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Dopaminergic Lesion in the Olfactory Bulb Restores Olfaction and Induces Depressive-Like Behaviors in a 6-OHDA Model of Parkinson's Disease

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Abstract
Olfactory impairments and depressive behavior are commonly reported by individuals with Parkinson's disease (PD) being observed before motor symptoms. The mechanisms underlying these clinical manifestations are not fully elucidated. However, the imbalance in dopaminergic neurotransmission seems to play an important role in this context. In patients and animal models of PD, an increase in the dopaminergic interneurons of the glomerular layer in olfactory bulb (OB-gl) is observed, which may contribute to the olfactory impairment. In addition, neuronal imbalance in OB is related to depressive symptoms, as demonstrated by chemical olfactory bulbectomy. In view of that, we hypothesized that a reduction in the number or density of dopaminergic neurons present in OB could promote an olfactory improvement and, in contrast, would accentuate the depressive-like behaviors in the 6-hydroxydopamine (6-OHDA) model of PD. Therefore, we performed single or double injections of 6-OHDA within the substantia nigra pars compacta (SNpc) and/or in the OB-gl. We observed that, after 7 days, the group with nigral lesion exhibited olfactory impairment, as well as the group with the lesion in the OB-gl. However, the combination of the lesions prevented the occurrence of hyposmia. In relation to depressive-like behaviors, we observed that the SNpc injury promoted depressive-like behavior, being accentuated after a double injury. Our results demonstrated the importance of the dopaminergic neurons of the OB-gl in different non-motor features of PD, since the selective reduction of these periglomerular neurons was able to induce olfactory impairment and depressive-like behaviors.

Keywords Depression · Dopaminergic neurons · Olfaction · Parkinson's disease

Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease [1, 2], accounting for 4.5–19 cases per 100,000 inhabitants per year [3, 4]. Olfactory impairment is the first pre-motor alteration of PD [5, 6], affecting more than 90% of the patients [7–9]. Currently, non-motor disturbances, such as depression, anxiety, hyposmia, constipation, and rapid eye movement (REM) sleep disorders, are gaining more attention in the literature since they appear before the motor signs [5, 10, 11].

Studies with relatives of PD patients had established that hyposmia may precede motor symptoms in 5 years [7], but other studies affirm that this disturbance is found decades before the motor onset [12]. In fact, it has been reported a significant increase in the number of the dopaminergic periglomerular interneurons located within the glomerular layer of the olfactory bulb (OB-gl), which are responsible for modulating olfactory transmission by inhibiting olfactory receptor cells and mitral/tufted neurons [8] in both humans [13, 14] and rats [15]. Hence, this mechanism raises the hypothesis that this increased dopaminergic activity could be a compensatory response to the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), possibly being related to olfactory impairment observed in such condition [5, 16].

Depression is a psychiatric comorbidity that affects 30–50% of PD patients [17–22] and is characterized by depressed mood, loss of interest, and fatigue [23]. Depressive symptoms are normally observed before motor alterations [11], and it is a

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well-known fact that there is a higher prevalence of PD-related depression compared to other neurodegenerative diseases [24]. There is evidence that several factors involved in the pathogenesis of PD, such as depletion of dopamine (DA), noradrenaline (NA) and serotonin (5-HT), and disruption of frontal-subcortical and limbic circuitries, may contribute to depression in these patients [20, 25–27].

Interestingly, several studies report a close relationship between depression and olfactory impairment in a non-Parkinsonism context [23, 28–30]. Indeed, depression leads to olfactory impairment by decreasing neurogenesis in the subventricular zone and, consequently, preventing neuroblast migration to olfactory bulb (OB) [31, 32]. In addition, depression affects granular and periglomerular interneuron activity [33], leading to a direct reduction of olfactory sensitivity [29]. Accordingly, bilateral olfactory bulbectomy is considered a model of depression in animals, since surgical removal or chemical injury results in hypothalamic and limbic alterations [32] leading to depressive-like behaviors and reduced nigral brain-derived neurotrophic factor levels [30, 34, 35]. Furthermore, nigrostriatal lesions are strongly associated with remarkable increases of 50 and 100% of dopaminergic periglomerular neurons, in rats and PD patients, respectively, both negatively impacting olfaction [13–15].

Here, we investigated if a 6-hydroxydopamine (6-OHDA) lesion, within the glomerular layer of the OB, would prevent this compensatory increment in density of periglomerular neurons generated by a nigrostriatal 6-OHDA-induced lesion as an early-phase model of PD. Therefore, we expect an olfactory improvement that could be related to the occurrence of depressive-like behaviors. Also, positive controls for anosmia were tested in order to identify if a blockage of the olfactory epithelium [15, 36, 37] would be able, itself, to induce depressive-like behaviors, according to this hypothesis.

Material and Methods

Ethics Statement

All the experiments were carried out in accordance with the guidelines of the Committee for the Care and Use of Laboratory Animals, United States National Institutes of Health. In addition, the protocol complies with the recommendations of Federal University of Paraná and was approved by the institutional ethics committee (approval ID no. 910).

Animals

Male Wistar rats ($n = 184$) from our breeding colony weighing 280–320 g at the beginning of the experiments were used. The animals were randomly housed in groups of five in polypropylene cages with wood shavings as bedding and maintained in a temperature-controlled room (22 ± 2 °C) on a 12:12 h light-dark cycle (lights on at 7:00 a.m.). The animals had free access to water and food throughout the experiment.

Experimental Design

The animals were randomly distributed in four experimental groups (Fig. 1a): 6-OHDA (−)SNpc/(−)OB-gl; 6-OHDA (−)SNpc/(+)OB-gl; 6-OHDA (+)SNpc/(−)OB-gl; 6-OHDA (+)SNpc/(+)OB-gl. Signals (+) or (−) indicate infusion of 6-OHDA or its vehicle (saline added 0.2% ascorbic acid), respectively. The experimental design shown in Fig. 1b–d indicates that on day 0, the animals underwent bilateral stereotaxic infusion of 6-OHDA in the SNpc, concomitantly to OB guide cannula implantation. Six days after, we performed microinfusions of 6-OHDA or vehicle within the OB. Afterwards, behavioral tests were performed according to the determined time-points: 7 and 14 days following SNpc lesions (Fig. 1b–d). In parallel, brain samples were collected on days 7 (Fig. 1b) and 14 (Fig. 1c) after SNpc lesioning. Positive controls of anosmia (intranasal solution of zinc gluconate + zinc acetate solution—Zicam) were assigned as follows: 6-OHDA (+)SNpc/Zicam(+) olfactory epithelium; 6-OHDA(−)SNpc/Zicam(+) olfactory epithelium. Zicam damages the olfactory epithelium and causes olfactory impairment [15, 36, 37], and the intranasal infusion was performed also 6 days after SNpc lesions. The third experiment, represented in Fig. 1d, shows the anhedonic-like behavior inflicted by the experimental conditions.

Stereotaxic Surgery

Rats were sedated with intraperitoneal xylazine (10 mg/kg; Syntec do Brasil Ltda, Brazil) and anesthetized with intraperitoneal ketamine (90 mg/kg; Syntec do Brasil Ltda, Brazil). The bilateral infusion of 2 μL of 6-OHDA (3 μg/μL) or its vehicle (saline containing 0.2% ascorbic acid) were made using an electronic infusion pump (Insight Instruments, Ribeirão Preto, Brazil) at a rate of 0.33 μL/min for 6 min (modified from [38]). For this, the following coordinates were used: (SNpc) (AP) = − 5.0 mm, (ML) = ± 2.1 mm e (DV) = − 8.0 mm, using bregma as reference [39]. Complementarily, a guide cannula was implanted in the olfactory bulb of each rat allowing a subsequent infusion of 1 μL of 6-OHDA (3 μg/μL) or vehicle (saline with 0.2% ascorbic acid) at a rate of 0.33 μL/min for 3 min. Coordinates with reference to bregma for implantation of guide cannula were (AP) = + 7.08 mm (ML) = 0.0 mm and (DV) = −3.6 mm [39].

Intranasal Administration of Zicam (Zinc Gluconate + Zinc Acetate Solution)

The administration of Zicam® Oral Mist (Matrixx Initiatives, Scottsdale, AZ, USA) was performed as previously reported

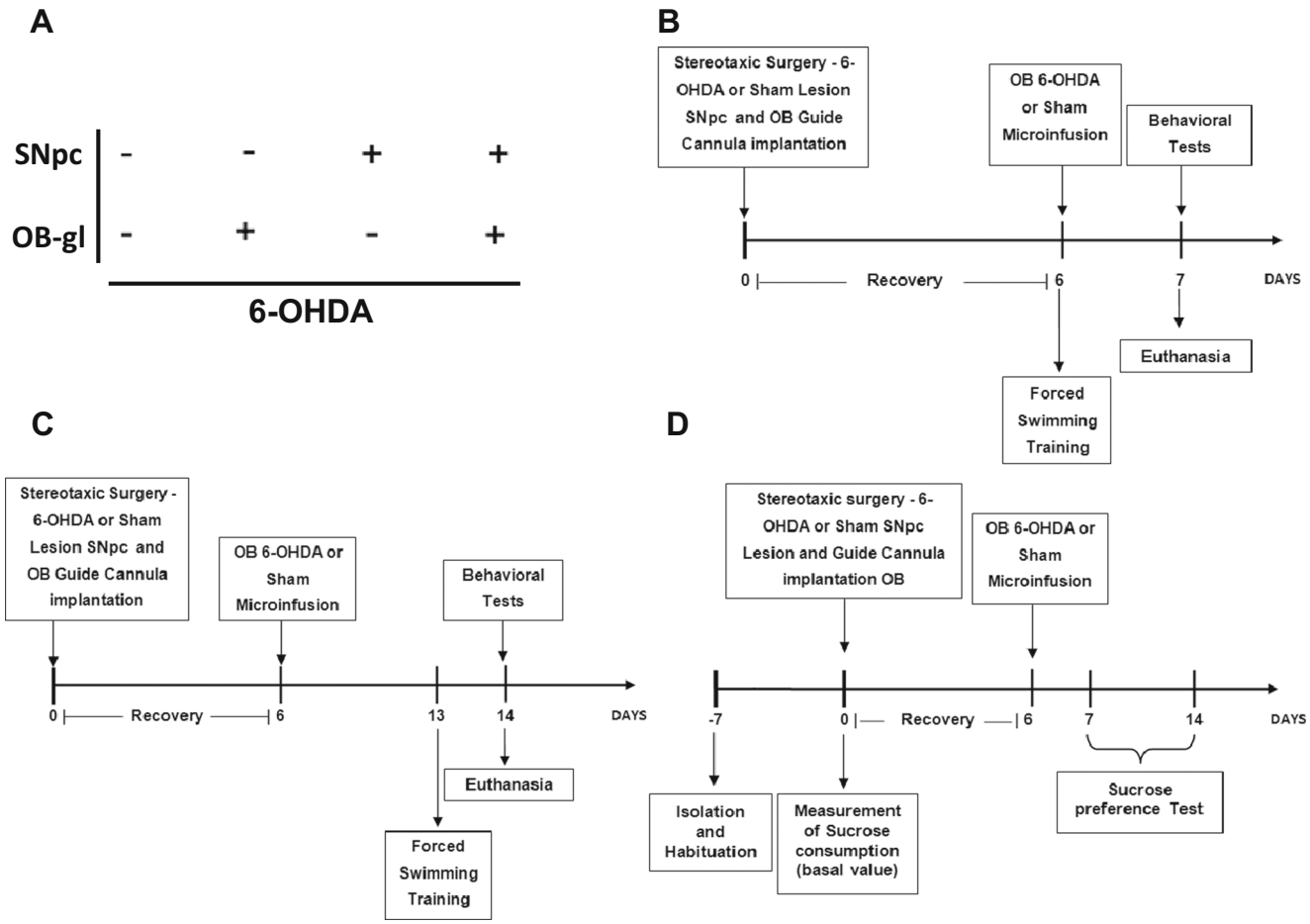

Fig. 1 Experimental design. **a** Representation of the experimental groups ($n = 184$, 8–12/group). **b** Behavioral tests and brain sample collection 7 days after intranigral 6-OHDA infusion and **c** 14 days after intranigral 6-OHDA infusion. **d** Sucrose preference test 7 or 14 days after intranigral 6-OHDA infusion. Legends: 6-OHDA, 6-hydroxydopamine; OB, olfactory bulb; SNpc, substantia nigra pars compacta

[15, 36, 37]: the animals were sedated with ketamine (90 mg/kg) and xylazine (3 mg/kg) administered intraperitoneally. After that, approximately 30 μL of Zicam solution was slowly delivered into the nasal cavity using a Hamilton syringe connected to a blunted 30-gauge needle through a polyethylene tube. The polyethylene cannula was inserted 15 mm past the right external nostril to help irrigate the olfactory epithelium. The procedure was repeated in the left nostril. During respiration, part of the solution was expelled through the nostril and dried with absorbent paper to allow the animal to continue breathing.

Olfactory Discrimination Task

This test was previously described and modified [15, 40–42]. The apparatus consisted of a box (60 × 40 × 50 cm) equally divided into two compartments, connected by a door that gives free access to the animal. Before the test, the animals were habituated to the apparatus for 2 min, with both compartments containing fresh sawdust. In the test, clean sawdust was added on one side of the box (non-familiar odor) and, on the other side of the box, we added sawdust in which the animals remained isolated for 48 h before testing (familiar odor). The rat was placed in the middle of olfactory discrimination box and we recorded, for 3 min, the time of investigation of each compartment. The animal that shows olfactory impairment tends to explore both compartments equally, indicating absence of discrimination. As a measure of discrimination, a "discrimination index (DI)" was calculated by dividing the difference in exploration time between the two compartments (non-familiar compartment − familiar compartment) by the total amount of exploration for both compartments (non-familiar compartment + familiar compartment). DI was then multiplied by 100 to express it as a percentage.

Modified Forced Swimming Test

This test is a modified version from previous studies [43, 44]. This test consists of an opaque plastic cylinder (diameter 20 cm; height 50 cm) containing water up to 30 cm (24 ± 1 °C); on day 1, the rats were placed in the cylinder for 15 min (training session) and 24 h later, they were placed back and tested for 5 min (test session). The test session was video recorded via a camera positioned above the cylinder for

subsequent analysis. The behaviors registered during the test session were immobility (when the rat stopped all active behaviors and remained floating in the water with minimal movements, with its head just above the water), swimming (when the rat moved throughout the swim cylinder, including crossing into another quadrant), and climbing (when the rat demonstrated upward movements of the forepaws along the cylinder walls). The time spent in each behavior was analyzed. The water was changed and the cylinder rinsed with clean water after each rat. Following the training and the test sessions, the animals were dried and placed in their home cages.

Open-Field Test

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 100 cm above the arena floor, providing illumination around 300 lx [45]. 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 was video recorded and the measures for traveled distance were computed online by an image analyzer (Smart Junior, PanLab, Harvard Apparatus, Spain).

Sucrose Preference Test

Sucrose preference is frequently used as a measure of anhedonia [46–48]. Eight days before administration of intranigral 6-OHDA, the animals were transferred to single-housing cages with free access to food. Each cage had two preweighed bottles of water on opposite sides during 24 h (training phase) to adapt the rats to drinking from two bottles. After training, one bottle was randomly switched to contain a 0.5% sucrose solution as described previously [49, 50]. Two days later, the bottles were reversed to avoid perseveration effects. The bottles were weighed before being offered to the animal and at the end of the experiment (1 week later). The sum of water consumption and sucrose consumption was defined as total intake. The percentage of sucrose intake was calculated according to the following equation: % sucrose preference = (sucrose intake / total intake) × 100. The tests began 1 week prior to neurotoxin exposure to provide baseline values and were completed at time-point 14 days.

TH Immunohistochemistry

Rats were deeply anesthetized with ketamine immediately after the behavior 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 1 week in that fixative solution at 4 °C. Subsequently, the brains were placed in 30% sucrose solution for 3 days and stored at − 80 °C freezer before sectioning. Six 40 μm sections per animal were taken from the olfactory bulb (+ 7.56 to + 7.08 mm, an interval of 480 μm) and six sections per animal were taken from the SNpc (− 4.92 to − 5.28 mm, an interval of 360 μm): bregma − 4.92 mm and − 5.28 mm. These sections were chosen because of their location in the mid-rostrocaudal part of the substantia nigra, which contains many dopaminergic neurons. Initially, the sections were incubated (30 min) in 0.1 M phosphate saline buffer (pH 7.4) and subsequently were submitted to endogenous peroxidase inhibition by incubation with 0.3% H_2O_2 in 0.1 M phosphate saline buffer (pH 7.4) for 10 min. After that, the sections were blocked in 10% 0.1 M normal goat serum in phosphate saline buffer (pH 7.4) and next, the sections were incubated with primary mouse anti-TH antibody, diluted in 0.1 M phosphate-buffered saline containing 0.3% Triton X-100 (1:1.000 in SNpc sections and 1:8.000 in OB sections; cat. no. AB152 Chemicon, CA, USA) overnight at 4 °C. Biotin-conjugated secondary antibody incubation (1:200 in both structures cat. no. S-1000 Vector Laboratories, USA), was performed for 2 h at room temperature.

After several washes in phosphate-buffered saline, antibody complex was localized using the ABC system (Vectastain ABC Elite kit cat. no. PK6101, Vector Laboratories, USA) followed by 3,3′-diaminobenzidine reaction with nickel enhancement. The sections were then mounted onto gelatin-coated slides and coverslipped after dehydration in ascending concentrations of ethanol-xylene solutions. OB manual cell counts and SNpc neuronal density determination were conducted making use of the software Image-Pro Express 6 and ImageJ, respectively. Quantifications (number of neurons within the OB and neuronal density of the SNpc) were performed in 6–10 tissue sections and an average count per section was determined for each animal. For each group, a mean value was calculated and compared with those of the other groups. The images were obtained using a motorized Axio Imager Z2 microscope (Carl Zeiss, Jena, DE), equipped with an automated scanning VSlide (Metasystems, Altlussheim, DE).

Statistical Analysis

Differences between groups in the ODT were analyzed by two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. Olfactory discrimination index, modified forced swimming test, open-field test, and TH immunohistochemistry were analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. Sucrose preference test was analyzed by two-way ANOVA with repeated measures followed by the Bonferroni post hoc test. Pearson's correlation coefficients (r) were calculated to establish relationships between histological 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

Olfactory Discrimination Task

We observed, at the time-point 7 days that the 6-OHDA (−)SNpc/(−)OB-gl group showed increased exploration ($P \leq 0.001$) of the familiar odor compared to the non-familiar. Similarly, the double-lesioned 6-OHDA (+)SNpc/(+)OB-gl group demonstrated an increased exploration of the familiar odor ($P \leq 0.001$) (Fig. 2a). In opposite, the 6-OHDA (−)SNpc/(+)OB-gl ($P > 0.99$) and the 6-OHDA (+)SNpc/(−)OB-gl groups ($P > 0.99$), as well as the positive controls for anosmia [6-OHDA (+)SNpc/(−) OB-gl/(+)Zicam and 6-OHDA (−)SNpc/(−)OB-gl/(+)Zicam] exhibited similar exploration times for both odors ($P > 0.99$), according to the lesion [F (5, 86) = 0.001; $P > 0.99$], odor [F (1, 86) = 24.97; $P < 0.0001$], and interaction [F (5, 86) = 5.868; $P = 0.0001$] factors (Fig. 2a). The analysis of the time-point 14 days demonstrated that the groups 6-OHDA (−)SNpc/(−)OB-gl ($P \leq 0.001$), 6-OHDA (−)SNpc/(+)OB-gl ($P \leq 0.01$), and 6-OHDA (+)SNpc/(−)OB-gl ($P \leq 0.0001$) spent significantly less time exploring the non-familiar odor compared to the familiar, as indicated by the odor [F (1, 86) = 24.97; $P \leq 0.0001$], lesion [F (5, 96) = 3.724; $P > 0.99$], and interaction [F (5, 86) = 5.868; $P = 0.0001$] factors (Fig. 2b). We did not find differences regarding exploration time between odors for the groups 6-OHDA (+)SNpc/(+)OB-gl ($P = 0.73$) and also for the positive controls for anosmia 6-OHDA (+)SNpc/(−)OB-gl/(+)Zicam ($P > 0.99$) and 6-OHDA (−)SNpc/(−)OB-gl/(+)Zicam ($P > 0.99$) (Fig. 2b).

Figure 2c, d shows the DIs obtained from the ODT at time-points 7 and 14 days, respectively. The group 6-OHDA (−)SNpc/(+)OB-gl exhibited a significant impairment in the DI compared to the 6-OHDA (−)SNpc/(−)OB-gl ($P \leq 0.05$) and the 6-OHDA (+)SNpc/(+)OB-gl ($P \leq 0.01$) as indicated

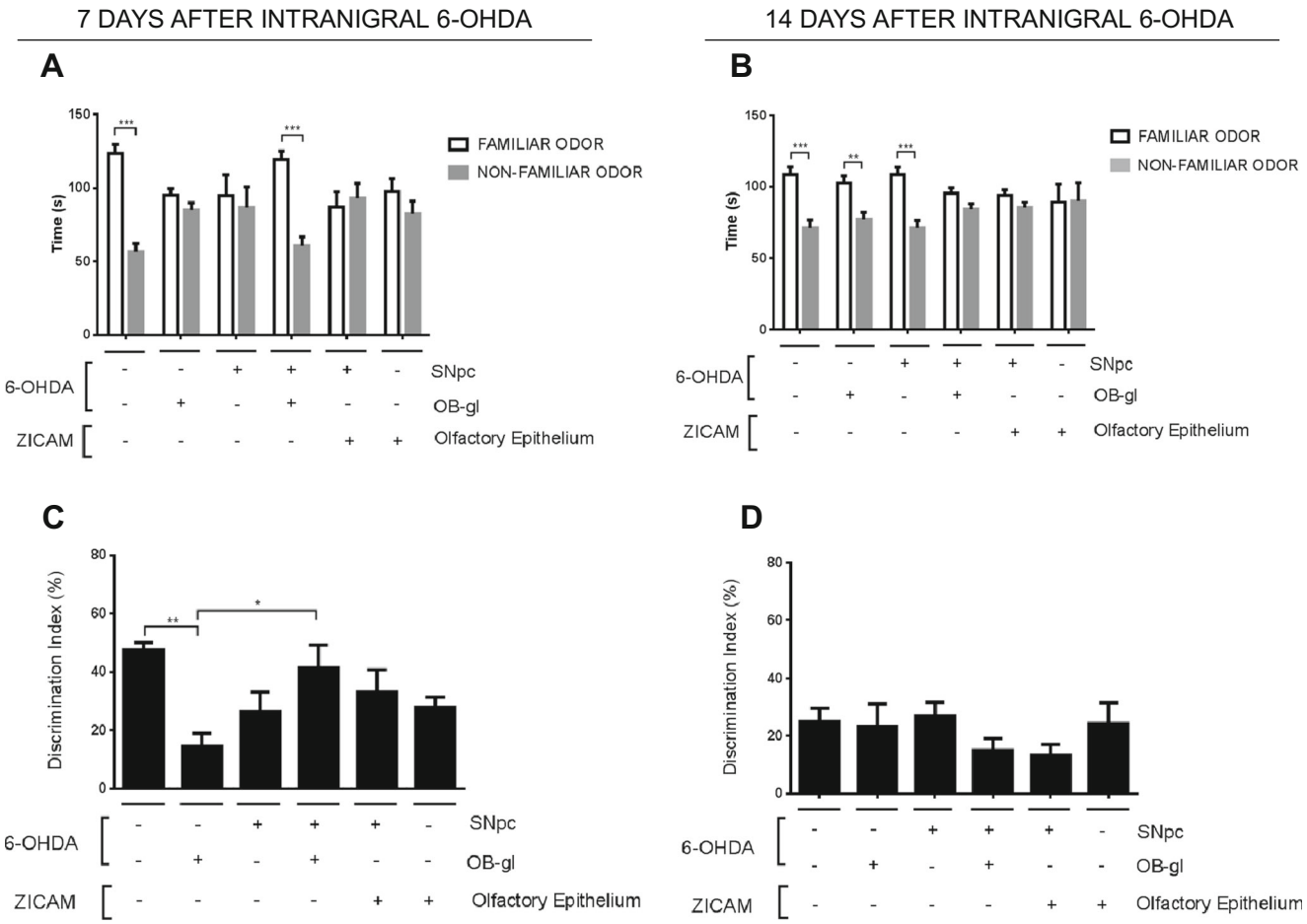

Fig. 2 Olfactory discrimination task analysis. **a**, **b** Time (s) spent in familiar and non-familiar compartments in the olfactory discrimination task (ODT) 7 and 14 days after 6-OHDA intranigral microinfusion, respectively. The bars represent the mean ± SEM ($n = 12$ per group), $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ comparing the mean time spent in familiar and non-familiar compartments. Two-way ANOVA followed by the Bonferroni post hoc test. **c**, **d** Olfactory discrimination index (DI)—from **a**, **b**, respectively. DI = (NF − F / NF + F) * 100, NF is the time spent in the compartment with non-familiar odor and F is the time spent in the compartment with familiar odor. The bars represent the mean ± standard error of the mean, $n = 12$ per group, $^{*}P \leq 0.05$; $^{**}P \leq 0.01$. One-way ANOVA followed by the Newman-Keuls post hoc test. Legends: 6-OHDA, 6-hydroxydopamine; OB-gl, olfactory bulb glomerular layer; SNpc, substantia nigra pars compacta; (+) presence of 6-OHDA lesion or Zicam administration; (−) absence of 6-OHDA lesion or Zicam (sham manipulation)

by the multiple comparisons [F (5, 46) = 4.155; $P = 0.0034$] (Fig. 2c). Notwithstanding, at time-point 14 days, we did not observe changes in the DI among the groups [F (5, 53) = 1.393; $P = 0.2419$] (Fig. 2d).

Modified Forced Swimming Test

The results from time-point 7 days evidenced a significant reduction of the swimming time in the group 6-OHDA (+)SNpc/(−)OB-gl when compared to 6-OHDA (−)SNpc/(−)OB-gl ($P \leq 0.05$) group and the positive control for anosmia 6-OHDA (−)SNpc/(−)OB-gl/(+)Zicam ($P \leq 0.01$) group (Fig. 3a). Also, the double 6-OHDA lesion group, i.e., 6-OHDA (+)SNpc/(+)OB-gl, presented a significant reduction of swimming time compared to the 6-OHDA (−)SNpc/(−)OB-gl ($P \leq 0.001$) and 6-OHDA (−)SNpc/(+)OB-gl ($P \leq 0.001$) groups, as well as for the positive control for anosmia 6-OHDA (−) SNpc/(−)OB-gl/(+)Zicam ($P \leq 0.001$) [F (5, 49) = 16.62; $P < 0.0001$] (Fig. 3a). The examination of the same parameter at time-point 14 days showed that all 6-OHDA-lesioned groups, that is, 6-OHDA (−)SNpc/(+)OB-gl, 6-OHDA (+)SNpc/(−)OB-gl, 6-OHDA (+)SNpc/(+)OB-gl, and 6-OHDA (+)SNpc/(−)OB-gl/(+)Zicam, presented a similar reduction ($P < 0.001$, for all groups) when compared

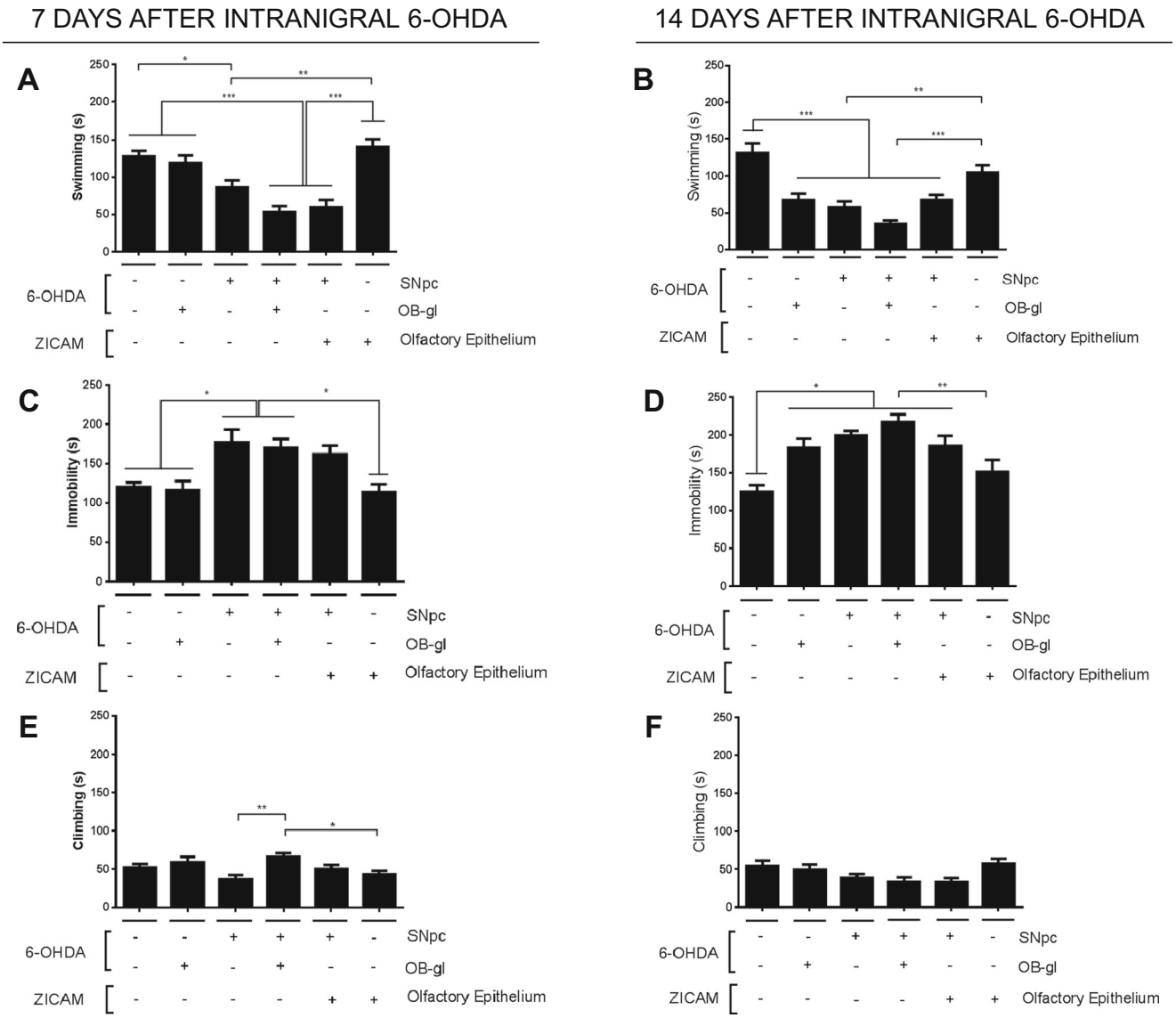

Fig. 3 Depressive-like behaviors during the modified forced swimming test. **a** Swimming time 7 days after intranigral 6-OHDA. **b** Swimming time 14 days after intranigral 6-OHDA. **c** Immobility time 7 days after intranigral 6-OHDA. **d** Immobility time 14 days after intranigral 6-OHDA. **e** Climbing time 7 days after intranigral 6-OHDA. **f** Climbing time 14 days after intranigral 6-OHDA. The bars represent the mean ± standard error of the mean ($n = 12$ per group), $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. One-way ANOVA followed by the Newman-Keuls post hoc test. Legends: 6-OHDA, 6-hydroxydopamine; OB-gl, olfactory bulb glomerular layer; SNpc, substantia nigra pars compacta; (+) presence of 6-OHDA lesion or Zicam administration; (−) absence of 6-OHDAlesion or Zicam (sham manipulation)

to 6-OHDA (−)SNpc/(−)OB-gl (Fig. 3b). Furthermore, the 6-OHDA (+)SNpc/(−)OB-gl and 6-OHDA (+)SNpc/(+)OB-gl groups exhibited reductions in this parameter [F (5, 49) = 17.45; $P \leq 0.0001$] (Fig. 3b) compared to the control for anosmia 6-OHDA (−)SNpc/(−)OB-gl/(+)Zicam group ($P \leq 0.01$ and $P \leq 0.001$, respectively).

As depicted in Fig. 3c, the immobility time of the groups 6-OHDA (+)SNpc/(−) OB-gl and 6-OHDA (+)SNpc/(+)OB-gl (at time-point 7 days) appears to be equally increased in comparison to the 6-OHDA (−)SNpc/(−)OB-gl ($P \leq 0.05$) and 6-OHDA (−) SNpc/(+)OB-gl ($P \leq 0.01$) groups. In addition, such increment in immobility was also observed in comparison to the control for anosmia 6-OHDA (−)SNpc/(−)OB-gl/(+)Zicam group ($P \leq 0.05$), [F (5, 48) = 6.665; $P \leq 0.0001$]. Figure 3d represents the immobility time obtained at time-point 14 days. It is noticeable that all the 6-OHDA-lesioned groups, that is, 6-OHDA (−)SNpc/(+)OB-gl, 6-OHDA (+)SNpc/(−)OB-gl, 6-OHDA (+)SNpc/(+)OB-gl, and 6-OHDA (+)SNpc/(−)OB-gl/(+)Zicam, present increased times of immobility when compared to the controls 6-OHDA (−)SNpc/(−)OB-gl ($P \leq 0.05$) and 6-OHDA (−)SNpc/(−)OB-gl/(+)Zicam ($P \leq 0.001$) [F (5, 55) = 7.989; $P \leq 0.0001$].

Complementarily, the climbing time of the groups, at time-point 7 days, showed to be increased in the 6-OHDA (+)SNpc/(+)OB-gl when compared to the 6-OHDA (+)SNpc/(−)OB-gl ($P \leq 0.01$) and the positive control for anosmia 6-OHDA (−)SNpc/(−)OB-gl/(+)Zicam ($P \leq 0.05$) [F (5, 41) = 3.948; $P = 0.0052$] (Fig. 3e). Moreover, the analysis of the time-point 14 days revealed significant differences in ANOVA among groups [F (5, 47) = 2.560; $P = 0.0396$], but did not show it in multiple comparisons (Fig. 3f).

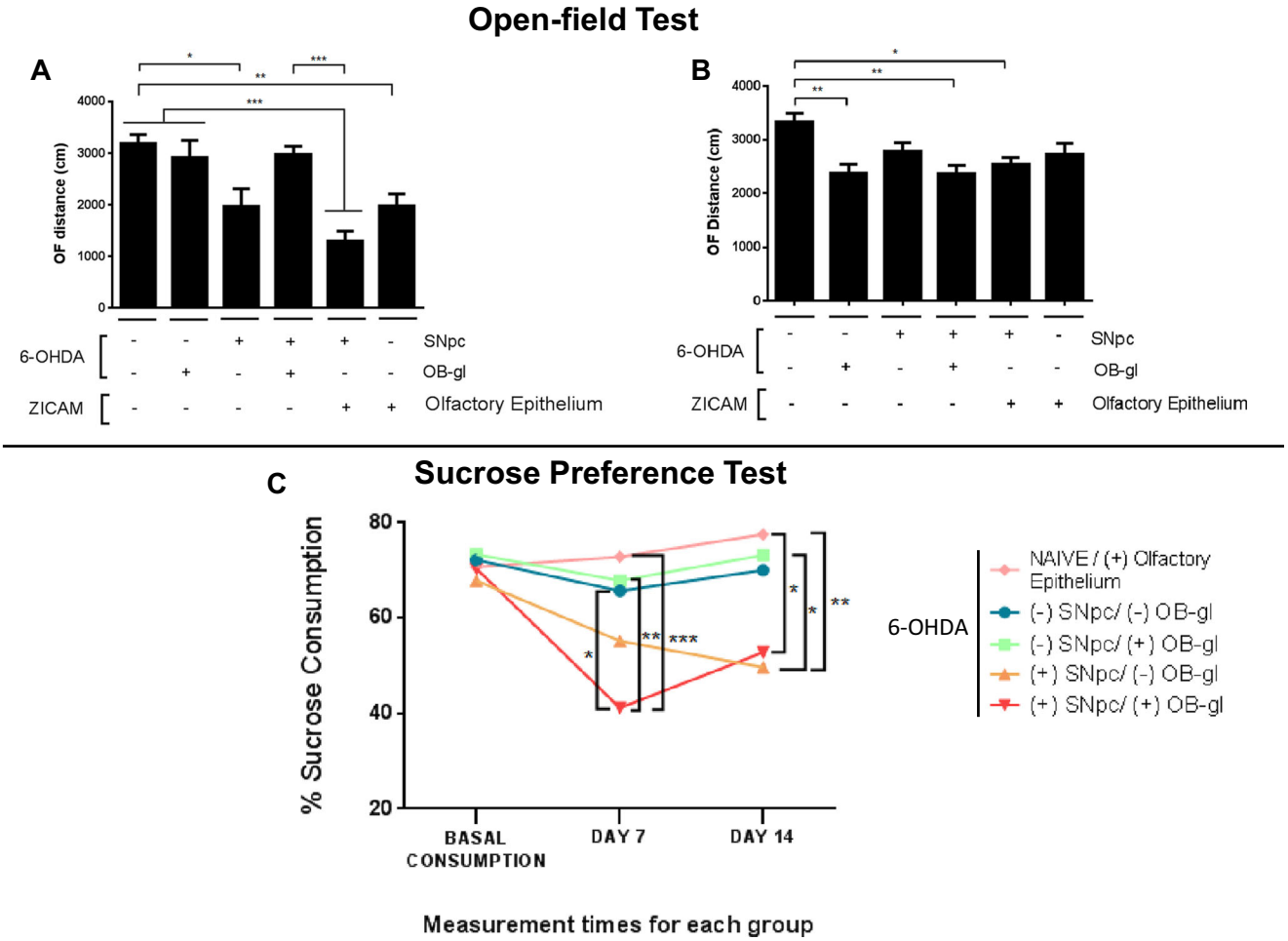

Fig. 4 Locomotor distance in the open-field test. **a** Seven days after intranigral 6-OHDA. **b** Fourteen days after intranigral 6-OHDA. The bars represent the mean ± standard error of the mean ($n = 12$ per group) $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. One-way ANOVA followed by the Newman-Keuls post hoc test. **c** Percentage of the sucrose consumption in the sucrose preference test. The symbols represent the mean ($n = 8$ per group), $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. Two-way ANOVA with repeated measures followed by the Bonferroni post hoc test. A naive (non-operated) group was included and only received intranasal Zicam. Legends: 6-OHDA, 6-hydroxydopamine; OB-gl, olfactory bulb glomerular layer; SNpc, substantia nigra pars compacta; (+) presence of 6-OHDA lesion or Zicam administration; (−) absence of 6-OHDA lesion (sham manipulation)

Open-Field Test

In the open-field test at time-point 7 days (Fig. 4a), significant reductions in the spontaneous locomotor behavior were observed in the 6-OHDA (+)SNpc/(−)OB-gl group ($P \leq 0.05$) and also in the two positive controls for anosmia 6-OHDA (−) SNpc/ (−)OB-gl/(+)Zicam ($P \leq 0.001$) and 6-OHDA (+)SNpc/(−)OB-gl/(+)Zicam ($P \leq 0.0001$) compared to the 6-OHDA (−)SNpc/(−)OB-gl. In addition, the locomotion of the group 6-OHDA (+)SNpc/(−)OB-gl/(+)Zicam was also reduced compared to 6-OHDA (−) SNpc/(+)OB-gl ($P \leq 0.001$) and 6-OHDA (+)SNpc/(+)OB-gl ($P \leq 0.001$), [F (5, 44) = 9.618, $P < 0.0001$]. Similarly, at time-point 14 days (Fig. 4b), reductions in locomotion were detected for the groups 6-OHDA (−)SNpc/(+)OB-gl ($P \leq 0.01$), (+)SNpc/(+)OB-gl ($P \leq 0.01$), and positive control for anosmia 6-OHDA (+)SNpc/(−)OB-gl/(+)Zicam ($P \leq 0.05$) compared to the 6-OHDA (−)SNpc/(−)OB-gl group, [F (5, 56) = 4.535, $P = 0.0015$].

Sucrose Preference Test

The analysis of sucrose preference (Fig. 4c) revealed that only 6-OHDA double-lesioned group, i.e., 6-OHDA (+)SNpc/(+)OB-gl, exhibited a significant reduction of sucrose consumption compared to the three groups: naive/(+)Zicam ($P \leq 0.001$), 6-OHDA (−) SNpc/(−)OB-gl ($P \leq 0.05$), and 6-OHDA (−)SNpc/(+)OB-gl ($P \leq 0.01$) for the time-point 7 days. At 14 days, the anhedonic behavior was still detected in the 6-OHDA (+)SNpc/(+)OB-gl group compared to naive/(+)Zicam ($P \leq 0.05$). At the same time-point, the group 6-OHDA (+)SNpc/(−)OB-gl also presented a significant reduction of sucrose consumption compared to naive/(+)Zicam ($P \leq 0.01$) and 6-OHDA (−)SNpc/(+)OB-gl ($P \leq 0.05$), according to the lesion [F (4, 25) = 6.639; $P = 0.0009$], time-point [F (2, 50) = 6.537; $P = 0.0030$], and interaction [F (8, 50) = 2.413; $P = 0.0275$] factors.

TH Immunochemistry within the SNpc

The analysis of the dopaminergic neurons in the SNpc revealed that 6-OHDA caused a pronounced neuronal loss at both time-points (see panels at Fig. 5). Figure 5e shows the quantification of TH-ir neuron density at time-point 7 days revealing that 6-OHDA (+)SNpc/(−)OB-gl and 6-OHDA (+)SNpc/(+)OB-gl exhibited significant reductions ($P \leq 0.001$ and $P \leq 0.05$, respectively) compared to the control 6-OHDA (−) SNpc/(−)OB-gl group. The same groups also exhibited significant decrease in TH-ir neurons compared to the 6-OHDA (−)SNpc/(+)OB-gl ($P \leq 0.0001$ and $P \leq 0.001$, respectively) [F (3, 49) = 17.26; $P < 0.0001$]. Likewise, at the 14 days time-point (Fig. 5j), the groups with 6-OHDA SNpc lesion: 6-OHDA (+)SNpc/(−)OB-gl and 6-OHDA (+)SNpc/(+)OB-gl also exhibited significant reductions in this parameter compared to the control group 6-OHDA (−)SNpc/(−)OB-gl ($P \leq 0.0001$ for both groups) and compared to the 6-OHDA (−)SNpc/(+)OB-gl ($P \leq 0.0001$for both groups as well). Remarkably, 6-OHDA (+)SNpc/(+)OB-gl demonstrated a further decrement in nigral TH-ir neurons compared to the 6-OHDA (+)SNpc/(−) OB-gl ($P \leq 0.05$), as indicated [F (3, 62) = 50.25; $P \leq 0.0001$].

TH Immunochemistry within the OB-gl

The dopaminergic neuronal population in the glomerular layer of the OB is represented in Fig. 6. As can be seen in Fig. 6e (time-point 7 days), it is indicated a significant decrease in TH-ir neurons in the group 6-OHDA (−) SNpc/(+)OB-gl compared to the groups 6-OHDA (−)SNpc/(−)OB-gl ($P \leq 0.0001$) and 6-OHDA (+)SNpc/(−)OB-gl ($P \leq 0.0001$) is present. Furthermore, 6-OHDA (+)SNpc/(+)OB-gl group also exhibited a significant neuronal reduction compared to 6-OHDA (−)SNpc/(−)OB-gl ($P \leq 0.001$). However, this 6-OHDA (+)SNpc/(+)OB-gl group presented an increased density of TH-ir neurons compared to the 6-OHDA (−)SNpc/(+)OB-gl ($P \leq 0.05$) [F (3, 22) = 28.23; $P \leq 0.0001$]. In relation to the time-point 14 days (Fig. 6j), the 6-OHDA (−)SNpc/(+)OB-gl group showed a significant reduction of this parameter compared to the 6-OHDA (−)SNpc/(−)OB-gl ($P \leq 0.05$) and 6-OHDA (+)SNpc/(−) OB-gl ($P \leq 0.01$) groups, [F (3, 16) = 6.428; $P = 0.0046$].

Statistical Correlations between Behavioral and Histological Parameters

Pearson's correlation coefficients (Table 1) revealed a negative correlation between the percentage of SNpc TH-ir neurons and immobility parameter of modified forced swimming test at time-points 7 ($r = -0.51$; $P = 0.002$) and 14 days ($r = -0.60$; $P < 0.0001$). Proportionally, a positive correlation between the percentage of SNpc TH-ir neurons and swimming parameter from the modified forced swimming test was found also at 7 ($r = 0.53$; $P = 0.001$) and 14 days ($r = 0.52$; $P = 0.0006$). The climbing parameter also showed a positive correlation with the percentage of SNpc TH-ir neurons, but only at 14 days ($r = 0.44$; $P = 0.005$).

In addition, positive correlations between the percentage of OB-gl TH-ir neurons and DI were identified at 7 ($r = 0.59$; $P = 0.002$) and 14 days ($r = 0.45$; $P = 0.03$). Complementarily, the percentage of sucrose consumption exhibited a negative correlation with immobility time in both time-points: 7 ($r = -0.51$; $P = 0.01$) and 14 days ($r = -0.44$; $P = 0.02$), positive correlation with swimming time at 7 ($r = 0.45$; $P = 0.02$) and 14 days ($r = 0.52$; $P = 0.008$), and a positive correlation with the percentage of SNpc TH-ir neurons ($r = 0.09$; $P = 0.67$ time-point 7 days) ($r = -0,055$; $P = 0.82$ time-point 14 days). Moreover, the percentage of OB-gl TH-ir neurons exhibited a negative correlation with

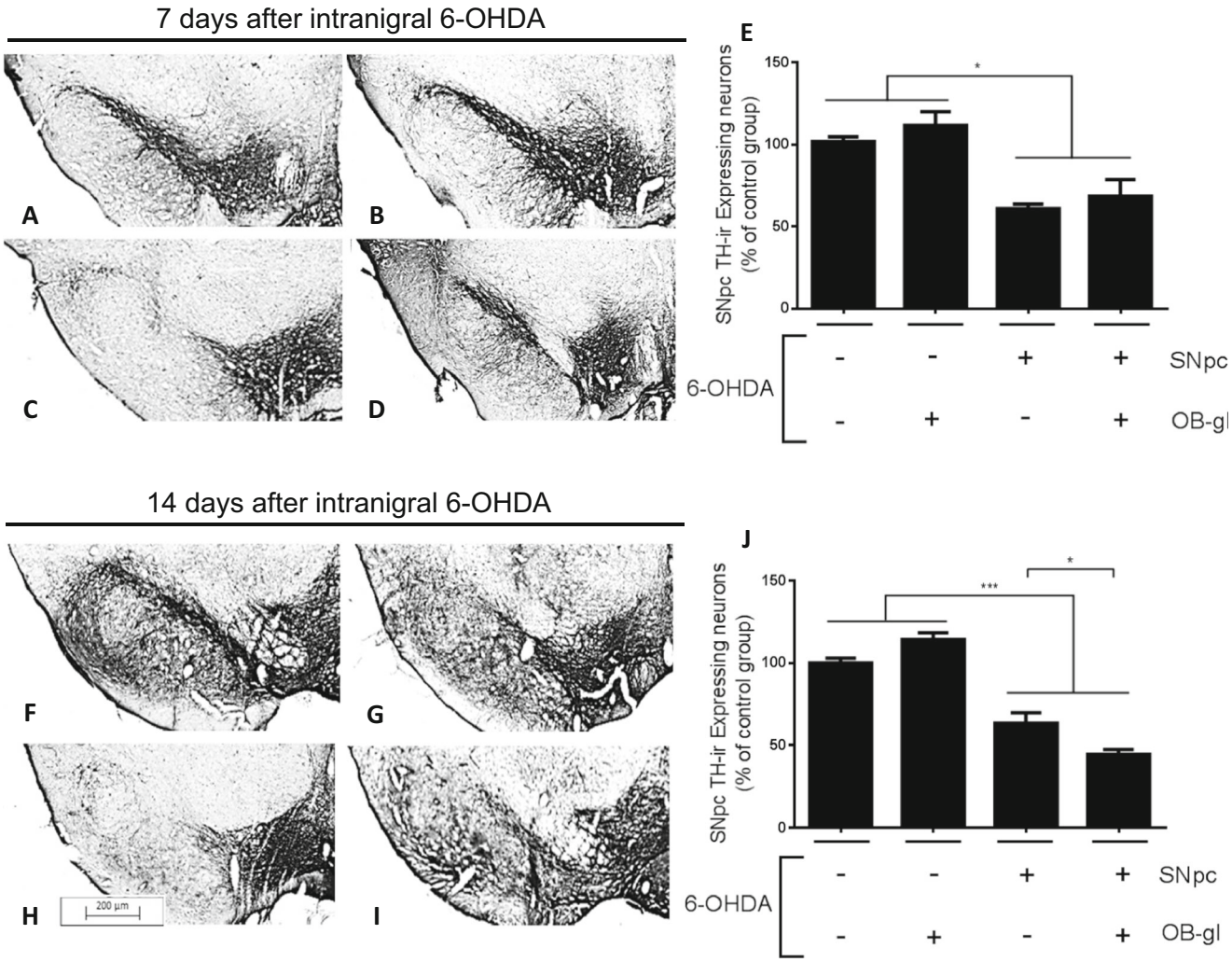

Fig. 5 Immunohistochemistry of TH-ir neurons in the SNpc. **a**–**d** Representative photomicrographies obtained 7 days after intranigral 6-OHDA microinfusion. **e** Quantification of SNpc TH-ir neurons 7 days after 6-OHDA. **f**–**i** Representative photomicrografies obtained 14 days after intranigral 6-OHDA. **j** Quantification of SNpc TH-ir neurons 14 days after 6-OHDA. **a**, **f** (−)SNpc/(−)OB-gl groups. **b**, **g** (−)SNpc/(+)OB-gl groups. **c**, **h** (+)SNpc/(−)OB-gl groups. **d**, **i** (+)SNpc/(+)OB-gl groups. The bars represent the mean ± standard error of the mean, ($n = 4$ animals, 6 slices/animal), $*P \leq 0.05$; $***P \leq 0.001$. One-way ANOVA followed by the Newman-Keuls post hoc test. Legends: 6-OHDA, 6-hydroxydopamine; OB-gl, olfactory bulb glomerular layer; SNpc, substantia nigra pars compacta; (+) presence of 6-OHDA lesion; (−) absence of 6-OHDA lesion (sham manipulation)

immobility time ($r = -0.44$; $P = 0.04$) and a positive correlation with swimming time ($r = 0.48$; $P = 0.02$) but only at 14 days. Ultimately, correlations between percentage of SNpc TH-ir neurons and percentage of OB-gl TH-ir neurons were not found.

Discussion

In the present study, we observed at the first time-point, 7 days after intranigral 6-OHDA microinfusion, that either SNpc or OB-gl dopaminergic lesions disrupted olfaction, like Zicam, in a very similar fashion. It has been reported an increased number of TH-ir neurons, within the glomerular layer, on post-mortem brains of patients with PD [13, 14] and in rotenone animal model [15] purportedly associated with impaired olfactory function. Curiously, at the earlier time-point, the group 6-OHDA (+)SNpc/(−) OB-gl did not present increase in TH-ir neurons in the OB-gl compared to the control group.

Therefore, this olfactory impairment without the increment in periglomerular cell density agrees with previous findings, which also report increase of dopaminergic neurons in OB-gl after intranigral 6-OHDA administration in rats [51]. Moreover, other reports showed an even more puzzling result that disputed the so-called TH-ir neuronal increase of OB-gl neurons in humans, once they did not replicated their previous study [13, 52]. In fact, our data showed that the olfactory impairment presented in the OB-gl-lesioned group may be due to periglomerular TH-ir neuronal loss compared to control. In this sense, our result stands that TH-ir periglomerular neurons have a key role in olfactory modulation; thus, a

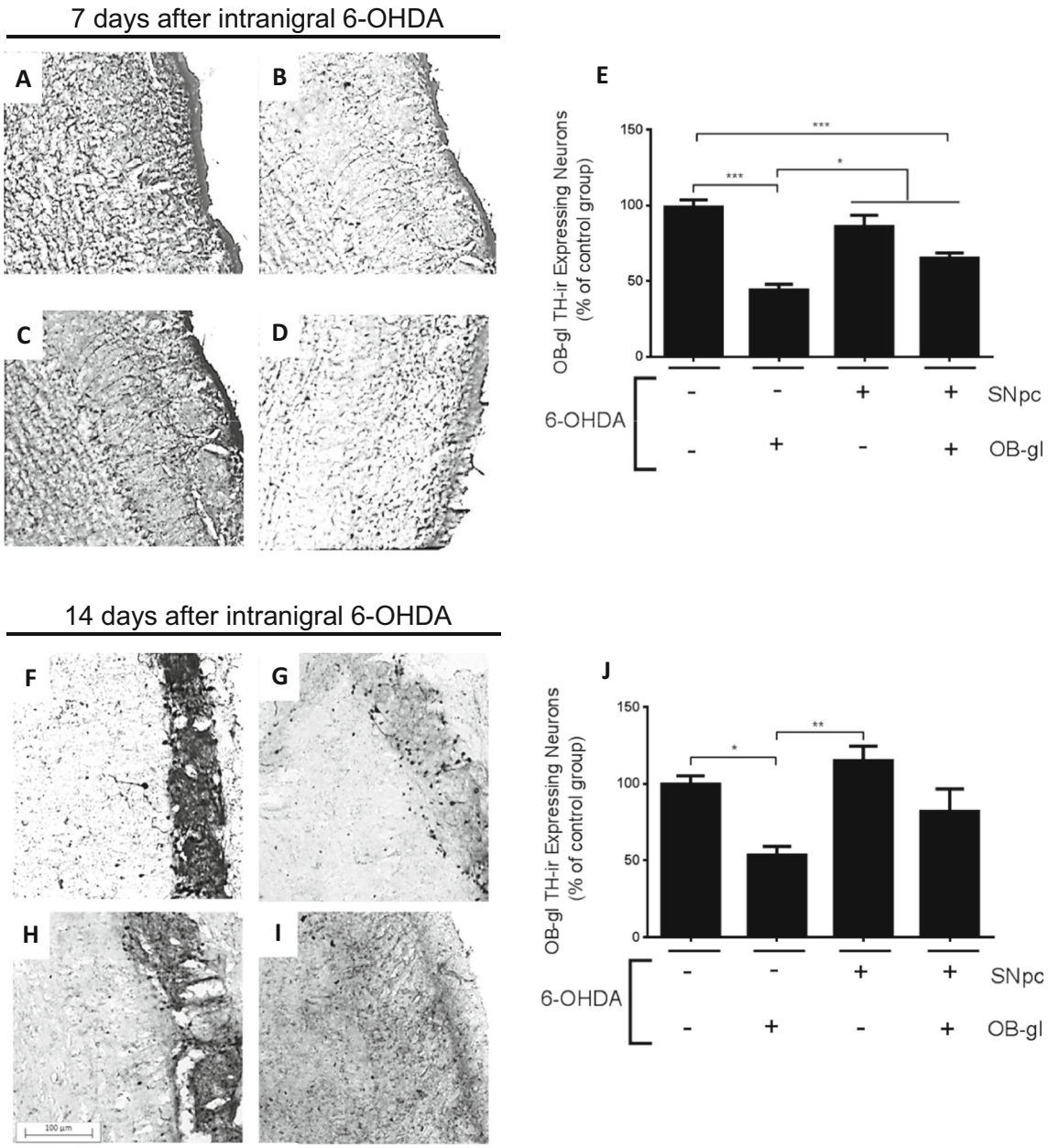

Fig. 6 Immunohistochemistry of TH-ir neurons in the OB-gl. **a**–**d** Representative photomicrographies obtained 7 days after intranigral 6-OHDA microinfusion. **e** Quantification of OB-gl TH-ir neurons 7 days after 6-OHDA. **f**–**i** Representative photomicrografies obtained 14 days after intranigral 6-OHDA. **j** Quantification of OB-gl TH-ir neurons 14 days after 6-OHDA. **a**, **f** (−)SNpc/(−)OB-gl groups. **b**, **g** (−)SNpc/(+)OB-gl groups. **c**, **h** (+)SNpc/(−)OB-gl groups. **d**, **i** (+)SNpc/(+)OB-gl groups. The bars represent the mean ± standard error of the mean ($n = 4$ animals, 6 slices/animal), $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. One-way ANOVA followed by the Newman-Keuls post hoc test. Legends: 6-OHDA, 6-hydroxydopamine; OB-gl, olfactory bulb glomerular layer; SNpc, substantia nigra pars compacta; (+) presence of 6-OHDA lesion; (−) absence of 6-OHDA lesion (sham manipulation)

decrease on these cells' density negatively impacts olfactory performance, as previously demonstrated [8]. In relation to the double-site 6-OHDA infusion, the OB-gl lesion seems to counteract the olfactory impairment caused by SNpc lesion.

In opposite, at time-point 14 days, both groups with lesions in the SNpc or in the OB-gl did not present detectable olfactory impairments, possibly as a result of compensatory mechanisms of the main olfactory pathway. Considering the OB-gl-lesioned group, we found that TH-ir periglomerular neurons were still decreased compared to the control and SNpc-lesioned group, however, not differing from the double-lesioned animals. Remarkably, a neurotoxic-induced lesion within the OB of mice is recovered by an increase of the newborn neurons coming from the subventricular zone, where they are stem cells [53, 54]. Such cells are recruited and then migrate through rostral pathway only differentiating in dopaminergic and GABAergic

Table 1 Pearson's correlations between different behavioral and histological parameters

Correlations	7 days after intranigral 6-OHDA groups	14 days after intranigral 6-OHDA groups
% SNpc TH-ir neurons × % OB-gl TH-ir neurons	$r = -0.35$; $P = 0.08$	$r = 0.054$; $P = 0.82$
% SNpc TH-ir neurons × DI	$r = -0.003$; $P = 0.98$	$r = 0.2017$; $P = 0.19$
% SNpc TH-ir neurons × immobility	$r = -0.51$; $P = 0.002$*	$r = -0.60$; $P < 0.0001$*
% SNpc TH-ir neurons × swimming	$r = 0.53$; $P = 0.001$*	$r = 0.52$; $P = 0.0006$*
% SNpc TH-ir neurons × climbing	$r = 0.19$; $P = 0.28$	$r = 0.44$;$P = 0.005$*
% OB-gl TH-ir neurons × DI	$r = 0.59$; $P = 0.002$*	$r = 0.45$; $P = 0.03$*
% OB-gl TH-ir neurons × immobility	$r = -0.059$; $P = 0.79$	$r = -0.44$; $P = 0.04$*
% OB-gl TH-ir neurons × swimming	$r = 0.11$; $P = 0.62$	$r = 0.48$; $P = 0.02$*
% OB-gl TH-ir neurons × Climbing	$r = -0.30$; $P = 0.15$	$r = -0.35$; $P = 0.12$
% sucrose consumption × DI	$r = -0.10$; $P = 0.63$	$r = 0.26$; $P = 0.21$
% sucrose consumption × immobility	$r = -0.51$; $P = 0.01$*	$r = -0.44$; $P = 0.02$*
% sucrose consumption × swimming	$r = 0.45$; $P = 0.02$*	$r = 0.52$; $P = 0.008$*
% sucrose consumption × % SNpc TH-ir neurons	$r = 0.43$; $P = 0.03$*	$r = 0.45$; $P = 0.02$*
% sucrose consumption × % OB-gl TH-ir neurons	$r = 0.09$; $P = 0.67$	$r = -0.055$; $P = 0.82$

*Significant correlations are indicated

interneurons at glomerular and granular layers, approximately 7 days post 6-OHDA injury [8]. Therefore, it is plausible to suggest that these newborn neurons could be able to repopulate (at least partially) the OB-gl, then recovering the olfactory sensory inputs. In addition, it has been described that animals could recover the sense of smell 8 days after a 6-OHDA OB-gl lesion, due to compensatory mechanisms triggered by the activation of the vomeronasal pathway and accessory olfactory system [55]. This system is being described as very primitive and is present in several mammals [56, 57], but only in humans fetuses [55]. In rodents, this system was first associated with detection of pheromones while common odors were perceived through the main olfactory pathway [55, 58, 59].

Regarding the extension of the nigral lesion, the percentage of TH-ir neurons, at time-point 7 days, was decreased only in association with intranigral 6-OHDA, as previously reported [48, 60–63], without the influence of OB-gl injury. However, the later time-point showed the occurrence of a possible synergic effect, probably due to a conceivable retrograde OB-gl lesion, affecting SNpc. In fact, this hypothesis is supported by the description of a direct axonal dopaminergic projection from the SNpc to the extern plexiform layer and granular layer of the OB, which promotes the perception of odorants and can mediate toxin-induced retrograde degeneration of dopaminergic SNpc neurons [51].

The absence of differences between TH-ir neurons in OB-gl of double-lesioned group and control group (unlike time-point 7 days) also may be interpreted as a compensatory increase in the number of the periglomerular neurons from OB-gl, possibly explaining the olfactory impairment, in agreement with our previous findings in a rotenone model of PD [15]. Of note, in this study, we demonstrated that the olfactory impairment at 7 and 14 days were particularly alike to the olfactory deficit inflicted by intranasal Zicam, which was used as a positive control of anosmia. This is an agent that has been described to promote a significant cytotoxicity to human, mouse, and rat nasal tissue given the potential development of long-lasting smell dysfunction [15, 36, 37]. Further, both positive controls for anosmia exhibited hypolocomotion at 7 days. This result could be related to a residual anesthetic effect due to intranasal Zicam administration; however, ketamine is supposed to produce hyperlocomotion in similar conditions [64–66]. Notwithstanding, this hypolocomotion was reversed at 14 days, suggesting an absence of locomotor bias.

As formerly described, intranigral 6-OHDA causes increase in the immobility time and consequent decreases in swimming time and also reduction in sucrose consumption at 7 [63, 67, 68], 14 [48, 69, 70], and other time-points already tested [71, 72]. Remarkably, double lesion produced depressive-like behaviors, most likely as decreased swimming, increased immobility, and anhedonic-like behavior at both time-points tested. This outcome is also interesting when compared to OB-gl lesion itself that only produced behavior despair, without anhedonia, at the later time-point, strengthening the notion of a maturation process of the retrograde lesion. It is characterized that depression causes alterations in olfactory circuits, reducing olfactory threshold, and identification and discrimination abilities in humans [28, 29]. Analogously, patients with congenital anosmia are more expected to exhibit signs of depression [23].

Our study originally demonstrated that a dopaminergic lesion of the OB-gl was able to produce depressive-like behaviors perhaps as a result of dysfunctions and/or compensatory mechanisms of the cortical–hippocampal–amygdala circuits that involve changes in synaptic strength and/or loss of spine density in these limbic areas [73, 74]. Besides, we found significant correlations between OB-gl TH-ir neuron counts and immobility time ($r = -0.44$; $P = 0.04$) and swimming time ($r = 0.48$; $P =$

0.02) at the later time-point, suggesting a direct association between these periglomerular neurons and depressive-like behaviors. Also, a correlation between OB-gl TH-ir neuron counts and DI of olfactory test is observed (at 7 days $r = 0.59$; $P = 0.002$ and at 14 days $r = 0.45$; $P = 0.03$). This reduction of dopaminergic neurons of OB-gl indicates a decrement of DI, suggesting that these neurons are key players on the functioning of the olfactory system. In accordance, the inhibition of the olfactory epithelium did not cause depressive-like behaviors such as helplessness, despair, or anhedonia. Our results demonstrated the importance of the dopaminergic neurons of the OB-gl in different non-motor features of PD, since the selective reduction of these periglomerular neurons was able to induce olfactory impairment (at acute time-point) and depressive-like behaviors (at later time-point). At the same time, the acute OB-gl lesion counteracted the olfactory impairment caused by the SNpc injury.

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Compliance with Ethical Standards

All the experiments were carried out in accordance with the guidelines of the Committee for the Care and Use of Laboratory Animals, United States National Institutes of Health. In addition, the protocol complies with the recommendations of Federal University of Paraná and was approved by the institutional ethics committee (approval ID no. 910).

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