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Research report

Nitric oxide synthesis inhibition increases proliferation of neural precursors isolated from the postnatal mouse subventricular zone

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Abstract

The subventricular zone (SVZ) of rodents retains the capacity to generate new neurons throughout the entire life of the animal. Neural progenitors of the SVZ survive and proliferate in vitro in the presence of epidermal growth factor (EGF). Nitric oxide (NO) has been shown to participate in neural tissue formation during development and to have antiproliferative actions, mediated in part by inhibition of the EGF receptor. Based on these findings, we have investigated the possible effects of endogenously produced and exogenously added NO on SVZ cell proliferation and differentiation. Explants were obtained from postnatal mouse SVZ and cultured in the presence of EGF. Cells migrated out of the explants and proliferated in culture, as assessed by bromodeoxyuridine (BrdU) incorporation. After 72 h in vitro, the colonies formed around the explants were constituted by cells of neuronal or glial lineages, as well as undifferentiated progenitors. Immunoreactivity for the neuronal isoform of NO synthase was observed in neuronal cells with long varicose processes. Cultures treated with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) showed an increase in the percentage of BrdU-immunoreactive cells, whereas treatment with the NO donor diethylenetriamine–nitric oxide adduct (DETA-NO) led to a decrease in cell proliferation, without affecting apoptosis. The differentiation pattern was also altered by L-NAME treatment resulting in an enlargement of the neuronal population. The results suggest that endogenous NO may contribute to postnatal neurogenesis by modulating the proliferation and fate of SVZ progenitor cells.

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

Keywords: Neural cell differentiation; Neuroblast; Nitric oxide synthase; Postnatal neurogenesis; Subventricular zone

1. Introduction

Specific regions of the mammalian brain retain the capacity to generate new neurons throughout the entire life of the animal [13,15,25]. In rodents, one of these regions is the subventricular zone (SVZ), located in the lateral walls of the lateral ventricles. The SVZ is a source of newly formed neuroblasts which, after migration through the rostral migratory stream, reach the olfactory bulb and differentiate as interneurons [26,27].

The fate of the SVZ stem cells and their early progeny is probably regulated by local factors and cell–cell contacts, which determine when and where a cell will proliferate, migrate or differentiate. Although these influences are presently under investigation, the proliferative effect of the epidermal growth factor (EGF) on these precursor cells has been well established, both in vitro [12,20,39] and in vivo [9,23].

Nitric oxide (NO) is an intercellular messenger with multiple functions in the cardiovascular, immunological and nervous systems [18,32]. In the brain, NO is synthesized by specific neurons expressing the neuronal isoform of NO synthase (nNOS) [2,40]. During development, the transient expression of nNOS in different brain areas

Abbreviations: BrdU, bromodeoxyuridine; DETA-NO, diethylenetriamine–nitric oxide adduct; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; 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; SVZ, subventricular zone

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[2,19,43] suggests that NO performs some function in embryonic neural tissue formation. A possible role of NO in neurogenesis might be proposed on the basis of its well-known antiproliferative action on many cell types [8,14,16,17,35], since growth arrest is a prerequisite for neuronal precursors to differentiate [37]. Indeed, Peunova et al. [38] have recently described that NO has an antiproliferative effect on the neuronal precursor population during development of *Xenopus*.

We have previously reported that, in the adult mouse brain, neuronal precursor cells in the SVZ and rostral migratory stream are surrounded by differentiated neurons expressing nNOS. Moreover, some of the neuronal precursors themselves express nNOS when they arrive to their final differentiation site in the olfactory bulb [33]. Due to this anatomical disposition, neuronal precursors could be under the influence of NO during their proliferation, migration and differentiation. Our group has also reported evidence of the capacity of NO to directly inhibit the EGF receptor (EGFR) tyrosine kinase thus inhibiting the mitogenic signal initiated by EGF [14,35]. These previous data, together with the above mentioned importance of EGF for SVZ precursor proliferation, raised the hypothesis that NO might modulate the proliferation and fate of the newly formed neurons in the postnatal and adult SVZ.

In this study, we sought to identify the phenotypic characteristics and the proliferative capacity of cells obtained from primary cultures of the postnatal mice SVZ, and to analyze whether endogenously produced and exogenous NO affected their proliferation and/or differentiation. We demonstrate here that, under our culture conditions, neuronal precursor cells express nNOS and that inhibition of NO synthesis increases the proliferation rate of SVZ cells and promotes a neuronal phenotype.

2. Materials and methods

Seven-day postnatal (P7) CD1 mice were used throughout the study. Care and handling of animals was carried out in accordance with the Guidelines of the European Union Council (86/609/EU) and the Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals.

2.1. Postnatal-mouse SVZ cultures and treatments

The SVZ of P7 mice were dissected out as described by Lois and Alvarez-Buylla [25]. Briefly, a coronal slice was cut from each mouse brain, beginning at the rostral opening of the third ventricle and extending 1.5–2 mm caudally. Then, two ≈ 100-μm wide dorsoventral tissue strips, containing the SVZs, were dissected out and minced with scalpels into micropieces. The explants were cultured onto 30-mm diameter coverslips coated with poly-D-lysine (0.5 mg/ml) and placed on 35-mm diameter dishes containing 800 μl of culture medium, which was renewed with 1 ml of fresh medium after 48 h. The medium composition was as follows: 65% (v/v) Dulbecco's modified Eagle's medium (DMEM), 25% (v/v) Earle's balanced salt solution, 10% (v/v) horse serum, 1 mM glutamine, 50 μg/ml gentamicin, 125 ng/ml fungizone, 6 mg/ml glucose and 20 ng/ml EGF. Cultures were maintained at 37 °C in a humidified atmosphere of 7% CO_2 in air. Approximately 60% of the explants adhered to the substrate and gave rise to colonies.

Explants obtained from a single brain (250–300) were distributed in four coverslips. All experiments were performed with paired samples, using both control and treated cells from the same animal. Treatments with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 0.1–0.6 mM) or the NO donor diethylenetriamine–nitric oxide adduct (DETA-NO; 10–60 μM), both dissolved in culture medium, were initiated at the seeding time. Unless otherwise indicated, control and treated cultures were maintained for 72 h. At that time, the cells were fixed for 10 min with 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) and were processed for immunocytochemistry.

2.2. Immunocytochemical studies

The following primary antibodies were used: rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, 1:500; DAKO), mouse monoclonal anti-GFAP (1:300; Sigma, St. Louis, MO), rabbit polyclonal anti-nestin (1:5000) [30], mouse monoclonal raised against the polysialylated form of the neural cell adhesion molecule (PSA-NCAM, 1:4000) [41], mouse monoclonal anti-βIII-tubulin (1:1000; Promega), rabbit polyclonal anti-neuronal specific enolase (NSE, 1:500; Polyscience), rabbit polyclonal anti-nNOS (1:5000 [45] and 1:250; Santa Cruz Biotechnology), mouse monoclonal anti-O4 (1:5; Boehringer Mannheim), mouse monoclonal anti-A2B5 (1:20) [46] and mouse monoclonal anti-BrdU (1:100; DAKO). The secondary antibodies used were: anti-rabbit IgG FITC (1:200), anti-mouse IgM Cy3 (1:100) and biotinylated anti-rabbit IgG (1:800), from Jackson ImmunoResearch; biotinylated anti-mouse IgG (1:250) and anti-mouse IgG FITC (1:1000), purchased from Sigma; and anti-mouse IgG Cy3 (1:4000) from Amersham Life Science.

For immunofluorescence, fixed cells were incubated for 30 min with a solution containing 2.5% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS), to block nonspecific antibody binding. Incubations with primary antibodies were carried out overnight at 4 °C in the blocking solution. Cells were then washed with PBS and incubated with the appropriate secondary antibodies for 1–2 h at room temperature. After washing with PBS, coverslips were mounted on slides with Vectashield and fluorescent signals were detected using a BX60 Olympus microscope fitted with appropriate filters.

For detection of NSE, O4 (an oligodendrocyte specific sulfatide) and nNOS, cells were treated with 2% H_2O_2

and 60% methanol in PBS for 10 min to block endogenous peroxidase activity, before overnight incubation with the primary antibodies. Then, cells were incubated with the corresponding biotinylated secondary antibodies and stained following the avidin–biotin–peroxidase method (ABC system, Vector Laboratories). Peroxidase activity was made visible using 0.01% (v/v) H_2O_2 as substrate and 0.05% (w/v) 3,3′ -diaminobenzidine as chromogen.

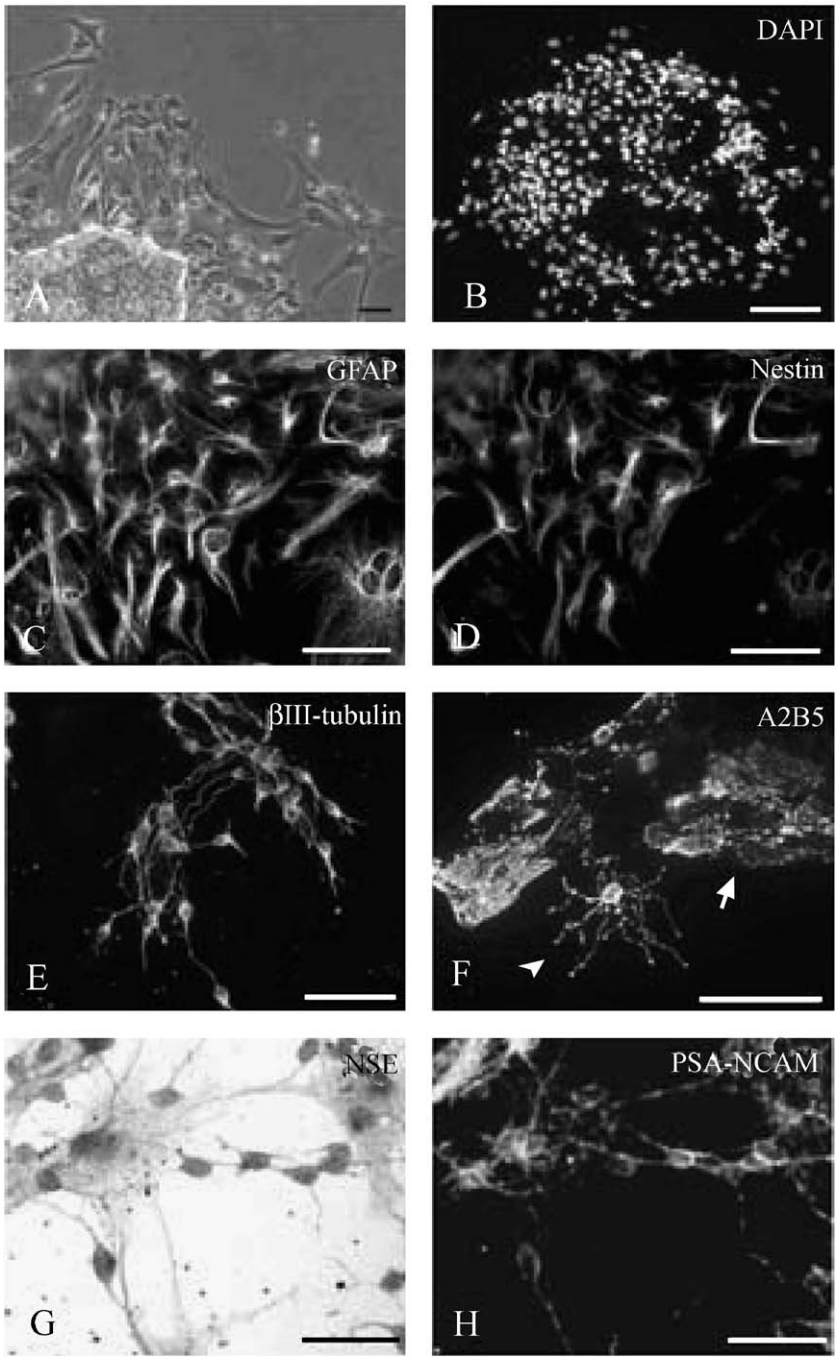

Fig. 1. Phenotypic characterization of postnatal mouse SVZ explants. (A) Phase-contrast photomicrograph showing part of a SVZ explant (outlined) with cells grown around the original tissue, after 3 days in culture. (B) Appearance of a 3-day SVZ explant after fixation and nuclear staining with DAPI. (C) Immunostaining for GFAP showing SVZ explant-derived astrocytes. (D) Nestin immunofluorescence in the same field shown in C. Note the high degree of co-localization with GFAP. (E) Immunostaining for the neuronal marker βIII-tubulin in cells grown around a SVZ explant. Small round neuronal cells with processes are visible. (F) The glial precursor marker A2B5 labeled cells with different morphologies: cells resembling oligodendrocytes (arrowhead) and flat cells without processes (arrow). (G) Expression of neuronal specific enolase (NSE) by SVZ-cultured cells. (H) Immunostaining with an antibody against PSA-NCAM of the same field shown in G; an almost complete co-expression of this antigen with NSE can be observed. Calibration bars: 25 μm in G and H, and 50 μm in the others.

Table 1
Distribution of the different phenotypes among total cells surrounding SVZ explants and among cells that incorporated BrdU in vitro

	Total cells (%)	$BrdU^+$ cells
GFAP	39.9 ± 3.9	25–35%
βIII-tubulin	18.8 ± 3.4	10–20%
Nestin	50.6 ± 1.5	45–55%

Percentage of GFAP-, βIII-tubulin- and nestin-positive cells among the total cell population (left column) and among cells that had incorporated BrdU in vitro (right column) in postnatal SVZ explants grown in medium containing 10% horse serum and 20 ng/ml EGF. Quantitative data are mean ± S.E.M., $n = 4–8$. Results from BrdU double labelings are given as semi-quantitative values (see Section 2).

Coverslips were dehydrated, mounted on slides with DePeX and analyzed under light microscopy.

For double staining experiments, incubations with the primary or secondary antibodies were performed simultaneously. All the secondary antibodies were adsorbed against several species, to prevent undesired cross-reactions. All double stainings were made visible by using fluorophores conjugated to the secondary antibodies, except double immunostaining for NSE and PSA-NCAM, where the respective secondary antibodies were biotinylated and Cy3-conjugated. Then, the ABC technique was applied after incubation with the secondary antibodies and cells were observed under both light and fluorescence microscopy after mounting on Vectashield.

Controls in which the primary antibodies were omitted resulted in no detectable staining. Counterstaining of the cell nuclei was performed with either 4′,6′-diamidino-2-phenylenindole dihydrochloride (DAPI; 0.1 μg/ml) or propidium iodide (0.5 μg/ml).

2.3. Bromodeoxyuridine (BrdU) incorporation

To analyze proliferation of SVZ cells in vitro, BrdU (10^{-4} M) was added to the cultures 24 h after seeding and was maintained until fixation (48 h afterwards). Nuclei that had incorporated BrdU were detected by immunofluorescence. Two different protocols were used for fixation and DNA denaturation. For quantitative experiments, in which propidium iodide was used to determine the total cell number in each coverslip, cells were fixed in 70% ethanol for 30 min, air dried, treated for 2 min with 0.07 N NaOH at room temperature and washed with PBS. For BrdU double stainings with GFAP, NSE, βIII-tubulin or nestin, cells were fixed with 4% PFA, and treated with 1 M HCl at 65 °C for 30 min. Immunofluorescence was performed as described, with the exception that the cells were incubated with the primary antibody for 1 h at room temperature.

2.4. Programmed cell death

The detection and quantification of apoptotic cells was performed in 72-h cultures using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining method, which takes advantage of the DNA fragmentation that occurs in cells undergoing programmed cell death. A commercial kit from Promega (Madison, WI) was used and the standard protocol provided by the supplier was followed. Apoptotic cells (fluorescein-12-dUTP DNA-labeled cells) were counted directly under a fluorescence microscope. Controls employed included the simultaneous processing of (a) explants in the absence of terminal deoxynucleotidyl transferase, to detect nonspecific staining (which was not found in any case), and (b) positive controls consisting of cells treated with DNase I, to generate DNA fragments that are targets for positive labeling.

2.5. Cell counting and statistics

The number of BrdU-positive nuclei, apoptotic cells or cells expressing the different phenotype-specific antigens were counted in each coverslip under fluorescence microscopy and expressed as percentage of the total number of nuclei, counterstained with propidium iodide or DAPI. All these measurements were performed in coded coverslips; the code was not broken until analysis of each experiment was completed.

The total number of cells surrounding the explants was counted by direct visual examination under phase-contrast microscopy, as previously described for olfactory epithelium and SVZ explants [28,29,44], at a magnification of 125 × on a Leitz Labovert microscope. Only adherent cells emerging out of the explants were counted and never those contained in the explant mass. The reliability of the procedure was tested by repetitively counting a series of explants. The standard error calculated from five consecutive countings of the same explant was always lower than 5% of the mean value obtained. The total number of cells was divided by the total number of explants in each coverslip, and this value was referred to as the average number of cells per explant.

Since explants were not homogeneous in terms of size, proliferation rate and phenotypic distribution, it was necessary to analyze a large number of them to obtain a representative population. Therefore, the quantitative data provided in this study resulted from the analysis of all the explants in each coverslip (varying from 35 to 65 explants, each of them surrounded by 20–300 cells). A minimum of three coverslips, with explants isolated from different animals, were analyzed for each condition.

To study the co-localization of distinct antigens in the same cell, 25–50 randomly selected fields per coverslip were visualized under the appropriate filters, at a magnifi-

Table 2
Immunocytochemical characterization of the two main phenotypes identified around the SVZ explants

Lineage	βIII-tubulin	NSE	GFAP	PSA-NCAM	A2B5	Nestin
Neuronal	+	+	–	+	–	–
Glial	–	–	+	–	+/–	+

cation of 400 × . Images were captured with a digital camera (DP10, Olympus) and analyzed using the Microimage software from Olympus. For the reasons explained, the results from co-localization studies were semi-quantitative and, therefore, are given as approximate values.

Data are presented as means ± S.E.M. of the values obtained from 3 to 12 animals. Comparisons between two values were done using the Student's *t*-test for paired samples. Differences were considered significant when $p < 0.05$.

2.6. Chemicals

Culture media, serum, gentamicin and fungizone were purchased from Life Technologies (Gaithersburg, MD),

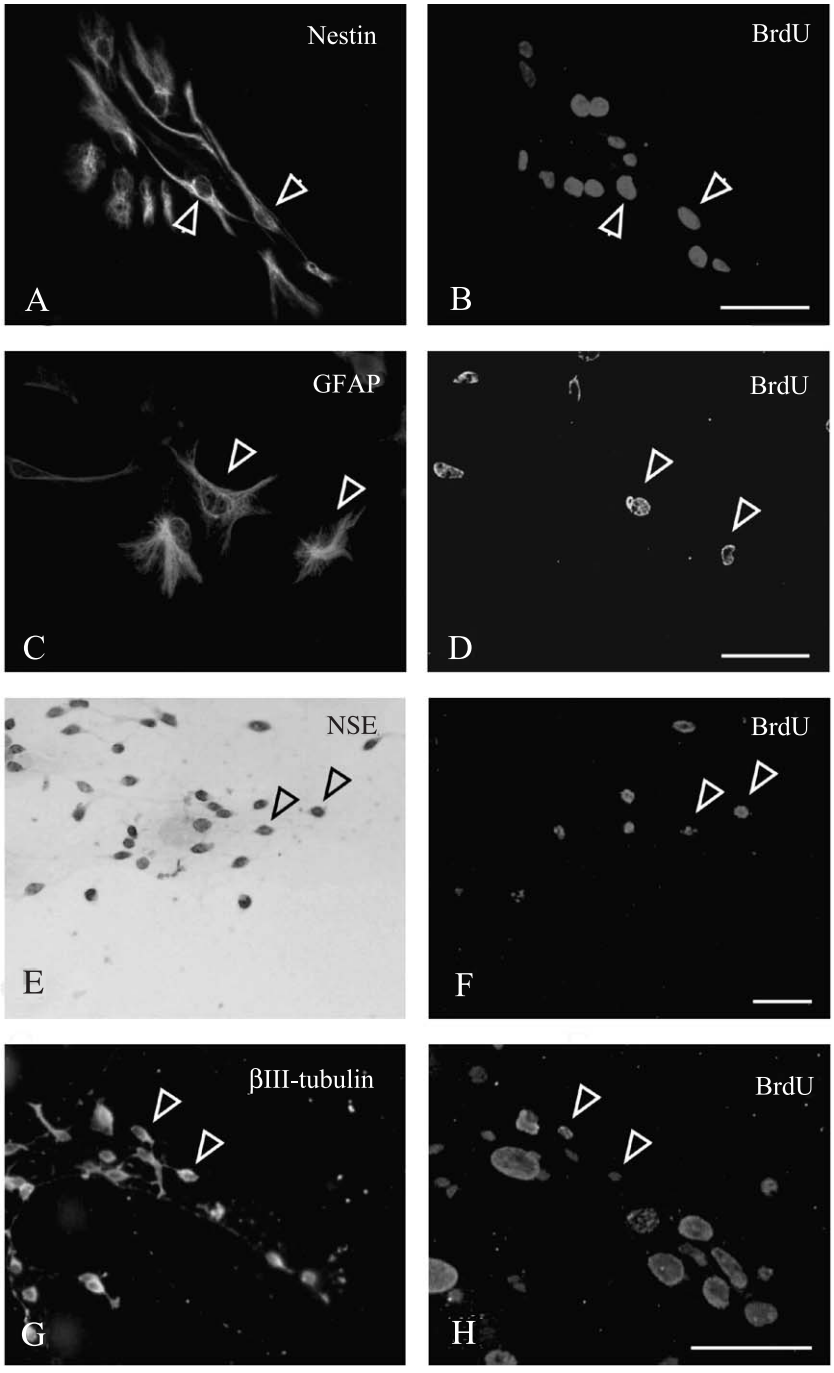

Fig. 2. SVZ-derived cells proliferate in vitro. Explants isolated from the postnatal mouse SVZ were cultured for 3 days in medium containing 10% horse serum and 20 ng/ml EGF. BrdU was added 24 h after seeding and maintained until fixation, to identify the nuclei of cells that underwent mitosis in vitro. BrdU was incorporated by nestin-positive cells, astrocytes and neurons as revealed by double immunostainings for nestin (A), GFAP (C), NSE (E) or βIII-tubulin (G) with BrdU (B, D, F and H, respectively). The same field is shown in each pair of double labeled sections. Arrowheads indicate examples of double labeled cells. Calibration bars: 25 μm in F and 50 μm in the others.

EGF from Becton Dickinson (Bedford, MA), Vectashield from Vector Laboratories (Burlingame, CA) and DePeX from Boehringer Ingelheim (Heidelberg, Germany). All other reagents were obtained from Sigma.

3. Results

3.1. Postnatal SVZ cells proliferated and differentiated in vitro in the presence of EGF

Three days after seeding, cell colonies of different sizes were formed around the explants (Fig. 1A–B). Both neuronal and glial lineage cells were identified on the basis of cell morphology and immunostaining for phenotype-specific antigens. Neuronal cells presented small round somas and one or more processes, and were positive for the neuronal markers βIII-tubulin (Fig. 1E) and NSE (Fig. 1G). These cells expressed in their membrane PSA-NCAM (Fig. 1H), a modified adhesion molecule characteristic of SVZ neuronal precursors in vivo and in vitro [10,24,42]. Astrocytes, containing GFAP, appeared as large flat cells forming a monolayer (Fig. 1C). More than 95% of the $GFAP^+$ cells also expressed nestin (Fig. 1C–D), an early antigen of neural precursors. The distribution of the most abundant markers in peri-explant cells is shown in Table 1, and Table 2 summarizes the immunocytochemical characterization of the neuronal and glial lineages identified in the SVZ explants. The early oligodendrocyte antigen A2B5 was also present in the explant cells but in a low percentage (< 10%). A2B5-labeled cells presented different morphologies and never co-localized with βIII-tubulin. Among them, there were round stellate oligodendrocyte-like cells, which were scarce (< 1%), and also immunoreactive for the O4 antigen (Fig. 1F), other cells that co-localized with GFAP (< 1%) and a third type of morphologically undifferentiated cells.

All cell types, including early precursors and neuronal and astrocytic lineage cells, proliferated in vitro, as assessed by immunocytochemical detection of BrdU in the nuclei of cells expressing nestin (Fig. 2A–B), GFAP (Fig. 2C–D), NSE (Fig. 2E–F) or βIII-tubulin (Fig. 2G–H). A semi-quantitative analysis of BrdU co-localization with these markers is shown in Table 1.

3.2. SVZ cells expressed nNOS in vitro

Immunocytochemical studies using an antibody which specifically recognizes nNOS were performed in postnatal mouse SVZ cultures 72 h after seeding. nNOS was expressed by cells of neuronal lineage, as demonstrated by their morphology—with small cell bodies and long varicose processes—(Fig. 3A–B) and by their co-staining with PSA-NCAM (Fig. 3C–D).

3.3. NOS inhibition enhanced proliferation of SVZ cells

To elucidate whether endogenously produced NO could affect the proliferation rate of SVZ cells, BrdU incorpora-

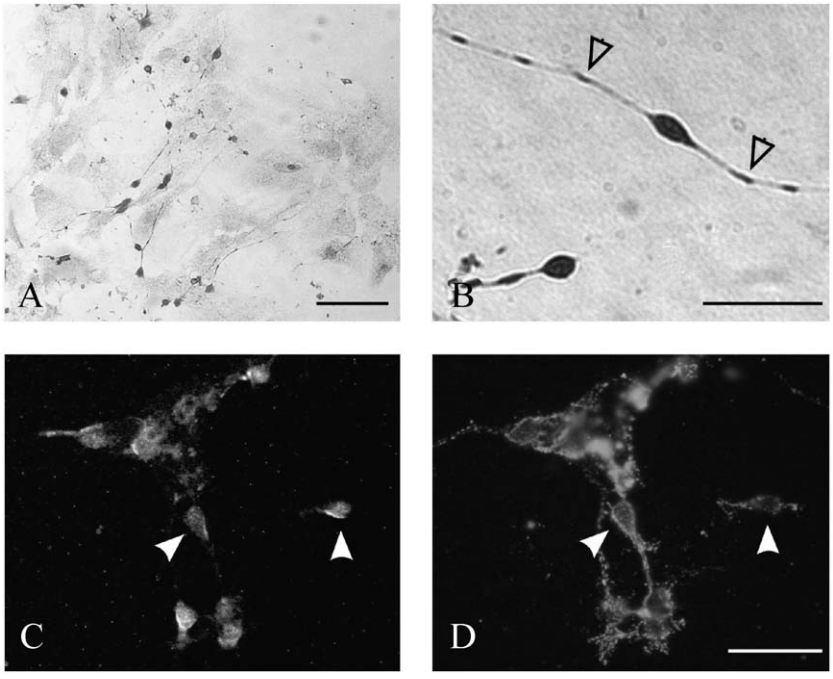

Fig. 3. Expression of nNOS in SVZ cultures. (A and B) nNOS immunocytochemistry in postnatal SVZ explants after 3 days in vitro. nNOS-positive cells presented morphological features of neurons such as long processes with varicosities (open arrowheads). (C and D) Double labeling for nNOS (C) and PSA-NCAM (D), visualized with secondary antibodies conjugated to FITC and Cy3, respectively. The field in D is the same as in C; a high degree of co-localization of nNOS and PSA-NCAM occurred in these cells. Filled arrowheads, examples of double-labeled cells. Calibration bars: 50 μm in A and 25 μm in the others.

tion was measured in cultures grown in the absence or presence of L-NAME (0.1–0.6 mM). $BrdU^+$ nuclei (Fig. 4A) were counted and expressed as percentage of the total number of cells, identified by nuclear staining with propidium iodide (Fig. 4B). As shown in Fig. 4C, treatment with 0.1 mM L-NAME produced a significant increase (30%) in BrdU incorporation, as compared with control cultures. The use of higher concentrations of L-NAME resulted in similar significant increases in cell proliferation (25.2% and 24.5%, for 0.3 and 0.6 mM, respectively; $n=3$). The consequences of NOS inhibition on DNA synthesis were in agreement with the enhanced average number of cells per explant observed in L-NAME treated versus untreated cultures (Fig. 4D).

3.4. NO donor addition decreased the proliferation of SVZ cells, without inducing apoptosis

Since NO may induce apoptosis in some neural cell cultures [3,4], the effect of a NO donor on programmed cell death was analyzed as an initial step. DETA-NO, a long half-life NO donor, was used for this purpose. As seen in Table 3, the percentage of apoptotic cells increased significantly with the concentration of the NO donor.

Next, we explored whether exogenous NO produced any effect on cell proliferation by adding DETA-NO to the cultures and analyzing BrdU incorporation as described. Cultures treated with 10 μM DETA-NO, a concentration that did not increase apoptosis in our cultures, showed a

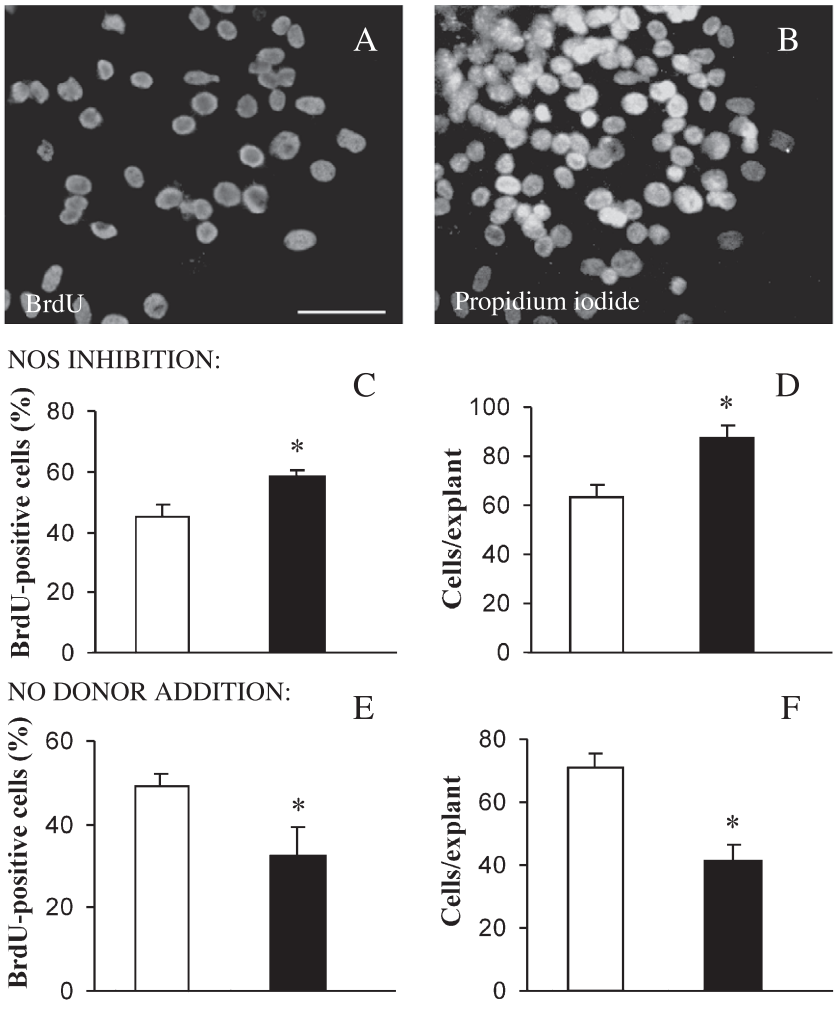

Fig. 4. Effect of NOS inhibition and NO donor addition on SVZ-derived cell proliferation. (A and B) Photomicrographs showing BrdU-labeled nuclei (A) and the total number of cell nuclei, as stained with propidium iodide (B), in the same field of a SVZ culture in control conditions. Calibration bar: 50 μm. (C and E) Effect of the NOS inhibitor L-NAME (C) and the NO donor DETA-NO (E) on the percentage of cells labeled with BrdU in SVZ-derived cultures. SVZ explants were seeded in culture dishes in medium containing 10% horse serum and 20 ng/ml EGF, in the absence (open bar) or presence (filled bar) of 0.1 mM L-NAME (C) or 10 μM DETA-NO (E). After 24 h in vitro, BrdU (10^{-4} M) was added to the culture medium. Two days later, cells were fixed, processed for BrdU immunocytochemistry and counted as described in Section 2. Data are the mean ± S.E.M. of the results obtained from four to six animals. At least 13,000 total cells were counted for each condition. *$p<0.05$, as compared with the values obtained in cultures without treatment. (D and F) Effect of L-NAME (D) and DETA-NO (F) treatments on the average number of cells per explant in SVZ-derived cultures after 3 days in vitro. Explants were seeded and cultured as indicated in C, except that BrdU was omitted in these experiments. Cells were counted by direct observation of the live cultures. Data are the mean ± S.E.M. of the results obtained from 10 animals; *$p<0.05$, compared with the values obtained in cultures without treatment.

Table 3
Effect of nitric oxide on apoptosis of SVZ-derived cells

	Apoptotic cells (%)
Control	9.7 ± 1.3
DETA-NO, 10 μM	9.5 ± 1.2
DETA-NO, 30 μM	13.1 ± 2
DETA-NO, 60 μM	16 ± 1.1*

Percentage of apoptotic cells in postnatal SVZ explants grown in the absence (control) or presence of the indicated concentrations of the NO donor DETA-NO. Apoptotic cells were identified using the TUNEL technique. The total number of cells was made visible by staining with propidium iodide. Data are mean ± S.E.M., $n = 3-6$, $*p < 0.05$, compared with the values obtained in control conditions.

significant decrease in the percentage of $BrdU^+$ cells (Fig. 4E), as well as in the number of cells per explant (Fig. 4F).

3.5. The phenotypic distribution of SVZ cells was modulated by endogenous NO

To analyze the influence of endogenous NO on the different phenotypes present in the cultures, a new series of experiments was performed in which SVZ explants were grown in the presence or absence of 0.1 mM L-NAME. Three days later, the cultures were analyzed by immunofluorescence to detect GFAP and βIII-tubulin in the same cell population, and nestin in independent samples. Cells labeled for each marker protein were counted and expressed as percentage of the total number of cells, as estimated by DAPI-counterstaining of nuclei. When endogenous NO formation was prevented by L-NAME, the percentage of cells expressing GFAP decreased significantly and a larger proportion of cells were positive for βIII-tubulin immunostaining (Fig. 5A). The percentage of nestin-positive cells did not significantly differ between the treated and control groups (47.7 ± 9% vs. 50.6 ± 1.5%, mean ± S.E.M., $n = 4$). Treatment of the cultures with 10 μM DETA-NO did not induce any significant change in the phenotypic distribution, although there was a tendency for an increase in the percentage of GFAP-positive cells and a decrease in that of cells labeled with βIII-tubulin (Fig. 5B).

4. Discussion

In this study, we demonstrate an effect of NO on the proliferation of a neural precursor population derived from the mammalian brain. We have shown that, in postnatal mouse SVZ-derived cell cultures, nNOS is expressed by neuronal precursors and that inhibition of NOS activity produces an enhancement of DNA synthesis as well as an enlargement of the subpopulation of cells expressing neuronal markers.

The anatomical relationship between nitrergic neurons and neuroblasts in the adult [33] and postnatal mouse SVZ (unpublished results) suggests that NO may participate in the control of neurogenesis. We have recently observed that systemic administration of NOS inhibitors to adult mice increases precursor cell proliferation in the SVZ and olfactory bulb [34]. To elucidate whether this effect of endogenous NO was exerted directly on the SVZ neural progenitors or was an indirect consequence of systemic NO deprivation, we have now used an in vitro system that resembles, as much as possible, the cell population existing in the SVZ in vivo. Postnatal mice were used to facilitate viability and proliferation of SVZ cells. Explants were maintained on a poly-lysine substrate to promote cell adhesion and differentiation and, in the presence of EGF, a growth factor that is essential for the survival and proliferation of SVZ cells [12,20,39]. Under these conditions, cells migrated out of the explants, proliferated in vitro as demonstrated by BrdU incorporation, and, after 3 days, differentiated into neuronal and glial cells, with a predominant commitment to the astrocytic phenotype.

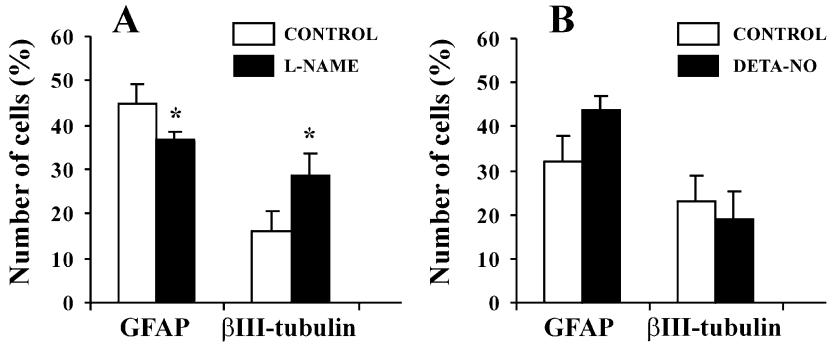

Fig. 5. Effect of L-NAME and DETA-NO on the phenotypic distribution of SVZ cultures. Explants were seeded in culture dishes with a medium containing 10% horse serum and 20 ng/ml EGF, in the presence or absence of the NOS inhibitor L-NAME or the NO donor DETA-NO. After 3 days in vitro, cells were fixed and analyzed by immunofluorescence to detect GFAP and βIII-tubulin in the same cell population. The total number of cells was evaluated by counterstaining with DAPI. (A) Percentage of GFAP-positive cells and βIII-tubulin-positive cells in SVZ-derived cells cultured in the absence (open bars) or presence (filled bars) of 0.1 mM L-NAME. Data are the mean ± S.E.M. of the results obtained from three to five animals. $*p < 0.05$, compared with the corresponding value obtained in the absence of L-NAME. (B) Percentage of GFAP-positive cells and βIII-tubulin-positive cells in SVZ-derived cells cultured in the absence (open bars) or presence (filled bars) of 10 μM DETA-NO. Data are the mean ± S.E.M. of the results obtained from four animals.

As reported by Doetsch et al. [10], the SVZ of adult mice contains migrating neuroblasts expressing PSA-NCAM (type A cells), astrocytes, which have been identified as the neural stem cells (type B cells) [11], and nestin-positive rapidly dividing precursors (type C cells), which generate neuroblasts. These three cell types were identified among periexplant cells in our culture system. Nestin hardly ever co-localizes with migrating neuroblasts in vivo [10] and never does it in the SVZ explants, where, in contrast, it frequently co-localizes with GFAP-positive cells. This high degree of co-localization of nestin or BrdU with GFAP indicates that a large proportion of the newly formed cells differentiated toward an astrocytic lineage. Therefore, as previously described for other culture systems [30,31], the transitions from progenitor stages to glial or neuronal lineages in the SVZ may have differential temporal regulations.

In SVZ cultures, nNOS was expressed in cells with small somata and long processes containing varicosities, a morphology characteristic of differentiated neurons. Although postmitotic nitrergic neurons are present in the SVZ from adult [33] and postnatal (unpublished observations) mice, these cells can not account for the nNOS staining observed in SVZ cultures, since they are scarce, and the magnitude of their processes would make unlikely their survival after the isolation procedure. Alternatively, nNOS was probably induced in neuroblasts generated in vitro and undergoing differentiation toward a mature neuronal phenotype. It is interesting to note that, in cerebral sections from adult mice, co-expression of PSA-NCAM and nNOS was observed in cells that had arrived at their final differentiation site within the olfactory bulb, and never in the SVZ, where neuroblasts replicate and initiate their migration [33]. The co-localization of these two markers in cultured SVZ cells is probably related to the rapid differentiation occurring in vitro, with more than 60% of the cells expressing specific neuronal or glial markers within 72 h. Taken together, these findings suggest that nNOS is expressed by cells undergoing neuronal differentiation, either in vivo in the olfactory bulb or in culture conditions. This is in agreement with the previously reported induction of different NOS isoforms that occurs in several cell culture systems or in brain tissue upon stimulation with differentiating agents such as NGF [21,37], TNFα [36] BDNF [6,22] or NT-3 and NT-4 [22].

We next investigated whether inhibition of NOS activity could affect the proliferation of SVZ-derived cells. A 3-day treatment of cultures with L-NAME led to significant increases in the percentage of BrdU-positive cells, indicating that endogenously produced NO had antiproliferative effects on SVZ cells. The cytostatic effect of NO was also made evident by the decrease in BrdU incorporation resulting from treatment of the cultures with the NO donor DETA-NO. In agreement with the results of BrdU incorporation, the average number of cells grown around each explant was increased under NOS inhibition and decreased upon the addition of NO. These results are in accordance with several previous reports, which demonstrated an inhibitory action of NO on the proliferation of cortical neuroepithelium progenitors [6] and neuroblastoma cells [35]. The latter study showed that, in the EGF-responsive neuroblastoma cell line NB69, NO induces cytostasis by mechanisms involving its direct interaction with the EGFR [35]. Taking into account that SVZ neuroblasts express EGFR [20,47] and respond to EGF by increasing their proliferation [9,20,23,39], we hypothesize that inhibition of the mitogenic signal initiated by EGF may be partially responsible for the NO antiproliferative effect.

The effects of NO on neuronal programmed cell death are controversial. In neuronal cell cultures, either protection [7] or induction [3–5] have been reported. In our postnatal mice SVZ cells, NO exerts a pro-apoptotic effect when present at concentrations larger than that required to inhibit cell proliferation. According to Beltrán et al. [1], the NO concentration produced by 100 μM DETA-NO is approximately 200 nM. Therefore, 10 μM DETA-NO is very likely to release NO at concentrations considered to be in a physiological range (10–100 nM). Thus, our results indicate that, although NO may affect both survival and proliferation of neuronal precursors, probably only the latter action occurs in physiological conditions. This is in agreement with our recent observation that systemic administration of NOS inhibitors to adult mice significantly increased the number of mitotic nuclei in the SVZ, without modifying the frequency of apoptotic nuclei in the same region [34].

Analysis of the phenotypic distribution in cultures treated or not with L-NAME revealed that endogenous NO had an heterogeneous effect on the different SVZ-derived cell lineages. NOS inhibition produced an increase in the percentage of cells expressing a neuronal phenotype. This finding is in agreement with a recent report by Peunova et al. [38], showing that NOS inhibition leads to an increase in the total number of cells in the *Xenopus* developing brain and to a preferential expression of neuronal markers. The enlarged number of cells expressing neuronal markers upon L-NAME treatment could be explained by induction of progenitor cell differentiation toward the neuronal phenotype and/or by a preferential proliferative action on precursor cells already committed to the neuronal lineage. The fact that an opposite effect was not evident upon DETA-NO treatment suggests that NO may have a permissive role on phenotypic determination, probably allowing the action of other(s) undetermined differentiating factor(s).

We can then conclude that NO is involved in the control of proliferation and differentiation of neural precursor cells in SVZ explants. Since neural precursors in the SVZ are naturally exposed to NO-producing neurons in vivo, the present results may be indicative of a direct action of NO on these cells during postnatal and adult neurogenesis.

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