Olfaction in female Wistar rats is influenced by dopaminergic periglomerular neurons after nigral and bulbar lesions

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Hyposmia is found in Parkinsonian patients decades before the onset of motor disorders. The same occurs with sleep disorders, especially infuencing rapid eye movement (REM) sleep, which affect a large percentage of people who have Parkinson's disease. These two disturbances presumably are closely related to a dopaminergic dysfunction. Therefore, we propose that selective lesions, induced by rotenone, of the periglomerular neurons within the olfactory bulb or of the nigrostriatal pathway could result in hyposmia. In addition, we hypothesized that REM sleep deprivation (REMSD) could have potential to generate a synergistic olfactory impairment in both lesion paradigms. The results indicated that rotenone-induced nigrostriatal lesions in female Wistar rats were associated with odor preference changes, similar to hedonic tone impairment, but without a supposed potentiation triggered by REMSD. The nigrostriatal injury negatively affected olfaction performance, which was counteracted, functionally, by REMSD. However, injury to periglomerular neurons was less influenced by REMSD, as olfactory performance was restored after rebound sleep.
We conclude that female rats present a pattern of olfactory discrimination/preference that is dependent on the activities of the nigrostriatal and the main olfactory pathways. *Behavioural Pharmacology* 00:000–000

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Introduction

Patients with Parkinson's disease (PD) often exhibit hyposmia and/or anosmia as an early phase sign that may be associated, according to studies in animal models, to disruptions in the main olfactory pathway (Tissingh *et al.*, 2001; Prediger *et al.*, 2006; Ansari and Johnson, 1975; Aurich *et al.*, 2017). Olfactory impairments are not usually considered as disabling as motor disturbances, but they are present in 90% of PD cases (Doty 2012; Doty *et al.* 1988). Remarkably, the topographical patterns of the first lesions develop at the olfactory bulb (OB) together with related portions of the anterior olfactory nucleus (Del Tredici *et al.*, 2002; Braak *et al.*, 2004). In addition to olfactory deficits, PD is also related to rapid eye movement (REM) sleep disorder, which is manifested in 25–50% of cases, significantly affecting the quality of life of these patients (Lima *et al.*, 2012; Lima, 2013). In fact, 24 h of sleep deprivation impairs olfactory performance in a smell identification task in humans (Killgore and McBride, 2006). Moreover, other studies have found similar olfactory impairment in patients with idiopathic REM sleep behavior disorder, suggesting a possible relation between this sleep phase and olfaction (Fantini *et al.*, 2006; Miyamoto *et al.*, 2009).

Despite the growing evidence of a dopaminergic influence in olfactory (Rodrigues *et al.*, 2014) and REM sleep mechanisms (Targa *et al.*, 2016, 2018), previously suggested by our group, and the recently described nigro-olfactory projection (Hoglinger *et al.*, 2015), several points still remain to be clarified. In this regard, tyrosine hydroxylase-immunoreactive (TH-ir) neurons, within the glomerular layer of the OB, appear to be the key players in odor discrimination, potentially contributing to the hyposmia/anosmia described in PD.

Hence, we propose that a selective lesion of these OB dopaminergic neurons could be associated with an early stage of the disease, thus producing hyposmia. We also hypothesize that the main olfactory pathway, which is potentially dependent on dopaminergic activity, might be drastically affected by a nigrostriatal lesion. In addition, REM sleep deprivation (REMSD) could also trigger, by itself (owing to dopaminergic D2 supersensitivity) (Tufik *et al.*, 1978; Tufik 1981a), some level of hyposmia, with a potential to generate a synergistic olfactory impairment in both lesion paradigms.

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Methods

Subjects

Female Wistar rats from Federal University of Paraná's biotery (Curitiba, Brazil) weighing 250–280 g were used. The animals were randomly housed in groups of five in polypropylene cages with wood shavings and maintained at 22 ± 2°C on a 12 : 12-h light–dark cycle (lights on at 07:00 h) with free access to water and food during all the experiments. The experiments were carried out according to the guidelines of Brazilian Guide for Care and Use of Laboratory Animals (COBEA). The protocols comply with the recommendations of Federal University of Paraná and were approved by the Institutional Ethics Committee (approval ID #852).

Experimental design

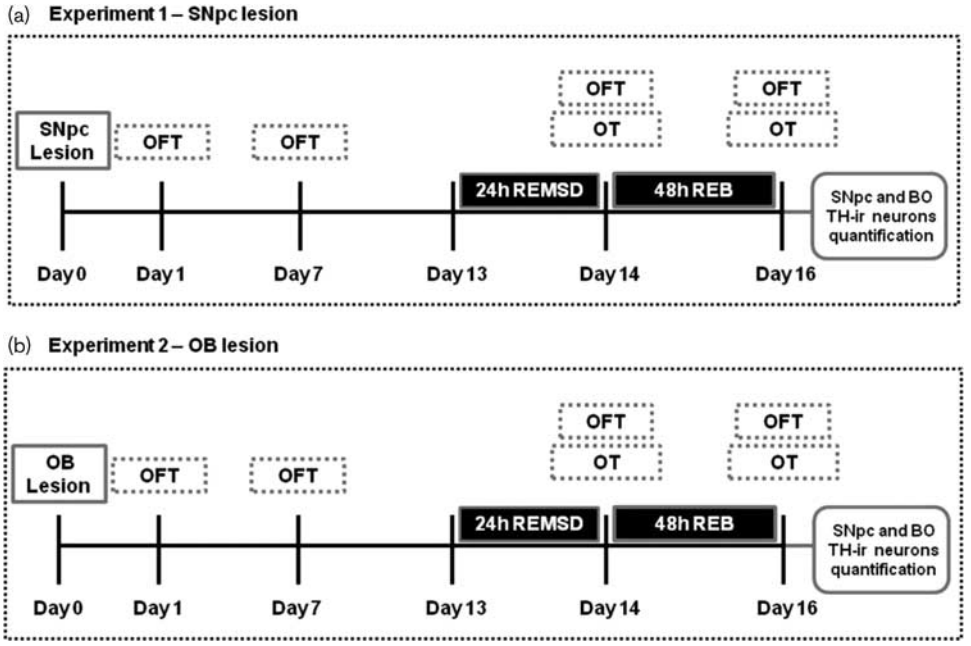
In experiment 1 (Fig. 1a), stereotaxic surgery was performed on day 0 for rotenone lesion or vehicle infusion (DMSO to sham groups) of the substantia nigra pars compacta (SNpc), a well-established animal model of PD (Noseda *et al.*, 2014; Rodrigues *et al.*, 2014; Targa *et al.*, 2016). On days 1 and 7 after the surgery, all the animals were recorded in the open-field test (OFT) for locomotor activity. Subsequently, the rats were distributed into four groups: sham control ($n = 12$), sham REMSD ($n = 12$), rotenone control ($n = 12$), and rotenone REMSD ($n = 11$). On day 13, a 24-h REMSD procedure was started. On day 14, rats were tested in the olfactory task (OT) and the OFT. Forty-eight hours after the REMSD procedure (rebound period), on day 16, the groups were retested for the same parameters. Immediately after the OT, 3–5 animals/group had their brains perfused for later analysis.

In experiment 2 (Fig. 1b), the animals were subjected to a dopaminergic neuron lesion in the OB. The neurotoxin rotenone was injected into the glomerular layer of the OB on day 0, and subsequently, all the procedures were conducted in the same way as the experiment 1. The animals were distributed into four groups: sham control ($n = 11$), sham REMSD ($n = 12$), rotenone control ($n = 12$), and rotenone REMSD ($n = 13$).

Stereotaxic surgery

The animals were sedated with xylazine (10 mg/kg, intraperitoneally; Syntec do Brasil Ltda, Brazil) and anesthetized with ketamine (90 mg/kg, intraperitoneally; Syntec do Brasil Ltda, Santana de Parnaíba, Brazil). In experiment 1, the following coordinates were used for the bilateral injury, with bregma as a reference: SNpc: anteroposterior = − 5.0 mm, mediolateral = ± 2.1 mm, and dorsoventral = − 8.0 mm (Paxinos and Watson, 2005). Needles were guided to the region of interest for a bilateral infusion of 1 µl of rotenone (12 µg/µl) or of dimethylsulfoxide (DMSO; Sigma-Aldrich, St Louis, Missouri, USA) using an electronic infusion pump (Insight Instruments, Ribeirão Preto, Brazil) at a rate of 0.33 µl/min for 3 min (Saravanan *et al.*, 2005; Moreira *et al.*, 2012; Dos Santos *et al.*, 2013). In experiment 2, the following coordinates were used, with bregma as a reference: OB: anteroposterior = + 7.08 mm, mediolateral = 0.0 mm, and dorsoventral = − 3.6 mm (Paxinos and Watson, 2005).

Fig. 1



(a, b) Schematic representation of the experimental design. OB, olfactory bulb; OFT, open-field test; OT, olfactory task; REB, sleep rebound; REMSD, REM sleep deprivation; SNpc, substantia nigra pars compacta; TH-ir, tyrosine hydroxylase-immunoreactive.

Needles were guided to the region of interest for an infusion of 1 µl of rotenone (12 µg/µl) or DMSO, according to the group.

Rapid eye movement sleep deprivation procedure

REMSD was performed using the single-platform method (Targa *et al.*, 2016). Rats were placed on a circular platform (6.5 cm in diameter) in a cage (23 × 23 × 30 cm) filled with water up to 1 cm below the platform level for 24 h. At the beginning of each REM sleep episode, the animal experiences a loss of muscle tonus and falls into the water, thus being awakened. This method effectively abolishes REM sleep (Machado *et al.*, 2004). The control group (non-sleep-deprived) was kept in individual cages with the same platforms except the presence of water that was substituted by sawdust. Free access to food and water was provided by placing chow pellets in a dispenser positioned inside the cage and water bottles on a grid located on top of the tank.

Nonsocial olfactory task

The apparatus consisted of a box (60 × 40 × 50 cm) divided into two compartments that were connected by a door. Initially the rats were habituated for 5 min with fresh sawdust in both compartments. Then, the test was performed by placing the rat in the middle of the box and the time of exploration of each compartment was recorded. Wistar rats exhibit a significant preference to explore the lemon odor, which is typically a nonsocial stimulus, able to selectively activate the main olfactory pathway (Aurich *et al.*, 2017). Therefore, lemon odor was presented to rats in 50-ml falcon tubes with several small holes (about 1 mm of diameter each) to allow the dissemination of the odor (Aurich *et al.*, 2017). Inside these tubes, there was a filter paper (3 × 1 cm) soaked with 100 µl of the odor essence (odor compartment) or water as a control odor (water compartment). The olfactory index (OI) was calculated by the following formula: [(lemon compartment − water compartment)/(lemon compartment + water compartment)] × 100 (Dos Santos *et al.*, 2013; Rodrigues *et al.*, 2014). OI values ranged from + 100 to − 100. Positive values correspond to a preference for the lemon odor.

Open-field test

The test was performed in a circular arena (1 m of diameter × 0.4 m wall) illuminated by four 60 W lamps, thus providing illumination around 300 lx. The animals were gently placed in the center of the arena and were allowed to freely explore the arena for 5 min. All the tests were video recorded, and the total distance was computed online by an image analyzer system (Smart junior, Pan Lab; Harvard Apparatus, Barcelona, Spain).

Tyrosine hydroxylase-immunohistochemistry within the substantia nigra pars compacta and olfactory bulb

Animals were deeply anesthetized with ketamine immediately after the OT on the 16th day and were intracardially perfused with saline, then with 4% of the formaldehyde in 0.1 mol/l phosphate buffer (pH 7.4). After that, brains were removed and were immersed for 48 h in that fixative solution at 4°C. Next, the samples were immersed in 30% sucrose solution for 3 days and finally frozen at − 80°C. Sections (40 µm) were obtained from the OB (+ 7.56 and + 7.08 mm an interval of 480 µm) and from SNpc (− 4.92 and − 5.28 mm, an interval of 360 µm) (Rodrigues *et al.*, 2014). The sections were incubated with primary mouse anti-TH antibody (1 : 500; Chemicon, California, USA) prepared in PBS containing 0.3% Triton X-100 overnight at 4°C. Biotin-conjugated secondary antibody incubation (1 : 200 anti-mouse; Vector Laboratories, Burlingame, California, USA) was performed for 2 h at room temperature. After several washes in PBS, antibody complex was localized using the ABC system (Vectastain ABC Elite kit; Vector Laboratories) followed by 3,3´-diaminobenzidine reaction with nickel enhancement. Cell counts for the glomerular layer of the OB were carried out by the software Image-Pro Express 6 (Media Cybernetics Inc., Bethesda, Maryland, USA), and neuronal density for the SNpc was performed with the software Image J 1.47v (US National Institutes of Health, Bethesda, Maryland, USA). The mean number of TH-ir neurons in each hemisphere was considered to be representative of the OB and SNpc neuronal cells in each animal. For each group, a mean value was calculated (percentage relative to the sham control), and compared with those of the other groups. The images were obtained through the use of a motorized Axio Imager Z2 microscope (Carl Zeiss, Jena, Germany), equipped with an automated scanning VSlide (Metasystems, Altlussheim, Germany).

Statistical analysis

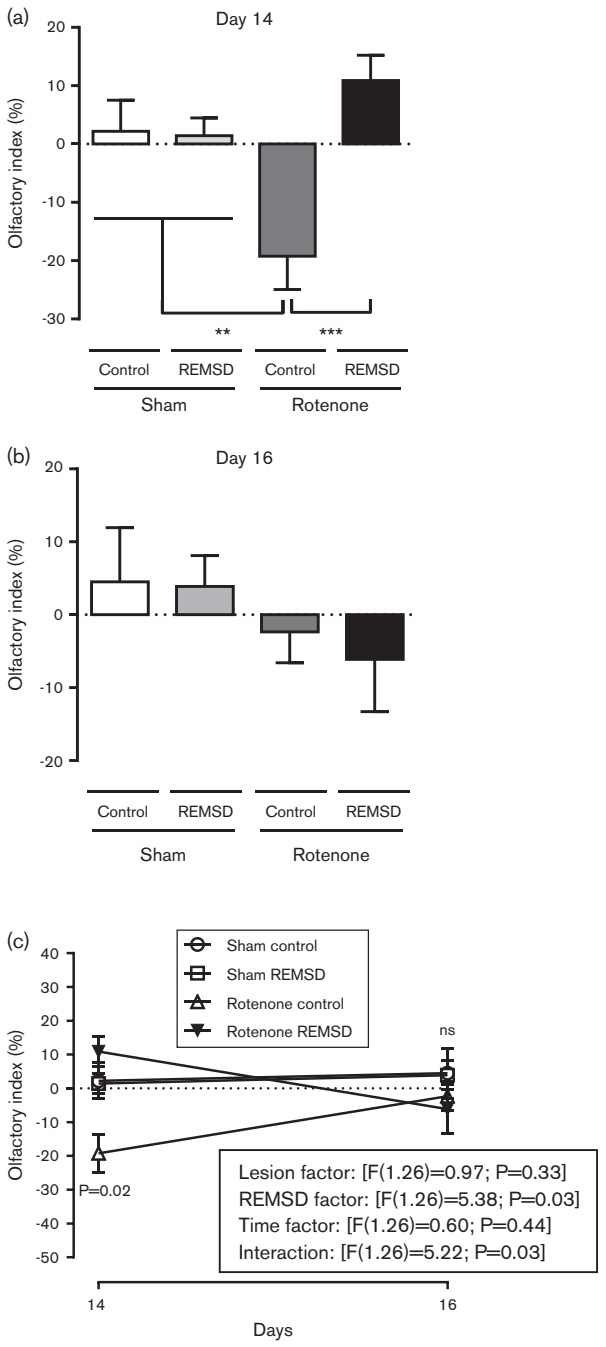
The OI and TH-immunohistochemistry were analyzed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test. Comparisons between time points were analyzed by three-way ANOVA (factors: lesion, REMSD, and time) followed by Bonferroni's post-hoc test. The OFT was analyzed by *t*-test on days 1 and 7, and the following days of this test were analyzed by ANOVA followed by the Newman–Keuls multiple comparison test. Values were expressed as mean ± SEM. The level of significance was set at P of less than or equal to 0.05.

Results

Olfactory index

The OI obtained for experiment 1 shows, after 24h of REMSD, that the rotenone control group presented a significant aversion to the lemon compartment, indicated by a significant negative OI compared with the sham control ($P < 0.05$), sham REMSD ($P < 0.05$), and rotenone REMSD ($P < 0.01$) groups [$F(3,36) = 5.81$, $P < 0.002$] (Fig. 2a). However, 48 h later, the rotenone control group did not demonstrate significant differences compared

Fig. 2



Olfactory index (OI) % obtained from experiment 1. (a) The OI 14 days after the nigral rotenone lesion. (b) The olfactory analysis performed 16 days after the nigral rotenone lesion. (c) The olfactory performance among the time points examined, demonstrating the influences of the lesion, time and rapid eye movement sleep deprivation (REMSD) factors. For (a, b) Two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. For (c), three-way ANOVA followed by Bonferroni's post-hoc test. The bars represent the mean ± SEM, $n = 11–13$/group, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

with the other groups [$F(3,34) = 0.65$, NS] (Fig. 2b). Comparative analysis of the time points (Fig. 2c) showed a significant influence of REMSD effect [$F(1.26) = 5.38$; $P < 0.05$], but not of the lesion [$F(1.26) = 0.97$; NS] or time [$F(1.26) = 0.60$; NS], and a significant interaction effect [$F(1.26) = 5.22$; $P < 0.05$], reflecting a significant decrease in the OI for the rotenone control group ($P < 0.02$) in this multiple comparison test.

In experiment 2, at the 14-day time point, both rotenone groups showed significant aversion to the lemon compartment, as shown by a negative OI ($P < 0.05$) compared with the sham control group [$F(3,34) = 4.00$; $P < 0.02$] (Fig. 3a). However, after 48 h of rebound (day 16), the rotenone REMSD group demonstrated a rather significant positive OI for the lemon odor compared with the sham REMSD group ($P < 0.05$) [$F(3,33) = 3.75$, $P < 0.02$] (Fig. 3b). Comparative analysis of the time points (Fig. 3c) indicated the existence of significant effects of rotenone administration within the OB [$F(1.31) = 13.85$; $P < 0.001$] and time [$F(1.31) = 20.14$; $P < 0.001$], but not of REMSD [$F(1.31) = 0.12$; NS]. Nevertheless, we failed to detect a significant interaction effect [$F(1.31) = 0.09$; NS].

Open-field test

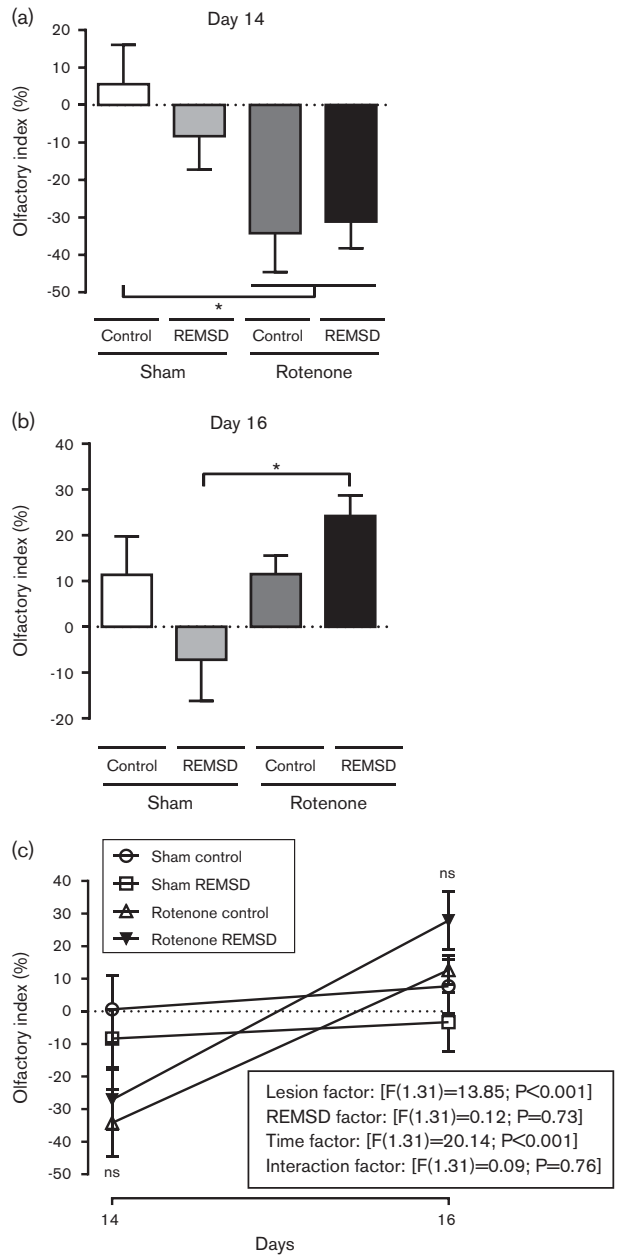
In experiment 1, the animals with nigral injury showed a decrease in the locomotor activity only on the first day after rotenone exposure, in comparison with the sham group ($P < 0.01$; $t = 2.96$) (Supplementary Fig. 1A, Supplemental digital content 1, *http://links.lww.com/BPHARM/A25*). However, there were no significant differences among the groups on the seventh ($P = 0.43$; $t = 0.79$) (Supplementary Fig. 1B, Supplemental digital content 1, *http://links.lww.com/BPHARM/A25*), 14th [$F(3,36) = 0.47$; NS] (Supplementary Fig. 1C, Supplemental digital content 1, *http://links.lww.com/BPHARM/A25*), and 16th [$F(3,37) = 2.81$; $P = 0.05$] (Supplementary Fig. 1D, Supplemental digital content 1, *http://links.lww.com/BPHARM/A25*) days after nigral lesion.

The results of experiment 2 (Supplementary Fig. 1E–H, Supplemental digital content 1, *http://links.lww.com/BPHARM/A25*) showed an absence of locomotor differences between groups on the first ($P = 0.85$; $t = 0.19$) (Supplementary Fig. 1E, Supplemental digital content 1, *http://links.lww.com/BPHARM/A25*), seventh ($P = 0.75$; $t = 0.32$) (Supplementary Fig. 1F, Supplemental digital content 1, *http://links.lww.com/BPHARM/A25*), and 14th [$F(3,43) = 2.45$, $P = 0.07$] (Supplementary Fig. 1G, Supplemental digital content 1, *http://links.lww.com/BPHARM/A25*) days after surgery. A similar result was also obtained on day 16, indicating an absence of locomotor differences between groups after the rebound period [$F(3,40) = 1.96$, NS]. Representative images of the trajectories of the animals from the groups are presented in Supplementary Fig. 2 (Supplemental digital content 2, *http://links.lww.com/BPHARM/A26*).

Tyrosine hydroxylase-immunohistochemistry

In the first experiment, the intranigral administration of rotenone significantly decreased the percentage of TH-ir

Fig. 3



Olfactory index (OI) % obtained from experiment 2. (a) The OI 14 days after the olfactory bulb rotenone lesion. (b) The olfactory analysis performed 16 days after the olfactory bulb rotenone lesion. (c) The olfactory performance for the time points examined demonstrating the influences of the lesion, time and rapid eye movement sleep deprivation (REMSD) factors. For (a, b), two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. For (c), three-way ANOVA followed by Bonferroni's post-hoc test. The bars represent the mean ± SEM, $n = 11–13$/group, $^*P < 0.05$.

neurons, compared with the sham control ($P < 0.001$) and rotenone REMSD ($P < 0.001$) [$F(3.18) = 11.3$; $P < 0.001$] (Fig. 4b). However, we did not observe significant differences in the percentage of TH-ir neurons in the OB (Fig. 4c) after the SNpc lesion [$F(3.18) = 0.48$; NS]. Similarly, in the second experiment, the rotenone infusion in the glomerular layer of the OB did not produce changes in the percentage of TH-ir neurons in the SNpc [$F(3.27) = 0.44$; NS] (Fig. 5c). However, rotenone within the OB caused a significant reduction in the percentage of TH-ir neurons in this structure [$F(3.25) = 10.5$; $P < 0.001$] (Fig. 5b). This effect was observed comparing all the experimental groups ($P < 0.05$) with the sham control group. Interestingly, REMSD decreased the TH-ir percentage, within the OB, of the rotenone REMSD group compared with the rotenone control ($P < 0.01$) and sham REMSD ($P < 0.05$) groups (Fig. 5b).

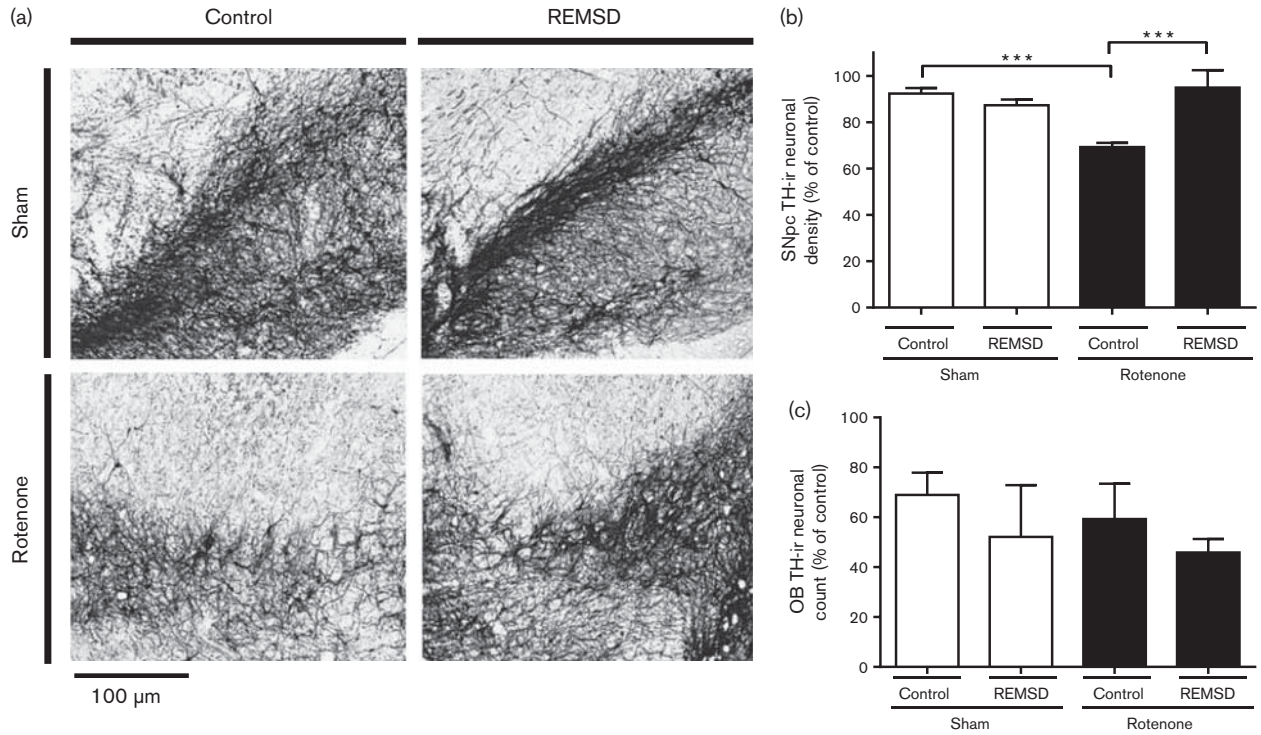
Discussion

In a previous report, we detected that male Wistar rats exhibited a significant preference to explore the lemon scent, which is typically a nonsocial odor, able to selectively activate the main olfactory pathway (Aurich *et al.*, 2017). Thus, in the current study, we conducted the same OT and observed that female sham control animals showed a similar pattern of odor preference. However, the rotenone nigral lesion disrupted this olfactory performance, which was partially counteracted by REMSD. This result is compatible with a recent finding that hedonic olfactory tone in PD is substantially reduced compared with controls, which means that range of pleasant odors is reduced in these patients, even when they were normosmic (Mrochen *et al.*, 2016). Furthermore, the dopaminergic bulbar lesion was able to create a more drastic impairment in the odor preference, which was not rescued by REMSD. In contrast, after sleep rebound, a significant preference for the lemon compartment was found, probably as a product of the combination of a bulbar lesion and compensatory REM sleep.

The effects on locomotor activity after the rotenone-induced nigral lesion were similar to previous findings in male rats 7 days after intranigral lesion (Rodrigues *et al.*, 2014). In fact, a reduced distance traveled in the OFT was restricted to the first day after the SNpc lesion and recovered at the subsequent time points. However, the dopaminergic OB lesion did not result in locomotor changes. Moreover, REMSD did not produce a detectable increase in the general activity of the animals, as was expected according to the typical dopaminergic supersensitivity generally attributed to REMSD (Tufik, 1981b; Lima *et al.*, 2008). This result may be related to the short period of REMSD tested.

Several studies have shown that intranigral rotenone infusion can cause a remarkable degeneration of dopaminergic neurons (Sherer *et al.*, 2003; Moreira *et al.*, 2012). Accordingly, our data indicated a similar pattern of dopaminergic degeneration of the SNpc, without a significant effect within the OB, when rotenone infusion was restricted to the nigrostriatal pathway. In fact, different studies have reported increases in the number

Fig. 4



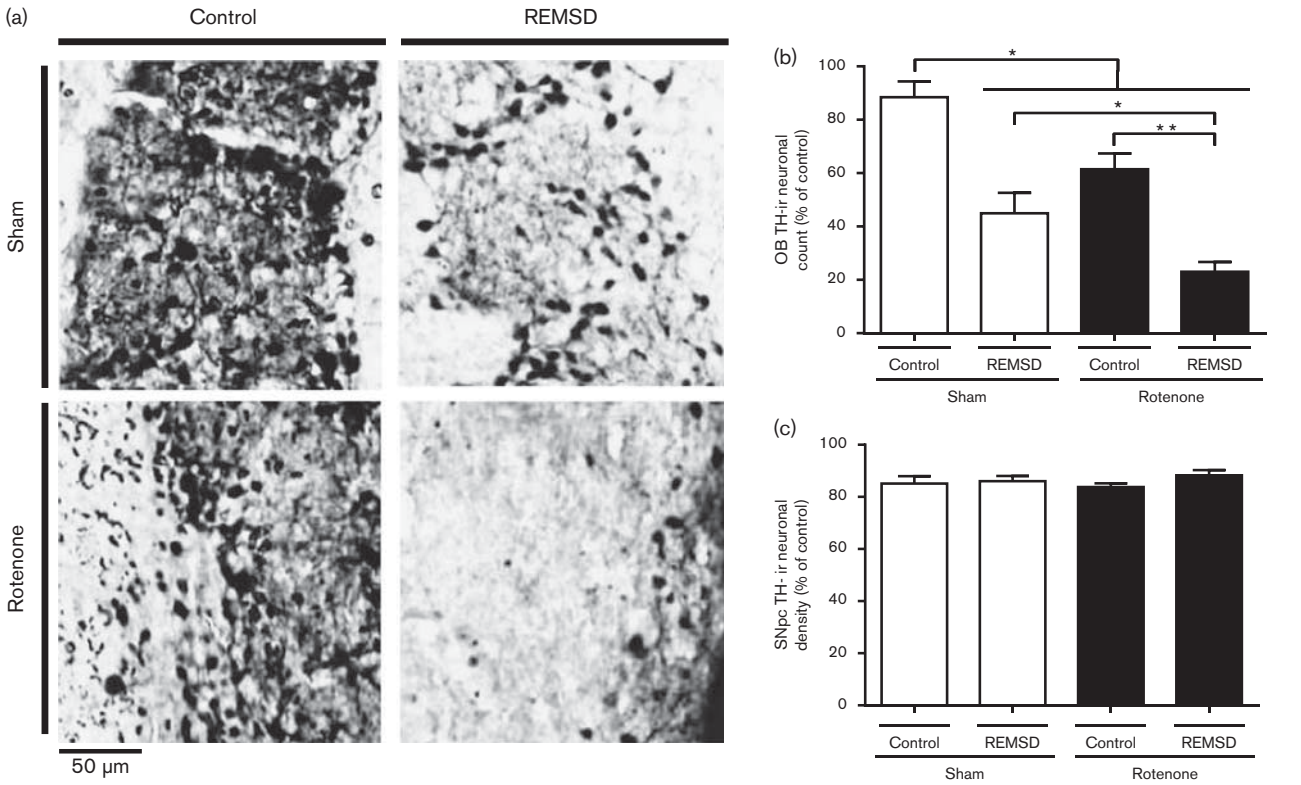
Percentage of tyrosine hydroxylase-immunoreactive (TH-ir) neuronal density in the SNpc obtained from experiment 1. (a) A representative panel of dopaminergic neurons within the SNpc (magnification × 20). (b) SNpc neuronal density analysis. (c) Olfactory bulb (OB) neuronal counting analysis. The bars represent the mean ± SEM, $n = 4–10$/group, $^{***}P \leq 0.001$. One-way analysis of variance followed by the Newman–Keuls multiple comparison test. REMSD, rapid eye movement sleep deprivation.

of dopaminergic neurons in the glomerular layer as a result of neurotoxin (6-hydroxydopamine) infusion in the SNpc (Winner *et al.*, 2006) or in the postmortem of patients with PD (Huisman *et al.*, 2004, 2008). Similarly, we have reported, for male Wistar rats, an increase of these periglomerular neurons after intranigral rotenone injury (Rodrigues *et al.*, 2014). However, in the current study, we did not observe a significant increase in the number of such neurons after SNpc rotenone administration. Nevertheless, this result accords with a postmortem PD study that shows no difference in the number of dopaminergic OB neurons in both human controls and patients with PD (Huisman *et al.*, 2008). Moreover, we did not observe a reduction in TH-ir neuronal density in the rotenone REMSD group, perhaps reflecting a certain level of a compensatory effect, possibly owing to an up-regulation of TH protein within the remaining neurons, as a product of the lesion itself. Moreover, we cannot discard the possible influence of dopaminergic D2 supersensitivity triggered on these nigrostriatal neurons. Thus, collectively, these results are corroborated by the comparative analysis of the olfactory performances on days 14 and 16, which indicated significant differences associated with REMSD and interaction factors.

In addition, the dopaminergic lesion of the TH-ir neurons in the OB did not reflect any detectable retrograde degeneration of TH-ir neurons in the SNpc. This is in accordance with previous studies that employed axotomy of the so-called 'nigro-olfactory' projection, suggesting the participation of unaffected collateralized striatal innervation avoiding the SNpc lesion (Hoglinger *et al.*, 2015). Notwithstanding, the rotenone infusion within the glomerular layer decreased the number of these TH-ir neurons, which was synergistically affected by REMSD, and not ameliorated by sleep rebound.

In the present study, we demonstrated that rotenone-induced nigrostriatal lesion, in female Wistar rats, is able to promote remarkable changes in odor preference that were counterbalanced by REMSD. Conversely, dopaminergic lesion of the periglomerular neurons showed an association with the rebound period, affecting, respectively, the olfactory performance and the number of TH-ir neurons within the OB. This is consistent with a report describing a deterioration in olfactory function during a short period of sleep deprivation (Killgore *et al.*, 2010). As a consequence, it is plausible to suggest that the plasticity of the nonsocial odor processing system, in female rats, depends on dopaminergic modulation.

Fig. 5



Percentage of tyrosine hydroxylase-immunoreactive (TH-ir) neuronal counting in the olfactory bulb (OB) obtained from experiment 2. (a) A representative panel of dopaminergic neurons within the OB (magnification ×40). (b) OB neuronal counting analysis. (c) SNpc neuronal density analysis. The bars represent the mean ± SEM, $n = 4–10$/group, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. One-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test. REMSD, rapid eye movement sleep deprivation.

Indeed, male PD volunteers performed significantly worse than females in an odor identification test (Liu, 2015).

Conclusion

Female rats present a pattern of olfactory discrimination/preference that is dependent on the nigrostriatal and the main olfactory pathways activities. A dopaminergic lesion of periglomerular neurons may be less associated with REMSD, to promote a 'pro-hyposmia' effect, than initially expected. However, damage to the nigrostriatal pathway negatively affected olfaction performance, which was counteracted by REMSD.

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Conflicts of interest

There are no conflicts of interest.

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