THE JOURNAL OF COMPARATIVE NEUROLOGY 506:339–346 (2008)

Age-Dependent Effect of Nitric Oxide on Subventricular Zone and Olfactory Bulb Neural Precursor Proliferation

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ABSTRACT

Nitric oxide (NO) synthase (NOS) is developmentally regulated in the embryonic brain, where NO participates in cell proliferation, survival, and differentiation. In adults, NO inhibits neurogenesis under physiological conditions. This work investigates whether the NO action is preserved all along development up to adulthood or whether its effects in adults are a new feature acquired during brain maturation. The relationship between nitrergic neurons and precursors, as well as the functional consequences of pharmacological NOS inhibition, were comparatively analyzed in the subventricular zone (SVZ) and olfactory bulb (OB) of postnatal (P7) and adult (>P60) mouse brains. The SVZ was markedly reduced between P7 and adults, and, at both ages, neurons expressing neuronal NOS (nNOS) were found in its striatal limits. In postnatal mice, these nitrergic neurons contained PSA-NCAM, and their projections were scarce, whereas, in adults, mature nitrergic neurons, devoid of PSA-NCAM, presented abundant neuropil. In the OB, local proliferation almost disappeared in the transition to adulthood, and periglomerular nitrergic neurons, some of which were PSA-NCAM positive, were found in postnatal and adult mice. Administration of the NOS inhibitor L-NAME did not affect cell proliferation in the SVZ or in the OB of postnatal mice, whereas it significantly enhanced the number of mitotic cells in both regions in adults. Thus, the NO action on SVZ neurogenesis is a phenomenon that appears after the postnatal age, which is probably due to the germinal layer size reduction, allowing exposure of the NO-sensitive neural precursors to the NO produced in the SVZ-striatum limits. J. Comp. Neurol. 506: 339–346, 2008.

Indexing terms: EGF receptor; neural stem cells; neurogenesis; nitric oxide synthase; PSA-NCAM, NADPH-diaforase

Nitric oxide (NO), a gaseous intercellular messenger synthesized in the brain by specific neurons expressing the neuronal isoform of NO synthase (nNOS), has been demonstrated to play a role in both embryonic nervous tissue formation and adult neurogenesis (for review see Estrada and Murillo-Carretero, 2005; Gibbs, 2003). In embryos, NO inhibits neural precursor cell division and promotes neuronal differentiation in developing *Drosophila* imaginal disks (Kuzin et al., 1996) and *Xenopus* tadpole optic tectum (Peunova et al., 2001). A similar antiproliferative effect of NO has been reported in vitro in mammalian tumoral cell lines of neural origin (Murillo-Carretero et al., 2002; Obregon et al., 1997; Peunova and Enikolopov, 1995; Phung et al., 1999) and in neuronal precursors isolated from the embryonic (Cheng et al., 2003) and neonatal (Ciani et al., 2004) brain. Additionally, several groups have shown that NO is a negative regulator of neurogenesis in the adult SVZ. Significant increases in SVZ cell proliferation have been demonstrated in nNOS knockout mice (Packer et al., 2003; Sun et al., 2005) as well as after pharmacological inhibition of nNOS activity (Cheng et al., 2003; Moreno-López et al., 2004; Packer et al., 2003; Romero-Grimaldi et al., 2006). The NO target

Grant sponsor: Fondo de Investigaciones Sanitarias; Grant number: 00/1080; Grant sponsor: Ministerio de Ciencia y Tecnología; Grant number: SAF2002-02131; Grant sponsor: Junta de Andalucía; Grant number: CTS-2005/883.

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Received 31 March 2007; Revised 11 June 2007; Accepted 2 October 2007
DOI 10.1002/cne.21556
Published online in Wiley InterScience (www.interscience.wiley.com).

cells within this region have been identified as the undifferentiated precursors, which express nestin and epidermal growth factor receptor (EGFR) but not the neuroblast markers PSA-NCAM or βIII-tubulin (Moreno-López et al., 2004; Romero-Grimaldi et al., 2006). It is not presently known whether the NO effects on embryonic and adult neurogenesis are distinct events or occur as a continuum all along brain development, including the early postnatal period, a time when considerable changes take place in both nNOS distribution and the organization of neurogenic sites.

During the first two weeks of age, nNOS expression disappears in the olfactory receptor neurons, whereas it progressively increases in periglomerular cells in the main olfactory bulb (OB; Chen et al., 2004). In other regions, such as hippocampus (Chung et al., 2004), striatum (Labuda et al., 2003; Murata and Masuko, 2003), and OB granular layer (Samama and Boehm, 1999), nNOS is transiently expressed during postnatal ages. Furthermore, conditions in which neurogenesis occurs also change drastically along the same period of time. Until the time of birth, stem cells in the ventricular zone (VZ) give rise to secondary progenitors that proliferate in the subventricular zone (SVZ). Postnatally, the VZ disappears as an anatomically defined entity, although astrocytes that retain stem cell properties remain in the lateral wall of the lateral ventricles, allowing continuous neurogenesis through adulthood in the SVZ (Doetsch et al., 1999; Morshead et al., 1994). Between P0 and P15, the radial glia, which constituted the majority of cells in the VZ at birth, almost disappear, and the proportion of ependymal cells and astrocytes increases to the level found in adults (Peretto et al., 2005; Tramontin et al., 2003). Considerable modifications also occur in OB neurogenesis during the first 3 weeks of age, when the number of neurons locally generated in the OB decreases and the number of new neurons reaching the OB from the SVZ increases (Lemasson et al., 2005).

This work comparatively analyses in postnatal and adult mice the organization of the neural precursor cells in the SVZ and OB, their anatomical relationship with nitrergic neurons in their neighborhood, and the functional influence of endogenously produced NO on precursor proliferation. We found that NO is a negative regulator of neurogenesis in the adult, but not in the postnatal, mouse brain and that this developmental difference probably is due to the relative anatomical disposition of the NO-producing neurons and their EGFR-expressing target cells at both ages.

MATERIALS AND METHODS

Postnatal (P1–P7) and adult (2–4 months old) CD1 mice were used throughout this study. Care and handling of animals were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), following the Spanish regulations (BOE 67/8509-12; BOE 1201/2005) for the use of laboratory animals.

Mice were injected with the broad-spectrum NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 90 mg/kg/day, i.p.), for 7 or 15 days in the case of adults and for 3 days (P3–P6) or 6 days (P1–P6) in the case of postnatal animals. Control mice received the same volume of phosphate-buffered saline (PBS), which was used as vehicle. The dose and administration route used produces a long-lasting significant inhibition of cerebral NOS activity in both postnatal (Virgili et al., 1999) and adult (Moreno-López et al., 2004) mice. At the end of the treatment period, mice were intraperitoneally injected with the thymidine analogue Br-deoxyuridine (BrdU) to obtain a specific and quantitative labeling of proliferating cells (Cameron and McKay, 2001). Adults received 200 mg/kg BrdU, distributed in three doses separated by 2.5-hour intervals, and were killed 2.5 hours after the last BrdU injection (7.5-hour cumulative labeling). Postnatal mice received 100 mg/kg BrdU in a single injection and were perfused 2 hours later.

Control and treated mice were deeply anesthetized with chloral hydrate (0.5 g/kg) and transcardially perfused with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, postfixed for 2 additional hours, and cryoprotected by immersion in 30% sucrose solution overnight. Serial coronal sections (30 μm thick) of the SVZ and OB were obtained with a cryostat and stored at –20°C in a cryoprotectant solution (glycerol: PBS, pH 7.4, 1:1 in volume).

NADPH-diaphorase staining

Brain sections were washed in 0.1 M phosphate buffer (PB) and incubated for 20 minutes in a shaking bath at 37°C in a solution containing 1 mM β-NADPH, 0.8 mM nitroblue tetrazolium, and 0.1% Triton X-100 in 0.1 M Tris buffer, pH 8. After extensive washing, the tissue was dehydrated, mounted with DePeX, and analyzed via light microscopy. Sections from postnatal and adult mice were always processed in parallel.

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 hours, followed by a 30-minute 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 minutes, to block endogenous peroxidase activity, then incubated for 30 minutes with a solution containing 2.5% (w/v) bovine serum albumin (BSA), 0.25% (w/v) sodium azide, and 0.1% (v/v) Triton X-100 in PBS (PAAT) to prevent nonspecific 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; catalog No. M0744) was carried out overnight at 4°C. After several rinses in PBS, sections were treated for 1 hour 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). The peroxidase reaction was made visible with diaminobenzidine (DAB; 0.25 mg/ml) and hydrogen peroxide (0.003%, v/v). Sections were then mounted on slides, dehydrated, coverslipped with DePeX, and analyzed under light microscopy.

Proliferating cells, detected by their BrdU-positive ($BrdU^+$) nuclei, were counted in selected regions of the SVZ and OB, in one of three sections with a ×40 objective (Nikon), by stereological unbiased methods, as previously described (Moreno-López et al., 2004). The sizes of the predetermined areas in which labeled nuclei were counted were as follows: postnatal SVZ, 61 × 61 μm; adult SVZ,

TABLE 1. List of Antibodies Applied

Antiserum	Immunogen	Source	Working dilution	Recognized bands in WB
Anti-nNOS rabbit polyclonal antiserum	Full-length recombinant rat brain nNOS (Riveros-Moreno et al., 1995)	Dr. J. Rodrigo, CSIC, Madrid, Spain	1:3,000	Single band of 155 kDa (Uttenthal et al., 1998; Moreno-López et al., 2004)
Anti EGFR sheep polyclonal IgG	Bacterially expressed GST fusion protein with residues 967 to the COOH terminus of the human EGF receptor.	Upstate Biotechnology (Lake Placid, NY; No. 06-129)	1:200	Single band of 170 kDa (manufacturer's technical information; Murillo-Carretero et al., 2002)
Anti-polysialic acid-NCAM (PSA-NCAM) mouse monoclonal IgM (clone 2-2B)	Viable meningococcus B (strain P355).	Dr. J. Rougon, Marseille, France	1:4,000	Specific men B polysaccharides and embryonic N-CAM (Rougon et al., 1986)
Anti-BrdU mouse monoclonal antibody (clone Bu20a)	Br-deoxyuridine conjugated to bovine serum albumin	Dako, Glostrup, Denmark, catalog No. M0744	1:100	Minimal cross-reactivity with other nucleosides, e.g., thymidine (manufacturer's technical information)

125 × 125 μm; OB, 250 × 250 μm. To preclude that differential overall intensity of staining might affect the quantitative results when BrdU immunohistochemistry was applied to different groups of mice on different days, tissue sections from control and treated mice were always processed in parallel. Analyses were performed on blind-coded slides.

Immunofluorescence staining

Double-immunofluorescence techniques were used to detect simultaneously nNOS and one of the following antigens: PSA-NCAM, the polysialylated form of the neuronal adhesion molecule, which is expressed by the SVZ neuronal progenitors in vivo (Rousselot et al., 1995) and in vitro (Matarredona et al., 2004); EGFR, which labels undifferentiated precursor cells both in vivo (Doetsch et al., 2002) and in vitro (Torroglosa et al., 2007); or BrdU, to label mitotic cells. Brain sections were washed in PBS and incubated for 30 minutes at room temperature in PAAT. Incubations with the two primary (overnight, 4°C) or secondary (1 hour, room temperature) antibodies were performed simultaneously. After washing with PBS, the sections were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA) and fluorescent signals were detected using a BX60 Olympus epifluorescence microscope and a Leica Spectra confocal microscope. Images were captured with a digital camera (DP50; Olympus), and imported into Adobe Photoshop 5.5 at 300 dpi. Red was transformed to magenta, and images were corrected for brightness and contrast but not otherwise manipulated.

Information on the primary antibodies used is provided in Table 1. The specificity of the antibody that recognizes nNOS was determined by immunoblots showing a single band of 155 kDa in NB69 cells and in whole mouse brain (Moreno-López et al., 2004; Murillo-Carretero et al., 2002). The EGFR antibody immunoprecipitated a 170-kDa protein in NB69 cells (Murillo-Carretero et al., 2002) and recognized a single 170-kDa band in immunoblots performed in neural precursor cell lysates (Torroglosa et al., 2007). By immunohistochemistry, EGFR was localized selectively in the SVZ of the adult mouse brain (Romero-Grimaldi et al., 2006), whereas PSA-NCAM was restricted to the SVZ, RMS, and OB (Moreno-López et al., 2000), thus confirming the specificity of these antibodies for morphological studies. Secondary antibodies used were FITC-labeled anti-rabbit IgG, Cy3-labeled anti-mouse IgM, Cy3-labeled anti-mouse IgG, and Cy3-labeled anti-sheep IgG, all from Jackson Immunoresearch (West Grove, PA). All the secondary antibodies were adsorbed against several species, to prevent undesired cross-reactions. Omission of either primary antibody resulted in no detectable specific staining in all cases.

Glutathione measurements

Control and L-NAME-treated postnatal and adult mice were killed by decapitation (postnatal) or cervical dislocation (adults), their brains were quickly removed, and the SVZ was dissected out and immediately frozen in liquid nitrogen. Total glutathione was measured by a colorimetric assay using the kit Biotech-420 (Oxis Research) and following the manufacturer's instructions. Protein concentration was determined with the Bradford method.

Statistical analysis

All experiments were repeated at least three times. Data are presented as mean ± SEM. The nonparametric Mann-Whitney U-test was used for statistical comparisons.

RESULTS

Cell proliferation and nitrergic innervation in the SVZ and OB of postnatal vs. adult brain

Proliferating cells, identified by their $BrdU^+$ nuclei, were found at a high density (~200,000/mm^3 after a 2-hour exposure to the thymidine analog) beside the dorsolateral wall of the lateral ventricle in the postnatal mouse brain (Fig. 1A). In the adult animal, proliferating cells were less abundant (~40,000/mm^3, after a 7.5-hour exposure) and were restricted to a thin area adjacent to the ependymal cells and extended through the limits between the striatum and the corpus callosum (Fig. 1C). Nitrergic neurons, identified with either NADPH-d histochemistry (Fig. 1B,D) or nNOS immunostaining (see Figs. 4,6), were similarly distributed in the limits between the SVZ and the striatum, in postnatal and adult mice. Nitrergic somas were alike in shape and size (21.8 ± 1.0 μm^2 and 22.9 ± 1.1 μm^2 in postnatal and adults, respectively; $n \geq 30$), although a higher density of nitrergic neuropil was observed in adult compared with postnatal brains (compare B and D in Fig. 1).

In the postnatal OB, abundant $BrdU^+$ nuclei were visualized in a position external to the glomerular layer

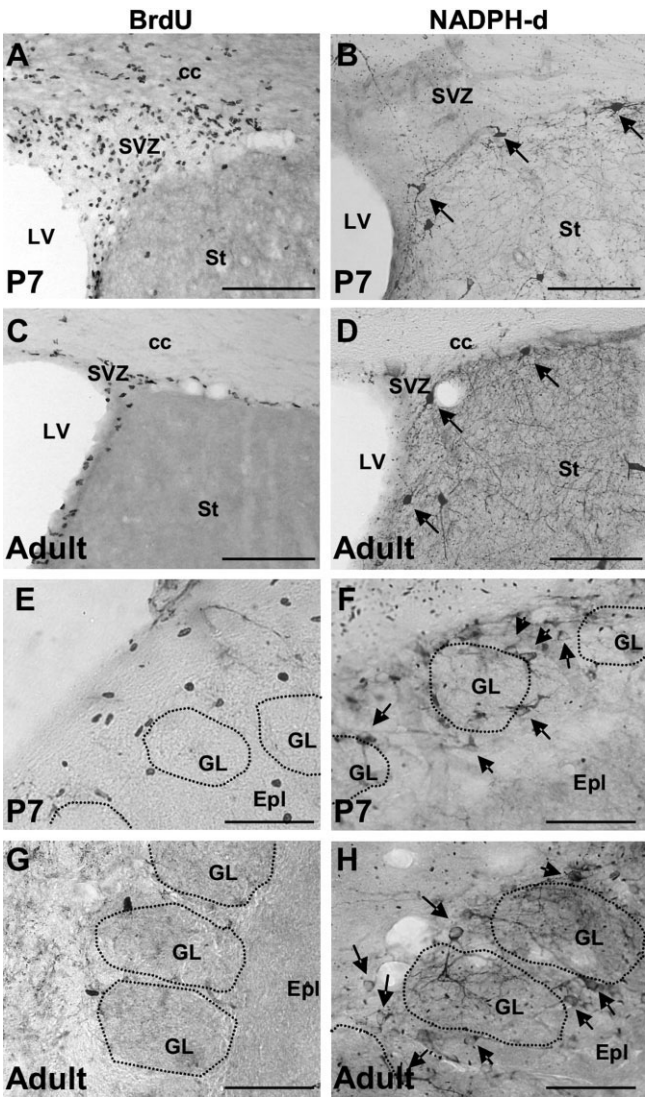

Fig. 1. Distribution of proliferating cells and nitrergic neurons in the subventricular zone (SVZ) and olfactory bulb (OB) glomerular layer of postnatal and adult mice. Photomicrographs showing SVZ (A–D) and OB (E–H) coronal sections of postnatal and adult mice, processed for BrdU immunohistochemistry or NADPH-diaphorase (NADPH-d) histochemistry. Some nitrergic cell bodies are indicated by arrows. Dotted lines label glomerulus limits. cc, Corpus callosum; Epl, external plexiform layer; GL, glomeruli; LV, lateral ventricle; St, striatum. Scale bars = 100 μm in A–D; 50 μm in E–H.

(Fig. 1E), although scattered positive nuclei were also present all over the granular cell layer (Fig. 2A). On the contrary, in adults, only a few stained nuclei were present around the glomeruli (Fig. 1G) and scattered in the granular layer, with a higher density observed exclusively in the central part of the granular cell layer, where the RMS enters the OB (Fig. 2E). At both ages, small, round cell bodies stained for NADPH-d histochemistry were found in a periglomerular position (Fig. 1F,H), although, in adults, these cells extended more processes, and their staining was darker than in postnatal mice. In the granular cell layer, medium-sized NADPH-positive neurons with few processes and slight to medium staining were frequently observed in postnatal brains (Fig. 2B,C) but were scarce in adults (Fig. 2F,G). In addition, a reduced number of large, heavily stained neurons with long processes were present in the central part of the OB of adult and postnatal mice (Fig. 2D,H).

Endogenous NO modulates cell proliferation in the adult but not in the postnatal brain

The density of $BrdU^+$ nuclei was quantified as an index of cell proliferation in the SVZ and OB of postnatal and adult mice untreated and after chronic inhibition of NOS activity, by systemic administration of L-NAME. As shown in Figure 3, 3–6 days of NOS inhibition did not modify the number of cells that incorporate BrdU in postnatal animals. On the contrary, adults presented a significant enhancement in the density of $BrdU^+$ cells in both regions. In the SVZ, 1 or 2 weeks of treatment produced approximately a 60% increase in cell proliferation, whereas, in the OB, the effect was time dependent over the period tested and reached approximately a 100% increase in the second week.

Anatomical relationship between precursor cells markers and nNOS in the SVZ and OB of postnatal and adult mice

Double immunostaining of nNOS and BrdU never showed colocalization of these markers within the same cell (Fig. 4A,B), which indicated that the proliferating neural precursors do not synthesize NO in vivo in the SVZ. On the contrary, the simultaneous immunohistochemical detection of PSA-NCAM and nNOS in this region revealed a different disposition of both markers in the two developmental stages. In the postnatal age, nitrergic neurons contained PSA-NCAM in their membranes (Fig. 4C–E). However, nitrergic neurons were devoid of PSA-NCAM in adults and their rich neuropil surrounded the PSA-NCAM-expressing neuronal progenitors (Fig. 4F–H). In the OB, colocalization of PSA-NCAM and nNOS was found in both postnatal (Fig. 5A–C) and adult (Fig. 5D–F) mice.

The modulatory effect of NO on adult neurogenesis is exerted specifically on cells that express EGFR in their membrane (Romero-Grimaldi et al., 2006), this is, the transit-amplifying cell population, or type C cells (Doetsch et al., 2002; Moreno-López et al., 2004). To analyze the anatomical relationship between the NO-producing cells and the NO-target cells, we used the simultaneous immunodetection of nNOS and EGFR. As shown in Figure 6, EGFR-positive ($EGFR^+$) cells were more numerous and occupied a larger extension in the SVZ of postnatal than adult brain, in such a way that only a fraction of the $EGFR^+$ cells were close to the nitrergic neurons in postnatal mice. On the contrary, all the $EGFR^+$ cells in the adult SVZ lay in the vicinity of NO sources (somas or neuropil) and, therefore, were probably exposed to NO. In neither case was colocalization of both molecules found in the same cell.

Postnatal and adult SVZ contain similar GSH concentration

GSH is an efficient NO scavenger in living cells, so changes in GSH concentration may explain different responsiveness to NO in diverse tissues. SVZ extracts from mice at postnatal days 1, 3, and 7, as well as adults, treated or not with L-NAME, were assayed to measure

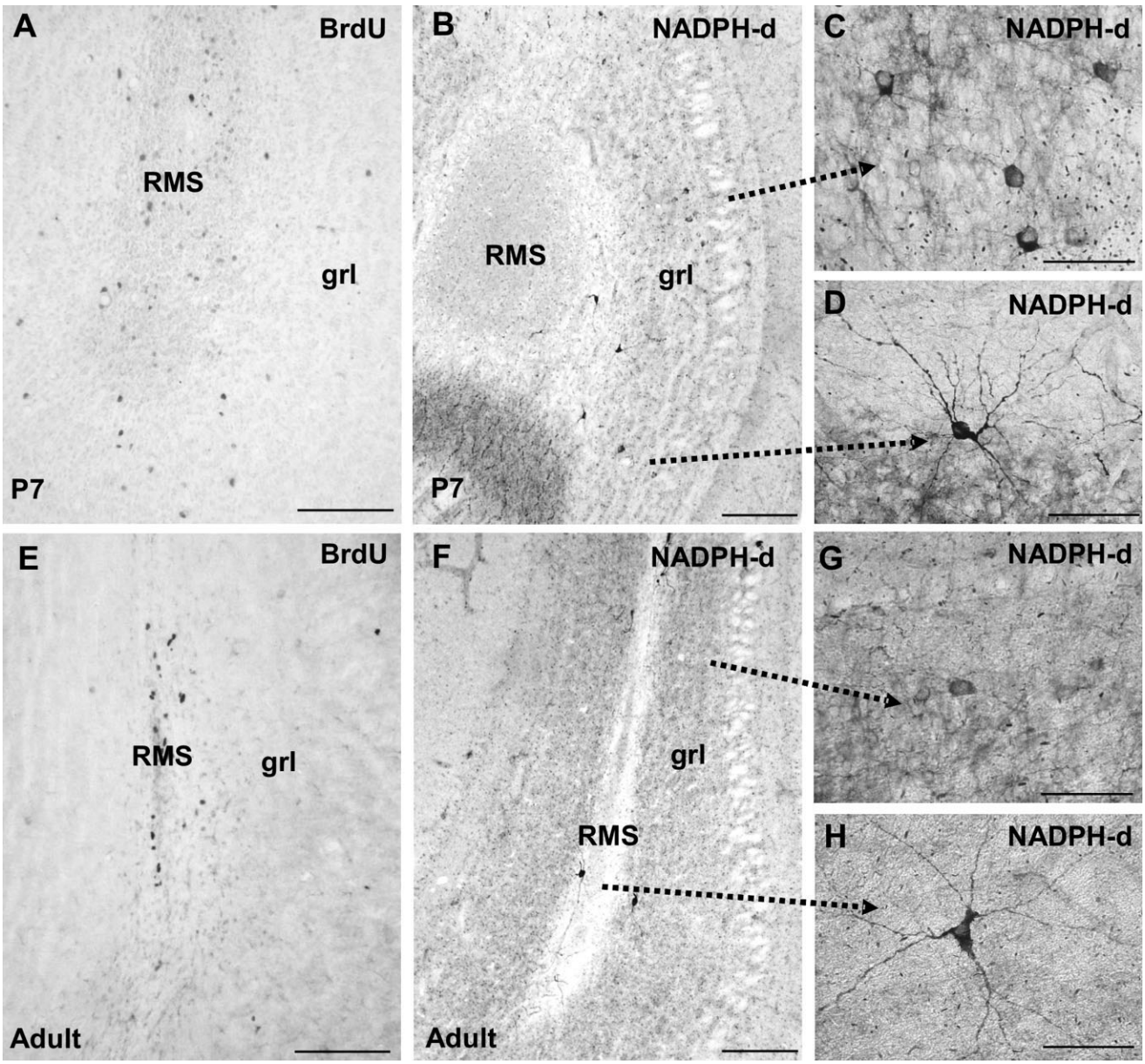

Fig. 2. A–H: Distribution of proliferating cells and nitrergic neurons in the olfactory bulb (OB) granular cell layer (grl) of postnatal and adult mice. Coronal sections of OB obtained from mice at the indicated ages, stained for BrdU immunohistochemistry or NADPH-diaphorase (NADPH-d) histochemistry. Arrows indicate the region from which the larger magnification images shown on the right were obtained. In the grl, round nitrergic cell bodies were more numerous and intensely stained in postnatal (C) than in adult (G) mice. In the central part of the OB, heavily stained neurons with abundant processes were visualized at both ages (D,H). RMS, rostral migratory stream. Scale bars = 100 μm in A,B,E,F; 50 μm in C,D,G,H.

GSH concentration. No significant differences were found between ages or conditions (in nmol/mg protein: P1, 33 ± 4; P3, 39 ± 7; P7, 34 ± 3; adult, 33 ± 3; L-NAME P7, 33 ± 3; L-NAME adult, 36 ± 2; n = 4–5).

DISCUSSION

In this work, we show that the regulatory action of NO on neurogenesis is not a continuum throughout ontogenic development but a property that is acquired after the postnatal period and that is probably a consequence of the cytoarchitectonic changes that take place at that age, leading to the adult SVZ organization. Although studies analyzing the effects of nNOS on neurogenesis at different developmental stages are scarce, several data indicate that neural precursors vary in their response to NO deprivation according to spatial and temporal cues. Temporally restricted changes in cell proliferation have been shown in the olfactory epithelium (Chen et al., 2004) as well as in the cerebellum (Ciani et al., 2006) during pre-

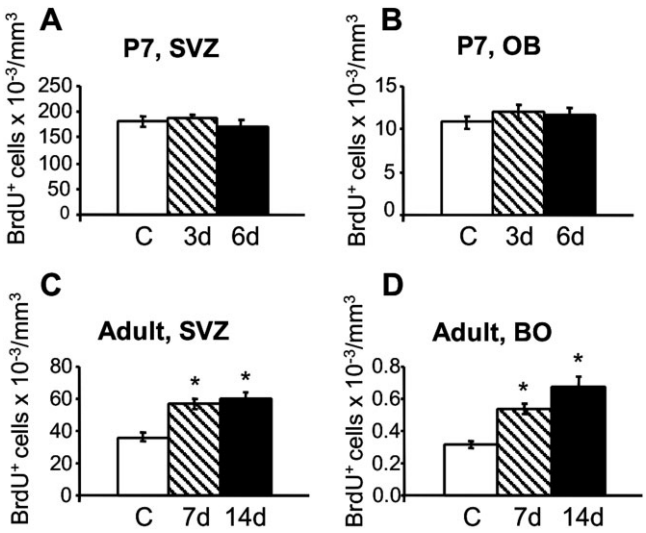

Fig. 3. Quantitative effects of NO synthesis inhibition on neural precursor proliferation. Density of $BrdU^+$ cells in the subventricular zone (SVZ; A,C) and olfactory bulb (OB; B,D) of postnatal and adult mice. Animals received daily intraperitoneal injections of L-NAME (90 mg/kg) for the indicated periods and then BrdU 2–7.5 hours before perfusion, as indicated in Materials and Methods. Data are presented as means ± SEM; $n \geq 5$. $^*P \leq 0.05$ compared with the vehicle-treated group.

natal and early postnatal periods, respectively. These differences, which correspond to specific neurogenetic periods within each region, may be caused by changes in the availability of either NO itself or any of its target molecules, such as soluble guanylyl cyclase in the case of the cerebellum (Ciani et al., 2006).

In the adult SVZ, neural precursors were sensitive to NOS inhibition, as we and others have previously demonstrated (Cheng et al., 2003; Moreno-López et al., 2004; Packer et al., 2003; Romero-Grimaldi et al., 2006); however, the postnatal SVZ cells did not modify their proliferation rate when NO synthesis was prevented by systemic administration of an NOS inhibitor. This finding indicates that the NO action in the SVZ is developmentally regulated and that the postnatal period is a transient situation in which the conditions allowing neurogenesis regulation by NO are not yet well established. Three reasons, discussed below, may account for the differential effect of NO in postnatal and adult SVZ neurogenesis: 1) reduced NO availability in the postnatal period, because of either decreased production or a higher concentration of NO scavengers, such as GSH; 2) reduced sensitivity of postnatal precursor cells to the antimitotic action of NO; or 3) decreased accessibility of NO to the neurogenic regions resulting from a different relative disposition of the NO sources and targets.

The use of both NADPH-d histochemistry and nNOS immunohistochemistry allowed the anatomical identification of nitrergic neurons in the limits between the striatum and the SVZ of postnatal and adult mice. Although cells bodies were similar in size and shape, two features differentiated the nitrergic neurons at both ages. nNOS-expressing postnatal cells contained PSA-NCAM in their membranes and presented a poorly developed neuropil, whereas, in adults, nitrergic neurons never contained PSA-NCAM and were provided with a rich neuropil. Colocalization of nNOS and PSA-NCAM in the postnatal period is not surprising, insofar as PSA-NCAM expression was diffuse and widespread at that age, with stronger immunostaining in the parenchyma surrounding the SVZ than in the SVZ itself, as previously reported (Bonfanti, 2006). The differential features were indicative of a higher degree of maturation of the NO-producing neurons in the adult SVZ. Functional consequences of such maturity might be that only adult nitrergic neurons were able to produce NO in sufficient concentration and/or at the adequate time in response to their more complex input system. Another reason for a reduced NO availability could be the presence in the tissue of a higher concentration of NO scavengers, among which GSH is the more abundant. However, no differences were observed when GSH was measured at different ages or after chronic NOS inhibition.

An alternative explanation of the differential effect of NO on postnatal and adult neurogenesis is that NO was unable to produce an antiproliferative response in postnatal precursor cells, which may not express some of the NO target proteins or have not yet developed the intracellular signaling pathways leading to cell cycle arrest in response to NO. However, it has recently been demonstrated that isolated postnatal SVZ precursor cells reduced their mitotic rate when exposed to NO (Matarredona et al., 2004; Torroglosa et al., 2007), through inactivation of the EGFR and the PI3-K/Akt pathway, a mechanism that is also operative in vivo, in adult mice (Torroglosa et al., 2007).

An analysis of the relative anatomical distribution in the SVZ of the nitrergic neurons and the NO target cells, identified by their expression of EGFR (Romero-Grimaldi et al., 2006), provided an additional clue that may explain why NO does not regulate neurogenesis in postnatal mice. Due to the larger volume in which $EGFR^+$ precursor cells are distributed in the postnatal SVZ and the restricted location of the nitrergic neurons in its lateral limits, a large proportion of the target cells might not be exposed to NO during postnatal development. The accessibility of NO to its target precursor cells might be further impaired by the above-mentioned less well developed neuropil of postnatal nitrergic neurons, a condition that causes a smaller penetration of their processes into the deepest regions of the SVZ.

In the OB, the cytoarchitectonic disposition might also explain the specific effect of NOS inhibition in the adult age, when both the scarce proliferating cells and the nitrergic neurons share a narrow compartment around the glomeruli. However, the differential proliferative effect of the NOS inhibitor in the adult vs. postnatal OB may be only the consequence of its differential effect in the SVZ. In adults, enhanced numbers of SVZ precursors progressively reach the OB along the treatment duration, while part of them are still able to incorporate BrdU. This interpretation is supported by the time-dependent effect of NOS inhibition in the OB and agrees with the hypothesis that, in this structure, local NO participates in synaptic remodeling rather than in neurogenesis (Chen et al., 2004).

An interesting observation was that proliferating $BrdU^+$ cells as well as PSA-NCAM-positive neuroblasts in the adult SVZ never expressed nNOS, which indi-

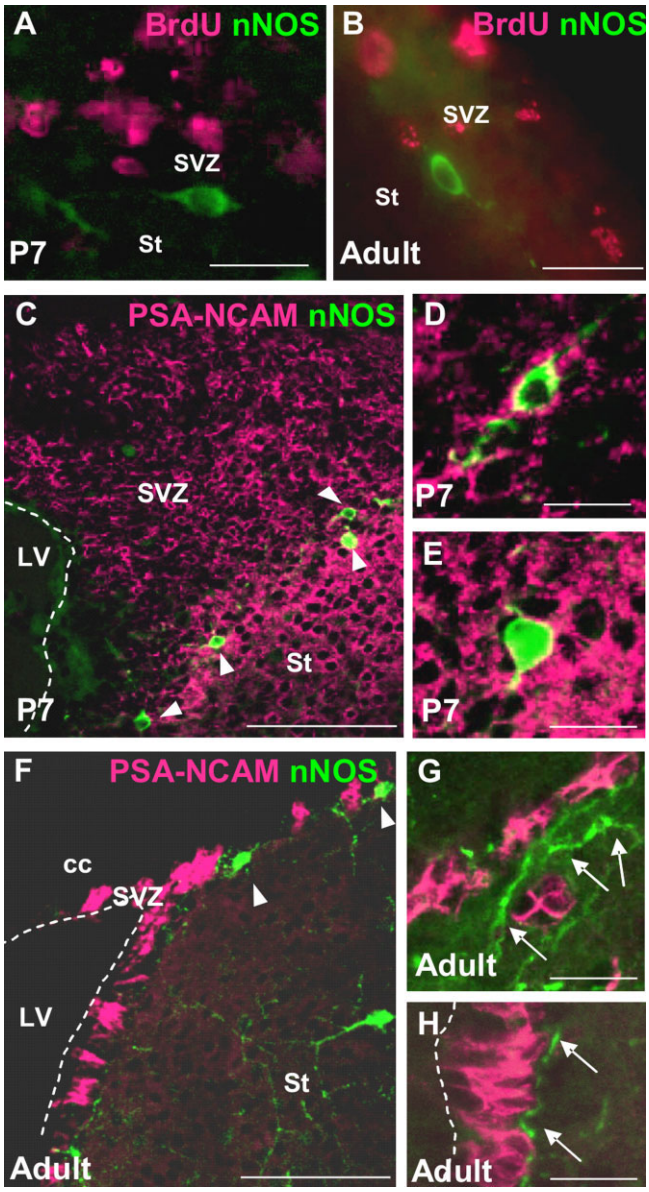

Fig. 4. Relationship between nNOS-expressing neurons and precursor cells in the postnatal and adult subventricular zone (SVZ). A,B: Fluorescence microscopic images of postnatal and adult mouse SVZ coronal sections doubly immunostained for BrdU and nNOS. BrdU was administered between 2 and 7.5 hours before brain perfusion. Larger number of proliferating cells can be seen in the postnatal compared with the adult SVZ. At both stages, nNOS was expressed by neurons in the proximity of the proliferating cells, but never by cells undergoing mitosis. C–H: Confocal images of the SVZ of postnatal and adult mice immunostained for nNOS and PSA-NCAM. Arrowheads in C and F indicate nitrergic cell bodies in the SVZ; in the postnatal brain, colocalization with PSA-NCAM can be observed in the higher magnification images shown in D and E. Arrows in G and H point to well-developed nitrergic neuropil in the adult SVZ. cc, Corpus callosum; LV, lateral ventricle; St, striatum. Scale bars = 20 μm in A,B; 100 μm in C,F; 10 μm in D,E,G,H.

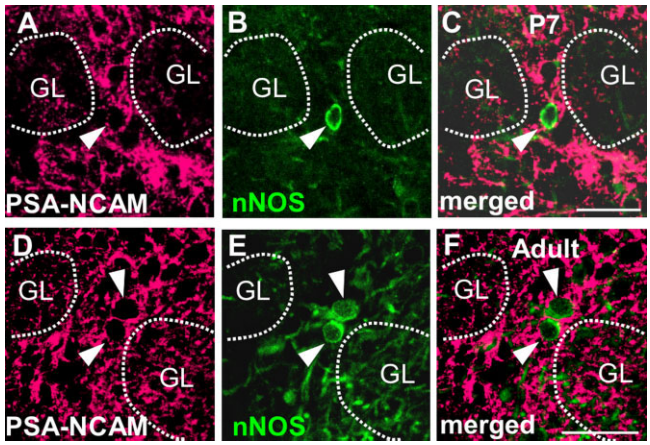

Fig. 5. Colocalization of nNOS and PSA-NCAM in the olfactory bulb (OB) glomerular layer of postnatal and adult mice. Confocal images of postnatal (A–C) and adult (D–F) OB immunostained for nNOS and PSA-NCAM. Arrowheads, periglomerular neurons showing nNOS in their cytoplasm and PSA-NCAM in their membranes; GL, glomeruli. Scale bar = 25 μm in C (applies to A–C); 25 μm in F (applies to D–F).

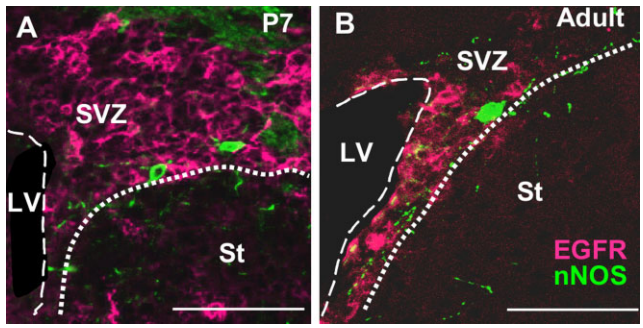

Fig. 6. Spatial relationship between nitrergic neurons and their epidermal growth factor receptor (EGFR)-expressing target cells in the subventricular zone (SVZ) of postnatal and adult mice. Confocal images of the SVZ of postnatal (**A**) and adult (**B**) mice immunostained for nNOS and EGFR. In the postnatal brain, a larger extension close to the dorsolateral wall of the lateral ventricle is occupied by EGFR-positive cells than in adulthood. However, nitrergic cell bodies and neuropil reach only a narrow strip of EGFR-positive cells close to the limits between the SVZ and the striatum (dotted line) in both postnatal and adult animals. Such a disposition makes difficult the access of NO to potential targets during the postnatal period. LV, lateral ventricle; St, striatum. Scale bars = 100 μm.

cates that NO is a molecule that participates in the SVZ neurogenic niche but is not synthesized by neural precursors. Colocalization of nNOS and PSA-NCAM in postnatal SVZ implies not that SVZ neuroblasts express nNOS but rather that nitrergic neurons during this period are still immature and forming their connexions, which is in agreement with their less well developed neuropil. In the OB, colocalization of nNOS and PSA-NCAM in periglomerular cell bodies in both postnatal and adult mice may represent a transitory nNOS expression concomitant with neuroblast differentiation (Cheng et al., 2003) or, alternatively be the consequence of PSA-NCAM production by mature nitrergic interneurons whose synaptic connections within the glomeruli are under continuous remodeling as a result of olfactory receptor neuron turnover.

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