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# Olfactory Training Prevents Olfactory Dysfunction Induced by Bulbar Excitotoxic Lesions: Role of Neurogenesis and Dopaminergic Interneurons

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## Abstract

Glutamatergic excitotoxicity is involved in pathologies affecting the central nervous system, including traumatic brain injury (TBI) and neurodegenerative diseases, such as Parkinson's disease (PD), in which olfactory dysfunction is an early symptom. Interestingly, our group has recently shown that bilateral administration of the glutamate agonist, *N*-methyl-D-aspartate (NMDA) in the olfactory bulbs (OBs) induces an olfactory dysfunction 1 week after lesions. Although a wide range of treatments have been attempted, no standard therapy has been established to treat olfactory disorders. Increasing evidence suggests a beneficial effect of olfactory training (OT) in olfactory function. However, the mechanisms underlying OT effects remain unknown. We investigated the effects of OT on the olfactory dysfunction induced by excitotoxicity in bilateral OB NMDA–lesioned animals. We compared OT effects with the ones obtained with neuroprotective therapies (pramipexole and MK801). We studied the underlying mechanisms involved in OT effects investigating the changes in the subventricular zone (SVZ) neurogenesis and in the number of periglomerular dopaminergic interneurons. One week after lesion, NMDA decreased the number of correct trials in the olfactory discrimination tests in the non-trained group ($p < 0.01$). However, OT performed for 1 week after lesions prevented olfactory dysfunction ($p < 0.01$). Pramipexole did not prevent olfactory dysfunction, whereas MK801 treatment showed a partial recovery ($p < 0.05$). An increase in SVZ neurogenesis ($p < 0.05$) associated with an increase in OB dopaminergic interneurons ($p < 0.05$) was related to olfactory function prevention induced by OT. The present results suggest a role for dopaminergic OB interneurons underlying the beneficial effects of OT improving olfactory dysfunction in bilaterally OB NMDA–lesioned animals.

**Keywords** Excitotoxicity · Olfaction · Olfactory training · Parkinson's disease · Traumatic brain injury · Dopamine · Neurogenesis

## Introduction

Olfactory dysfunction is estimated to affect 3–20% of the population [1–3] negatively influencing the quality of life, enjoyment of food, reducing challenges with maintaining personal hygiene, impacting on physical and mental well-being, and social relationships [4–6]. Risk of olfactory dysfunction increases with age and may result from upper respiratory infections, sinonasal disease, and head trauma as well as degenerative diseases [1, 3, 7–11], being olfactory dysfunction considered an early marker for Parkinson's disease (PD), Alzheimer's disease, or dementia with Lewy bodies [3, 12, 13].

The involvement of glutamate in olfactory function has been suggested [14–16]. Glutamatergic *N*-methyl-D-aspartate (NMDA) receptor subunits and vesicular glutamate transporters are extensively expressed throughout the olfactory bulbs (OB) and olfactory epithelium [14, 17, 18]. The activation of NMDA receptors results in intracellular calcium

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overload and reactive oxygen species that are considered key factors of glutamate excitotoxicity [19, 20]. Excitotoxicity is involved in several pathological conditions affecting the central nervous system, including traumatic brain injury (TBI) and neurodegenerative diseases in which olfactory dysfunction is an early symptom [21–24]. Interestingly, our group has recently shown that the bilateral administration of the glutamate agonist, NMDA, in the OBs induces olfactory dysfunction 1 week after lesions [15, 16].

Although a wide range of treatment modalities including corticosteroid, theophylline, and antibiotics have been attempted [25–27], no standard therapy has yet been established to treat effectively olfactory disorders [7, 28–30]. Moreover, pharmacological treatment of PD fails to restore olfactory function in PD patients [31].

An increasing body of preclinical [22, 32] and clinical [28–30, 33–36] evidence supports the beneficial effect of olfactory training (OT) in olfactory function. In this line, wine tasters have an increased ability to correctly identify odors when compared with non-trained healthy normal subjects [37]. Moreover, repeated exposure to androstenone significantly increased the sensitivity of young normal subjects [38]. In addition, several studies have suggested promising therapeutic effects of OT in patients with post-infectious, posttraumatic, and PD olfactory loss [28, 31, 34, 38–40]. All these data suggest that the olfactory system has the plasticity to recover with training [39–42]. However, although the efficacy of the OT has been shown, the molecular mechanisms underlying OT remain poorly understood.

The main purpose of this study was to investigate the effects, and their underlying molecular mechanisms, of OT on the olfactory dysfunction induced by excitotoxicity in bilateral OB NMDA–lesioned animals. In addition, a comparative study between the effects of OT and the ones induced by the neuroprotective treatment with the dopamine agonist, pramipexole [43], or with the glutamate antagonist, MK-801 [44], on the olfactory dysfunction induced by excitotoxicity has been performed.

## Material and Methods

### Ethics Statement

All experiments were carried out following the European (2010/63/UE) and Spanish (RD 53/2013) regulation for the care and use of laboratory animals and approved by the local Government (Generalitat de Catalunya). The Ethic's Committee of our institution approved this study.

### Animals and Experimental Design

Male Sprague-Dawley rats (260–280 g Charles River) were housed individually in standard laboratory cages on a 12-h light/dark cycle with free access to food and water. During the training/discrimination test periods, rats were maintained in a food-deprivation schedule designed to keep rats' body weight at approximately 85% over the behavioral testing period.

Two sets of experiments were performed (Fig. 1). In the first set, the effects of OT on the olfactory dysfunction induced by excitotoxicity in bilaterally OB NMDA–lesioned animals were investigated. In the second set, the effects of neuroprotective pharmacological treatments on the olfactory dysfunction induced by OB NMDA–induced lesions were studied.

Animals were olfactory trained for 5 days prior to the first olfactory discrimination (Test-1), and then, animals received a bilateral NMDA OB administration, as described below. Control (sham-lesioned) animals received a bilateral infusion of vehicle. After lesions, animals were randomly distributed in several groups with or without OT, or pharmacological treatment for 1 week. To evaluate the effects of OT or neuroprotective therapies on the olfactory function, the olfactory discrimination test was repeated at the end of OT or treatments (Test-2). Animals were sacrificed after the last olfactory discrimination test in order to perform the immunohistological studies.

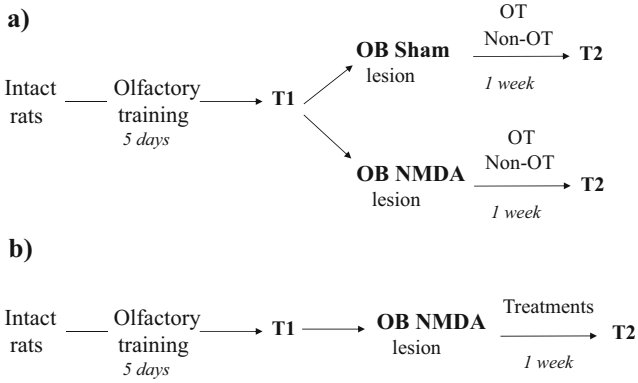


**Fig. 1** Experimental design: intact rats were olfactory trained for 5 days. Before lesions, an odor discrimination test (T1) was performed in all animals. Animals were randomly distributed in experimental groups receiving three bilateral NMDA (1.5 μl of a saline solution of 12 mg/ml) or vehicle (sham) OB administrations. Two sets of experiments were performed: **a** in order to investigate the effect of olfactory training (OT) on olfactory dysfunction induced by the excitotoxic lesions, NMDA and sham-lesioned animals were randomly distributed in different groups that were trained, or non-trained, for 1 week after lesions. A second odor discrimination test (T2) was performed 1 week after lesions; **b** in order to investigate the effect of neuroprotective treatments on olfactory dysfunction induced by the excitotoxic lesion, NMDA-lesioned animals were randomly distributed in three different groups that received treatment with: the dopamine agonist pramipexole, the NMDA glutamate antagonist MK801 or saline (control group). A second odor discrimination test (T2) was performed 1 week after lesions. Animals were sacrificed 1 day after the last olfactory test

## NMDA Lesions and Experimental Groups

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p., $n = 47$) and placed in a stereotaxic frame with the incisor bar positioned at 4.5 mm below the interaural line. Bilateral OB lesions were induced by the administration of three injections of NMDA (1.5 μl of a saline solution of 12 mg/ml, in each injection, pH 7.0) injected at 30 μl/h rate over a 3-min period [15, 16, 45, 46]. The coordinates for OB used were from bregma: A: + 8.0 mm, L: ± 1.5 mm, V: − 4.5 mm (for injections 1 and 2) or − 5 mm (for the third injection), according to the atlas of Paxinos and Watson (1986, [47]) [15, 16, 48–50]. Sham-lesioned animals received the same volume of vehicle in both OB ($n = 7$). The infusion needle was kept in place for two additional minutes after each infusion ended in order to minimize backflow. It has been previously confirmed that the maximal volume (4.5 μl of NMDA solution) injected does not spread to other areas of the brain [15, 16].

In order to investigate the effects of OT on the olfactory dysfunction induced by OB NMDA lesions, 24 h after lesion, animals were randomly distributed in the following groups with or without training for 1 week after NMDA-induced lesions: (a) non-trained sham-lesioned ($n = 7$), (b) trained sham-lesioned ($n = 5$), (c) non-trained NMDA-lesioned ($n = 6$), and (d) trained NMDA-lesioned ($n = 5$).

In order to investigate the effects of neuroprotective treatments on the olfactory dysfunction induced by OB NMDA lesions, 24 h after lesion, animals were randomly distributed in the following groups daily treated for 1 week after NMDA-induced lesions with (a) pramipexole (1 mg/kg, sc, $n = 6$), (b) pramipexole (2 mg/kg, sc, $n = 6$), (c) MK801 (0.1 mg/kg, ip, $n = 6$), and (d) MK801 (0.5 mg/kg, ip, $n = 5$), or (e) saline ($n = 5$).

## Olfactory Training and Discrimination Tests

### Odors Stimuli

Olfactory stimuli consisted of powdered odorants (cinnamon and vanilla) mixed in sand (4 g of odorant in 80 g of sand) and presented in clear plastic cups (3-cm diameter and 3-cm high) as previously described [15, 16, 51, 52]. The odors were chosen based on previous reports describing these odorants to perform olfactory discrimination tests [51, 53–57]. Our research group has previously reported this methodology when the olfactory dysfunction in the OB NMDA–lesioned animal model was described [15, 16].

A food reward, a piece of Froot Loop cereal, was buried beneath the surface of the sand in order to eliminate any potential visual cues [15, 16, 50].

### Olfactory Training

All testing occurred during the light portion of the rat's light/dark cycle. All the animals were trained for 5 days prior to the first discrimination test in the following manner: On the first day, each animal—in its own cage—was presented with a small cup containing 80 g of sand. On the second day, the animal was presented with a reward on top of the sand in the cup [58]. From the third day onwards, the animal was trained in a custom testing cage (Fig. 2) [49] where the discrimination tests took place, and the positive smell (4 g of cinnamon) was added to the cup with sand and the reward. During the following 2 days, the difficulty in finding the reward was increased by burying the reward in the sand. On the fifth day, the animal was placed at the start chamber, the cups were kept at the test chamber, and a divider panel was put in place. The 5-day interval of time is the one that has been previously reported as required for the animals to habituate to the experimental chamber and to learn the rewarded odor [59, 60]. Our research group has previously reported this methodology when the olfactory dysfunction in the OB NMDA–lesioned animal model was described [15, 16]. Rats capable of daily finding the reward were considered trained animals [15, 16, 52]. For the OT performed during the week after NMDA OB lesions, animals completed daily olfactory exposure in the test chamber [48, 61].

### Olfactory Discrimination Tests

The olfactory discrimination task involved conditioning subjects to discriminate between two simultaneously presented odors, one rewarded (cinnamon) and one not rewarded (vanilla), between which the subject must choose to obtain the reward [15, 16, 48, 62]. Each test consisted of 15 consecutive discrimination trials using the same pair of odorants.

All discrimination olfactory testing occurred in a custom methacrylate cage that was split into a star chamber and a test chamber by an opaque divider panel [15, 16, 49, 63]. Each test began placing the rat in the start chamber with the divider in place. Once the divider was raised, the rat entered the test chamber, which contained two differently scented cups filled with sand one of which (cinnamon) contained a reward. Once the rat had entered the test chamber, the divider was lowered. During each trial, the pot in which the rat dug first was recorded. Self-correction after initially digging in the incorrect cup was permitted but the trial was recorded as incorrect. Trials were terminated after 2 min if the rat failed to dig [15, 16, 63]. The amount of time that the rat spent to achieve and actively investigating the correct odorant was measured with a stopwatch. Active investigation was defined as directed sniffing within 1 cm of the odor source presented [15, 16, 48, 49].

To control for the possibility of rats learning directly from detecting the rewarded cup based on spatial location, both cups were moved to different locations after every trial [15,

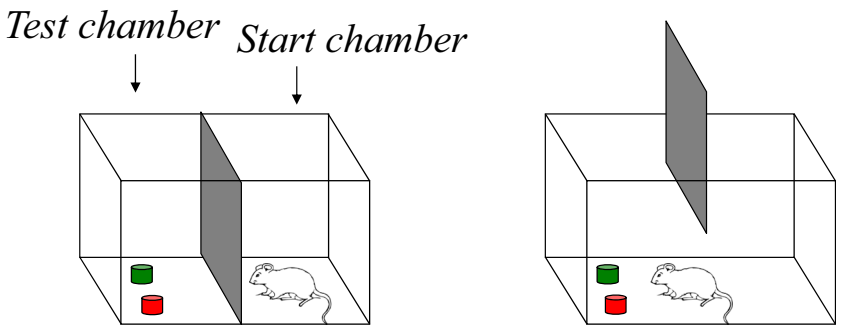


**Fig. 2** Experimental setup for odor discriminatory tests: all olfactory discrimination tests occurred in a transparent observational cage, which was split into a test chamber and a start chamber by an opaque divider panel. Left: before each trial, the rat was placed in the start chamber with the divider panel in place. Right: once the divider panel was raised, the rat entered the test chamber to dig in either a rewarded and scented (cinnamon) or non-rewarded and scented (vanilla) cup. Each test consisted of 15 trials. Trials 5, 10, and 15 were performed without any reward present to control for the possibility that rats might locate the reward through its own odor rather than by learning the association with a training odorant

16, 58]. To control for the possibility of rats locating the cereal reward through its own odor rather than by learning the association with a training odorant, we performed every fifth trial without any reward present (trials 5, 10, and 15) [15, 16, 63]. Olfactory discrimination tests were performed before (Test-1) and 1 (Test-2) week after NMDA lesions (Fig. 1). Rats that failed to dig during Test-1 were not included in the studies. Data for discrimination tests was shown as the number of correct trials (in the study of the effect of OT on the olfactory dysfunction) or the percentage of correct responses (in the study of pharmacological treatments effects), and the time used to achieve the correct cup.

## Tissue Collection

The day after the last olfactory test, the animals were sacrificed under an overdose of pentobarbital anesthesia. Brains and OB were quickly removed from the skull. Brains were frozen on dry ice, and the OB were embedded in cryoprotective media (OCT compound, Tissue-Tek) and frozen on dry ice. They were cut in coronal 14-μm-thick sections in a cryostat and kept at − 80 °C until needed.

## Immunohistochemistry

SVZ and OB sections from trained and non-trained animals were processed for immunohistochemistry, according to a standard peroxidase-based method [15, 16]. Briefly, sections were thawed and dried at room temperature, fixed with acetone for 10 min at 4 °C and immersed in 0.3% hydrogen peroxide in PBS for 10 min to block the endogenous peroxidase. Sections were incubated with goat, rabbit, or horse serum for 20 min and incubated overnight at 4 °C with NeuN (1:500, MAB377 Millipore), the monoclonal anti-Polysialylated-Neural Cell Adhesion Molecule (PSA-NCAM, 1:500, MAB5324, Millipore), the polyclonal anti-Ki67 (1:200, sc-7846, Santa Cruz Biotech Inc.), and the monoclonal anti-Tyrosine Hydroxylase (TH, 1:5000, MAB5280, Millipore) antibodies. Sections were incubated with their respective biotinylated secondary antibodies (1:1000, Vector Laboratories, Ltd., UK) for 30 min, followed by avidin-biotinylated peroxidase complex (Vectastain® Peroxidase Standard PK-4000, Vector Laboratories Ltd., UK) for 30 min and 3-3′-diaminobenzidine and 0.01% hydrogen peroxide for 40–60 min. Slides were washed with PBS, dehydrated in ascending alcohol concentrations, cleared in xylene, and coverslipped in DPX mounting medium.

Immunohistochemical quantifications were performed with an Olympus® BX41 microscope connected to a Color View IIIu being the data acquired using the Cell F software program (Olympus Soft Imaging Solutions GmbH, Germany). All the parameters such as light exposure and magnification were kept constant across all measurements to avoid confounding variables. SVZ counts were made along the lateral wall of the lateral ventricle adjacent to the striatum, beginning where the ventricle opens beneath the corpus callosum and extending caudally. SVZ PSA-NCAM and Ki67 positive cells/mm$^2$ were quantified in three coronal sections (from + 1.20 to + 0.5 mm relative to bregma, 20 ×) [15, 46, 64]. The optical density (OD) for OB TH immunohistochemistry was quantified using the NIH ImageJ 1.48v software in three coronal sections (from + 1.20 to +0.5 mm, and + 6.70 to + 5.70 mm, relative to bregma, respectively) [15, 65, 66]. Data were expressed as the percentage of non-trained sham animals.

## Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) followed by Dunnett's $t$ test for multiple comparisons or paired Student's $t$ test when required. The level of statistical significance was set at $p < 0.05$ for all analyses.

## Results

### Histological Characterization of the Bilateral OB Lesion Degree Induced by NMDA Administration

To estimate the extent of cellular damage, OB sections from sham and NMDA-lesioned animals were processed for NeuN immunohistochemistry (Fig. 3). Sham-lesioned animals showed a normal laminar structure. As expected, bilateral OB NMDA injections resulted in neural injury as indicated by cell loss in all OB layers. Laminar organization had deteriorated being the cell loss greatest in the central portion of the bulb at the point of delivery of the injection and tapered thereafter in each direction (Fig. 3).

### Effects of OT on Olfaction Dysfunction Induced by Bilateral OB Lesions Induced by NMDA

Before bilateral OB administrations (Test-1), no differences were observed in the number of correct responses between NMDA and sham-induced lesions, or between trained and non-trained animals (Fig. 4a).

One week after OB lesions (Test-2), sham-lesioned animals did not show differences in the number of correct responses when compared with their respective Test-1 (Fig. 4a). Non-trained NMDA-lesioned animals showed olfactory dysfunction decreasing the number of correct responses by 79% ($p < 0.01$) (Fig. 4a). However, trained NMDA-lesioned animals did not show significant changes in the number of correct responses when compared with their respective Test-1. Moreover, trained NMDA-lesioned animals showed an increased number of correct responses when compared with the non-trained group ($p < 0.01$) (Fig. 4a). When considering the patterns of total errors (errors plus corrections), non-trained NMDA-lesioned animals showed a higher percentage of errors than trained NMDA-lesioned animals that showed a higher percentage of corrections (Fig. 4b).

In addition, 1 week after sham-lesion (Test-2), animals did not show differences in the time spent to obtain the correct odor (Fig. 4c). Non-trained NMDA-lesioned animals showed a significant increase in the time spent to obtain the correct odor ($p < 0.01$ vs their respective Test-1) (Fig. 4c). However, trained NMDA-lesioned animals did not show changes in the time spent to obtain the correct odor when compared with their respective Test-1 (Fig. 4c). Moreover, trained NMDA-lesioned animals showed a lower time spent when compared with non-trained NMDA-lesioned group ($p < 0.05$) (Fig. 4c).

### Effects of Neuroprotective Therapies on Olfaction Dysfunction Induced by Bilateral OB Lesions Induced by NMDA

Before NMDA bilateral OB administration (Test-1), no differences were observed in the number of correct trials between saline-, pramipexole-, or MK801-treated groups (Figs. 5a and 6a). As expected, animals that received saline for 1 week after NMDA-induced lesions (Test-2), showed a significant decrease in the number of correct trials ($p < 0.01$) (Figs. 5a and 6a).

Treatment with the DA agonist Pramipexole for 1 week did not induce a significant increase in the number of correct trials in NMDA-lesioned animals (Fig. 5a). When considering the patterns of total errors (considered as errors plus corrections), no significant differences were observed in the percentage of errors or corrections after pramipexole treatment (Fig. 5b). The low tested dose of pramipexole (1 mg/kg) induced a significant decrease in the time that the animals spent to obtain the correct odor when compared with saline-treated animals ($p < 0.05$) (Fig. 5c).

Treatment with a low dose (0.1 mg/kg), but not a higher dose (0.5 mg/kg), of the NMDA glutamate antagonist MK801 induced a significant, although partial, increase in the number of correct trials (Fig. 6a). When considering the patterns of total

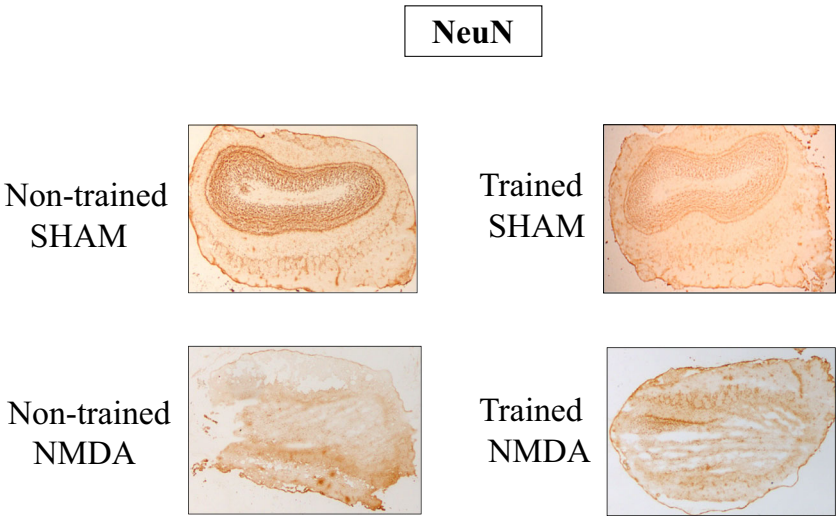


**Fig. 3** Histological characterization of excitotoxic lesion degree induced by bilateral OB NMDA administration: representative NeuN-immunoreactive staining from 14-μm coronal OB sections. Animals received bilateral NMDA (1.5 μl of a saline solution of 12 mg/ml, three injections) or vehicle (sham group) OB administrations

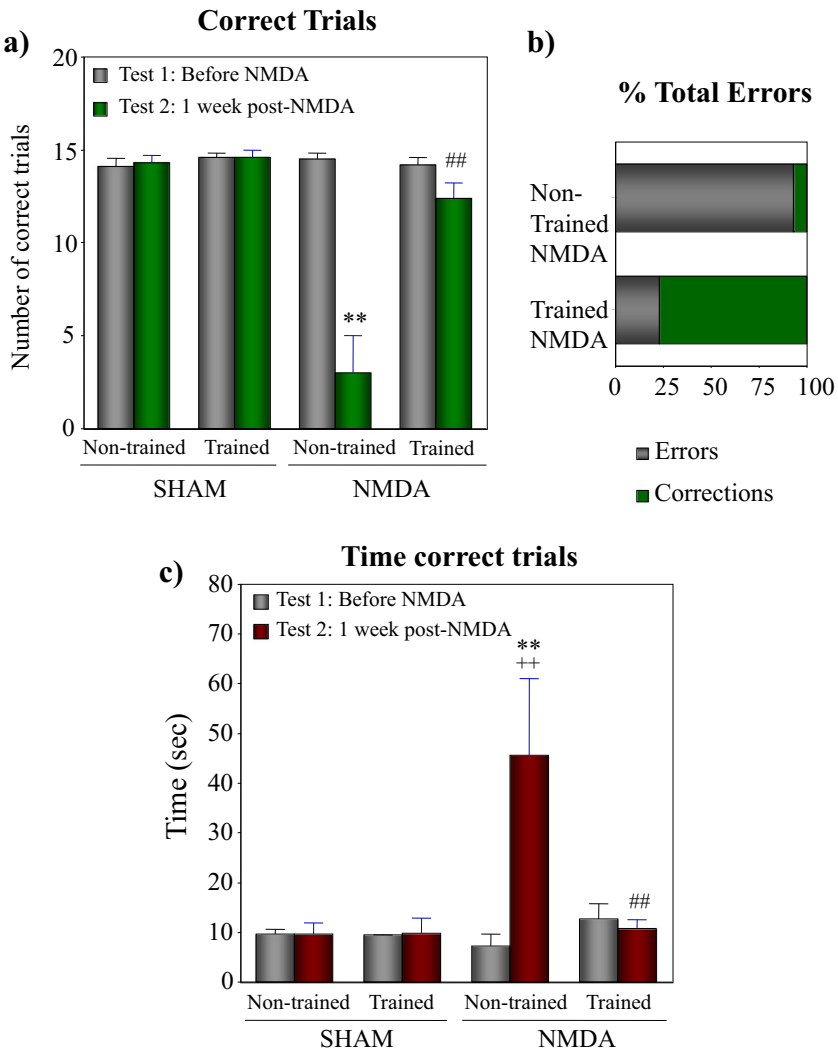


**Fig. 4** Effect of olfactory training (OT) on the olfactory dysfunction induced by bilateral OB NMDA administration. The parameters of the olfactory discrimination tests evaluated were as follows: **a** number of correct trials, **b** pattern of a total number of errors (considered as errors plus corrections), and **c** investigation time spent to achieve the correct odor in the olfactory discrimination tests. Animals received bilateral NMDA (1.5 μl of a saline solution of 12 mg/ml, three injections) or vehicle (sham-lesioned groups) OB administrations. Trained animals received olfactory training for 1 week after lesions. Olfactory discrimination tests were performed before (Test-1), and 1 (Test-2) week after NMDA lesions. Each test consisted of 15 trials. Data are expressed as mean ± SEM. $^{**}p < 0.01$ vs Test-1 (before lesion); $^{\#\#}p < 0.01$ vs non-trained NMDA-lesioned group Test-2; $^{++}p < 0.01$ vs non-trained sham Test-2

errors, no significant differences were observed in the percentage of errors or corrections after MK801 treatment (Fig. 6b). The low tested dose of MK801 (0.1 mg/kg) induced a significant decrease in the time that the animals spent to obtain the correct odor when compared with saline-treated animals ($p < 0.05$) (Fig. 6c).

## Effects of OT on SVZ Neurogenesis and in OB Glomerular TH+ Cell Population

One week after sham lesions, the immunohistochemistry for PSA-NCAM showed an increase, although statistically non-significant, in the number of SVZ migrating neuroblasts in the trained sham-lesioned animals when compared with the non-trained sham group (Fig. 7a). One week after NMDA lesions, non-trained NMDA-lesioned animals did not show changes in the number of SVZ migrating neuroblasts when compared with the non-trained sham-lesioned group (Fig. 7a). However, the number of PSA-NCAM positive cells in the trained NMDA-lesioned group showed a significant increase by 54% when compared with the non-trained NMDA group ($p < 0.05$), and by 70% when compared with the non-trained sham group ($p < 0.01$) (Fig. 7a).

One week after sham lesions, the immunohistochemistry for Ki67 showed a significant increase in SVZ neuroblast proliferation in the trained sham-lesioned animals when compared with the non-trained sham group ($p < 0.05$) (Fig. 7b). One week after NMDA lesion, non-trained NMDA-lesioned did not show changes in SVZ neuroblast proliferation when compared with the non-trained sham-lesioned group (Fig. 7b). However, the number of Ki67 positive cells in the trained NMDA-lesioned group showed a significant increase by 64% when compared with the non-trained NMDA group ($p < 0.05$), and by 74% when compared with the non-trained sham group ($p < 0.01$) (Fig. 7b).

One week after sham lesions, the immunohistochemistry for TH showed an increase, although statistically non-significant, in the number of TH-positive cells at the level of OB glomerular layer in the trained sham-lesioned animals when compared with the non-trained sham group. One week after NMDA lesions, the number of TH-positive cells showed a significant increase by 71% in the trained NMDA-lesioned

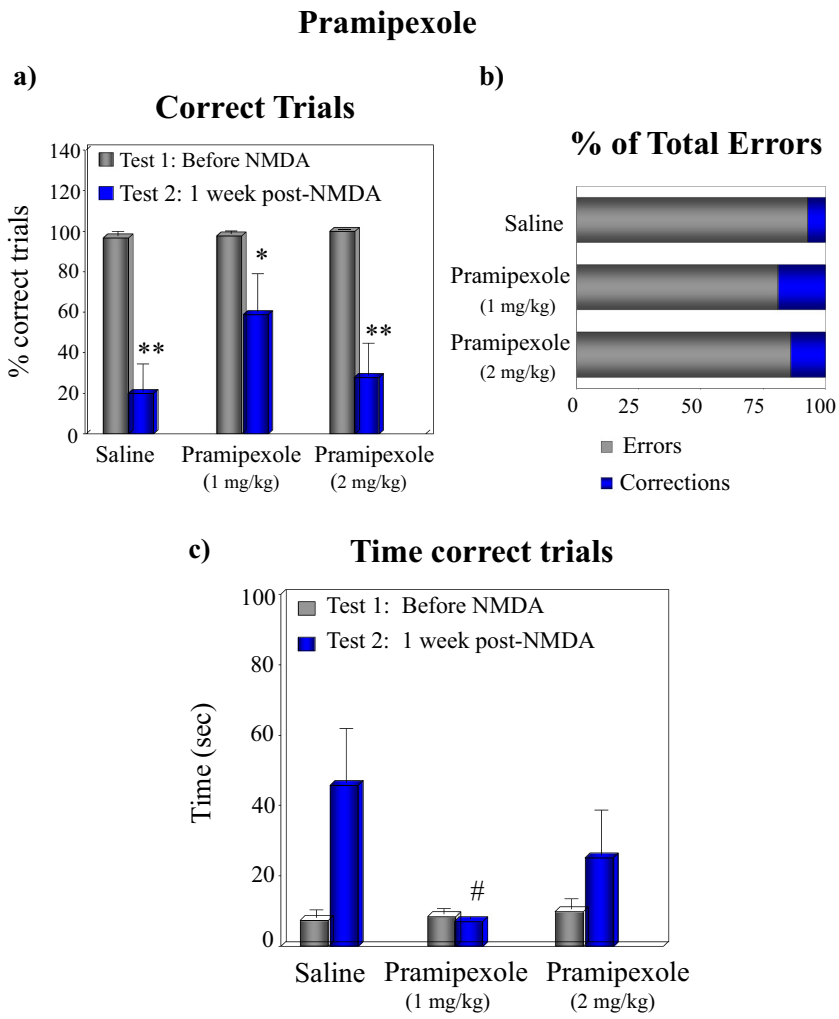


**Fig. 5** Effect of pramipexole treatment on the olfactory dysfunction induced by bilateral OB NMDA administration. The parameters of the olfactory discrimination tests evaluated were as follows: **a** number of correct trials, **b** pattern of a total number of errors (considered as errors plus corrections), and **c** investigation time spent to achieve the correct odor in the olfactory discrimination tests. Animals received bilateral NMDA (1.5 μl of a saline solution of 12 mg/ml, three injections) OB administrations, and randomly distributed in three groups that were treated with: pramipexole (1 or 2 mg/kg) or saline, for 1 week after lesions. Olfactory discrimination tests were performed before (Test-1), and 1 (Test-2) week after NMDA lesions. Each test consisted of 15 trials. Data are expressed as mean ± SEM. $*p < 0.05$, $**p < 0.01$ vs Test-1 (before lesion), $^{\#}p < 0.05$ vs saline-treated group Test-2

animals when compared with the non-trained sham-lesioned group ($p < 0.05$) (Fig. 8).

## Discussion

We investigated the effect of OT, and its underlying mechanisms, on the olfactory dysfunction induced by bilateral excitotoxic OB NMDA lesions in rodents during the first week after lesion. In addition, we have compared the OT effects on the olfactory dysfunction with the ones obtained after treatment with the dopamine agonist pramipexole, or the NMDA glutamate antagonist MK801.

The elimination of OB neurons induced by NMDA lesion was verified by immunostaining for the nuclear protein NeuN, a neuronal marker [15, 16, 45]. NMDA infusions produced a cell loss [15, 16, 45, 67] that was greatest at the point of the injection, diminishing thereafter in each direction. In agreement with our previous results, excitotoxic OB lesions induced a decrease in the olfactory function evidenced by a diminished number of correct trials in the discrimination olfactory tests 1 week after NMDA-induced lesion in rats [15, 16]. In addition, an increment in the time used to obtain the correct odor was observed 1 week after OB lesions [15]. In agreement with our previous data [15], non-trained sham-lesioned animals did not show olfactory dysfunction indicating that the decrease in olfactory function observed after NMDA lesions is not due to a loss of memory of the preoperative training.

Our present results showed that OT prevented the development of the olfactory dysfunction when performed for 1 week after bilateral OB NMDA lesions. Interestingly, the trained NMDA-lesioned group showed a similar number of correct trials than before lesion, and an increased number of correct trials when compared with the non-trained group. Moreover, OT decreased the time spent on the animals to achieve the correct odor.

In the present study, pramipexole treatment did not induce a significant improvement in olfactory function in animals with bilateral OB NMDA–induced lesions. Pramipexole is a non-ergoline dopamine agonist that has been shown to activate D2/D3 receptors and to have neuroprotective properties in neurodegenerative diseases, such as PD, in which glutamate-induced excitotoxicity is involved [68–70].

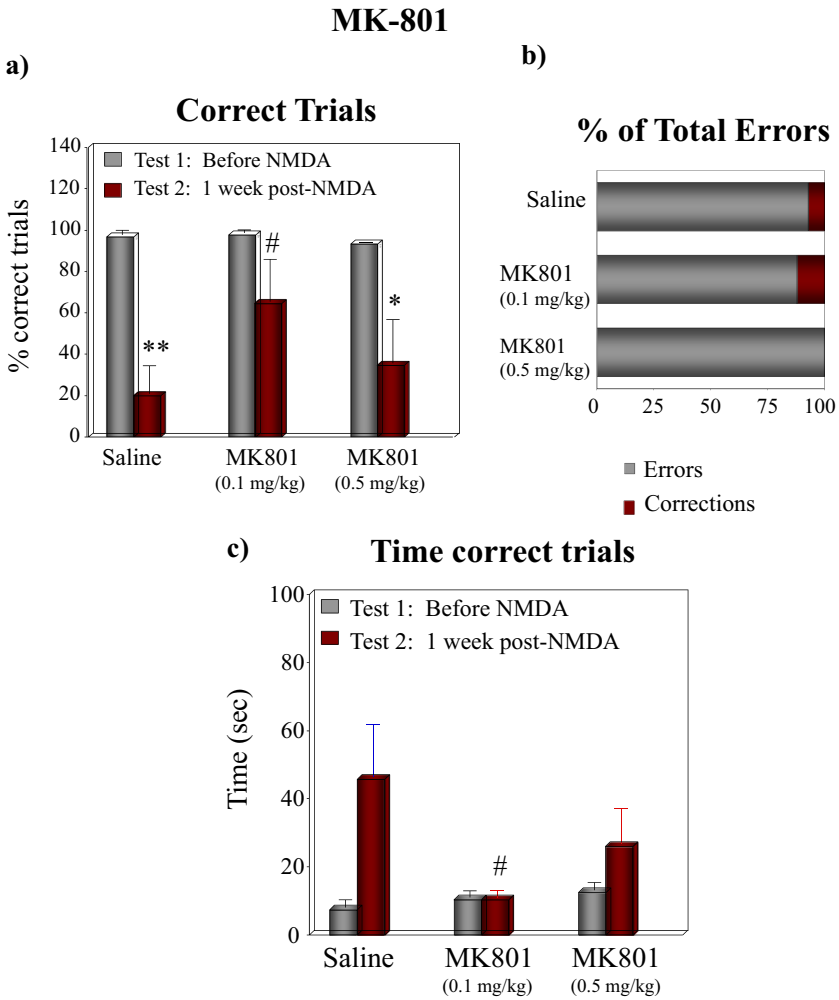


**Fig. 6** Effect of MK-801 treatment on the olfactory dysfunction induced by bilateral OB NMDA administration. The parameters of the olfactory discrimination tests evaluated were as follows: **a** number of correct trials, **b** pattern of a total number of errors (considered as errors plus corrections), and **c** investigation time spent to achieve the correct odor in the olfactory discrimination tests. Animals received bilateral NMDA (1.5 μl of a saline solution of 12 mg/ml, three injections) OB administrations and were treated with MK-801 (0.1 or 0.5 mg/kg) or saline for 1 week after lesions. Olfactory discrimination tests were performed before (Test-1), and 1 (Test-2) week after NMDA lesions. Each test consisted of 15 trials. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs Test-1 (before lesion), $^{\#}p < 0.05$ vs saline-treated group Test-2

However, the role of pramipexole in olfactory function was still unknown. The present results are in agreement with previous clinical data showing that the olfactory dysfunction occurring in PD does not respond to dopaminergic medication, such as levodopa [31, 71, 72]. An explanation for the lack of effects may be the differential effect of dopaminergic receptors subtypes in the olfactory system. In this line, previous studies have demonstrated, in rodents, that D1 receptor activation facilitates odor discrimination and detection threshold sensitivity, while D2 receptor does the opposite [31, 73, 74].

Our present results showed that a lower, but not higher, dose of MK801 induced a partial recovery of the olfactory dysfunction. In agreement with our results, the post-injury treatment with MK801 has little effect on post-injury neurologic scores [75]. It has been described that the outcome with drugs affecting NMDA receptor-mediated functions is completely dependent on the time of treatment initiation. When given shortly after the injury, NMDA antagonists consistently improve outcome, however, when treatment is initiated more than 1 h after injury, results may be negative [76, 77]. This strong time dependence suggested that NMDA receptors might undergo significant dynamic changes in availability and functionality relative to time after injury. Among other contributing factors to the observed results in the present study is the short therapeutic window for the efficacy of the NMDA antagonists [78].

Our results are in agreement with previous studies suggesting that repeated exposure to odors increases olfactory sensitivity and that the olfactory system has the plasticity to recover with training [33, 39, 41, 42]. Evidence of OT-induced plasticity exists in the neural circuitry in healthy controls, in patients with olfactory dysfunction, and in animal models [33, 79–81]. A number of studies have suggested promising therapeutic effects of OT in patients with post-infectious [28, 30, 34, 39, 40, 82], posttraumatic [10, 28, 34, 39], PD [41, 83] olfactory loss, as well as in older and younger healthy volunteers [79, 80, 84]. The observation that patients with training benefit mainly improve their identification and discrimination scores has suggested a central effect of the training procedure [28, 42]; however, electrophysiological changes at the level of the olfactory epithelium have been recently described [84].

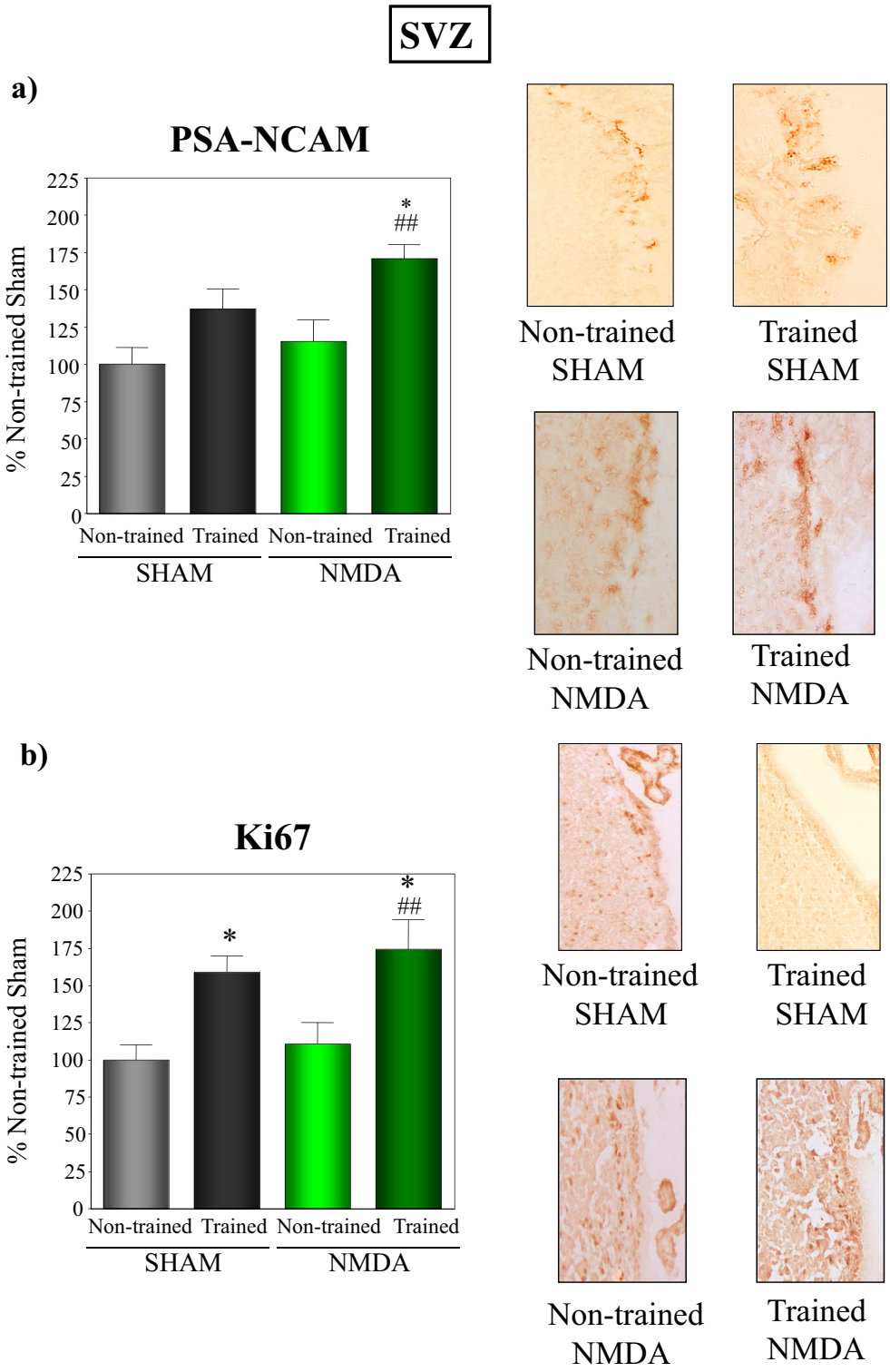


**Fig. 7** Effect of olfactory training (OT) on SVZ cell population and neuroprogenitor proliferation formation at 1 week after lesions expressed as the percentage of the non-trained sham-lesioned group. Animals received three bilateral NMDA (NMDA-3, 1.5 μl of a saline solution of 12 mg/ml) or vehicle OB administrations. Trained animals received olfactory training for 1 week after lesions. **a** PSA-NCAM and **b** Ki67 immunohistochemistry. Left: quantification of PSA-NCAM and Ki67-positive cells. Right: representative immunohistochemical staining of SVZ coronal sections. Data are expressed as mean ± SEM. $^{*}p < 0.05$ vs respective non-trained groups; $^{\#\#}p < 0.01$ vs non-trained sham group

However, although OT has been introduced as a promising treatment for patients with olfactory dysfunction, less is known about the molecular mechanisms underlying its beneficial effect improving olfactory dysfunction. The OB is the first brain region involved in the processing of olfactory information, being highly plastic, undergoing cellular/molecular dynamic changes that may be modulated by sensory stimulus [85]. The OB plasticity is due to the fact that it receives newly generated neurons throughout life that are continuously added to different layers and integrated into pre-existing OB circuits

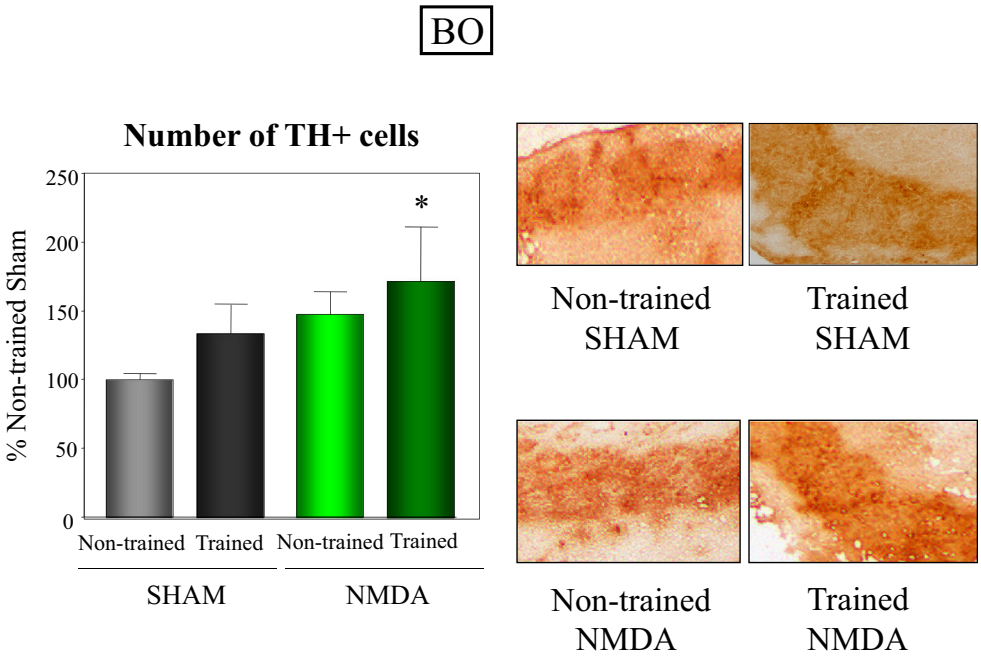


**Fig. 8** Effect of olfactory training (OT) on periglomerular OB dopaminergic TH-positive cell population at 1 week after lesions expressed as the percentage of the non-trained sham-lesioned group. Animals received three bilateral NMDA (1.5 μl of a saline solution of 12 mg/ml) or vehicle OB administrations. Left: quantification of TH+ cells. Right: representative immunohistochemical staining of OB coronal sections. Data are expressed as mean ± SEM. *$p < 0.05$ vs non-trained sham group

[86–91]. The new cells originate from a periventricular region called the subventricular zone (SVZ) and migrate via the rostral migratory stream (RMS) to populate the OB throughout life [86, 90, 92, 93]. The newly generated cells acquire the electrophysiological properties of bulbar interneurons [94], being differentiated into gamma-aminobutyric acid (GABA) ergic and dopaminergic local interneurons, granule and periglomerular cells, before integrating into the olfactory functional circuitry [86, 92, 94, 95], suggesting that they may sustain olfactory functions [93, 96, 97].

In the present study, the improvement in the olfactory function induced by OT in bilateral OB NMDA–lesioned animals was associated with an increase in SVZ neurogenesis since neural precursor cells proliferation and migrating neuroblasts were increased. Our results suggest that OT stimulates SVZ cell proliferation and migration, resulting in the recovery of the olfactory function. These observations are in agreement with previous observations showing that a simple manipulation of olfactory experience, such as odor-enrichment and odor-discrimination learning, can modify the main OB neural network by increasing the number of newborn neurons, regulating the maturation and survival of adult-born OB interneurons [95, 98–100]. A learning period of only 6 days has been shown to be clearly sufficient for substantially better survival rates of newly generated neurons [95]. In addition, in unilaterally naris-occluded mice, the apoptotic rate of newborn GCs is increased on the closed side of the OB [99, 100]. Consistent with this observation, odor detection and odor-discrimination learning are reportedly impaired in mice with diminished adult neurogenesis in the OB [101]. In addition, it has been described that odor deprivation and odor-enriched environments suppress and facilitate, respectively, dendritogenesis and spinogenesis in newborn OB interneurons [96, 100, 101]. Our present results are also in agreement with our previous data showing that the spontaneous recovery of the olfactory deficit occurring 2 weeks after OB NMDA lesion is also associated with an increase in SVZ neural precursor cell proliferation and migrating neuroblasts [15].

In our study, the improvement in the olfactory function and the increase in SVZ neurogenesis induced by OT in NMDA-lesioned animals have been found associated with an increase in the number of dopaminergic tyrosine hydroxylase (TH)–positive interneurons in the periglomerular OB layer. TH is the limiting enzyme required for the synthesis of dopamine and is a marker for mature dopaminergic interneurons in the glomerular layer of the OB [15, 46, 100, 102, 103]. Our results suggest a relevant role for the periglomerular dopaminergic neurons in the recovery of olfactory function induced by OT. Several pieces of data indicate high plasticity of the OB dopaminergic neurons in response to the manipulation of the olfactory pathway. Indeed, TH expression in dopaminergic neurons is strongly and reversible downregulated in animals subjected to odor deprivation by either chemical or surgical sensory deafferentation of the OB [103–105]. In the same line, naris occlusion selectively reduces the number of adult-generated dopaminergic cell OB [100, 103], which is recovered after naris reopening, suggesting that periglomerular dopaminergic neurons depend on olfactory input for their survival [99]. A relevant role of the periglomerular dopaminergic interneurons in olfactory function has been previously suggested. Thus, a paradigm of 2 months olfactory enrichment with different aromatic fragrances, previously shown to affect OB neurogenesis and olfactory memory, resulted in a selective increase in the TH-positive dopaminergic population [85]. In

addition, the present observation is in agreement with our recent previous data showing that the spontaneous recovery of the olfactory deficit that occurs 2 weeks after OB NMDA–induced lesion is also associated with an increase in dopaminergic bulbar neurons [15].

In summary, the present results show that OT reverses the olfactory dysfunction observed in bilateral OB NMDA–lesioned animals during the first week after lesion. The improvement in olfactory function induced by OT was associated with the presence of an increase in SVZ neurogenesis, since an increase in progenitor cell proliferation and migrational neuroblasts was observed. In addition, an increase in OB periglomerular dopaminergic TH-positive neurons was found associated with the improvement of the olfactory function and with the increase in SVZ neurogenesis. The present results suggest that the increase in dopaminergic interneurons may be involved in the improvement of discriminatory olfactory function achieved by OT, reinforcing the notion that plasticity of the dopaminergic neurons is required for OB circuit functions.

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