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Chronic inhibition of nitric oxide synthesis enhances both subventricular zone neurogenesis and olfactory learning in adult mice

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Abstract

The ability to generate new neurons during the course of adult life is preserved in the subventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus in the mammalian brain. These two regions constitute specifically regulated neurogenic niches, and provide newborn neurons involved in olfactory and spatial learning, respectively. Nitric oxide (NO) is a negative regulator of neurogenesis in the subventricular zone, whereas its role in the dentate gyrus remains controversial. Using systemic administration of NO synthase (NOS) inhibitors to chronically inhibit NO production, we increased neural precursor proliferation in the subventricular zone as well as neurogenesis in the olfactory bulb, without modifying the number of mitotic cells or the granular cell layer thickness in the dentate gyrus. The same treatment specifically improved olfactory learning performance, whereas spatial learning and memory was unchanged, thus demonstrating that olfactory memory is closely associated with the level of ongoing neurogenesis in the subventricular zone–olfactory bulb. The anatomical specificity of the NOS inhibitor actions was not due to differences in the availability of NO, as demonstrated by immunohistochemical detection of neuronal NOS and S-nitrosylated proteins in both regions. Remarkably, the distinct NO sensitivity might result from a differential expression of epidermal growth factor receptor in precursor cells in both regions, as the proliferative effect of NOS inhibitors in the subventricular zone was restricted to the cells that expressed this receptor.

Introduction

In the adult brain, new neurons are generated from neural stem cells in the dentate gyrus (DG) of the hippocampus and in the subventricular zone (SVZ) surrounding the lateral ventricles (Gage, 2000; Alvarez-Buylla *et al*., 2002). In the DG, precursors divide in the subgranular zone (SGZ) and become mature neurons in the granular cell layer (GCL). In the SVZ, newly generated neuroblasts migrate tangentially within the rostral migratory stream (RMS), and then radially in the main olfactory bulb (OB) to differentiate into inhibitory granular and periglomerular interneurons that become integrated in olfactory circuits (Belluzzi *et al*., 2003; Carleton *et al*., 2003). Although the physiological role of continuous neuronal replacement in these two regions is not fully understood, adult neurogenesis may represent a new form of neuronal adaptation involved in OB and hippocampus-dependent learning processes, respectively. A first line of evidence supporting this hypothesis is that neurogenesis can be modulated by environmental conditions that concomitantly modify learning performance. Thus, animals exposed to odour-enriched environments exhibit an increased number of newly generated neurons in the OB, and enhanced olfactory memory (Rochefort *et al*., 2002). In contrast, nostril occlusion impairs bulbar neurogenesis (Murray & Calof, 1999). In line with this, exercise or complex environments increase the number of newly generated neurons in the DG and improve performance in a spatial navigation task (Kempermann *et al*., 1997; Kempermann & Gage, 1999; van Praag *et al*., 1999). Conversely, conditions known to specifically impair neurogenesis result in cognitive deficits. For instance, mice null for the neural cell adhesion molecule NCAM, or for transforming growth factor (TGF)α, exhibit reduced neurogenesis in the SVZ and altered fine olfactory discrimination (Gheusi *et al*., 2000; Enwere *et al*., 2004). Also, adrenal steroids and stress, which are negative regulators of hippocampal neurogenesis, have been associated with impaired performance in hippocampal-dependent learning tasks (Bodnoff *et al*., 1995; Krugers *et al*., 1997). However, it has not been demonstrated yet whether substances promoting adult neurogenesis also increase learning and/or memory. Similarly, it is not clear whether specific modulation of the degree of neurogenesis, either in the SVZ or the SGZ, results in selective changes of performance in olfactory and spatial learning tests, respectively.

Nitric oxide (NO) produced by nitrergic neurons is a negative regulator of adult neurogenesis. Systemic or local administration of NO synthase (NOS) inhibitors enhances precursor proliferation in the SVZ (Cheng *et al*., 2003; Packer *et al*., 2003; Moreno-López *et al*., 2004; Sun *et al*., 2005), whereas its effects on the DG are variable, depending on the experimental conditions (Park *et al*., 2001, 2003; Packer *et al*., 2003; Moreno-López *et al*., 2004; Sun *et al*., 2005). In this work, we have chronically inhibited NO synthesis in adult mice, using a paradigm that selectively enhances precursor proliferation in the SVZ–OB system, without affecting the DG. We found that these animals performed better in olfactory, but not spatial, memory tests.

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Finally, we report that the selective responsiveness of SVZ precursors is due to their expression of epidermal growth factor (EGF) receptors, a molecular target of NO in cultured cells (Murillo-Carretero *et al.*, 2002).

Materials and methods

Subjects

Adult CD1 male mice (2–4 months old) were used throughout the study. Care and handling of animals were in accordance with the Guidelines of the European Union Council (86/609/EU), following the Spanish regulations (BOE 67/8509-12, 1988) and the French Ethical Committee recommendations for the use of laboratory animals. During the treatment period, animals were allowed access to food and water *ad libitum*, and were kept on a 12 h light : dark cycle at constant temperature.

Materials

The odours used were: 1-hexanol, +limonene, +carvon, valeric acid, m-xylol, 1-pentanol, butyraldehyde and isoaminobutyl, from Fluka (Buchs, Switzerland), cineole and anisole, from Sigma (Saint Louis, MO, USA), +terpinel 4-ol, from Interchim (Montlucon, France).

Administration of NOS inhibitors

Mice were injected with the broad-spectrum NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME; 90 mg/kg/day, i.p.) or the inactive stereoisomer D-NAME (90 mg/kg/day, i.p.) for 1–5 weeks. As previously reported (Moreno-López *et al*., 2004), *ex vivo* measurement of cerebral NOS activity indicated that this L-NAME administration protocol maintained NOS activity under 45% of the control value all over the treatment duration. The relatively specific nNOS inhibitor 7-nitroindazole (7-NI) was also administered for 4 days, at doses reported to significantly decrease cerebral NOS activity (30 mg/kg, i.p.) (Yoshida *et al*., 1994). Due to the transient effect of this drug, injections were repeated every 12 h. In all cases, control animals received the corresponding vehicle [phosphate-buffered saline (PBS) for L- and D-NAME, or 10% dimethylsulphoxide in 1, 2-propanediol for 7-NI] with the same time sequences.

Bromodeoxyuridine (BrdU) administration

To determine the role of endogenous NO on neural precursor proliferation, mice were treated with either L-NAME or D-NAME for 7 days, or with 7-NI for 4 days. The last day of treatment, the thymidine analogue BrdU was administered to label proliferating cells, as previously described (Moreno-López *et al*., 2004). A total of 200 mg/kg, distributed in three doses separated by 2.5-h intervals, was administered intraperitoneally, and the animals were killed 2.5 h after the last BrdU injection (7.5 h cumulative labelling).

Immunohistochemistry

Control and treated mice were deeply anaesthetized with chloral hydrate (0.5 g/kg, i.p.) and transcardially perfused with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, postfixed for two additional hours and cryoprotected by immersion in 30% sucrose solution overnight. Serial coronal sections (30 μm thick) of the SVZ, OB and hippocampus were obtained using a cryostat and stored at −20 °C in a cryoprotectant solution (glycerol and PBS, pH 7.4, 1 : 1 in volume). For BrdU detection, free-floating cerebral sections were subjected to DNA denaturation by treatment with a solution containing 50% (v/v) formamide, 150 mM NaCl and 15 mM sodium citrate at 65 °C for 2 h, followed by a 30-min incubation in 2 N HCl at 37 °C (Kuhn *et al*., 1997). For single BrdU immunohistochemistry, sections were first treated with 2% H_2O_2 and 60% (v/v) methanol in PBS for 30 min, to block endogenous peroxidase activity, and then incubated for 30 min with a solution containing 2.5% (w/v) bovine serum albumin, 0.25% (w/v) sodium azide and 0.1% (v/v) Triton X-100 in PBS (PAAT) to prevent non-specific antibody binding; this solution was also used to dilute the primary and secondary antibodies. Incubation with the primary anti-BrdU antibody (1 : 100, mouse monoclonal; Dako, Glostrup, Denmark) was carried out overnight at 4 °C. After several rinses in PBS, sections were treated for 1 h at room temperature with a biotinylated anti-mouse IgG secondary antibody (1 : 250, Sigma), and exposed to the avidin-biotin-peroxidase complex (Pierce, Rockford, IL, USA). The peroxidase reaction was made visible with diaminobenzidine (0.25 mg/mL) and hydrogen peroxide (0.003%, v/v). Sections were then mounted on slides, dehydrated, coverslipped with DePeX and analysed under light microscopy.

Immunofluorescence was used to reveal the presence of doublecortin or S-nitrosylated proteins, as well as to detect simultaneously nNOS and the polysialylated form of NCAM (PSA-NCAM), or BrdU and the EGF receptor (EGFR). For that, free-floating brain sections were treated, when necessary, for DNA denaturation, and incubated with PAAT for 30 min at room temperature. Incubations with the two primary (overnight, 4 °C) or secondary (1 h at room temperature) antibodies were performed simultaneously, when necessary. After washing with PBS, the sections were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA, USA), and fluorescent signals were detected using a BX60 Olympus epifluorescence microscope and a Leica Spectra confocal microscope. The primary antibodies used were: goat polyclonal doublecortin (1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-nitrosothiol (S-NO) 1 : 400, AlphaDiagnostics, San Antonio, TX, USA, rat monoclonal anti-BrdU antibody (1 : 100, Harlan, Indianapolis, IN, USA), sheep polyclonal anti-EGFR (1 : 200; Upstate Biotechnology, Lake Placid, NY, USA), rabbit polyclonal anti-nNOS (gift from Dr J. Rodrigo, CSIC, Madrid, Spain), and mouse monoclonal anti-PSA-NCAM (IgM 1 : 4000, Chemicon, Temecula, CA, USA). Secondary antibodies used were: anti-rabbit IgG labelled with FITC or Cy5, anti-rat IgG labelled with Cy5, anti-goat IgG labelled with Cy5 and anti-sheep IgG labelled with Cy3, all of them from Jackson ImmunoResearch, West Grove, PA, USA, and anti-mouse IgM labelled with Cy3, from Sigma. All the secondary antibodies were adsorbed against several species, to prevent undesired cross-reactions. Omission of primary antibodies resulted in no detectable staining in all cases.

Quantification

Proliferating cells, detected by their BrdU-positive nuclei, were counted by optical microscopy (Nikon) using a 40 × objective, by stereological unbiased methods, as previously described (Moreno-López *et al*., 2004). Sections were obtained between the following coordinates: 0.38–1.42 mm rostral to Bregma for the SVZ, and 1.22–3.80 caudal to Bregma for the DG, according to Franklin & Paxinos (1997). For the SVZ, $BrdU^+$ nuclei were counted in predetermined areas in the dorsal, medial and ventral lateral wall of the lateral

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ventricles, in every three sections, and expressed as the number of labelled nuclei per mm^3. In the DG, all nuclei were counted in the hilus, SGZ and GCL, in every six sections, and expressed as total number per DG. The GCL volume was calculated from the areas measured in the same sections after counterstaining with neutral red, using the software MicroImage from Olympus.

The number of doublecortin-positive cells was counted in four areas (250×250 μm) of the periglomerular region, in confocal serial images of OB coronal sections counterstained with propidium iodide (five sections per mouse; five mice per condition). To calculate the density of newborn cells expressing EGFR, the percentage of $BrdU^+$ cells that were also $EGFR^+$ was quantified in confocal serial images of the SVZ (four sections per mouse; three–four mice per condition), and this percentage was applied to the total number of $BrdU^+$ cells counted in the SVZ of the same animal. The relative S-nitrosylation in control and treated animals was estimated by comparison of the mean fluorescence intensity using the Leica confocal analysis software LCS Lite (four sections per mouse; three–four mice per condition). Tissue sections from control and treated mice were always processed in parallel and quantified in blind-coded slides.

Behavioural studies

Mice were randomly assigned to two experimental groups, receiving i.p. injections of L-NAME or D-NAME at a dose of 90 mg/kg/day, for 4 (spatial learning) to 5 (olfactory memory) weeks. Animals were housed in groups of two–three per cage during the treatment period. Tests were always performed at least 12 h after L-NAME injections, to avoid the transient increase in blood pressure produced by this drug (Moreno-López *et al*., 2004).

Olfactory memory was tested during the last 2 weeks of treatment following the procedure described by Rochefort *et al*. (2002). A total of 31 mice were used in this experiment. Animals were exposed to odours by placing a glass Pasteur pipette containing odorized filter paper through the centre of one side of the mesh top of the animal's home cage so that the odorized paper was approximately 8 cm from the floor. All odours (1-hexanol, +limonene, +carvon, valeric acid, m-xylol, 1-pentanol, cineole, +terpinel 4-ol, butyraldehyde, anisole and isoaminobutyl) were dissolved in mineral oil (10^{-3} M) and freshly prepared before each experiment. In all experiments, we recorded the time that the animals spent sniffing or investigating the odour. One day before tests were run, animals were familiarized with the procedure by exposing them to mineral oil, and then to odour stimuli different from those used in the test session. Mice were submitted to two odour presentations (5 min per presentation) of the same odorant with intervals of 0.5, 4, 8, 12 and 16 h. Intervals were tested in random order in separate sessions spaced by 24 h, using different odours. Different odours were used in each test and counterbalanced across the different delay conditions. We considered that animals were able to recognize an olfactory stimulus when they spent significantly less time investigating the odour during the second presentation. To assess the specificity of odour recognition, the same animals were presented, in a separate experiment, with a first odour, followed 8 h later by exposure to a different one. Throughout all experiments, observers were blind to the treatment conditions.

To assess spatial memory, a total of 23 mice were tested in the Water maze. A circular tank (83 cm diameter) filled to a depth of 40 cm with white-coloured water at 23 ± 2 °C was used. The escape platform (8 cm diameter) was submerged 1 cm below the surface of the water. The pool was located in a room with multiple cues on all sides. Each mouse was tested during four daily trials on 5 successive days. The position of the platform was kept constant during training. During the four trials, each mouse was started once from three start positions and allowed to search for the platform. The order of start positions was randomized. The trial ended either when the animal climbed onto the platform or when a maximum of 60 s had elapsed. After the animal found the platform, it was allowed to rest on it for 10 s. If the mouse had not found the platform at the end of the trial, it was led to it and allowed to rest for 10 s. The time taken to escape onto the submerged platform (escape latency) was recorded to assess performance. A probe test was administered after the training trials on day 6. During this test the platform was removed from the pool. Each animal was started in a position opposite the location of the training platform position and allowed to swim for 90 s. The time spent by the animal in the different quadrants was recorded.

Statistics

Anatomical data were compared between the two groups using Student's *t*-tests for each location. Data obtained from the olfactory memory task and water maze were analysed using a three-way ANOVA and Student's *t*-tests for pairwise comparisons. Logarithmic transformations were applied for skewed distributions of duration. Levels of significance were set at 0.05. Data are presented as means ± SEM.

Results

Differential effects of chronic NOS inhibitors on SVZ and DG precursor proliferation

Intraperitoneal administration of 7-NI, a selective nNOS inhibitor that has no systemic vascular effects, during 4 days to adult mice significantly enhanced the number of newborn cells in the SVZ ($t = 4.51$, $P < 0.01$), without any noticeable effect in the SGZ, hilus or GCL of the DG of the same animals (respectively, $t = 0.59$, $t = 0.68$ and $t = 0.12$, $P > 0.05$) (Fig. 1A–D and G). Similarly, systemic administration of another NOS inhibitor, L-NAME, for 15 days, led to significant increases in the number of proliferating cells in the SVZ ($t = 2.64$, $P < 0.02$), but not in the hippocampus (respectively, $t = 0.06$, $t = 1.81$ and $t = 1.45$, $P > 0.05$) (Fig. 1E, F and H). As a control, we checked that administration of the inactive enantiomer D-NAME, for the same period of time, did not modify precursor proliferation in the SVZ or in the DG ($n = 4$; data not shown). Finally, we measured the GCL volumes and found that L-NAME was without effect ($t = 2.01$, $P > 0.05$) (Fig. 1I). Together, these results indicate that the systemic administration of NOS inhibitors enhances the rate of proliferation of the SVZ precursors but not the number of newly generated neurons in the SGZ of the DG.

To assess whether the increased precursor proliferation in the SVZ resulted in enhanced OB neurogenesis, the density of cells that express the immature neuron marker antigen doublecortin was measured in the glomerular layer of mice treated with L-NAME or vehicle for 15 days. Chronic NOS inhibition produced a significant increase in the doublecortin-positive cell population in the OB periglomerular region ($t = 3.11$, $P < 0.05$) (Fig. 2), thus indicating that a larger number of migrating neuroblasts have reached this region, from the SVZ.

SVZ and DG precursors were equally exposed to NO sources

The lack of effect of systemically administered NOS inhibitors in the hippocampus might result from the absence of local production of NO in the proximity of the SGZ. To test this hypothesis, the

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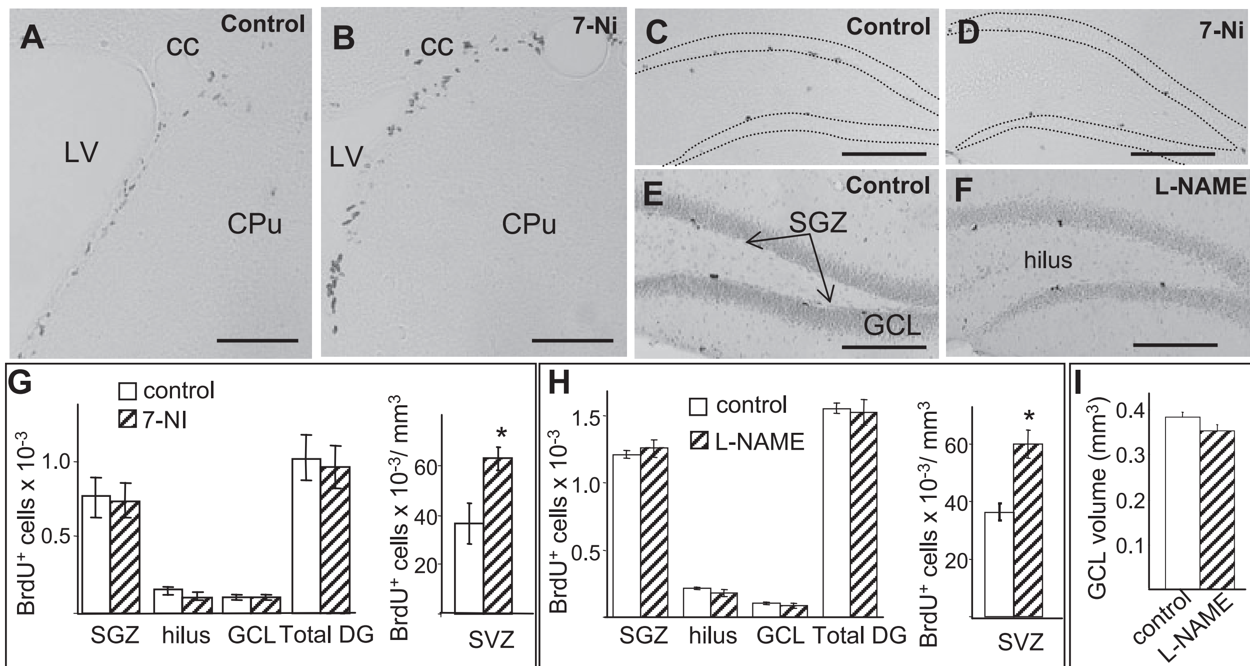

FIG. 1. Chronic NOS inhibition enhanced cell proliferation in the subventricular zone (SVZ), but not in the dentate gyrus (DG) of the hippocampus. Images of the SVZ (A and B) and DG (C–F) of adult mice treated (B, D and F) or not (A, C and E) with the indicated NOS inhibitor, processed for immunohistochemical detection of bromodeoxyuridine (BrdU). Sections in (E) and (F) were counterstained to visualize the granular cell layer (GCL). cc, corpus callosum; CPu, caudate-putamen; LV, lateral ventricle, (G) The number of BrdU-positive cells in the subgranular zone (SGZ), hilus, GCL, total DG and SVZ of mice injected with vehicle or with 7-nitroindazol (7-NI) for 4 days. (H) The number of BrdU-positive cells in the same regions as in (G) in mice injected with vehicle or with N-nitro-L-arginine methyl ester (L-NAME) for 15 days. (I) GCL volume in mice treated with vehicle or L-NAME for 15 days. Means ± SEM, $n = 9$ (H), 3 (G and I). *$P < 0.05$. Scale bars, 100 μm.

anatomical relationship between neural precursors and nitrergic neurons was comparatively analysed in the SVZ and the DG. We used double immunohistochemistry to detect PSA-NCAM, which selectively labels neuroblasts in the adult neurogenic areas (Rousselot *et al*., 1995), and nNOS. As shown in Fig. 3A and F, in both regions, neuroblasts were generated at a distance from nitrergic neurons that was short enough to allow NO interaction with these cells (Wood & Garthwaite, 1994). Furthermore, the presence of S-nitrosylated proteins, taken here as an index of NO release (Sunico *et al*., 2005), was revealed by immunofluorescence in both regions (Fig. 3B, D and G). The decreased nitrosothiol labelling observed in mice treated with 7-NI ($t = 4.21$, $P < 0.001$ for the SVZ and $t = 2.93$, $P < 0.02$ for the DG; Fig. 3C, H and I) or with L-NAME (Fig. 3E) assessed the efficacy of our treatment aimed at inhibiting chronically the NOS activity. Together these data demonstrate that SVZ and DG precursors are closely and equally exposed to NO-producing neurons.

SVZ but not DG proliferating precursors expressed the EGFR, a requisite for the proliferative effect of systemic NOS inhibitors

In previous works, we have demonstrated that NO reduces the proliferation of the SVZ transit amplifying cells (Moreno-López *et al*., 2004), which are endowed with the EGFR (Doetsch *et al*., 2002). *In vitro,* we have also shown that NO directly inhibits the tyrosine kinase activity of the EGFR in neural cells (Murillo-Carretero *et al*., 2002), thus suggesting that the EGFR might be an important molecule mediating the NO-induced reduction of precursor proliferation. We performed a comparative study of the EGFR expression in proliferating cells in the SVZ and DG using double immunofluorescence to detect BrdU and EGFR simultaneously. In the DG, the number of $BrdU^+/EGFR^+$ cells represented a very low percentage of the total $BrdU^+$ cells, whereas a majority of proliferating cells expressed this receptor in the SVZ ($t = 12.16$, $P < 0.001$ for EGFR-positive cells and $t = 13.36$, $P < 0.001$ for EGFR-negative cells, compared with values in the DG) (Fig. 4). Analysis of BrdU and EGFR co-localization in the SVZ was also performed in mice treated with 7-NI, L-NAME or the corresponding vehicles. As shown in Fig. 5, 7-NI produced a significant increase in the density of $BrdU^+/EGFR^+$ cells ($t = 4.99$, $P < 0.01$), whereas the population of $BrdU^+/EGFR^-$ cells was not modified by NOS inhibition ($t = 1.77$, $P > 0.05$). Similar results were obtained using L-NAME (56 ± 1% and 66 ± 3% of the proliferating cells were $EGFR^+$ in control and treated animals, respectively; $t = 3.50$, $P < 0.05$; 1600 $BrdU^+$ cells from three control mice and 1000 $BrdU^+$ cells from two treated mice were analysed). Thus, these results indicate that the anti-proliferative NO effect was exerted selectively on the cell population that express EGFR, and suggest that the different distribution of this receptor in the two neurogenic areas may account for the selective sensitivity of SVZ precursors to NOS inhibitors.

FIG. 3. Neural precursors in the subventricular zone (SVZ) and dentate gyrus (DG) are equally exposed to nitric oxide (NO) sources and exhibit NO synthase (NOS) activity-dependent S-nitrosylated proteins. Images of the SVZ (A–E) and DG (F–H) of adult mice, immunostained to simultaneously visualize the modified adhesion molecule PSA-NCAM (red) and neuronal NOS (green) (A and F), or the presence of S-nitrosothiol groups (blue) in control and 7-nitroindazol (7-NI) treated animals (B–E and G–H). Cc, corpus callosum; CPu, caudate-putamen; GCL, granular cell layer; LV, lateral ventricle; SGZ, subgranular zone. Scale bars, A–C, 25 μm; D–H, 50 μm. (I) S-nitrosothiol (S-NO) mean fluorescence intensity measured in the SVZ and DG of control and 7-NI treated mice. Means ± SEM, $n = 4–7$; *$P < 0.05$.

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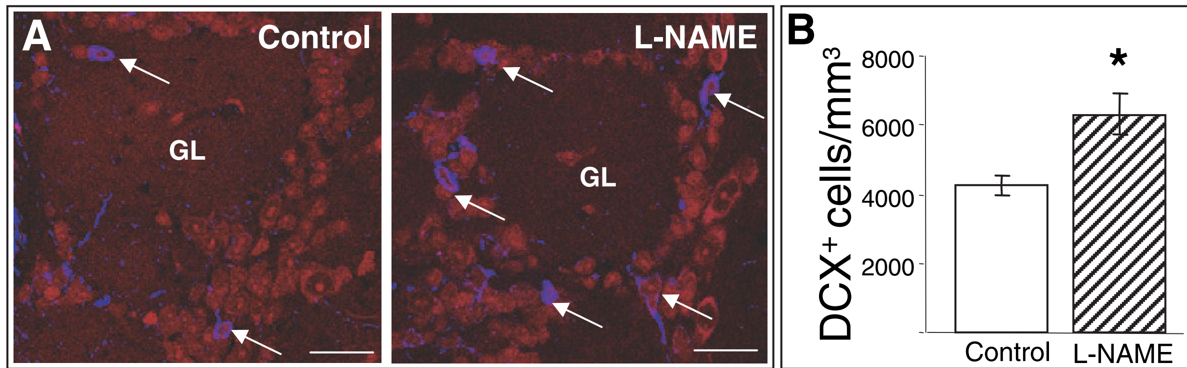

FIG. 2. Chronic NOS inhibition enhanced neurogenesis in the OB. (A) Confocal images of the OB glomerular layer of adult mice treated or not with N-nitro-L-arginine methyl ester (L-NAME) for 15 days, processed for immunohistochemical detection of the young neuron marker doublecortin (blue). Nuclei were stained with propidium iodide (red). GL, glomeruli. Arrows: doublecortin-positive cell somas. Scale bars: 20 μm. (B) Density of doublecortin-positive (DCX^+) cells in the OB glomerular layer of control and L-NAME-treated animals. Means ± SEM, $n = 5$; $*P < 0.05$.

FIG. 3.

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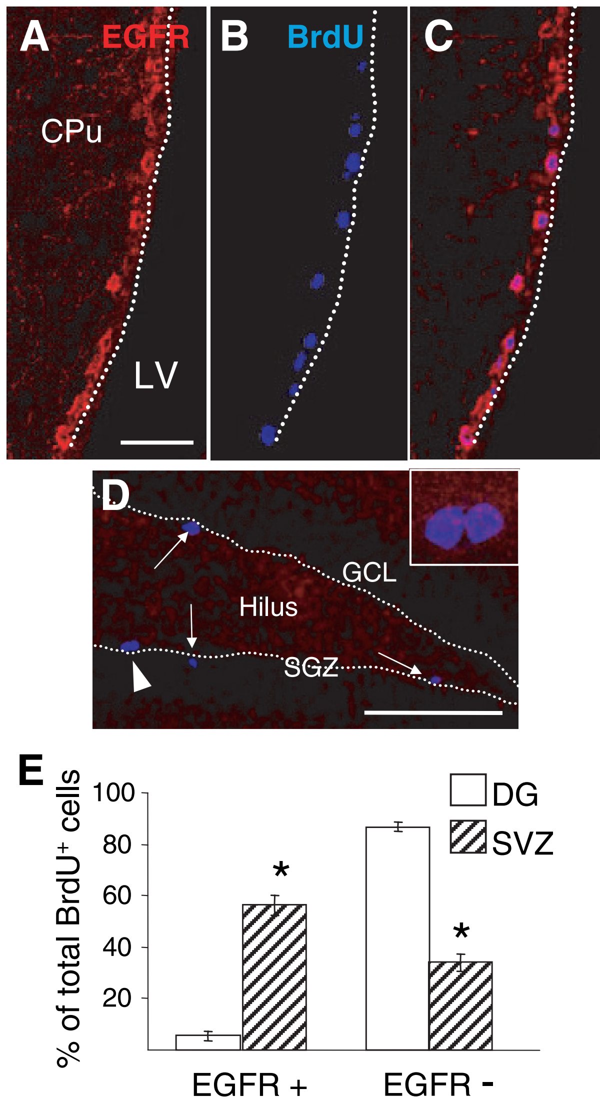

FIG. 4. Neural precursors in the subventricular zone (SVZ), but not in the dentate gyrus (DG) express the epidermal growth factor receptor (EGFR). Confocal images of the SVZ (A–C) and DG (D) of adult mice, immunostained to visualize EGFR (red) and BrdU (blue). Arrows: $BrdU^+$ cells in the subgranular zone (SGZ); arrowhead: pair of $BrdU^+$ nuclei in the SGZ, shown at a higher magnification in the inset. CPu, caudate-putamen; GCL, granular cell layer; LV, lateral ventricle; SGZ, subgranular zone. Scale bars: 25 μm (A–C); 50 μm (D). (E) Relative number of $BrdU^+$ cells that do or do not express the EGFR in the DG and SVZ. Means ± SEM. Total $BrdU^+$ nuclei counted: 240 in the DG, $n = 4$; 1600 in the SVZ, $n = 3$. $*P < 0.05$ as compared with values in the DG.

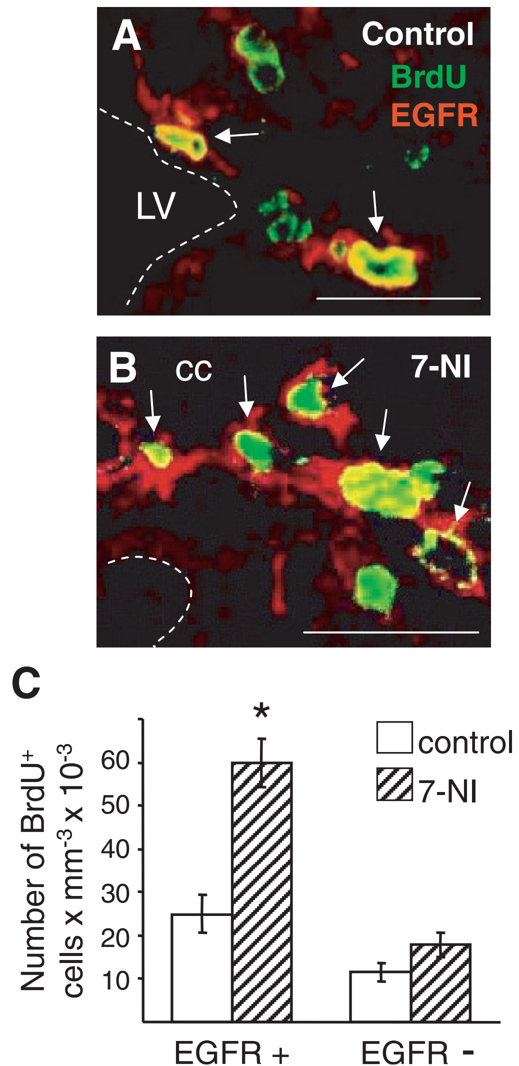

FIG. 5. Chronic NOS inhibition enhances proliferation of SVZ precursors endowed with the epidermal growth factor receptor (EGFR). Confocal images of the SVZ of a control mouse (A) and a mouse treated with 7-nitroindazol (7-NI; B), double stained for bromodeoxyuridine (BrdU; green) and EGFR (red). Arrows: newborn cells ($BrdU^+$) that express EGFR. Scale bars, 20 μm. (C) Density of $EGFR^+$ and $EGFR^-$ newborn cells in the SVZ of mice injected with vehicle (control) or 7-NI for 4 days. Means ± SEM, $n = 4$; $*P < 0.05$.

Chronic NOS inhibition improved olfactory learning

To assess whether the increase of SVZ neurogenesis induced by chronic NOS inhibition functionally impacted olfactory performance, mice were tested in an odour memory task. Animals treated with L-NAME and D-NAME were exposed to the same odour at different time intervals. ANOVA with repeated measures (two treatments × five intervals × two presentations) on the duration of odour investigation revealed no effect of the treatment factor ($F_{1,6} = 0.09$; $P > 0.05$), a significant effect of the interval factor ($F_{4,24} = 4.44$, $P < 0.01$), of the exposure factor ($F_{1,6} = 145.23$, $P < 0.001$), as well as an interaction between treatment and interval factors ($F_{4,24} = 3.87$, $P < 0.05$) and an interaction between interval and exposure factors ($F_{4,24} = 4.19$, $P < 0.05$). D-NAME-treated mice showed less interest in investigating the odour during the second exposure at 30 min, 4 h and 8 h after the first exposure (respectively, $t = 4.56$, $P < 0.001$; $t = 5.45$, $P < 0.001$ and $t = 4.35$, $P < 0.01$), but not at 12 h or 16 h (respectively, $t = 2.07$ and $t = 1.16$, $P > 0.05$; Fig. 6A). In contrast, L-NAME-treated mice showed a significant reduction in investigation duration when tested after 30 min, 4 h, 8 h and 12 h (respectively, $t = 5.63$, $P < 0.01$; $t = 3.83$, $P < 0.01$; $t = 4.59$, $P < 0.001$ and $t = 4.87$, $P < 0.001$; Fig. 6B). No significant change in investigation duration was found in this group when the same odour was presented 16 h after the first presentation ($t = 0.35$, $P > 0.05$).

To ensure that the decrease in investigation time observed at different time intervals was specific to familiar odours and reflected odour memory only rather than non-specific processes (for instance olfactory satiation), the specificity of odour recognition was assessed. When mice of both groups were presented with an odour, followed 8 h later by exposure to a different odour, they spent similar times in

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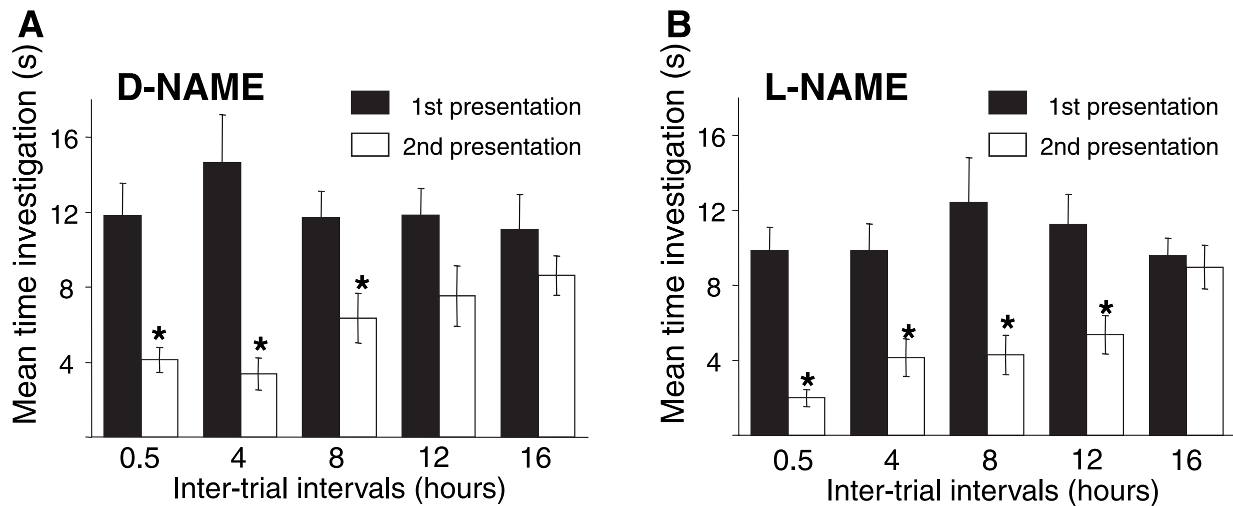

FIG. 6. Mice exposed to chronic NOS inhibition have longer olfactory memory. Effect of different intertrial intervals on odour recognition in animals treated with D-NAME (A) or N-nitro-L-arginine methyl ester (L-NAME) (B). Each bar represents the mean time (± SEM) spent investigating a given odour on the first exposure (filled columns) and on the second exposure (open columns). Means ± SEM, $n = 15–16$; $^{*}P < 0.05$ compared with the first presentation.

investigating the two odours (ANOVA, presentation, $F_{1,21} = 0.84$, $P > 0.05$; group × presentation, $F_{1,21} = 3.32$, $P > 0.05$, data not shown). Also, the overall duration of investigation when each odour was presented for the first time was not different between the two groups (ANOVA, $F_{9,65} = 1.407$, $P > 0.05$). Together, these results indicate that chronic inhibition of the NOS activity allows mice to hold the odour traces for a longer time compared with control conditions.

Chronic NOS inhibition did not modify spatial learning

The better performance of L-NAME-treated animals in odour memory tests could be produced by either a selective action on olfactory learning or alternatively a general improvement of attention and/or cognitive capability. To test the two possibilities, mice were tested for their spatial learning ability using the Morris water maze. Figure 7A shows that both L-NAME- and D-NAME-treated animals showed a significant reduction in the time needed to escape onto the hidden platform over sessions ($F_{4,84} = 12.68$, $P < 0.001$), without any difference found between the two experimental groups (groups, $F_{1,21} = 0.07$, $P > 0.05$ and groups × sessions, $F_{4,84} = 1.13$, $P > 0.05$). During the probe trial (Fig. 7B), animals from both groups spent more time at the expected target location (i.e. the training quadrant) than in others (Newman–Keuls, $P < 0.05$). This clear bias for the target quadrant was statistically similar in both groups (groups × quadrants, $F_{3,63} = 1.84$, $P > 0.05$). Thus, the similar performance in spatial learning indicates that the behavioural effects of NOS inhibition are specific for olfactory memory.

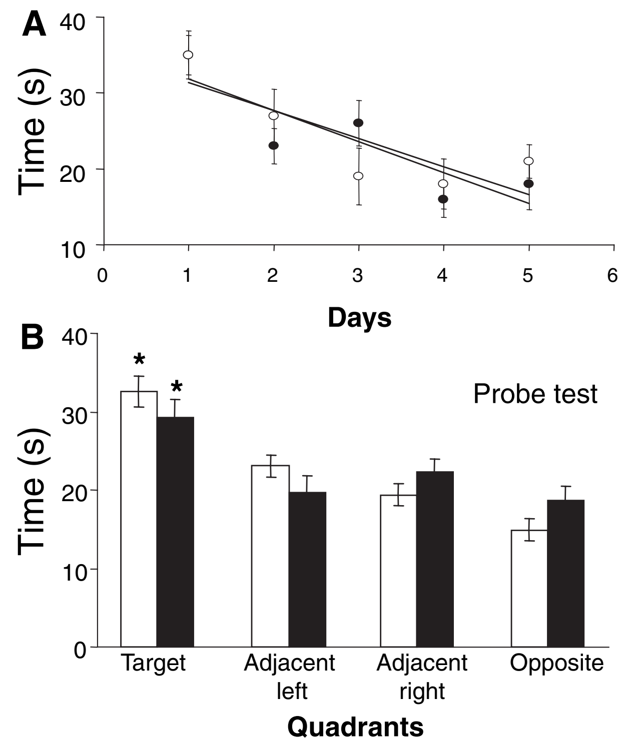

FIG. 7. Chronic NOS inhibition does not modify spatial memory in the water maze task. (A) Latency to climb onto the platform in a random starting version of the place navigation task. D-NAME- (open circles) and L-NAME- (filled circles) treated mice showed a similar reduction in escape latency over the 5 days of training. (B) Time spent in each quadrant by D-NAME- (open columns) or L-NAME- (filled columns) treated mice after 5 days of training. Means ± SEM, $n = 11–12$. $^{*}P < 0.05$ when time spent in the quadrant where the platform had been located during training (target) was compared with that spent in any other quadrant for each group. No differences were found between the two treatments.

Discussion

In the present study, we demonstrate that systemic administration of NOS inhibitors in adult mice enhanced neural precursor proliferation selectively in the SVZ, without any modification in SGZ cell proliferation, or in the GCL volume, in the hippocampus. Concomitantly, treated mice performed better in olfactory, but not in spatial memory, tests, thus indicating that the increase in proliferation of SVZ precursors has specific consequences on olfactory learning. Because neural precursors in both the SVZ and DG were equally exposed to NO production, the specificity of NOS inhibitor action might result from a different sensitivity of SVZ precursors to NO. This assumption

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is supported by the selective effect of NOS inhibition on the cell population that express the EGFR in the SVZ and the scarcity of cells provided with this receptor in the DG.

Several laboratories, using different protocols that include local or systemic administration of NOS inhibitors, as well as nNOS knock-out mice, have recently demonstrated that endogenous NO is a negative regulator of cell proliferation in the SVZ of adult rodents (Cheng *et al*., 2003; Packer *et al*., 2003; Moreno-López *et al*., 2004; Sun *et al*., 2005). However, results obtained in the hippocampus were variable, as both increases (Packer *et al*., 2003; Park *et al*., 2003; Sun *et al*., 2005) and no changes in cell proliferation (Park *et al*., 2001, 2003; Moreno-López *et al*., 2004; Sun *et al*., 2005) have been reported after chronic inhibition of NO production (reviewed in Estrada & Murillo-Carretero, 2005). The present results confirm our previous study; using systemic administration of NOS inhibitors, cell proliferation was not modified in the DG as a whole, nor in any of its various layers: the SGZ, GCL and hilus, whereas the same animals presented a significant upregulation of precursor proliferation in the SVZ. Furthermore, systemic administration of NOS inhibitors caused an increase in the number of immature neurons around the glomeruli in the OB, a destination site of the neuroblasts generated in the SVZ, without modification of the GCL volume, where the DG neural precursors become integrated.

Two alternative or complementary hypotheses may explain the anatomical specificity of the NOS inhibitor effects on precursor proliferation in the adult brain: NO is not sufficiently available in the SGZ, and/or the DG and SVZ precursors respond differently to changes in NO concentration. To be regulated by NO, this short half-life mediator must be produced locally in the proximity of the neural precursors. Furthermore, because 7-NI is as effective as L-NAME, it is possible to conclude that the NO that modulates precursor proliferation is synthesized by nitrergic neurons expressing nNOS. A careful comparison of paired sections obtained from the same animal and exposed to the same staining procedure revealed that dividing cells in the DG were close enough to nitrergic neurons to be exposed to NO actions, as previously shown for the SVZ (Moreno-López *et al*., 2000a). This was confirmed by the use of an antibody that specifically recognizes S-NO, therefore allowing the visualization of S-nitrosylated proteins in brain sections (Sunico *et al*., 2005). S-nitrosylated proteins were detected throughout the SVZ and in the hilus and SGZ, in the hippocampus, thus indicating that NO reached the neurogenic areas in physiological conditions. Furthermore, the significant decrease in S-NO staining in 7-NI- and L-NAME-treated mice ensured the efficacy of NOS inhibitors in both regions.

The hypothesis of a different responsiveness to NO in the SVZ and DG is supported by the diversity of cellular/molecular mechanisms by which neural stem cells produce intermediate precursors and then immature neurons in the two regions (Doetsch *et al*., 1999; Alvarez-Buylla & Garcia-Verdugo, 2002; Kempermann *et al*., 2004; Seri *et al*., 2004), and also by the modulators known to affect specifically one of the two neurogenic niches (Shingo *et al*., 2003; Mechawar *et al*., 2004; Abrous *et al*., 2005). Although, in both cases, stem cells are represented by astrocytes, the SVZ generates a highly proliferative cell population, the transit amplifying cells or type C cells that, later on, differentiate into migrating neuroblasts (Doetsch *et al*., 1997), whereas in the DG, only a few mitosis occur in the intermediate cell population, the D cells (Kempermann *et al*., 2004; Seri *et al*., 2004). Analysis of the protocols used when NO effects on neurogenesis were investigated (Park *et al*., 2001, 2003; Packer *et al*., 2003; Moreno-López *et al*., 2004; Sun *et al*., 2005) suggest that neural precursors in the two neurogenic niches may have different sensitivities to NO. Moderate decreases in NO concentration, such as those caused by systemic administration of NOS inhibitors, were sufficient to modify cell proliferation in the SVZ, whereas drastic reductions in NO production, such as those reached in nNOS knock-out mice or in animals intracerebroventricularly injected with NOS inhibitors, were needed to enhance proliferation and/or survival of DG neural precursors. A reasonable explanation is that neural precursors in the DG are more sensitive to the NO anti-mitotic action, whereas those in the SVZ require higher NO concentrations to reduce their proliferation rate.

Type C cells in the SVZ express the EGFR (Doetsch *et al*., 2002), whose stimulation increases precursor mitotic rate in this area (Craig *et al*., 1996; Kuhn *et al*., 1997; Tropepe *et al*., 1997; Doetsch *et al*., 2002). Furthermore, Waved-1 mutant mice, which express reduced quantities of TGF-α, the predominant endogenous ligand for EGFR in adulthood, present reduced olfactory neurogenesis and poor performance on fine olfactory discrimination tasks (Enwere *et al*., 2004). The selective sensitivity of the EGFR-expressing cells to NOS inhibitors is functionally relevant, as NO directly inhibits the EGFR tyrosine kinase activity in several cell lines (Estrada *et al*., 1997; Ruano *et al*., 2003), including neuroblastoma cells (Murillo-Carretero *et al*., 2002), as well as in cultured SVZ precursors (Torroglosa *et al*., 2006) by a mechanism involving S-nitrosylation (Murillo-Carretero *et al*., unpublished results). Such a mechanism of action, which requires relatively high concentrations of NO, may explain why partial NOS inhibition increases proliferation selectively in the SVZ. In the DG, whose precursor cells do not express EGFR, NO may affect proliferation by mechanisms other than S-nitrosylation that can be operative at very low NO concentration and that would only be released when NO production is severely compromised. Because in physiological conditions, nNOS regulation by cell signalling events results in up and down fluctuations in local NO concentration, rather than in the total disappearance of this intercellular mediator, NO can be considered a modulator of neurogenesis in the SVZ in the normal behaving brain.

Whatever the mechanism, the selective effect of systemic NOS inhibitors on SVZ precursor proliferation provided a good model for analysing whether increased neurogenesis only in the SVZ may have functional consequences for olfactory abilities. Evaluating the behavioural consequences of increasing the population of new olfactory interneurons, by enhanced SVZ-progenitor proliferation, is an important question that has received few interests so far. For instance, studies aimed at exploring the potential link between adult neurogenesis and olfactory learning/memory have been concentrated more on newborn neurons survival rather than the production of neuronal progenitors in the SVZ (reviewed in Lledo *et al*., 2006). When transgenic models were used for such a quest, they all displayed a reduced neurogenesis. Nevertheless, similar to our results, a recent study has reported a stimulation of the production of neuronal progenitors in the SVZ, but not in the DG, by pregnancy or prolactin (Shingo *et al*., 2003). They have shown that this effect led to a specific increase in the number of newborn bulbar interneurons, yet the functional consequences were not analysed.

Because the two neurogenic regions are involved in very distinctive learning tasks, both olfactory memory and spatial learning were tested in mice after 3–4 weeks of chronic inhibition of NOS activity, a time at which all types of adult-generated neurons have been recruited in the DG (Overstreet *et al*., 2004; Overstreet, 2005) and bulbar (Petreanu & Alvarez-Buylla, 2002; Belluzzi *et al*., 2003; Carleton *et al*., 2003) circuits. After chronic inhibition of NOS activity, we observed a temporal association between the enhancement of SVZ neurogenesis and olfactory performance. This correlation supports several reports showing striking parallels between the degree of

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neurogenesis and learning and memory performance (reviewed in Lledo *et al*., 2005). For instance, it has been shown that NCAM-mutant mice, which have reduced bulbar neurogenesis, displayed impaired olfactory discrimination (Gheusi *et al*., 2000). In ageing mice a decrease in bulbar neurogenesis, without a reduction in the total number of neurons, is also associated with impaired olfactory discrimination (Enwere *et al*., 2004). Similarly, a substantial reduction of hippocampal cell proliferation induced by anti-mitotic drug treatment impairs hippocampal memory, while the recovery of cell production is associated with the ability to acquire new trace memories (Shors *et al*., 2001). In the present work, the similar performance of L-NAME- and D-NAME-treated mice in the learning of a hippocampus-dependent task correlated well with the lack of effect of these treatments on DG precursor proliferation. This result also assessed the specificity of NOS inhibition on olfactory learning processes, ruling out that the observed behavioural changes were due to some non-specific learning-related aspects such as attention or motivation. Together, these studies indicate that adult-generated neurons are potentially involved in specific memory formation.

It could be argued that mechanisms other than neurogenesis may affect olfactory learning in NOS-treated animals, based on the proposed involvement of NO in long-term potentiation and synaptic remodelling (Schuman & Madison, 1991; Williams *et al*., 1993; Southam *et al*., 1996). However, the participation of NO in these events has been demonstrated in acute or short-term experiments in which the effects of minutes to hours of NOS inhibition were evaluated. Furthermore, in these short-term experiments, NOS inhibition reduced synaptic plasticity in both the OB and the hippocampus, and impaired learning performance in both olfactory and spatial-based tasks (Bohme *et al*., 1993; Prendergast *et al*., 1997; Prast & Philippu, 2001; Majlessi *et al*., 2003). We therefore propose that NO might be a positive regulator for olfactory and spatial memory through a fast synaptic action, and a negative regulator for olfactory memory by inhibition of SVZ neurogenesis on a slower time scale. Therefore, after long-term NOS inhibition, the latter mechanism might be predominant.

It is interesting to note that the inhibitory effects of NOS inhibition on olfactory learning behaviour have been demonstrated, so far, using social and litter recognition (Bohme *et al*., 1993; Kendrick *et al*., 1997), or associative learning tasks (Samama & Boehm, 1999), all of them emotionally relevant. This is important because of the involvement in these processes of the accessory OB (AOB) (Doty, 2001), endowed with an abundant nitrergic neuropil, and where population renewal by SVZ neuroblasts is scarce (Moreno-López *et al*., 2000b). To avoid the participation of the AOB in our behavioural experiments, only emotionally irrelevant odorants were used in this work.

In conclusion, our results indicate that the effect of endogenous NO on adult neurogenesis, under physiological conditions, was selectively exerted in the SVZ, and that the NO behavioural effects specifically concerned olfactory learning. The ability to adjust the degree of adult neurogenesis in a restricted forebrain area may bring into bulbar neuronal networks a degree of neuronal adaptation unmatched by synaptic plasticity alone.

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Abbreviations

7-NI, 7-nitroindazol; AOB, accessory olfactory bulb; BrdU, bromodeoxyuridine; DG, dentate gyrus; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GCL, granular cell layer, L-NAME, N-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; OB, olfactory bulb; PBS, phosphate-buffered saline; PSA-NCAM, polysialylated form of the neuronal cell adhesion molecule; RMS, rostral migratory stream; SGZ, subgranular zone; S-NO, nitrosothiol groups; SVZ, subventricular zone; TGF, transforming growth factor.

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