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Review

Role of nitric oxide in subventricular zone neurogenesis

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Abstract

A possible role of nitric oxide (NO) in adult neurogenesis has been suggested based on anatomical findings showing that subventricular zone (SVZ) neuroblasts are located close to NO-producing cells, and on the known antiproliferative actions of NO in many cell types.

Experiments have been performed in rodents with systemic and intracerebroventricular administrations of the NO synthase (NOS) inhibitor L-NAME. NOS inhibition leads to significant increases in the number of proliferating cells in the SVZ and olfactory bulb (OB). NO exerts its cytostatic action preferentially on the cell population expressing nestin but not βIII-tubulin, which may correspond to the type C cells described in the SVZ. The negative effect of NO on SVZ cell proliferation has also been confirmed in SVZ primary cultures.

An inhibition of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) is described as one of the molecular mechanisms responsible for the antiproliferative effect of NO in SVZ cells. Biochemical data supporting this conclusion has been obtained using the neuroblastoma cell line NB69, which endogenously expresses the EGFR. In these cells, the antimitotic action of NO occurs upon inhibition of the EGFR tyrosine phosphorylation, probably by a direct S-nitrosylation of the receptor.

The latest published reports on NO and neurogenesis indicate that NO physiologically participates in the control of adult neurogenesis by modulating the proliferation and fate of the SVZ progenitor cells. These effects might be partially due to a direct inhibition of the EGFR by S-nitrosylation.

Theme: Development and regeneration
Topic: Genesis of neurons and glia

Keywords: Neural precursor; Cell proliferation; Nitric oxide synthase; Adult neurogenesis; Epidermal growth factor

Contents

Abbreviations: BrdU, Bromodeoxyuridine; DETA-NO, Diethylenetriamine-nitric oxide adduct; DG, Dentate gyrus; EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor; EPO, Erythropoietin; GFAP, Glial fibrillary acidic protein; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, Nitric oxide; NOS, Nitric oxide synthase; NSE, Neuronal specific enolase; PSA-NCAM, Polysialylated form of the neuronal cell adhesion molecule; SNAP, S-nitroso-N-acetylpenicillamine; SVZ, Subventricular zone

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1. Nitric oxide in the nervous system

1.1. General aspects

Nitric oxide (NO) is a short-life diffusible gas that acts in an autocrine and/or paracrine manner in different tissues. NO has important functions in the immunological, cardiovascular and nervous systems [28,46]. Many effects of NO are mediated by its interaction with transition metal-containing proteins, which results in stimulation (e.g., soluble guanylate cyclase) or inhibition (e.g., cytochrome oxidase) of their intrinsic activity; also, the formation of nitrosothiol adducts in specific cysteine residues (or S-nitrosylation) is currently considered a post-translational mechanism of protein regulation by NO.

NO is synthesized by the enzyme nitric oxide synthase (NOS) that catalyzes the conversion of L-arginine and oxygen into NO and citrulline [46,57]. A heterogeneous

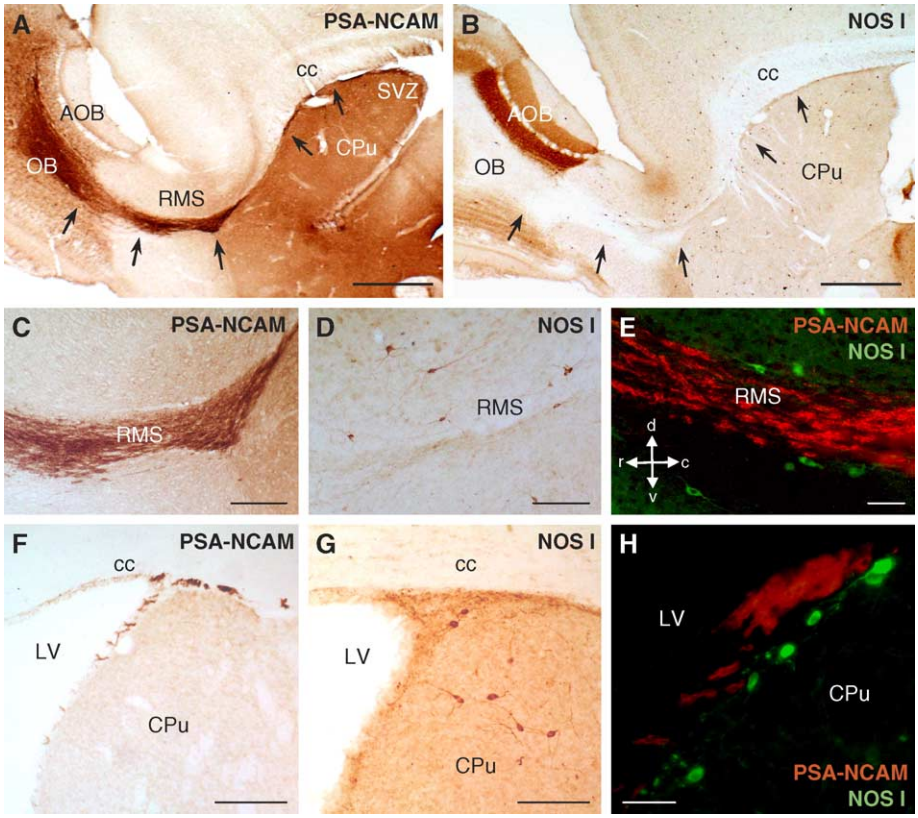

Fig. 1. Relationship between nitrergic cells and neuronal precursors in the subventricular zone (SVZ) and rostral migratory stream (RMS). Photomicrographs of adult mouse sagittal (A–E) and coronal (F–H) sections labeled for the neuronal progenitor marker PSA-NCAM (A, C, E, F, and H) and neuronal nitric oxide synthase (NOS I or nNOS) (B, D, E, G, and H). (A–B) Photomicrographs showing the SVZ, RMS and olfactory bulb (OB) in a sagittal view. (C–E) Higher magnification photomicrographs showing sagittal views of the RMS. (F–H) Photomicrographs showing coronal views of the SVZ. Note in all the photographs the proximity of nitrergic neurons and neuronal precursors, and, in the double labelings shown in E and H, the absence of colocalization of NOS I with PSA-NCAM in the same cell. AOB, accessory olfactory bulb; c, caudal; cc, corpus callosum; CPu, caudate/putamen; d, dorsal; LV, lateral ventricle; OB, olfactory bulb; r, rostral; v, ventral. Calibration bars: 750 μm in A and B; 150 μm in C, D, F, and G; and 50 μm in E and H. The details of the immunohistochemistry are described in Moreno-López et al. [49].

group of cofactors (NADPH, FAD, FMN, haem group, tetrahydrobiopterine and calmodulin) is required for the activity of this enzyme [30]. So far, three different NOS isoforms have been identified in mammals: neuronal NOS (nNOS or NOS-I), inducible NOS (iNOS or NOS-II), and endothelial NOS (eNOS or NOS-III). The principal form expressed in the brain is nNOS and its expression is restricted to neurons. The other isoforms, eNOS and iNOS, are found in other cell types in the CNS [17,32]. NO synthesized by nNOS acts as a modulator of synaptic activity [5,47] and has important roles in neuronal differentiation, survival, and synaptic plasticity [33]. In addition, NO is also involved in CNS pathological processes such as ischemic neuronal death and neurodegeneration [16,23].

1.2. Involvement of NO in proliferation and differentiation

NO exerts antiproliferative actions on a wide variety of cell types [26,27,44,52,62,74], including cells of neuronal lineage [50,59]. In addition, a role of NO in neural cell differentiation is supported by two lines of evidence. First, several tumoral cell lines, including human neuroblastoma and PC12 cells, express different isoforms of NOS when they are exposed to differentiating factors [29,55,67], thus suggesting that these enzymes may contribute to the

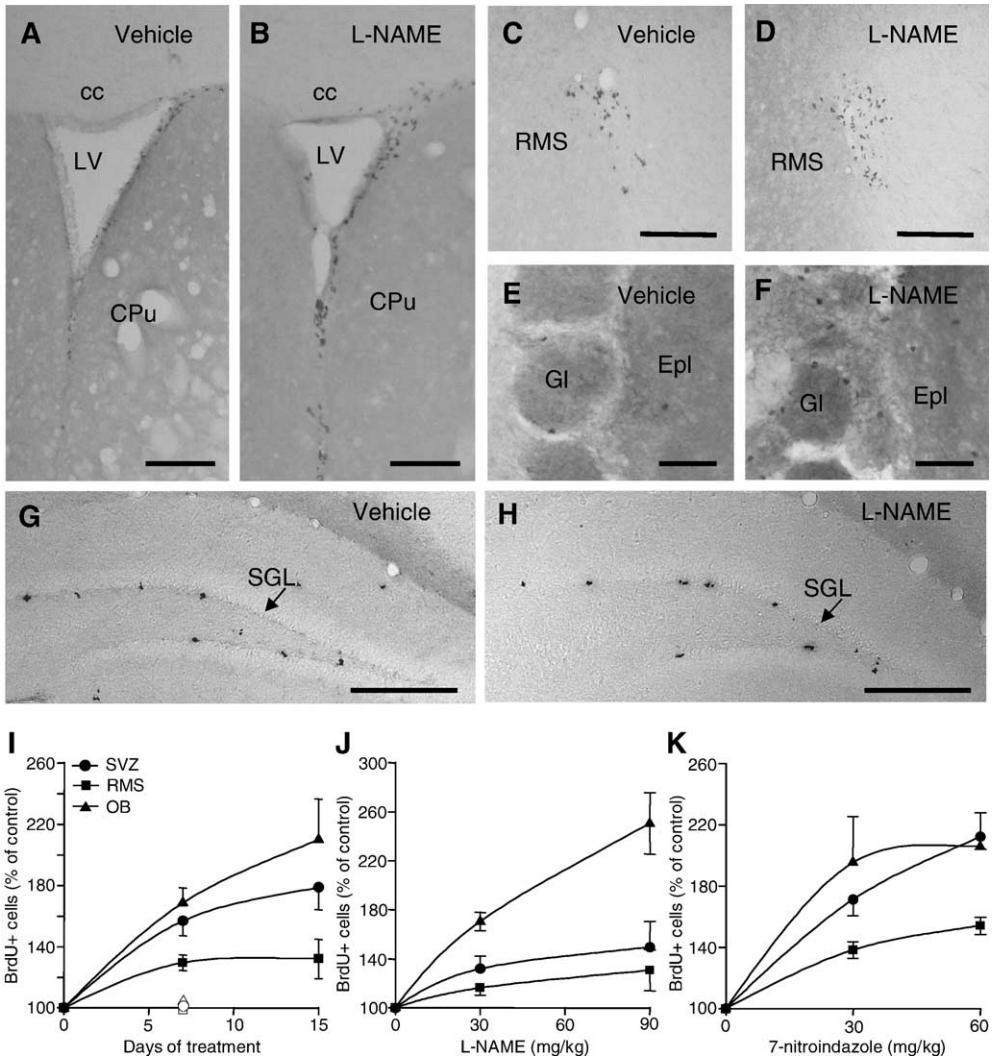

Fig. 2. Effect of NO synthesis inhibition on the proliferation of neural precursors in the adult forebrain. Photomicrographs showing coronal sections processed for BrdU immunohistochemistry, to point out recently divided cells in the subventricular zone (SVZ, A, B), rostral migratory stream (RMS, C, D) glomerular layer (Gl) of the olfactory bulb (E, F), and dentate gyrus of the hippocampus (G, H), obtained from two mice treated for 7 days with vehicle or L-NAME (90 mg/kg/day). cc, corpus callosum; CPu, caudate/putamen; Epl, external plexiform layer; SGL, subgranular layer; LV, lateral ventricle. Calibration bars: A–D, 100 μm, E–F, 50 μm. G–H, 150 μm. I–K: number of bromodeoxyuridine-positive ($BrdU^+$) cells in the SVZ, RMS and OB of mice treated with 90 mg/kg/day L-NAME for 7 or 15 days (I), treated for 15 days with different doses of L-NAME (J), or receiving different doses of 7-nitroindazole (7-NI) for 4 days (K). The effects of D-NAME injections are shown with open symbols in I. After completion of the treatments, the animals were i.p. injected with 200 mg/kg BrdU, distributed in three injections separated by 2.5 h intervals (7.5 h cumulative labeling). Results are expressed as percentage of the values obtained in animals injected with vehicle and are presented as means ± SEM. At least four mice per group were used. All groups treated with L-NAME or 7-NI were significantly different from controls ($P < 0.05$, non-parametric Mann–Whitney U test). From Moreno-López et al. [49]. Copyright 2004 by the Society for Neuroscience.

differentiation process. Second, inhibition of NOS activity prevents or delays differentiation in these cell lines [53,59,65] and in neural progenitor cells [11]. Since cell cycle arrest and differentiation are closely linked phenomena, it is possible that NO, due to its anti-mitotic action, acts as a permissive factor allowing the subsequent action of the differentiating agents.

NO might then have a role in the maturation process that occurs during both developmental and adult neurogenesis, since it seems to be required to induce the switch from immature proliferative neural cells towards well-differentiated neurons. In order to consider such a possibility, a morphological evidence should be provided: are neuronal progenitors able to synthesize NO and/or are they exposed to a NO source in vivo?

2. Anatomical relationships between NO-producing cells and neuronal precursors. Functional implications

2.1. Development and postnatal period

Expression of nNOS is dynamic, both during development and postnatally [6,13,54]. For instance, nNOS is widely expressed in the cortical plate during embryogenesis [6], whereas it is restricted to discrete neuronal populations in adult rodents [7,8]. In the developing brain of *Xenopus* tadpoles, cells expressing the neuronal isoform of *Xenopus* NOS are located close to the zone of dividing precursors [60]. Relevantly, prevention of NO formation during this period leads to excessive cell division and to brains with perturbed organization [60]. Important consequences are also derived from NOS inhibition during development in *Drosophila* and in rodents [22,61].

In a recent paper, Chen et al. [10] have demonstrated different roles for NO in the olfactory system depending on the developmental stage. During embryonic and early postnatal periods, nNOS is expressed in neuronal precursors, and endogenous NO promotes proliferation of olfactory receptor precursors. However, in the adult, nNOS appears restricted to periglomerular interneurons in the olfactory bulb, where it influences glomerular organization and connectivity.

Therefore, spatio-temporal NO production during development and postnatally has important functional implications for a correct balance between proliferation and differentiation.

2.2. Adult age

It is well accepted that, in the adult brain of many animal species, there are two main neurogenic areas: the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus (DG) of the hippocampus (for review, see Ref. [25]). The SVZ is located along the lateral walls of the lateral ventricles [18] and, in contrast to what occurs in the

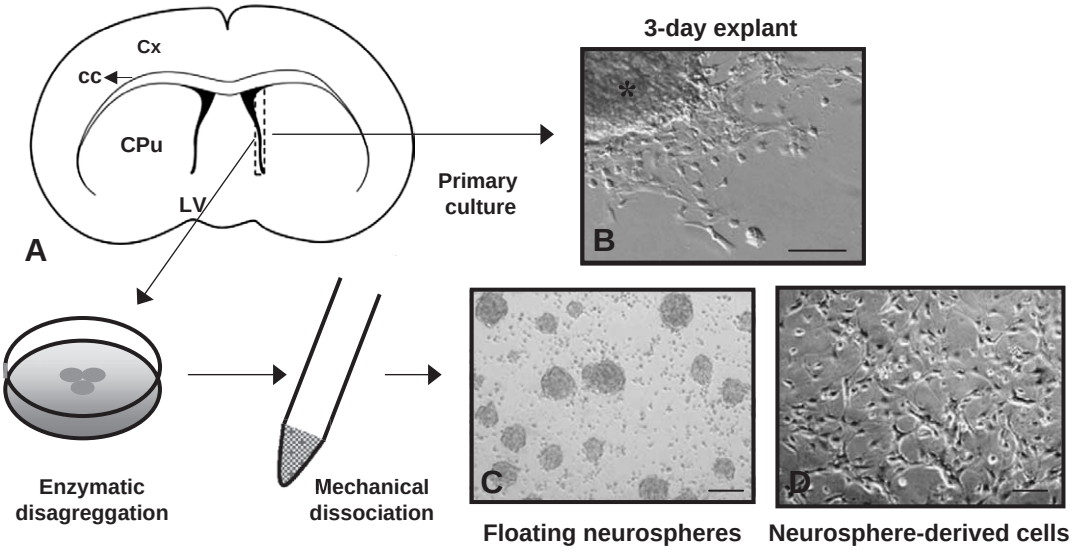

Fig. 3. Subventricular zone (SVZ) cultures used to analyze the effect of NO on cell proliferation in vitro. (A) Schematic drawing of a coronal section showing in dashed lines the tissue dissected out for the SVZ cultures. Cx, cortex; cc corpus callosum, Cpu, caudate/putamen, LV, lateral ventricle. (B) Phase-contrast photomicrograph showing a 3-day SVZ explant. Explants obtained from P7 mice were cultured on poly-D-lysine-coated dishes in medium containing 10% horse serum and epidermal growth factor (EGF, 20 ng/ml) at 37 °C and 5% CO_2. The cells grew around the original tissue (asterisk) and had a limited life span. See Matarredona et al. [45] for details. (C) Phase microscopy image of floating neurospheres formed from SVZ cells that were isolated essentially as described by Reynolds and Weiss [64]. Briefly, postnatal SVZ dissected tissue was enzymatically dissociated in medium containing 1 mg/ml trypsin and 0.2 mg/ml kinurenic acid. After centrifugation, the cells were resuspended and mechanically disaggregated with a fire polished Pasteur pipette. The dissociated cells were resuspended in a defined medium supplemented with 20 ng/ml EGF and 25 μg/ml insulin and maintained in an atmosphere of 5% CO_2, at 37 °C. After 1–2 days, cell aggregates known as neurospheres were formed. Every 4–5 days, neurospheres were centrifuged, resuspended in fresh medium, mechanically dissociated, and seeded at a density of 10^5 cells/ml to obtain new neurospheres. (D) Phase-contrast photomicrograph of neurosphere-derived cells seeded on a poly-ornithine substrate for 3 days. Calibration bars: 100 μm.

hippocampus, where neurons are born and differentiate in the same location, the SVZ neuroblasts migrate tangentially along the rostral migratory stream (RMS) to reach the olfactory bulb (OB) where they mature into local interneurons [37,42,58].

A close proximity between nitrergic neurons and neuronal precursors has been described in the SVZ and DG of adult mice [48] (Fig. 1). In the RMS, NOS-positive cells form a loose network around the migrating neuroblasts (Fig. 1). This special anatomical distribution has also been described in adult rats [56]. The NO-producing cells surrounding the neuroblasts are well-differentiated and present abundant varicose processes that intermingle with the chains of precursor cells.

Neuroblasts, identified by PSA-NCAM immunolabeling, never express nNOS in the adult SVZ, but do so in the OB, specifically in the granular layer and around the glomeruli. Interestingly, it is in the OB where the final differentiation of these migrating neuroblasts occurs. Thus, it may happen that these double-labeled cells represent an intermediate stage of differentiation of neuroblasts towards a nitrergic phenotype or, alternatively, that differentiating neurons transitionally express nNOS whatever their final phenotype might be. As previously stated, different types of cell cultures express different NOS isoforms when they are induced to differentiate using several differentiating agents [29,55,67].

Increasing evidence suggests that continuous replacement of neurons in the OB may have a role in the adjustment of olfactory circuitry as the environment or relevance of odor change. NO production within the OB may mediate a correct OB cell turnover which is necessary for the development of normal olfactory-guided behaviors. Indeed, NOS knock-out mice have been described to present alterations in behaviors directly related to olfactory learning (aggressiveness or sexual behaviour) [51].

The morphological distribution of neuronal precursors and nitrergic neurons suggests that NO plays a role in adult

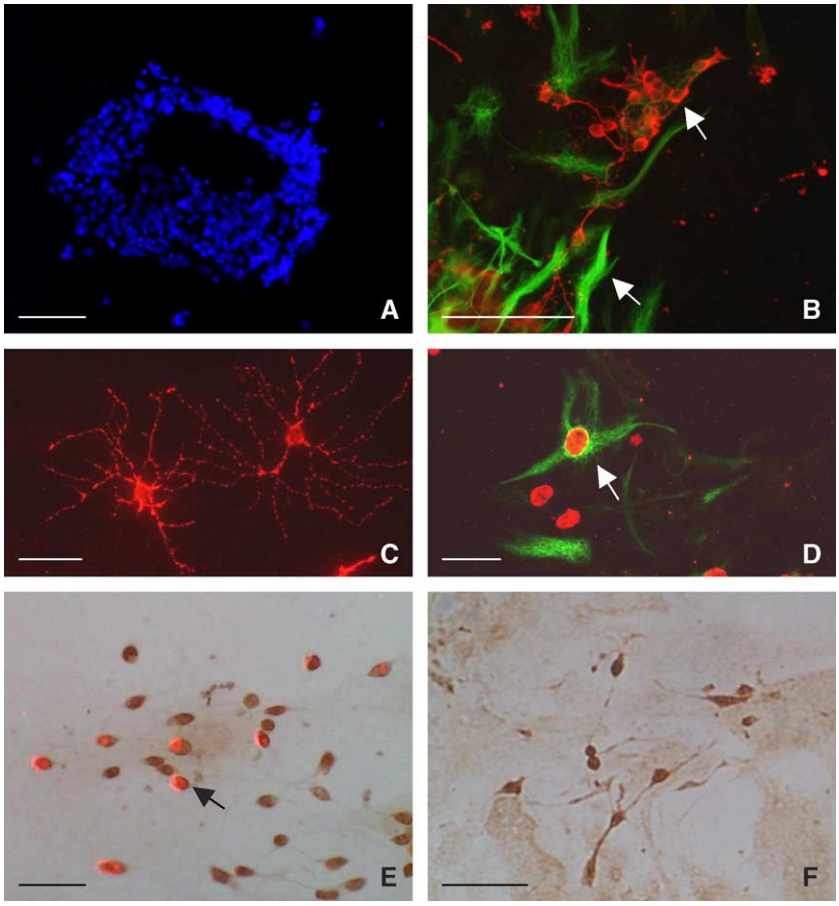

Fig. 4. Phenotypic characterization of postnatal mouse SVZ explants. (A) Appearance of a 3-day SVZ explant after fixation and nuclear staining with DAPI. (B) Double immunostaining for βIII-tubulin (red) and GFAP (green) showing SVZ explant-derived neurons and astrocytes. (C) SVZ-derived oligodendrocytes immunolabeled with the marker A2B5. (D and E) SVZ-derived cells proliferate in vitro. Bromodeoxyuridine (BrdU) was added 24 h after seeding and maintained until fixation (48 h later) to identify the nuclei of cells that underwent mitosis in vitro. BrdU was incorporated by astrocytes and neurons as revealed by double immunostainings for GFAP and neuronal specific enolase (NSE) with BrdU (D and E, respectively). Arrows indicate examples of double labeled cells. (F) nNOS immunocytochemistry in a SVZ explant after 3 days in vitro. nNOS-positive cells presented morphological features of neurons such as long processes with varicosities. Calibration bars: 50 μm in A and B and 25 μm in the others. The details of the immunohistochemistry are described in Matarredona et al. [45].

neurogenesis. NO may act in a paracrine manner when released from cell layers adjacent to the progenitor cell population in the SVZ, and as an autocrine mediator in OB neuroblasts, during their final maturation stages toward a differentiated phenotype.

3. Effects of NO on neural precursor proliferation in different neurogenic systems

Recently, three independent articles have convincingly demonstrated a function of endogenous NO as a negative regulator of adult neurogenesis in physiological conditions [11,49,56].

To analyze the effect of NO on cell proliferation within the neurogenic areas, experiments have been performed by inhibiting NO synthesis with L-NAME, a non-selective competitive inhibitor of NOS activity [63]. Packer et al. [56] administered L-NAME into the lateral ventricle of adult rats with osmotic minipumps. Their study was complemented by using animals in which the nNOS had been genetically inactivated. They showed that reduction of NOS activity increases neurogenesis in both the SVZ and the hippocampal DG.

Moreno-López et al. [49] carried out experiments using systemic administrations of L-NAME and, in parallel, of 7-nitroindazole (7-NI), a selective nNOS inhibitor which has no vascular effects and does not increase blood pressure. After the treatments, the number of proliferating cells increased in the SVZ and OB. Interestingly, under these conditions, no effects were detected in the DG (Fig. 2). The possibility that endogenous NO may have an effect on programmed cell death was ruled out after demonstrating by the TUNEL technique that the number of apoptotic cells after L-NAME administration was not significantly different from that observed in control animals [49,56]. The distribution of postmitotic cells throughout the SVZ-OB neurogenic area did not differ between control and L-NAME-treated animals, which also excludes an effect of NO on cell migration [49].

In contrast with the results obtained with the intracerebroventricular administrations of L-NAME, an effect on neurogenesis in the hippocampal DG was not found with the systemic administration experiments. The different response to NOS inhibition observed in the SVZ and DG has two possible explanations. It might be due to a difference in the availability of endogenous NO in the two regions, or to a difference in sensitivity to NO. There is some data supporting this latter possibility. First, SVZ precursors increase their proliferation in response to epidermal growth factor (EGF), whereas DG precursors do not [39]. Second, expression of the EGF receptor (EGFR) is much more prominent in the SVZ than in the DG [35]. And third, NO exerts antiproliferative actions on several cell lines by direct inhibition of the EGFR tyrosine kinase activity [24,50]. All

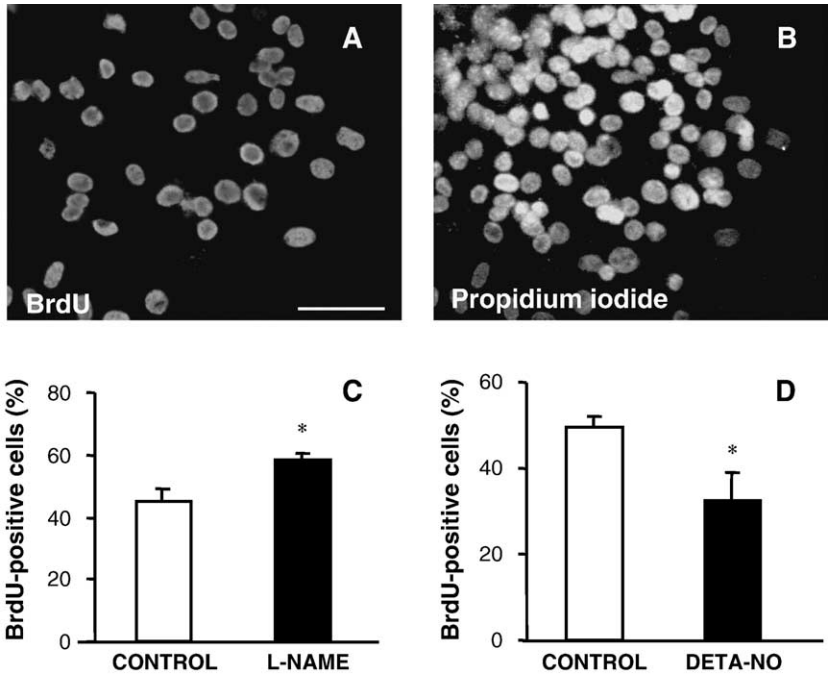

Fig. 5. Effect of NOS inhibition and NO donor addition on SVZ-derived cell proliferation. (A and B) Photomicrographs showing bromodeoxyuridine (BrdU)-labeled nuclei (A) and the total number of cell nuclei, as stained with propidium iodide (B), in the same field of a SVZ culture in control conditions. Calibration bar: 50 μm. (C and D) Effect of the NOS inhibitor L-NAME and the NO donor DETA-NO on the percentage of cells labeled with BrdU in SVZ explants. Explants were seeded in the absence (open bar) or presence of 0.1 mM L-NAME (C, filled bar) or 10 μM DETA-NO (D, filled bar). After 24 h in vitro, BrdU (10^{-4} M) was added to the culture medium. Two days later, cells were fixed, processed for BrdU immunohistochemistry and counted as described in Ref. [45]. Data are the mean ± SEM of the results obtained from 4 to 6 animals. *$P < 0.05$, as compared with the values obtained in cultures without treatments (Student's t test). Reprinted from Ref. [45]. Copyright (2004), with permission from Elsevier.

these data suggest that EGFR may be one of the molecular targets for the antiproliferative effect of NO on the SVZ neuronal precursors.

Additional evidence for a direct effect of NO on SVZ neurogenesis was obtained by using SVZ primary cultures and subsequently analyzing the effects of endogenous and exogenously-added NO on the proliferation of the SVZ-derived cells [45]. Explants from the SVZ of postnatal mice were cultured in the presence of EGF, a condition in which cells migrate out of the explants, proliferate, and differentiate into neuronal and glial cells after 3 days of culture (Figs. 3 and 4). Inhibition of NOS activity with L-NAME during this culture period led to a significant increase in cell proliferation (Fig. 5). The cytostatic effect of NO was also corroborated by a decrease in bromodeoxyuridine (BrdU) incorporation after culture treatments

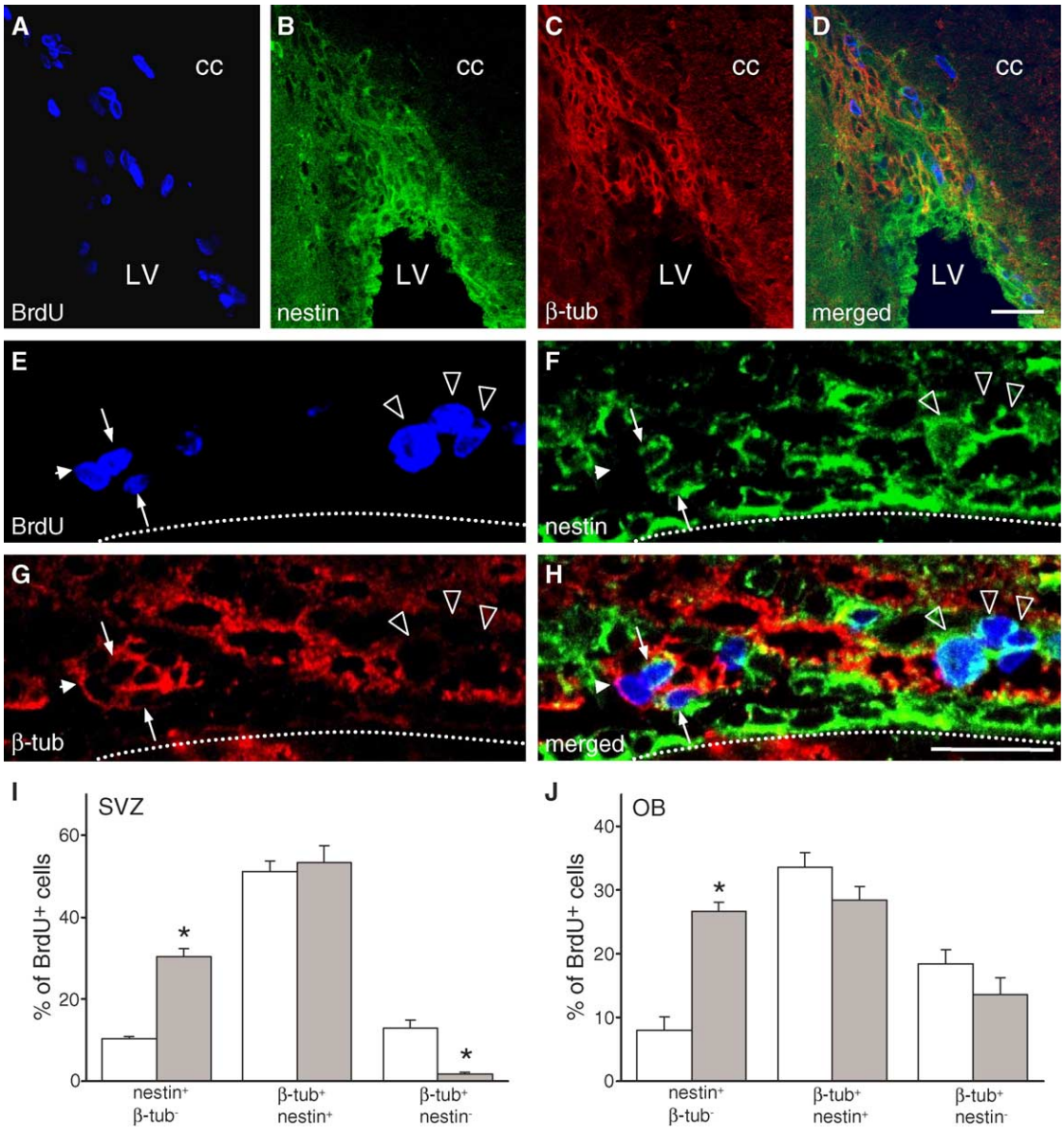

Fig. 6. Effect of NOS inhibition on the expression of nestin and βIII-tubulin (β-tub) by proliferating cells. Confocal photomicrographs of a coronal section through the subventricular zone triple-labeled for BrdU (A), the precursor marker nestin (B), and the young-neuron marker βIII-tubulin (β-tub; C), obtained from an adult mouse treated with L-NAME (90 mg/kg/day) for 15 days, and then injected with BrdU. The merged image is shown in D. (E–H) High magnification confocal photomicrographs of a coronal section through the lateral wall of the lateral ventricle, showing recently divided cells expressing nestin (empty arrowheads), β-tub (filled arrowhead) and double-labeled for nestin and β-tub (arrows). The merged image is shown in H. The dotted line indicates the limits of the lateral ventricle. The section was obtained from the same mouse as in A. Calibration bars: 25 μm. (I and J) Percentage of recently divided cells that were characterized as nestin$^+$/βIII-tubulin$^-$, nestin$^+$/βIII-tubulin$^+$ or nestin$^-$/βIII-tubulin$^+$, in the subventricular zone (SVZ; I) and olfactory bulb (OB, J). The analysis of the recently divided cells that were positive for GFAP has not been included since cells double labeled for BrdU and GFAP were found only occasionally in the SVZ and OB. Mice were treated for 15 days with 90 mg/kg/day L-NAME (filled bars) or with vehicle (empty bars), and then injected with BrdU as described in Ref. [49]. For each group of mice, a total of 1165 BrdU$^+$ cells were analyzed in the SVZ and 714 in the OB. Data are expressed as means ± SEM. n =3. *P < 0.05 (non-parametric Mann–Whitney U test). From Ref. [49]. Copyright 2004 by the Society for Neuroscience.

with low concentrations of the NO donor DETA-NO (Fig. 5). No effects on apoptotic cell death were found after treatment of the cultures with L-NAME or with DETA-NO [45], which indicates that, also in vitro, the effect of NO on this SVZ cell population was due to interaction with cell proliferation and not with cell survival.

Another series of experiments were carried out in SVZ-derived multipotent transit amplifying cells, which generate neurospheres in vitro under stimulation by EGF [20,65] (Fig. 3). By using this culture system, stem cell properties are maintained over multiple subcultures and the SVZ-derived cell population is amplified, thus, allowing subsequent investigation of the NO mechanisms of action. Cells obtained from neurospheres were used for quantification of BrdU incorporation, once adhered to a substrate. Again, both exogenously-added and endogenously-produced NO reduced proliferation of neurosphere-derived cells (unpublished results).

This antiproliferative effect of nNOS-derived NO contrasts with the promoting effects on neurogenesis produced after brain injury events by iNOS-derived NO or NO donors [43,75,77]. Indirect mechanisms, such as the stimulation of growth factors, may mediate the positive effect on neurogenesis found after the general increase in cerebral NO concentration obtained in these conditions. For instance, NO stimulates the secretion of vascular endothelial growth factor [76], which has been shown to induce neurogenesis both in vivo and in vitro [36].

4. Target cells for NO effect in the SVZ

Several cell types have been described in the SVZ: migrating neuroblasts (A cells), astrocytes (B cells), immature precursors (C cells) and, finally, ependymal cells (E cells) [19]. Type A cells are arranged as chains parallel to

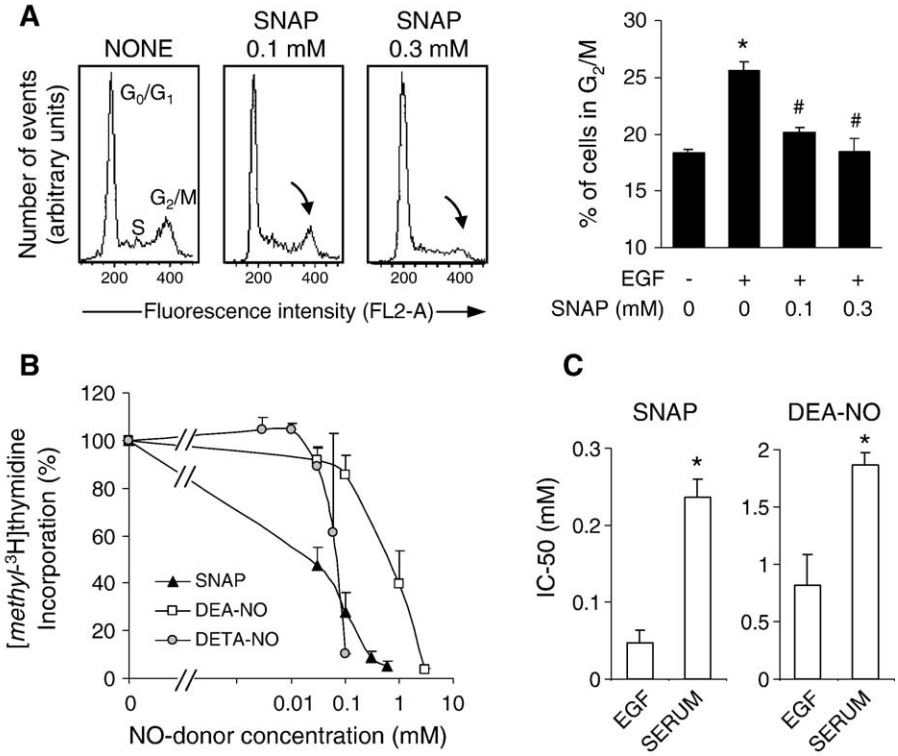

Fig. 7. Effect of NO donors on EGF-induced proliferation in EGF-responsive NB69 human neuroblastoma cells. (A) Effect of the NO donor SNAP on cell cycle in EGF-treated NB69 cells. NB69 cells were cultured for 24 h in serum-free medium containing EGF (human recombinant, 10 ng/ml), in the absence or presence of the NO donor SNAP at the indicated concentrations. Afterwards, cells were detached, fixed, and their DNA was stained with propidium iodide. DNA content of individual cells was measured by flow cytometry, and frequency histograms were used to quantify the relative amount of cells in each of the cell cycle phases. Left panel, typical examples of histograms obtained for each experimental condition, as indicated. Right panel, quantification of the percentage of cells with double DNA content, characteristic of G_2/M phases of the cell cycle. SNAP inhibited the progression through the cell cycle induced by EGF, promoting growth arrest in the G_0/G_1 phases. Data are the mean ± SEM; $n = 3$; $^{*}P < 0.05$ when compared to control cultures; $^{\#}P < 0.05$ when compared to cultures treated with EGF (Student's t test). (B) Incorporation of [*methyl*-^{3}H]thymidine into DNA of NB69 cells grown for 48 h in serum-free medium containing EGF (as above), in the absence or presence of the indicated concentrations of three different NO donors, SNAP, DEA-NO, and DETA-NO. Data are expressed as percentage of the values obtained in the absence of NO donors; mean ± SEM, $n = 3$. As shown in the graph, the three NO donors tested inhibited cell proliferation in a concentration-dependent manner. (C) Calculated concentrations of NO donors required to inhibit by 50% (IC_{50}) [*methyl*-^{3}H]thymidine incorporation into the DNA of NB69 cells cultured for 48 h in medium with either 10 ng/ml EGF or 15% fetal calf serum (as indicated). $^{*}P < 0.05$ as compared with EGF-treated cultures (Student's t test). Cells treated with EGF were more sensitive to the NO antiproliferative effect than cells whose proliferation was stimulated by serum. Further details in Murillo-Carretero et al. [50].

the walls of the ventricle. Astrocytes ensheath the A cell chains in the SVZ and RMS. Type C cells are highly proliferative cells closely associated with the chains of migrating neuroblasts. All of these cell types have been carefully characterized by their ultrastructure, morphology, and molecular markers.

The paracrine antiproliferative action of NO on the SVZ may be exerted on any (or all) of these described cell types. The analysis of phenotypic features among the BrdU-positive cells revealed a priority action of NO on the cell population expressing nestin but not βIII-tubulin (Fig. 6). This population may correspond to the type C cells.

NO may then physiologically control the size of the precursor population before these cells are committed to a specific neuronal phenotype. The final destination of the SVZ progenitors is the OB, where they complete their maturation process resulting in the disappearance of the young-neuron marker βIII-tubulin and the progressive appearance of the mature-neuron marker NeuN [14]. In the experiments using systemic NOS inhibition, a delay in the appearance of NeuN was observed, suggesting a role for NO in facilitating the neuronal differentiation process [49]. Then, NO may control the proliferation rates of the more immature proliferating population in the SVZ and RMS and may have a permissive role in its phenotypic determination in the OB, probably by allowing the action of other undetermined differentiating factors.

5. A possible molecular mechanism for the antiproliferative action of NO in the subventricular zone

5.1. S-nitrosylation of the epidermal growth factor receptor

NO can interact with cysteine residues to form nitrosothiol adducts leading to alterations in the activity of proteins. This reversible process of protein S-nitrosylation is thus considered to be a physiological signalling mechanism for NO [34]. A considerable number of endogenous S-nitrosylated proteins has been found [69] including metabolic enzymes, ion channels, structural proteins, signaling proteins, or proteins involved in apoptosis.

Some of the proteins susceptible to S-nitrosylation are involved in the control of cell proliferation [31,70]. For example, S-nitrosylation of ornithine decarboxylase inhibits proliferation of smooth muscle cells [3,4], and S-nitrosylation of the retinoblastoma (Rb) protein may be responsible for the cell cycle-arrest activity of the Rb pathway in Drosophila [40]. But, to date, no evidence has been provided for a role of S-nitrosylation as the mechanism responsible for the antiproliferative action of NO in the SVZ.

The EGFR is a membrane tyrosine kinase that belongs to the ErbB family of receptors. These molecules remain as monomers until they are activated by specific ligands that induce homo or heterodimerization, and this triggers the activation of their intrinsic tyrosine kinases and the cross-phosphorylation of some of their tyrosine residues [73].

There are some cues that point to EGFR being a candidate molecular target for the antiproliferative effect of NO in the SVZ: (1) SVZ cells express EGFR in humans [72] and in rodents [20,38]. In fact, in the mouse SVZ, it is selectively expressed in type C cells [20]. (2) The proliferation of SVZ progenitors is increased after EGFR activation by specific ligands such as EGF, transforming growth factor-α or heparin-binding EGF-like growth factor in vitro and/or in vivo [15,35,39,64,71]. (3) NO reversibly inhibits the EGFR tyrosine kinase activity in neural cells [50].

The effects of NO on the EGFR were first described in a transfected murine cell line that overexpresses the human EGFR [24] and then in the neuroblastoma cell line NB69 that naturally expresses the EGFR ([50], and Fig. 7). NB69 cells exposed to NO decreased their proliferation in a cGMP-independent way and this cytostatic effect was more evident when the cells were exposed to EGF (Fig. 7). In addition, NO donors reversibly inhibited the EGF-induced tyrosine phosphorylation of the EGFR molecule (Fig. 8), a consequence of the EGFR tyrosine kinase inhibition. Therefore, the antiproliferative effect of NO in NB69 cells is mediated, at least in part, by direct inhibition of the EGFR activity. Neuroblasts of the SVZ have a phenotypic profile similar to that described for NB69 cells: EGFR-positive, TuJ1-positive and GFAP-negative. We then hypothesize that a similar mechanism probably operates physiologically in the SVZ-OB.

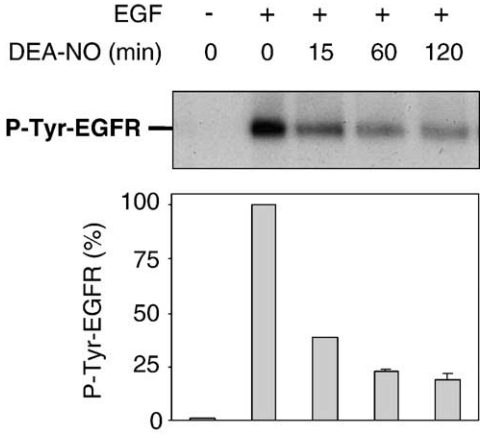

Fig. 8. Effect of NO donors on phosphorylation of the epidermal growth factor (EGF) receptor (EGFR) in NB69 cells. NB69 cells were serum-starved for 24 h, incubated with 1 mM DEA-NO for 15–120 min (as indicated), and pulse-stimulated with 10 ng/ml EGF for 2 min. Next, cells were lysed and the same amount of total protein from each sample were used to immunoprecipitate the EGFR, which was analyzed by SDS-PAGE and Western blotting, using an anti-phosphotyrosine antibody to determine the level of activation of the receptor. Control samples in the absence of EGF and DEA-NO were also included. A representative blot is shown and, below it, the densitometric relative quantification of phosphorylated EGFR bands (P-Tyr-EGFR); $n = 3$. Addition of EGF to the cells produced a dramatic activation of the EGFR, which was inhibited in those cells that were previously treated with DEA-NO. Further details in Murillo-Carretero et al. [50].

Additional unpublished data (Murillo-Carretero et al., submitted for publication) indicates that the EGFR molecule can be S-nitrosylated in vitro in the presence of NO. Therefore, it is possible that, in the SVZ, NO inhibits the EGFR by S-nitrosylation and interrupts its mitogenic signal, with the subsequent cessation of SVZ precursor proliferation. Nevertheless, knowing that NO affects gene expression of proteins involved in the regulation of cell cycle [31] and exerts antiproliferative actions on other cell types by mechanisms independent of S-nitrosylation, we cannot rule out that other additional mechanisms may mediate the antiproliferative effect of NO on the SVZ progenitors.

6. Conclusions

We have provided evidence for the participation of NO in the control of SVZ cell proliferation and differentiation. To date, not many endogenous autocrine-paracrine molecules have been proposed as having a determinating role in adult neurogenesis. Some of the most relevant ones include: bone morphogenetic protein [41], ephrin [12], erythropoietin (EPO) [68], p27Kip1 [21], serotonin [2,9], and IGF-1 [1].

It cannot be excluded that the effects of these factors in the control of neurogenesis may be inter-related or dependent on each other. For instance, EPO receptors co-localize with EGF receptors and the effects mediated by EPO in the SVZ are very similar to those described for NO [68]. A relation between NO and the cell-cycle inhibitor p27Kip1 might also be possible since, in smooth muscle cells, NO upregulates the expression of this factor [66].

The elucidation of the mechanisms that control adult neurogenesis from endogenous neural precursors/stem cells may allow the development of neuronal replacement therapies for neurodegenerative diseases and other central nervous system injuries.

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