ORIGINAL ARTICLE

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Absence of a synergic nigral proapoptotic effect triggered by REM sleep deprivation in the rotenone model of Parkinson´s disease

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ABSTRACT

Excitotoxicity has been related to play a crucial role in Parkinson's disease (PD) pathogenesis. Pedunculopontine tegmental nucleus (PPT) represents one of the major sources of glutamatergic afferences to nigrostriatal pathway and putative reciprocal connectivity between these structures may exert a potential influence on rapid eye movement (REM) sleep control. Also, PPT could be overactive in PD, it seems that dopaminergic neurons are under abnormally high levels of glutamate and consequently might be more vulnerable to neurodegeneration. We decided to investigate the neuroprotective effect of riluzole administration, a N-methyl-D-aspartate (NMDA) receptor antagonist, in rats submitted simultaneously to nigrostrial rotenone and 24h of REM sleep deprivation (REMSD). Our findings showed that blocking NMDA glutamatergic receptors in the SNpc, after REMSD challenge, protected the dopaminergic neurons from rotenone lesion. Concerning rotenone-induced hypolocomotion, riluzole reversed this impairment in the control groups. Also, REMSD prevented the occurrence of rotenone-induced motor impairment as a result of dopaminergic supersensitivity. In addition, higher Fluoro Jade C (FJC) staining within the SNpc was associated with decreased cognitive performance observed in rotenone groups. Such effect was counteracted by riluzole suggesting the occurrence of an antiapoptotic effect. Moreover, riluzole did not rescue cognitive impairment impinged by rotenone, REMSD or their combination. These data indicated that reductions of excitotoxicity, by riluzole, partially protected dopamine neurons from neuronal death and appeared to be effective in relieve specific rotenone-induce motor disabilities.

Keywords: Excitotoxicity; Neuroprotection; Riluzole; REM sleep deprivation; Intranigral rotenone; Parkinson's disease.

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INTRODUCTION

Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder, especially prominent in aging societies[1,2]. PD is characterized by progressive dopaminergic neuronal loss within the substantia nigra pars compacta (SNpc) that tends to happen more broadly in the ventrolateral layer followed by medial ventral and dorsal layers[3,4]. PD pathogenesis is a complex and multifactorial process, both genetic features and environmental stressors converge and compromising neuronal activity[5].

The precise intrinsic mechanism of the dopaminergic neuronal death remains unknown, however, several lines of evidence highlighted the importance of enhanced glutamatergic neurotransmission in basal ganglia and related structures in the PD development[6]. In PD, some glutamatergic systems are somewhat hyperactive, such as corticostriatal and subthalamo-nigral pathways, potentially contributing to dopaminergic neuronal death, through the excitotoxicity process[2,7,8].

In this sense, pedunculopontine tegmental nucleus (PPT) is one of the major source of glutamatergic afferences to nigrostriatal pathway[9]. Remarkably, the basal ganglia, including SNpc, and PPT share numerous similarities in projections to cortex, thalamus amygdala and brainstem[10]. Accordingly, there is a hypothesis that PPT could play an excitotoxic role in dopaminergic neurons, suggesting some level of participation in PD pathogenesis, since PPT demonstrated to be highly active in the *6-hydroxydopamine* (6-OHDA) animal model[2,11,12].

Furthermore, mutual interactions between PPT and SNpc are associated with REM sleep generation. Thus, nigral degeneration has been related with significant disruption in REM sleep in both animal models[13-16] and PD[17]. In this context, PPT could play a role in neurodegenerative process, triggering excitotoxicity within the SNpc, contributing to the occurrence of sleep disorders[2].

Therefore, it is considered that reduced nigrostriatal activity would result in a compensatory PPT firing, resulting in increased glutamatergic neurotransmission to SNpc, with potential excitotoxic mechanisms. Based on these observations, we promoted nigrostriatal lesions with the neurotoxin rotenone[18], associated with REM sleep deprivation (REMSD) and/or riluzole administration, a NMDA receptor antagonist with clinical neuroprotective applications, p.e., in amyotrophic lateral sclerosis[19]. Our hypothesis is also based on a 24 h REMSD-induced down-regulation of tyrosine hydroxylase (TH) expression, within the nigrostriatal pathway[20-23], possibly promoting an increased PPT activity, negatively impacting SNpc neurons through excessive glutamatergic signaling.

MATERIAL AND METHODS

Animals and house conditions

All of the experiments performed in this study were approved by the ethics committee of Federal University of Paraná (approval ID#918) and conducted according to the guidelines of ethics and experimental care and use of laboratory animals (SBCAL). Male Wistar rats weighing 280-230 g at the beginning of the experiment were used. The animals were randomly housed in groups of 5 in polypropylene cages and maintained in a temperature controlled room ($22\ °C \pm 2°C$) with a 12h light-dark cycle (lights on at 7:00 am). Bottles of water and pellets of food were available throughout the entire experiment.

Experimental design

Before the stereotaxic surgeries the animals were distributed randomly in two groups: sham (n=48) and rotenone (n=48). Afterwards, the animals were redistributed in eight groups (n=12/group): control sham vehicle, control sham riluzole, control rotenone vehicle, control rotenone riluzole, REMSD sham vehicle, REMSD sham riluzole, REMSD rotenone vehicle and REMSD rotenone riluzole. On the day 0, the animals underwent stereotaxic surgery for bilateral guide cannulas implantation within the SNpc. On day 7 the animals received a bilateral intranigral infusion of 1μl of rotenone (12 μg/μl) or equal volume of its vehicle dimethylsulfoxide (DMSO) and then the rats were subjected to 24 h of REM sleep deprivation (REMSD) or were kept in their home cages (control). Immediately after this period, the animals received a single bilateral intranigral infusion of 1μl of riluzole (10μg/μl) or equal volume of DMSO, subsequently (30 minutes after) they were tested in the open field (OFT) and in the object recognition test (ORT). At the end of these experiments, the animals were intracardially perfused (under deep anesthesia) for tissue fixation and the brains were removed for subsequent confocal microscopy analysis of apoptotic cells within the SNpc.

Stereotaxic surgery and intranigral infusions

The animals were sedated with intraperitoneal xylazine (10 mg/kg; Syntec do Brasil Ltda, Brazil) and anaesthetized with intraperitoneal ketamine (90 mg/kg; Syntec do Brasil Ltda, Brazil). The following coordinates were used for bilateral guide cannulas implantation, bregma as a reference: SNpc (AP) = - 5,0 mm, (ML) = ± 2,1 mm e (DV) = - 7,8 mm[24].

Bilateral intranigral infusions of 1 μl of Rotenone (12μg/μl), 1 μl of riluzole (10μg/μl); Sigma-Aldrich®, St. Louis, MO, USA, or 1 μl of DMSO (Sigma-Aldrich®) were all made through the guide cannulas, at a rate of 0.33 ml/min for 3 min, with the assistance of an electronic infusion pump (Insight Instruments, Ribeirão Preto, SP. Brazil).

REMSD procedure

REMSD was attained by means of the single platform method. The animals were individually placed in a circular platform (6.5 cm in diameter) inside of a tank (23 x 23 x 35 cm) filled with water up 1 cm below the platform surface for 24 h. Once the animal experiences a REM sleep episode, it loses its muscular tonus and falls into the water, being awakened. This procedure has demonstrated effectiveness in ablation of REM sleep without affecting NREM sleep[25]. Throughout the study, the experimental room was maintained at controlled conditions

(22 ± 2 ◦C, 12:12 h light/dark cycle, lights on 7:00 a.m.). The control group (non-sleep deprived) was maintained in the same room during the period, but isolated in their usual home cages, to mimic a possible effect of isolation caused by the procedure. Water and food were available during the entire experiment.

Open Field Test (OFT)

The apparatus consists of a circular arena (1 m of diameter) limited by a 40 cm high wall and illuminated by four 60 W lamps situated 48 cm above the arena floor, providing illumination around 300 lx[26]. The animals were gently placed in the center of the arena and were allowed to freely explore the area for 5 min. During the experiments, the open field test was video recorded and the measures of locomotion of the groups were computed by an image analyzer system (Smart junior, PanLab, Harvard Apparatus, Spain).

Object Recognition Test (ORT)

The apparatus consists of an open box (width × length × height = 80 cm × 80 cm × 50 cm) made of wood and covered with a black opaque plastic film. The illumination on the floor of the box apparatus was around 186 lx. The objects to be discriminated were available in triplicate copies and were made of a biologically neutral material such as glass, plastic, or metal. The objects were weighted so that the animals could not move them around in the arena. They are not known to have any ethological significance for the rats and they had never been associated with any reinforcement[27]. The object recognition test consists of two phases, a sample phase (3-min duration) and a choice phase (3-min duration). In the sample phase, two identical objects are exposed in the back corners of the open box, 10 cm away from the sidewall.

The rat is placed in the open box facing away from the objects for 3 times with a 15 min retention interval between the times. After 24 h, the rat is reintroduced to the open box and the choice phase is started. In the choice phase, two different objects are exposed in the same locations that were occupied by the previous sample objects. One of the objects is identical to the object seen in the sample phase and the other is a novel object, the total time spent in exploring the two objects was video recorded. The frequencies of approaches of each object are recorded. The exploration is recorded only when the rat touches the object with its nose or that the rat's nose is directed toward an object at a distance ≤ 2 cm. As a measure of discrimination, "discrimination index (DI)" was calculated by dividing the difference in number of explorations between the two objects (object novel - object familiar) by the total amount of exploration for both objects (object novel + object familiar). DI was then multiplied by 100 to express as a percentage.

Fluoro Jade C (FJC) staining within SNpc

Animals were deeply anesthetized with ketamine (100 mg/kg) immediately after the behavioral tests and were intracardially perfused with saline first, then with 4% of the fixative solution formaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed from the skulls and were immersed

for 48 hours in that fixative solution at 4ºC. Subsequently, the brains were placed in 30% sucrose solution for 3 days and were freeze at -80ºC before sectioning. Four 40 µm sections per animal were taken from the SNpc (-4.92 mm and -5.28 mm). The coordinates were obtained from[24].

Prior to staining, sections were mounted from distilled water onto gelled slides. Gelatin coated slides were prepared by immersion in a 60 ℃ solution of 1% pig skin gelatin (Sigma-Aldrich; type A, 300 Bloom) and then oven dried overnight at the same temperature. The sections were mounted onto the slides from distilled water and then air dried for at least 30 min on a slide warmer at 50 ℃. Slides bearing frozen cut tissue sections were first immersed in a solution containing 1% sodium hydroxide in 80% ethanol for 5 min. They were then rinsed for 2 min in 70% ethanol, for 2 min in distilled water, and then incubated in 0.06% potassium permanganate solution for 10 min. Slides were then transferred for 10 min to a 0.0001% solution of Fluoro Jade C as indicated by the fabricant (Thermo Fischer Scientific). The proper dilution was accomplished by first making a 0.01% stock solution of the dye in distilled water and then adding 1 ml of the stock solution to 99 ml of 0.1% acetic acid vehicle. The working solution was used within 2 h of preparation. The slides were then rinsed through three changes of distilled water for 1 min per change. Excess water was drained onto a paper towel, and the slides were then air dried on a slide warmer at 50 ℃ for at least 5 min. The air dried slides were then cleared in xylene for 5 min and then coverslipped with Fluoromount-G (SouthernBiotech, Birmingham, Alabama, USA) non-fluorescent mounting media.

The images were analyzed in Nikon Confocal Microscope A1RSi+MP (Nikon Instruments Inc.; Tokyo, Japan), using 20x lenses. For FJC, 488 nm laser was used for excitation and the imagens was obtained using a 500-550nm band pass filter. The Imaging Software Nis Elements 4.20 (Nikon) was used for z-stack visualization and to generate maximum projectin image.

Statistical analysis

Homogeneity of variance was assessed by the Bartlett test and normal distribution of the data was assessed by the Kolmogorov-Smirnov test. Differences between groups in the OF, ORT and discrimination index were analyzed by two-way analysis of variance (ANOVA) followed by the Tukey's post hoc test. Fluorescence intensity was analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. Pearson's correlation coefficients (r) were calculated to establish relationships between fluorescence intensity and behavioral parameters. Values were expressed as mean ± standard error of mean (SEM). The level of significance was set at $p \leq 0.05$.

RESULTS

Open Field Test (OFT)

As can be seen in Figure 1, the control rotenone vehicle group presented an impaired locomotion in comparison to the control sham vehicle group ($p \leq 0{,}05$), as indicated by the group factor [$F_{(3.54)} = 2.21$; $p \leq 0.09$]. Interestingly, the same effect

was not observed in the control rotenone riluzole group when compared to the control sham vehicle ($p \geq 0.96$) and control sham riluzole ($p \geq 0,99$) groups. Complementarily, the rotenone REMSD groups did not exhibit reductions in locomotion when compared to the control sham ($p \geq 0.95$) and REMSD sham ($p \geq 0.97$) groups, according to the treatment [F (1.54) = 0.063; $p = 0,8$] and interaction [F(3,54) =1.59; $p = 0,20$] factors.

Object Recognition Test (ORT)

Regarding the cognitive analysis, we found that the control sham vehicle group showed an increment in the time exploring the novel object in comparison to the familiar ($p \leq 0.01$) during the choice phase, as well as the control sham riluzole group ($p \leq 0.01$), by means of the treatment [F(7.10)=3.17; $p \leq 0.01$] and interaction [F(7.10)=3.63; $p \leq 0.001$] factors (Fig. 2A). Conversely, the control rotenone groups demonstrated a similar exploration time for both objects, indicating memory impairment as showed by the object [F(1.10)=2.57; $p = 0.1$] factor. Besides, it was detected that REMSD, itself, produced a remarkable impairment in the object recognition, since all the sleep deprived groups explored both objects equally.

Figure 2B shows the discrimination index (DI) obtained from the time of objects exploration recorded for each control and REMSD groups. Accordingly, the control rotenone vehicle exhibited a significant reduction in this parameter when compared to the control sham vehicle ($p \leq 0.05$). As observed in the previous parameter, REMSD, per se, promoted a noteworthy decrease in this index for all the groups that were sleep deprived. Therefore, we observed a significant decrease in the DI for the REMSD sham vehicle ($p \leq 0.01$) and REMSD sham riluzole ($p \leq 0.05$) groups when compared to their respective controls [F(3.57)=10.84; $p < 0.0001$].

Fluoro Jade C (FJC) staining within SNpc

FJC labeling can sensitively and selectively stain degenerating neurons. In this sense, Figure 3 depicts the differences on fluorescence intensity among the groups. In fact, the groups control sham vehicle (Fig. 3A), REMSD sham
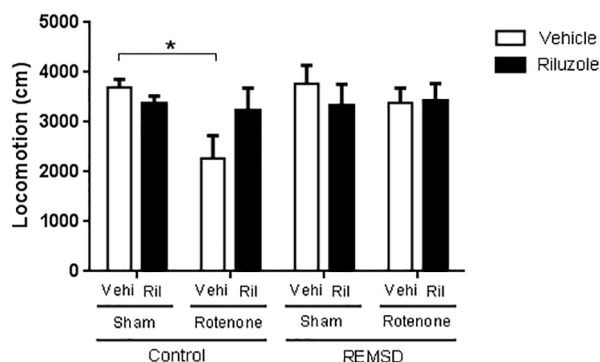

Figure 1. Locomotion parameter obtained from the open field test 8 days after surgery, 30 minutes after drug (riluzole) or vehicle (DMSO) micro infusion. Comparison between control and REMSD groups. Values are expressed as mean ± SEM. *$p \leq 0,05$. Two-way ANOVA followed by Tukey's post hoc test.
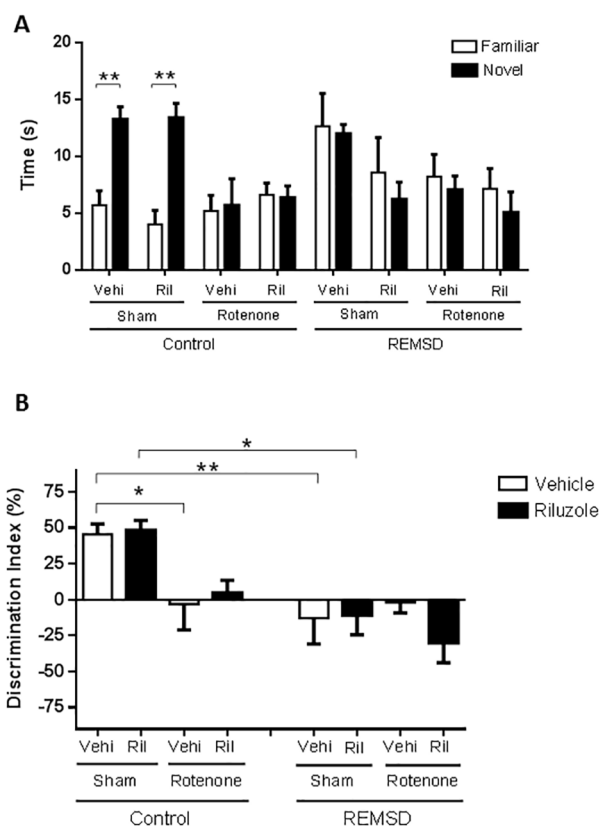

Figure 2. Cognitive effects produced by riluzole administration after REMSD, detected in the object recognition test. (**A**) Time (s) exploring the familiar and novel object 8 days after surgery, 30 minutes after drug (riluzole) or vehicle (DMSO) micro infusion. (**B**) Discrimination index calculated by (N-F/N+F)* 100, N is the time exploring novel object and F is the time exploring familiar object. Comparison between control and REMSD groups. Values are expressed as mean ± SEM. *$p \leq 0,05$, ** $p \leq 0,01$. Two-way ANOVA followed by Tukey's post hoc test.

vehicle (Fig. 4A), control sham riluzole (Fig. 3B) and REMSD sham riluzole (Fig. 4B) did not exhibit FJC labeling. In opposite, the control rotenone vehicle (Fig. 3C) and REMSD rotenone vehicle (Fig. 4C) groups have shown remarkable fluorescence labeling compared to the control sham vehicle and REMSD sham vehicle groups. Conspicuously, we detected that riluzole treatment produced a prominent reduction in the FJC fluorescence intensity as can be seen in the control rotenone riluzole (Fig. 3D) and REMSD rotenone riluzole (Fig. 4D) groups.

DISCUSSION

In the present study we observed that blocking NMDA glutamatergic receptors, within the SNpc, immediately after an acute REMSD challenge, partially protected dopaminergic neurons from the rotenone lesion and the excitotoxicity purportedly inflicted by PPT increased activity. Such limited protection was also manifested as an absence of memory preservation observed in the rotenone-treated groups. Besides, sleep deprivation generated massive memory impairment in all the groups subjected to this condition, hampering the identification of any possible protective effect of the drug in this context. Nevertheless, we detected an interesting effect of riluzole preserving the locomotor activity of the control
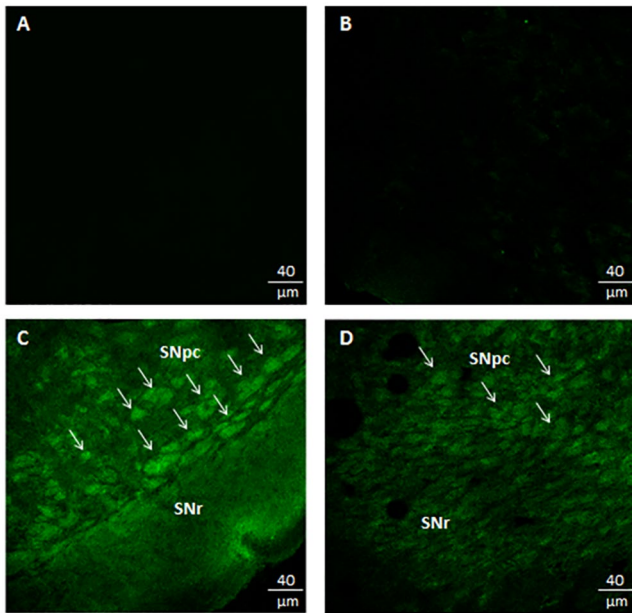
Figure 3. Comparison of Fluoro-Jade C (FJC) staining in the SNpc between: (**A**) Control sham vehicle, (**B**) Control sham riluzole, (**C**) Control rotenone vehicle, (**D**) Control rotenone riluzole. Arrows indicate FJC-positive neurons.
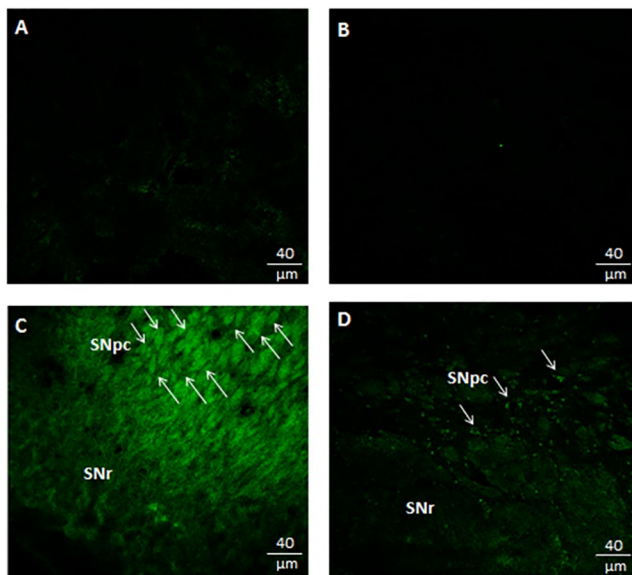

Figure 4. Comparison of Fluoro-Jade C (FJC) staining in the SNpc between: (**A**) REMSD sham vehicle, (**B**) REMSD sham riluzole, (**C**) REMSD rotenone vehicle, (**D**) REMSD rotenone riluzole. Arrows indicate FJC-positive neurons.

rotenone-treated rats. In addition, REMSD counteracted the locomotion deficit induced by the lesion due to the occurrence of a vastly reported effect of dopaminergic supersensitivity[22,28].

Riluzole has been consistently demonstrated to provide a multitude of neuroprotective effects in neurodegenerative disorders, such as amyotrophic lateral sclerosis[1]. Moreover, analogous effects were reported in studies from animal models of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 6-hydroxydopamine, showing reduction in the dopaminergic neuronal loss, covered by a variety of administration protocols[1,7,29,30]. It is described that the pharmacological mechanism of riluzole

is based on the noncompetitive blockade of NMDA receptors, reduction of glutamate release from presynaptic terminals, inhibition of voltage-gated sodium channels and inhibition of protein kinase C activity, hence counteracting excitotoxic processes[7,31].

Rotenone is a neurotoxin that produces massive inhibition of mitochondrial complex I, increase of reactive oxygen species and selective degeneration of the dopaminergic neurons, reproducing key pathological features of PD[18,32,33]. Evidence suggest that rotenone might induce neuronal damage via excitotoxic mechanisms[34] potentiating NMDA-mediated currents in the dopaminergic neurons[35]. Similar results were found in human postmortem tissues, indicating that mitochondrial alterations are important in the pathogenesis of sporadic PD[36].

The localization and activity profile of the ionotropic glutamate receptors in the SNpc suggest that these receptors may provide a positive feedback mechanism triggered by PPT activation[2,37]. As a result, a putative reciprocal connectivity between those structures may exert a potential influence on the mechanism of REM sleep[2,15,16]. This raises an intriguing possibility, for which there is currently no further evidence, that reducing the glutamatergic firing from PPT to SNpc, this could generate neuroprotective effects and influence the rate of the progression of PD[2]. In view of that, our FJC-staining investigation showed some level of neuroprotection inflicted by riluzole particularly when fluorescence is compared to the rotenone groups. This result suggests the occurrence of and antiapoptotic effect[38]. Besides, REMSD did not inflict increased or synergic neuronal death associated to rotenone lesions, as originally expected. One plausible explanation is that the nigrostriatal damage may have been substantially extensive and the REMSD effect on neuronal death could not be observed.

In fact, rotenone produces a considerable reduction on the percentage of SNpc tyrosine hydroxylase immunoreactive neurons[18,33]. Furthermore, it seems to be more potent when compared to other neurotoxins requiring lower doses to produce the lesion[39]. Thus, additional studies, with lower neurotoxin dosages, will be necessary to completely refute the current hypothesis; hence, we cannot entirely exclude the potential deleterious effects of REMSD as demonstrated by other studies[40-42].

Regarding the rotenone-induced hypolocomotion, riluzole rescued this impairment in the control groups. This is in accordance to previous reports that described a positive effect of riluzole in the motor performance in PD animal models[43,44]. However, we did not found locomotion differences between the sham REMSD groups, neither in the rotenone REMSD groups. Probably due to REMSD is able to elicit a locomotion increase, per se, associated to the well-known supersensivity of dopaminergic receptors[20,28,45].

Concerning the cognition, ORT is a familiarity-based memory task correlated with the human episodic-like memory[46] which is compromised in early-stages PD patients[47]. In light

of this, given the correlation between sleep disturbances and cognitive impairments, it is possible to consider that sleep disorders observed in PD patients, might be considered as an early marker for dementia processes[48]. Accordingly, our results demonstrated that riluzole did not affected memory processes of control groups and also not rescued the impairment impinged by rotenone, REMSD or their combination. Furthermore, REMSD generated remarkable memory impairment, corroborating with previous reports[49,50]. Such result is opposite to other studies that tested riluzole in different chronic and systemic protocols showing levels of improvement of cognitive performance[51,52]. This discrepancy could be related to our experimental design that projected riluzole administration to a period of high neurotoxic condition, i.e., 24 h after rotenone and immediately after the end of REMSD (challenge to induce PPT activation). Complementarily, decreased FJC labeling within the SNpc could be associated with increased cognitive performance in controls, but not in REMSD groups, possibly due to massive memory disruption inflicted by sleep loss. These data indicate improvement of memory performance in the control animals with lower dopaminergic neuronal loss, as an outcome of the excitotoxicity blockade.

In summary, the intranigral administration of riluzole partially protected the dopaminergic neurons from the rotenone-induced lesion, particularly preventing the occurrence of locomotor, but not declarative-like memory deficits. The data also indicate absence of a synergic excitotoxic mechanism triggered by a supposed PPT overactivity towards SNpc, through reciprocal projections. Although, we cannot completely exclude this potential association because more studies will be necessary to identify the levels of glutamatergic PPT activation inflicted by different protocols of REMSD, perhaps in a more gradual and chronic situation, mimicking PD.

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