (DA) is the main neurochemical alteration (Höglinger et al., 2004), although other neurotransmitters such as noradrenalin and serotonin are also decreased. The deficit of dopamine is correlated with a complex onset of motor symptoms (Fahn & Jankovic, 2007).

Several PD animal models have tried to resemble particular neuroanatomical, neurochemical, or neurobehavioral abnormalities found in humans; however, only a few have addressed the whole constellation of symptoms. Since its first total synthesis in 1953 by Woodward (Nicolaou & Sorensen, 1996), reserpine (RES) has become an invaluable agent to reproduce Parkinsonism experimentally. The ubiquitous action of the drug on several amines (both centrally and peripherally) does not emulate the underlying pathology of PD, although it is a useful model to study behavioral and motor symptoms generated by DA depletion. Manifold clinically used antiparkinsonian drugs (e.g., DA agonists, L-DOPA + benzerazide, amantadine and trihexyphenidyl) have shown to improve motor impairments caused by RES (Carlsson, Lindquist, & Magnusson, 1957; Menzaghi, Whelan, Risbrough, Rao, & Lloyd, 1997; Millan et al., 2004), while other medications (e.g. central anticholinergics) are ineffective in this respect (Fischer & Heller, 1967).

The administration of RES (5 mg kg^{-1}) intraperitoneally for five consecutive days in mice, (Shen et al., 2007; Shen et al., 2008) irreversibly blocks the uptake and storage of norepinephrine and DA into synaptic vesicles by inhibiting the vesicular monoamine transporter (VMAT); as a consequence of this, severe striatal dopamine depletion is generated (LaHoste, Yu, & Marshall, 1993) along with spines and

glutamatergic synapses of striatopallidal medium spiny neurons pruning (50%) (Day et al., 2006).

The changes in neurochemical balance generated by RES are associated with behavioral deficits predominantly in motor activity. Studying the behavioral deficits in animal models of PD is of particular interest in order to study the connection between DA depletion and the corresponding behavioral alterations; to then be able to test new therapeutic treatments based on the construct and face validity comprehension of the model. Here, important insights into the relationship between appearance, extent of behavioral deficits and neurologic changes with relevance to both the animal model and the human PD itself have been gained.

Being behavioral testing a critical application to assess effectiveness of new potential therapeutics, several issues have been traditionally examined in other well-known rodent models of PD, such as the unilateral 6-OHDA-brain injection and the systemic MPTP model, even though behavioral changes are present, they do not resemble closely PD-like motor symptoms as the RES model does. The purpose of this study is to provide an overview of the variety of tests used to study behavioral changes in mice which have been under a RES regimen, to delineate the interaction between behavioral changes and the degree of DA insufficiency and to analyze factors of the experimental design, which appear to be important when conducting behavioral analyses in RES-treated mice and DA-deficient mice in general.

Material and Methods

Animal studies

All procedures were approved by the Universidad Panamericana animal care and bioethics committee and performed in accordance with United States NIH guidelines. CD1 (*vivarium*, Autonomous National University of Mexico) adult male mice (4-6 months old, n= 32) were used for all experiments. They were housed individually in polypropylene cages with wood shavings as bedding and under controlled conditions of light (12-h light/dark cycle) and temperature (22 ± 2 °C). The animals had free access to water and food the whole time. Two groups were established, the positive control (reserpine group, n=16) and negative control group (glucose solution, n=16).

Dopamine depletion

Mice were treated with RES for 5 consecutive days to generate acute DA depletion (Shen et al., 2007; Shen et al., 2008). RES (Sigma-Aldrich) was dissolved in 1% glacial acetic acid at a concentration of 2.5 mg/ml. This solution was diluted with dH_20 by a factor of 10, to obtain a final concentration of 0.1% glacial acetic acid and 250 µg/mL RES (Kreitzer & Malenka, 2007). This RES solution was injected i.p. at a concentration of 5 mg/kg. Injection of 5% glucose solution i.p. (10 ml) was used as control.

Behavioral testing procedures (BTP)

Muscular rigidity

For the examination of muscular rigidity, the mouse was suspended by its forelimbs on a metal rod (0.25 cm in diameter) located approximately 20 cm above the surface. The time the animal remains on the rod (maximum 1 min) was registered (Jolicoeur, Rivest, & Drumheller, 1991). To evaluate rigidity in a bracing task, the number of steps taken with each forelimb when the

mouse is pushed sideways over a distance of 50 cm was recorded (Lindner, Plone, Francis, & Emerich, 1996).

Palpebral ptosis

The anomalous sagging of the upper lid, caused by partial or total decrement in levator muscle function was scored in the following way: 4, eyes completely closed; 2, half-open eyes; and 0, wide–open eyes; with 1 and 3 indicating intermediate values (Janssen, Niemegeers, & Schellekens, 1965).

Tremor

Also described as shivering, the phasic tremor of the whole body was evaluated visually in mice utilizing the rating scale: 0, no tremor; 1, occasional isolated twitches; 2, moderate or intermittent tremor associated with short periods of calmness; and 3, pronounced continuous tremor (Coward, Dogget, & Sayers, 1977).

Akinesia/bradykinesia

In the impaired ability to initiate movements or akinesia test, the mouse was held by the tail so that he is standing by his forelimbs and moving on his own. The number of steps taken with both forelimbs was recorded for 30 s (Lindner et al., 1996). The existence of bradykinesia (movements in parkinsonian mice are slower than observed in healthy controls) was measured by placing the animal's forepaws on a horizontal wooden bar (0.7 cm in diameter), 4 cm above the tabletop. The time until the mouse removed both forepaws from the bar was recorded, with a maximum cut off time of 3 min (Betancur et al., 2001; Sedelis, Schawarting, & Huston, 2001).

Gait alteration

The alteration of the march was assessed only once by recording footprints (can be done by marking mice's paws with ink). Successive walking over a sheet of paper allows analysis of gait patterns and pathways (Barlow et al., 1996).

Righting reflex

The righting reflex was evaluated by turning the mouse onto its back five times. Normal mice immediately turn themselves over, to right themselves onto all four feet. Righting reflex was scored as follows: 0, no impairment; 1, on side one to two times; 2, on side three to four times; 3, on side five times; 4, on back one to two times; 5, on back three to four times; 6, on back five times; 7, sluggish when placed on back; and 8, righting response absent when on back and tail pinched (Crawley & Paylor, 1997).

Spontaneous locomotor behavior and abnormal involuntary movements

We used a rectangular box (40x50x63 cm) whose floor was divided into 20 (10x10) small rectangles. The mice were placed in the right corner of the open field and were allowed to walk without restraint inside the area for 5 min. The immobility duration (time of total absence of paw movements), rearing frequency (partial or total elevation onto hind limbs), locomotion frequency (number of squares crossed), and latency duration (time spent to leave the central circle) were determined, using hand-operated counters (Tadaiesky, Andreatini, & Vital, 2006). A rating scale for abnormal involuntary movements in rodents was used (Lee, Cenci, Schulzer, & Bjorklund, 2000). The open field was uncontaminated with a 5% water-alcohol solution before behavioral testing to eradicate possible bias due to smells left by previous mice.

Drinking and eating behavior

Weight control was measured daily. In acute cases of adipsia and aphagia, diet was supplemented with oral administration of Nutrical (Evsco Pharmaceuticals, Buena, NJ, USA) and lactated Ringer's solution

(Pet Nutrition Products, Apopka, FL, USA) was subcutaneously injected when mice showed signs of dehydration (Sedelis et al., 2001).

Swim test

Each mouse was introduced individually into a pool (45 cm long; 22 cm wide diameter and 20 cm high) filled with 10 –cm-deep water (21-23^{o} C) (Yacoubi et al., 2003). Two days of 12 one-minute training trail in which the mouse strived for a secure location (platform) sited in the middle of one of the quadrants of the pool. The trials began at one of the four starting locations in bins of three. When the mouse found the platform, it stayed on it for thirty seconds and then was removed from the pool. Following the final training examination on day 2 and a 5-min period, the mouse underwent a 1 min probe trial in which the platform was removed from the pool, and began from a unique starting location directly on an opposite platform. During the trial, all movements and loops were registered and measured for 60 s that the mouse remained in the pool (Wilcoxon, Nadolski, Sumarut, Chassande, & Redei, 2007). All tests were recorded with a digital video camera (Sony, New York, NY, USA), using the computer software Doc-ITrLS Image Analysis (ultra-violet products Ltd., Cambridge, UK) to analyze the angles of their limbs.

Nest Building test

Mice habitually start to build a nest within hours when provided with appropriate material (nesting squares manufactured from pulp virgin cotton fibber and shacks) a behavior that is thought to be thermoregulatory (Wilcoxon et al., 2007). In our study, a folded tissue towel is put into every home cage after treatment. Nest building was video-recorded every day.

Statistical Analysis

Data were expressed in media and SEM for parametric values, and median, maximum and minimum values for non-parametric measures. Parametric measures (rigidity, akinesia/bradikinesia, motor alterations, drinking and eating behavior, and swimming) were performed with Student-T test for independent values and for non-parametric measures (palpebral ptosis, tremor, and righting reflex) were used a U-Mann-Whitney test for compared both groups. The level of significance set at $p < 0.05$ and power of 0.10.

Results

Parkinsonian symptoms were evaluated and recorded blindly by two raters and videotaped daily since the beginning of the study and for the following 8-day period since reserpine administration. Major and significant parkinsonian symptoms were observed. Whole body phasic tremor, arched posture and palpebral ptosis appeared abruptly within the first three days of reserpine administration. Each symptom was evaluated individually using a different rating scale. At the third day of reserpine administration, 78.14% of motor manifestations were found (Figure 1A). However, well-established Parkinsonism was assessed at the fifth day (95.14%) (Figure 1, A to C).

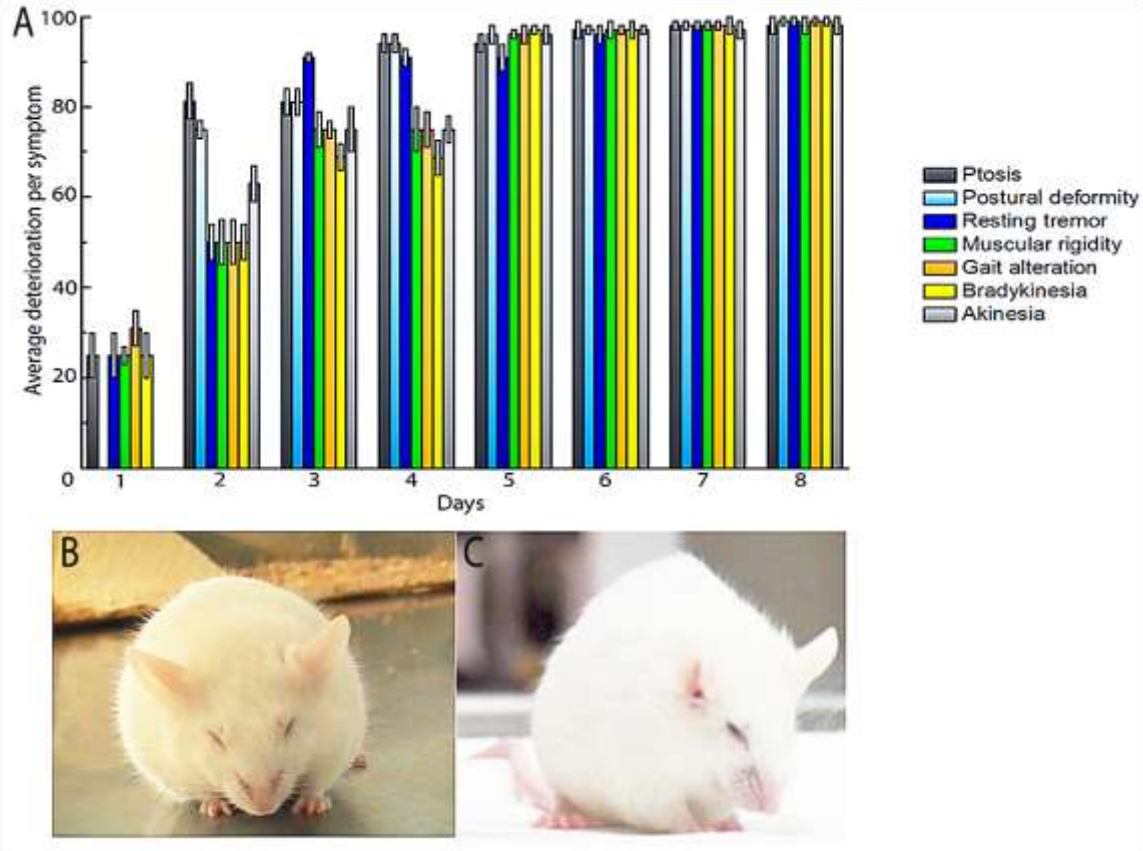

Figure 1. Parkinsonian symptoms following reserpine regimen. (A). Major parkinsonian symptoms were observed. Results are expressed in percentage of symptomatological evolution. (B) and (C). The images show an adult mouse with Parkinsonism. Arched posture, bilateral ptosis and piloerection can be observed at Day 5 after reserpine administration.

Applying a rating scale for abnormal involuntary movements in rodents (Lee et al., 2000) (Figure 2A), reserpine recipients exhibited a significant increase in abnormal involuntary movements scale (Figure 2A). Behavioral testing (Tadaiesky et al., 2006) was continued on the two study groups mice exhibited a significant decrease in locomotion and rearing frequencies (Figure 3, A and D), in addition to an increase in immobility time and latency to start the movement compared to the control group (Figure 3, B and C). There was evidence that reserpine treatment impaired mice ability to feed, hence leading to body weight loss**.** Significant body weight decreases were measured (Figure 2B). In severe cases of adipsia and aphagia, soft food and water were placed in easy access in order to assist nutrition and hydration. The average weight loss was of 1.08 g daily ($p < 0.01$) (Table 1). In the swim test, mice showed progressive inability to complete the test, fatigue, increased passive floating and altered hindlimbs angles while swimming; normal mice used in average 3 s (30 cm) to get to the secure area and presented in average 0.60 loop s^{-1} and 25 hindlimb movement s^{-1} with an angle of 30^{o} (active swimming), compared with mice treated

with reserpine that showed 0.10 loop s^{-1} and 6.45 hindlimb movement s^{-1} with an angle of 85°.

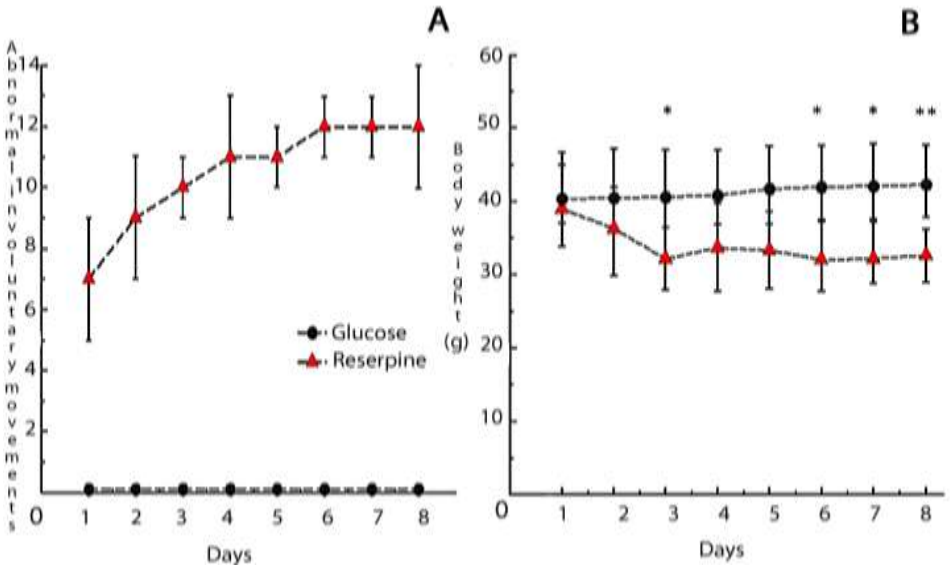

Figure 2. Behavioral response to reserpine treatment. A. Adult CD1 mice receiving five doses of the reserpine (5 d) showed deterioration from 6 to 12 on the rating scale for abnormal involuntary movements in 8 days. Behavioral scores for the negative controls and reserpine recipients presented significant differences P < 0.01. B. Weight response to different drugs administration per experimental group. Significant weight loss was observed in positive controls compared with negative controls (*P < 0.05, **P < 0.01).

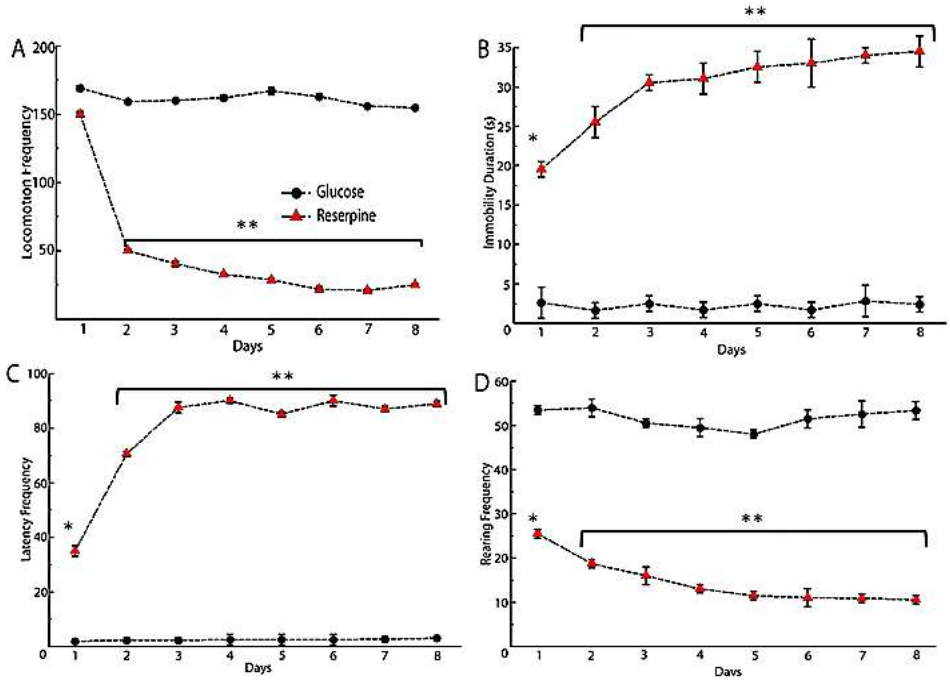

Figure 3. Motor alterations after reserpine administration. Effects of reserpine on frequency of locomotion (A), immobility duration (B), latency duration (C) and rearing frequency during 8 days (*P < 0.05, **P < 0.01).

Table 1.
The average weight loss after reserpine regimen

Reserpine group			
Animal (n=16)	**Original weight (g)**	**Weight loss per day (g)**	**Day of death**
1	34.3	-0.7	6th
2	34.5	-0.86	6th
3	33.9	-1.11	8th
4	35.3	-0.88	6th
5	34.7	-1.00	7th
6	37.8	-1.51	5th
7	36.9	-1.18	7th
8	34	-0.31	8th
9	41	-1.53	7th
10	43	-0.76	6th
11	42	-1.38	8th
12	43	-1.01	8th
13	44	-1.23	5th
14	45	-1.46	6th
15	42	-1.18	6th
16	42	-1.32	5th
General Average	38.9	-1.08	6.5

Nest building was impaired by the reserpine regimen; mice stopped constructing appropriate nests starting from the second day of the regimen, while controls did well on this task. The probable causes are a loss of thermoregulatory control and motor alterations. All animals died within 8 days (n=3, day 5; n=6, day 6; n= 3, day 7; and n=4, day 8) with 75% (n=12) of accumulated death on the seventh day (Table 2). Controls remained alive during all of the study. The effects of reserpine are reversible if a single dose of it is injected, but if rodents are submitted to a constant administration regimen, all individuals will die even if nutritional and thermoregulatory support is provided. When RES is given at the aforementioned dose in this rodent specie, it will be able to emulate cardinal symptoms of idiopathic PD including rigidity, tremor, akinesia and posture/gait abnormalities. The reserpine model of dopamine depletion has predictive validity, notwithstanding the fact that construct validity has always been controversial.

Discussion

Animal models are created to allow the study of aspects of human pathology. Construct validity, predictive validity, concurrent validity and face validity are some of the desirable features of a good model. In this study, we essayed to evaluate the predictive validity of RES regimen in mice submitted to a set of procedures as a model of motor–PD association. It was found that the administration of RES to mice induces a reduction in the spontaneous locomotor activity, a frequently used index

of motor impairment in animal studies of PD (Menzaghi et al., 1997). RES acts at the level of intraneuronal storage vesicles of monoamines (DA, noradrenaline and serotonin) through magnesium- and ATP dependent mechanisms with the consequent depletion of these monoamines in nerve terminals (Carlsson, 1975). The doses administrated in the present study were effective in inducing a significant reduction in monoamine levels in the CNS of mice (Kasahara, Nagatani, Takao, & Hashimoto, 1993).

Table 2.
Mortality rate in the first 8 days after reserpine regimen

Reserpine group		
Mortality percentage in the first 8 days	**Percentage and animal numbers**	**Accumulated mortality (%)**
5th day	18.75% n=3	18.75%
6th day	37.5 n=6	56.25%
7th day	18.75% n=3	75%
8th day	25% n=4	100%

Motor impairment

An essential factor for the manifestation of parkinsonian signs is the degree of striatal DA reduction, since a loss of 80% is commonly assumed to be necessary to view symptoms across species (Heikkila & Sonsalla, 1992). In human PD, this amount is associated with mild symptoms, while for severe symptoms near-total depletions are obliged (Zigmond, Abercrombie, Berger, Grace, & Stricker, 1990). Thus, when analyzing behavioral consequences of RES-induced nigrostriatal damage in mice, a significant deficiency is required to obtain longer-lasting motor functional deficits (Sedelis et al., 2000a y b). This can be achieved by administering 5 doses of RES (5mg/Kg); but then lethality (which is also dose-dependent) is assured within the next 8 days.

The appearance of bradykinesia, tremor and muscular rigidity induced by RES administration provides a useful animal model of Parkinsonism. The decrease in locomotor activity could be secondary to hypothermia (Bourin, 1990) (due to serotonin depletion in neurons located in the medium raphe), since 2.5-5-mg/kg of RES simultaneously induced a decrease in locomotor activity and temperature. However, the reversal of hypothermia by noradrenergic drugs (e.g., desipramine) was not followed by reversal of akinesia (Dutra, Andreazza, Andreatini, Tufik, & Vital, 2002). As cited above, one important criticism of the reserpine model is its nonspecific effect on the monoaminergic neurotransmission, but there are some findings suggesting that other brain nucleus (*locus coreuleus*, basalis nucleus of Meynert, *vagus motoris dorsalis*, reticular formation, medium raphe)

and neurotransmitter pathways (e.g., serotonin, acetylcholine and noradrenaline) are involved in PD pathogenesis, thus the reserpine model may result a good parameter for evaluating those correlations.

Conduct alterations

Experimental situations can be decisive, because highly challenging testing conditions are sometimes required to 'unmask' deficits. The induction of stress, such as transportation handling (Sundstrom, Henriksson, Mohammed, & Souverbie, 1994) or contact to cold water (Weihmuller, Hadjiconstantinou, & Bruno 1988), can increase behavioral differences between glucose against RES-treated mice. That stress has an influence on dopaminergic systems and is evident since the finding that rats liberate more DA in *nucleus accumbes*, *striatum* and medial frontal cortex when exposed to stress (Abercrombie, Keefe, DiFrischia, & Zigmond, 1989). A comparable mechanism in animals with nigrostriatal injuries might 'overcharge' the nigrostriatal system and its compensatory capacity (Zigmond & Stricker, 1989). Here we demonstrated that the effect of reserpine at the dose used in the present study was due to major depression and motor impairment. Other proofs have also been presented by some authors to give a reason for the high prevalence of depressive symptoms in PD patients, like the increased total scores on depression scales due to motor impairment (Skalisz et al., 2002). This consolidated the similarities between the model and the clinical condition; however moderate stress was sometimes correlated with a lesser gain in body weight than in control animals, with the suggestion that this parameter casts the change in body weight more than depression. Significant decrease in body weight is also a symptom of major depression, thus an association between these symptoms (depression and decrease body weight) is to be expected. The present data suggest that the administration of reserpine reproduces some of the clinical signs and symptoms of the depression-motor impairment association present in some PD patients, indicating an initial good validity of the model to study non-motor symptoms of the disease and potential therapeutics.

Validity of the reserpine model for testing anti-parkinsonian treatments

Reserpine induces severe striatal dopamine depletion by inhibiting the vesicular monoamine transporter (VMAT) and therefore allowing monoamine oxidase-B (MAO-B) to degrade it, considerably reducing monoamine levels both centrally and peripherally (Kreitzer & Malenka, 2007). If a drug or any other kind of treatment is going to be applied in this model, it needs to work either as a reserpine antagonist, MAO-B antagonist, dopamine receptor agonist, restore dopamine levels or prolonging dopamine half life. Any other type of approaches using this model will not be useful, like anticholinergics that only antagonize rigidity but not tremor and bradykinesia induced by reserpine. However, to really consolidate a treatment as effective in treating PD, it needs to be proven in two different animal models at least. Nonetheless, assessment of functional recovery using this model is critical, because it reproduces faithfully dopamine depletion symptoms seen in PD, something not achieved completely by other models.

Conclusion

Results presented suggest that the administration of reserpine to mice analyzed with functional and behavioral tests shows

an adequate validity as an animal model for the study of the motor (tremor, rigidity, postural abnormalities, bradykinesia, akinesia and gait alterations) signs of PD. However, we have to take in count that it has a nonspecific effect on other amines peripherally, and that may interfere in some aspects of the behavioral analysis.

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