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Recovery of Olfactory Function After Excitotoxic Lesion of the Olfactory Bulbs Is Associated with Increases in Bulbar SIRT1 and SIRT4 Expressions

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Abstract

Excitotoxicity consists in a cascade of intracellular events initiated by an excessive release of glutamate and hyperactivation of glutamatergic receptors that is involved in several pathologies, including traumatic brain injury and neurodegenerative diseases such as Parkinson's disease. Both disorders are a common cause of olfactory dysfunction. We previously reported a role for glutamate excitotoxicity in olfactory dysfunction showing an olfactory deficit 1 week after lesion and a spontaneous recovery 2 weeks after excitotoxicity lesion of the olfactory bulbs (OBs). The olfactory dysfunction recovery was associated with an increase in subventricular zone neurogenesis and an increase in the OB glomerular dopaminergic interneurons. However, the underlying molecular mechanisms involved in the OB dopaminergic differentiation and olfactory recovery are still unknown. To investigate the role of silent information regulator family proteins sirtuins (SIRTs), a family of NAD+-dependent histone deacetylases, on the olfactory function recovery, we examined the OB SIRT (SIRT1, SIRT2, and SIRT4) expressions after OB excitotoxic lesions in rodents. *N*-methyl-D-aspartate (NMDA) OB administration induced a decrease in the number of correct choices in the discrimination tests 1 week after lesions ($p < 0.01$) and a spontaneous recovery of the olfactory deficit 2 weeks after lesions ($p < 0.01$) associated with an increase in OB SIRT1 and SIRT4 expression. Our results point out for the first time the association between recovery of olfactory function and the increase in bulbar SIRT1 and SIRT4 expression suggesting a role for these SIRTs in the pathophysiology of recovery of loss of smell.

Keywords Excitotoxicity · Olfaction · Dopamine · Sirtuins · Parkinson's disease · Traumatic brain injury · Neurogenesis

Introduction

Excitotoxicity consists in a cascade of intracellular events initiated by an excessive release of glutamate and hyperactivation of glutamatergic receptors involving both, intracellular pathways and cell-to-cell interactions, and promoting neuronal death [1–3]. The activation of glutamatergic *N*-methyl-D-aspartate (NMDA) receptors and resultant intracellular calcium overload, accumulation of mitochondrial calcium, and reactive oxygen species (ROS) are considered pivotal factors of glutamate excitotoxicity [4–6]. An accumulation of extracellular glutamate is involved in several pathological conditions affecting the central nervous system, including traumatic brain injury (TBI) and neurodegenerative diseases, such as Parkinson's disease (PD) [7–14], in which olfactory dysfunction is an early symptom [15–21].

The possible involvement of glutamate in olfactory function has been suggested [22]. NMDA receptor subunits and vesicular glutamate transporters are extensively expressed throughout the olfactory bulbs (OBs) and olfactory epithelium [22–26]. Within the OB, glutamate is released by axons of olfactory sensory neurons [22, 23], and by mitral and tufted cells, which establish dendrodendritic synapses with several types of interneurons [24, 25]. In addition, our group has recently showed that the bilateral administration of the glutamate agonist, NMDA, in the OBs induces an olfactory dysfunction with a spontaneous recovery [27] in rodents. An increase in subventricular zone (SVZ) neurogenesis was found in association with the olfactory recovery [27]. Moreover, a role for the

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periglomerular dopaminergic (DAergic) interneurons on the recovery of olfactory function was pointed out since an increase in the number of DAergic neurons was found [27]. However, the molecular mechanisms involved in olfactory recovery and the DAergic neuronal differentiation in the OB are still unknown. The knowledge of these mechanisms will allow develop therapeutic strategies focused on these molecular targets in order to improve olfactory dysfunction in neurological diseases.

Silent information regulator family proteins, sirtuins (SIRTs), are a family of NAD+-dependent histone deacetylases believed to play an important role in longevity, aging, metabolism, and cellular stress resistance [28–33]. SIRTs interact with multiple signalling proteins and transcription factors [32–34]. In mammals, seven SIRTs have been identified (SIRT1–SIRT7) [32, 35, 36], being localized in different subcellular compartments: nucleus, cytoplasm, and mitochondria according to their substrates and functions [37]. SIRT1 is a nuclear protein [38, 39], whereas SIRT2 is a cytoplasmic protein [40] and both, SIRT1 and SIRT2, can shuttle between nucleus and cytoplasm under different stimulus [41–43]. SIRT3–SIRT5 are mitochondrial sirtuins, whereas SIRT6 and SIRT7 are nucleolar [39]. Some SIRTs also possess additional enzymatic activities such as ADP-ribosylation, the ability to remove a wide array of other lysine modifications, and/or lack detectable deacetylation capability (SIRT4) [44, 45].

SIRTs are stress response genes that are activated when an organism is subjected to insults such as hypoxic or oxidative stress [34–46]. All SIRTs are present in the brain in a highly regulated, spatiotemporal pattern, and through these functions, SIRTs modulate fundamental mechanisms in aging-related neurodegenerative diseases, including protein aggregation, mitochondrial homeostasis, stress responses, and inflammatory processes [29, 47–50]. Moreover, SIRTs and their pharmacological activators/inhibitors have been shown to possess neuroprotective properties in a variety of pathological conditions such as PD, Alzheimer's disease (AD) and Huntington's disease (HD) among others [51–53], being a promising therapeutic target in neurodegeneration.

Although SIRTs expression have been reported in the OBs, their role in excitotoxicity and olfactory function is still unknown. In order to investigate the possible role of SIRTs on the olfactory function recovery, we examined the OB SIRTs (SIRT1, SIRT2, and SIRT4) expression 1 or 2 weeks after bilateral NMDA-induced OB excitotoxic lesion in rodents.

Material and Methods

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 them to keep rats at approximately 85% of their body weight over the behavioural testing period [27].

Animals were olfactory trained for 5 days. A first olfactory discrimination test (test-1) was performed after training and then distributed in two groups that received NMDA or vehicle (Sham) OB administrations, as described below. The olfactory discrimination tests were repeated one (test-2) and 2 weeks (test-3) after lesions (Fig. 1a). Animals were sacrificed after test-2 or test-3 in order to perform the immunohistochemical studies.

NMDA Lesions

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and were 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, $n = 13$) or saline (Sham-lesioned group, $n = 7$), injected at 30 μl/h rate over a 3-min period [27, 54, 55]. 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 3rd injection), according to the atlas of Paxinos and Watson (1986, [56]) [27, 57–59]. In all cases, 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 volume (4.5 μl of NMDA solution) injected in the NMDA-lesioned group does not spread to other areas of the brain [27, 60].

Olfactory Training and Discrimination Tests

Odour Stimuli

Olfactory stimuli consisted of powdered odourants (cinnamon and vanilla) mixed in sand (4 g of odourant in 80 g of sand) and presented in clear plastic cups (3 cm diameter and 3 cm high) as previously described [27, 61–64]. 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 [27, 61].

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: The first day each animal—in its own cage—was presented with a small cup containing 80 g of sand. The second day, the animal was

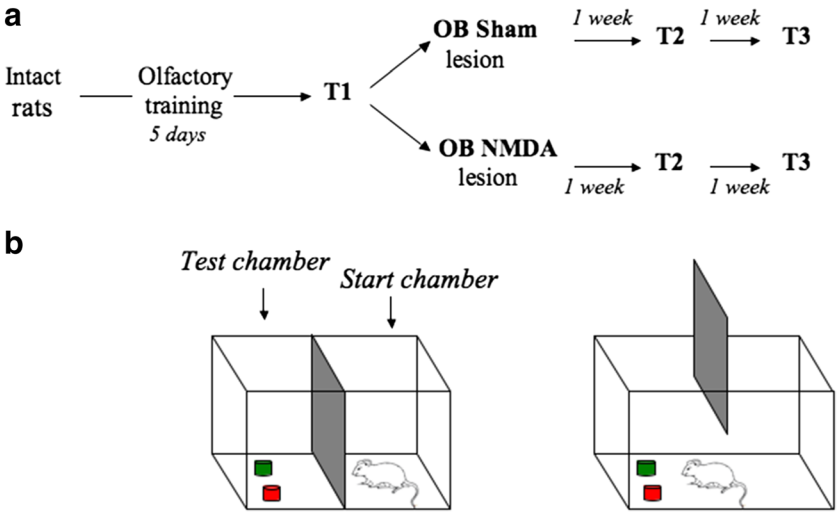

Fig. 1 **a** Experimental design: Intact rats were olfactory trained for 5 days. Before lesions, an odour discrimination test (T1) was performed in all animals. Animals were randomly distributed in experimental groups receiving three bilateral NMDA (1.5 μl of a saline solution of 12 mg/ml) or vehicle (Sham) OB administrations. One (T2) and two (T3) weeks after NMDA and Sham lesions, odour discrimination tests were performed. Animals were sacrificed after T2 or T3 olfactory tests. **b** Experimental setup for odour 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 odour rather than by learning the association with a training odourant

presented with a reward on top of the sand in the cup [60]. From the third day onwards, the animal was trained in a custom testing cage (Fig. 1b) [58] 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 rats capable of daily finding the reward were considered trained animals [27, 63, 64].

Olfactory Discrimination Tests

The olfactory discrimination task involved conditioning subjects to discriminate between two simultaneously presented odours, one rewarded (cinnamon) and one not rewarded (vanilla), between which the subject must choose to obtain the reward [27, 60, 65]. Each test consisted of 15 consecutive discrimination trials using the same pair of odourants.

All discrimination olfactory testing occurred in a custom methacrylate cage that was split into a start chamber and a test chamber by an opaque divider panel (Fig. 1b) [27, 58, 66]. 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 [27, 66]. The amount of time that the rat spent to achieve and actively investigating the correct odourant was measured with a stopwatch. Active investigation was defined as directed sniffing within 1 cm of the odour source presented [27, 57, 58].

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 [27, 60]. To control for the possibility of rats locating the cereal reward through its own odour rather than by learning the association with a training odourant, we performed every fifth trial without any reward present (trials 5, 10, and 15) [27, 66]. 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 choices (trials).

Tissue Collection

The day after the last olfactory test (1 or 2 weeks after NMDA lesions), the animals were sacrificed under an overdose of pentobarbital anaesthesia. OBs were quickly removed from the skull, 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° until needed.

Histological Analysis

Nissl Staining

OB sections were thawed at room temperature for 30 min, fixed with acetone for 10 min at 4 °C, and rinsed twice in phosphate-buffered saline (PBS) for 5 min each. Sections were then dehydrated in ascending alcohol concentrations and cleared in xylene. The samples were then hydrated in alcohol followed by distilled water. Once the sections were hydrated, they were immersed in a thionin acetate solution for 20 s followed by an immersion in a differentiating solution for another 20 s. Immediately after, the sections were rinsed twice in distilled water. Lastly, the samples were dehydrated in ascending alcohol concentrations, cleared in xylene, and coverslipped in dibutylphthalate polystyrene xylene (DPX) mounting medium.

Immunohistochemistry

OB sections were processed for immunohistochemistry, according to a standard peroxidase-based method [27]. 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 the monoclonal anti-neuronal nuclear antigen (NeuN, 1:500, MAB377 Millipore), the polyclonal anti-glial fibrillary acidic protein (GFAP, 1:500, AB5804 Millipore), the polyclonal anti-sirtuin-1 (SIRT1, 1:100, Santa Cruz Biotechnology, Inc. #sc-15,404), the polyclonal anti-sirtuin-2 (SIRT2, 1:100, Santa Cruz Biotechnology, Inc. #sc-20,966), and the polyclonal anti-sirtuin-4 (SIRT4, 1:100, Santa Cruz Biotechnology, Inc. #sc-135,053) 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 variable. Quantifications of OB SIRT1, SIRT2, and SIRT4 immunohistochemistries were undertaken by assessing the intensity of staining (five grades): 0 = no detectable immunoreactive cells, 1 = very low density of positive cells, 2 = moderate density of positive cells, 3 = high, but not maximal density of positive cells, and 4 = maximal density of positive cells, as previously reported [67].

Statistical Analysis

Data from olfactory discriminatory tests were analysed by ANOVA followed by Dunnett's t test for multiple comparisons. In the immunohistochemical analysis, differences between two experimental groups were determined by unpaired Student's t test. The level of statistical significance was set at $p < 0.05$ for all analyses.

Results

Histological Characterization of the Bilateral OB Lesions Induced by NMDA Administration

To estimate the extent of cellular damage, OB sections from Sham and NMDA-injected animals were processed for Nissl stain and immunohistochemistry for NeuN and GFAP (Fig. 2). Sham animals showed a normal laminar structure and absence of glial activation (Fig. 2). In the NMDA-lesioned OB, 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. NMDA injections resulted in neural injury as indicated by neuronal loss and increased GFAP immunoreactivity (Fig. 2).

Effect of Bilateral OB Lesions Induced by NMDA on Olfaction Function

Before NMDA or saline bilateral OB administration (test-1), no differences were observed in the number of correct responses (Fig. 3) between NMDA and Sham-lesioned groups.

One week after saline administration (test-2), sham animals did not show changes in the number of correct responses (Fig. 3) when comparing with their respective test-1. However, NMDA-lesioned animals showed an olfactory dysfunction decreasing the number of correct responses by 62% when compared with their respective test-1 ($p < 0.01$) and with Sham-lesioned group ($p < 0.01$) (Fig. 3).

Two weeks after NMDA or saline administration (test-3), Sham-lesioned animals did not show significant changes in the number of correct responses (Fig. 3). A reversion of the olfactory dysfunction was observed in the NMDA-lesioned group, increasing the number of correct responses when compared with their respective test-2 ($p < 0.01$) (Fig. 3).

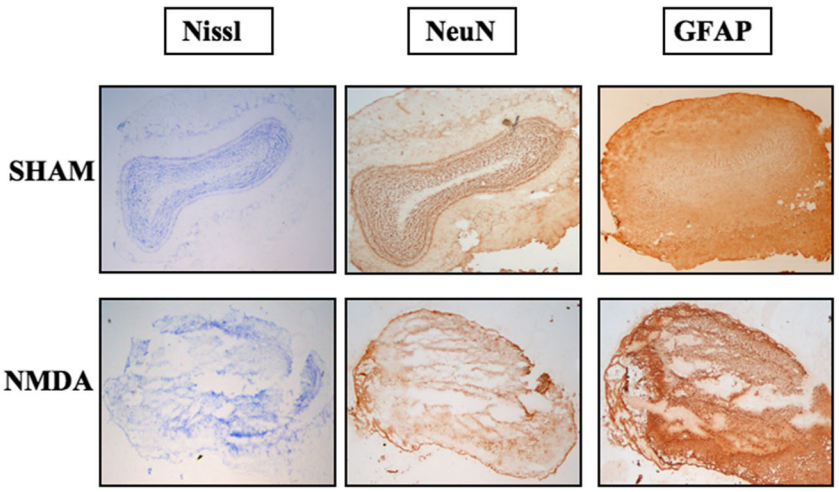

Fig. 2 Histological characterization of excitotoxic lesion degree induced by the OB NMDA administration. Representative Nissl, NeuN, and GFAP-immunoreactive staining, performed 2 weeks after lesions, from 14-μm coronal OB sections. Animals received bilateral NMDA (1.5 μl of a saline solution of 12 mg/ml, three injections) or vehicle OB administrations

Effects of Bilateral OB NMDA-Induced Lesions on SIRT Expression

To investigate the potential role of SIRTs in the recovery of olfactory function, we assessed the changes in the OB sirtuins expression induced by the NMDA administration by immunohistological studies for SIRT1, SIRT2, and SIRT4 1 and 2 weeks after lesions (Fig. 4).

A markedly SIRT1 expression was predominantly found in mitral and granular cells layers in Sham-lesioned animals (Fig. 4). An increase in the expression of SIRT1 in the affected OB layers was observed 2 weeks, but not 1 week, after NMDA lesions (Fig. 4).

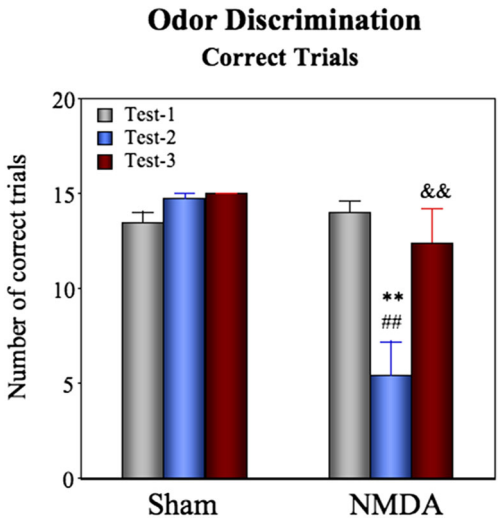

Fig. 3 Effect of bilateral NMDA-induced excitotoxic OB lesions on odour discrimination expressed as the number of correct choices. Animals received bilateral NMDA (1.5 μl of a saline solution of 12 mg/ml, three injections) or vehicle OB administrations. Olfactory discrimination tests were performed before (test-1), 1 (test-2) and 2 (test-3) weeks after NMDA ($n = 8$) or Sham lesions ($n = 7$). Each test consisted of 15 trials. Data are expressed as mean ± SEM. $^{**}p < 0.01$ vs Sham; $^{\#\#}p < 0.01$ vs test-1; $^{\&\&}p < 0.01$ vs test-2

A light SIRT2 expression was present in mitral layer, being moderate expressed in the internal plexiform layer, in Sham-lesioned animals (Fig. 4). No changes in SIRT2 expression were observed in NMDA-lesioned OBs (Fig. 4).

A markedly SIRT4 expression was predominantly expressed in mitral layer in Sham-lesioned animals (Fig.4). An increase in the expression of SIRT4 in the affected OB layers was observed 2 weeks, but not 1 week, after NMDA lesions (Fig. 4).

Discussion

In order to investigate the possible involvement of SIRTs in the recovery of olfactory function, we studied the OB expression of SIRT1, SIRT2, and SIRT4 one and 2 weeks after bilateral OB NMDA-induced lesions. In agreement with our previous results, the present results confirm that bilateral NMDA OB administration induces a dose-related decrease in the olfactory function 1 week after NMDA-induced lesion, and a spontaneous recovery of the olfactory deficit 2 weeks after lesions [27]. In the present study, we demonstrate that an increase in OB SIRT1 and SIRT4, but not SIRT2, expression was associated with the recovery of the olfactory function occurring 2 weeks after bilateral NMDA-induced OB excitotoxic lesion.

Loss of OB neurons was verified by immunostaining for the nuclear protein NeuN, a neuronal marker [54]. As previously described for unilateral and bilateral NMDA OB administrations, NMDA infusions produced cell loss [27, 54, 68]. NMDA-injected animals had profuse GFAP positive immunoreactivity seen in all cell layers of the bulbs, indicative of cellular inflammation.

Our present results showed that bilateral NMDA OB administration induced a decrease in the olfactory function evidenced by a diminished number of correct trials in the

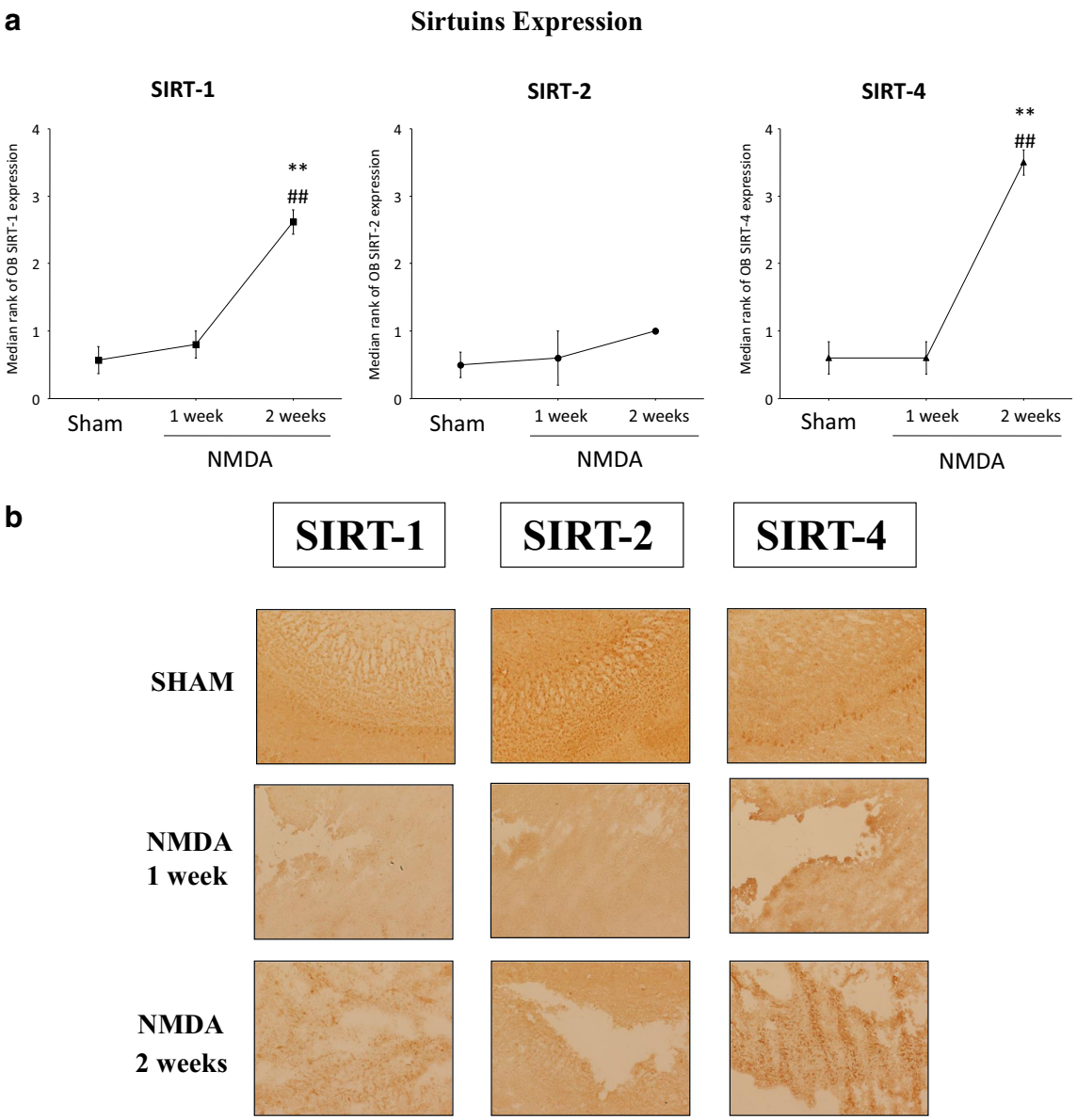

Fig. 4 Effect of bilateral NMDA-induced excitotoxic OB lesions on OB SIRT1, SIRT2, and SIRT4 expression 1 ($n = 5$) and 2 ($n = 8$) weeks after lesions. Animals received bilateral NMDA (1.5 μl of a saline solution of 12 mg/ml, three injections) or vehicle OB administrations. **a** Quantification of OB SIRT1, SIRT2, and SIRT4 expressions. **b** Representative SIRT1, SIRT2, and SIRT4 staining of OB coronal sections. Data are expressed as mean ± SEM. $^{**}p < 0.01$ vs Sham, $^{\#\#}p < 0.01$ vs NMDA 1-week group

discrimination olfactory tests 1 week after NMDA-induced lesion in rats, suggesting that the NMDA-induced glutamate excitotoxicity may be the cause of the olfactory deficit [27]. A spontaneous reversion of the olfactory deficit was obtained 2 weeks after NMDA-induced lesions, as previously reported [27]. The presence of an olfactory function recovery in the bilateral OB NMDA-lesioned animals performed in the present study allows to specifically investigate the molecular mechanisms involved in the recovery of olfactory neural circuits.

In the present study, SIRT1 expression was found in the mitral and granular cell layer in the OB. Interestingly, 2 weeks after the excitotoxic OB lesion induced by the bilateral administration of NMDA, an increase in OB SIRT1 expression was found in association with a spontaneous recovery of the olfactory function. Our results suggest that SIRT1 expression may be related with the olfactory improvement, suggesting a neuroprotective or neurorestaurative role for SIRT1.

SIRT1 is the best-characterized SIRT, being widely expressed in the adult brain, especially in the cortex, hippocampus, hypothalamus, and cerebellum [33, 48, 69]. Most of this expression is neuronally localized [69, 70], although SIRT1 is also found in neural stem cells, neural progenitor cells [71, 72], cultured microglia and astrocytes, as well as glial cells [67, 73, 74]. SIRT1 has been reported as an important regulator of several physiological processes including

transcription, apoptosis, cell survival, DNA repair, inflammation, and oxidative stress through the deacetylation of intracellular signalling molecules and histones [29, 43, 67], promoting neurite outgrowth, axon development [72], and regulating long-term potentiation, learning, and memory [35, 75]. In addition, SIRT1 activation is involved in stem cells differentiation and synaptic plasticity [33, 36, 48, 49, 69].

Supporting our results, growing evidences suggest a beneficial role for SIRT1 in excessive glutamate-associated acute neuronal injuries resulting from traumatic brain injury, ischemia, and in a range of neurodegenerative diseases [33, 43, 67, 76–83]. Thus, as occurring in the present study, SIRT1 was upregulated in the peri-infarct area in mice models of cerebral ischemia [83]. Moreover, although a report did not show neuroprotective effects in SIRT1 transgenic mice [84], mice overexpressing SIRT1 or treatment with the SIRT1 activators reduced infarct volume in animal models of ischemia [78, 85–87]. In this line, activation of SIRT1 has been shown to exhibit neuroprotective activities in PD models [43, 52, 73, 88], Alzheimer's disease (AD) [89, 90], Huntington's disease (HD) [79, 91], amyotrophic lateral sclerosis [92], and prion diseases [93, 94]. In PD models, SIRT1 over-expression suppresses the formation of α-syn aggregates [43, 48, 95]. Interestingly, the SIRT1 activator resveratrol, together, with quercetin, prevented the decrease in DAergic neurons induced by MPP+ in organotypic midbrain slice cultures [88]. Moreover, SIRT1 activation has been associated with the neuroprotection induced by curcumin against glutamate excitotoxicity in cortical neurons [6]. On the other hand, pharmacological SIRT1 inhibition by sirtinol or salermide or SIRT1 siRNA exacerbates brain injury by increasing oxidative stress, neuroinflammation, and apoptosis [33, 67, 77] and worsened the outcome increasing infarct volume [82, 83]. All these observations indicate that SIRT1 plays an important role in excitotoxic neurodegeneration, and therefore, its overexpression or pharmacological activation may be protective in neurodegenerative diseases and acute nervous system injury [33, 67, 77, 83]. It is worth to consider that the increase in OB SIRT1 expression, observed 2 weeks after bilateral OB NMDA-lesion, might have a role in the recovery of the olfactory function.

Interestingly, the increase in OB SIRT1 expression observed in the present study might be related with the increase in the number of OB DAergic interneurons associated to the recovery of olfactory function in NMDA-lesioned animals previously reported by our group [27]. Thus, it is worth to consider that the OB is one of the few brain structures receiving a supply of newly generated cells through adult life by the subventricular zone (SVZ) progenitor niche, which might contribute to the plasticity of the sense of smell [96–99]. It has been shown that SIRT1 is expressed by proliferating adult SVZ neural precursors [100], and it has been reported that SIRT1 plays an important role in differentiation of neural precursor cells [70, 101] through translocation into the nucleus [70, 102]. Moreover, SIRT1 upregulates tyrosine hydroxylase (TH) expression, a marker for DAergic neurons [70, 102, 103]. Thus, resveratrol increases TH expression and DA levels in the nucleus accumbens and in the striatum in mice [104], whereas the SIRT1 inhibitor, nicotinamide, reduced the neuronal differentiation and TH levels in cultured neuroblastoma cells [102]. In addition, SIRT1 was found to positively modulate the Wnt signalling pathway [105], which is deeply implicated DA neurogenesis and differentiation [49, 106].

In the present study, sham-lesioned animals showed a moderate SIRT2 expression in the internal plexiform layer in the OB where the deep short-axon cells that integrate centrifugal cholinergic inputs are located [107]. Two weeks after the excitotoxic OB lesion induced by the bilateral administration of NMDA, no changes in OB SIRT2 expression were found. Our results suggest that SIRT2 expression may be not related with the olfactory improvement. The lack of modification of OB SIRT2 expression after NMDA lesions observed in the present study is in agreement with the observation that modulation of SIRT2 activity may be important only in the context of particular neuronal stress [108]. It is now considered that SIRT2 may play different roles in different cell types and during different stages of development/aging.

In the present study, SIRT4 expression was mainly found in the mitral layer. Interestingly, 2 weeks after the excitotoxic OB lesion induced by the bilateral administration of NMDA, an increase in OB SIRT4 expression was found in association with a spontaneous recovery of the olfactory function. Our results suggest that SIRT4 expression may be related with the olfactory improvement, suggesting a neuroprotective or neurorestaurative role for SIRT4.

In agreement with our present results, SIRT4 has been described up-regulated following treatment with the excitotoxin kainic acid [109]. An anti-excitotoxic role for SIRT4 promoting proper glutamate transport capacity has been described [109]. SIRT4 interacts with and represses glutamate dehydrogenase (GDH) activity to downregulate the metabolism of glutamate and glutamine to limit ATP production [110, 111]. SIRT4-mediated blockade of glutamine catabolism is an essential component of DNA repair response [6, 82]. Moreover, loss of SIRT4 increases sensitivity to excitotoxic insults leading to a more severe reaction of kainic acid and decreasing glutamate transporter GLT1 expression and overall brain function [82, 109]. All these observations might suggest a role for SIRT4 expression in the recovery of olfactory function observed in the OB NMDA-lesioned animals.

In summary, our present results point out for the first time the association between an increased bulbar SIRT1 and SIRT4 expression and the recovery of the olfactory dysfunction induced by the bilateral OB excitotoxic lesion. These observations suggest a role for these SIRTs in the pathophysiology of

recovery of loss of smell. Although further investigations will be required for the rational use of SIRT1 and SIRT4 activators as a therapeutic target on olfactory dysfunction, current data shed new light on the molecular pathways that might be involved in the recovery of olfactory function.

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Compliance with Ethical Standards

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 Ethics Committee of our institution approved this study.

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