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Nitric oxide acts in a positive feedback loop with BDNF to regulate neural progenitor cell proliferation and differentiation in the mammalian brain

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Abstract

Nitric oxide (NO) is believed to act as an intercellular signal that regulates synaptic plasticity in mature neurons. We now report that NO also regulates the proliferation and differentiation of mouse brain neural progenitor cells (NPCs). Treatment of dissociated mouse cortical neuroepithelial cluster cell cultures with the NO synthase inhibitor L-NAME or the NO scavenger hemoglobin increased cell proliferation and decreased differentiation of the NPCs into neurons, whereas the NO donor sodium nitroprusside inhibited NPC proliferation and increased neuronal differentiation. Brain-derived neurotrophic factor (BDNF) reduced NPC proliferation and increased the expression of neuronal NO synthase (nNOS) in differentiating neurons. The stimulatory effect of BDNF on neuronal differentation of NPC was blocked by L-NAME and hemoglobin, suggesting that NO produced by the latter cells inhibited proliferation and induced neuronal differentiation of neighboring NPCs. A similar role for NO in regulating the switch of neural stem cells from proliferation to differentiation in the adult brain is suggested by data showing that NO synthase inhibition enhances NPC proliferation and inhibits neuronal differentiation in the subventricular zone of adult mice. These findings identify NO as a paracrine messenger stimulated by neurotrophin signaling in newly generated neurons to control the proliferation and differentiation of NPC, a novel mechanism for the regulation of developmental and adult neurogenesis.

Introduction

During embryonic development, neurons of the mammalian cerebral cortex are generated from proliferating neuroepithelial cells in the telencephalic ventricular zone (VZ) (Takahashi et al., 1995). Initially, cells in VZ are highly proliferative, and as neurogenesis proceeds, some daughter cells exit the cell cycle, migrate away from the VZ into the cortical plate (CP), and differentiate into neurons (McConnell, 1991, 1995; Takahashi et al., 1993, 1994). In the adult brain, neural stem cells are concentrated in specific regions of the CNS, including the subventricular zone of the cerebral cortex and the subgranular region of the hippocampal dentate gyrus (Gage, 2000). Adult neural stem cells undergo a similar transition from proliferation to differentiation, and may provide a reservoir of cells capable of forming new neurons that may eventually integrate into neuronal circuits (Garcia-Verdugo et al., 1998).

Little is known about the cellular mechanisms that determine when neural progenitor cell (NPC) proliferation stops and differentiation begins, although the signaling molecules that regulate the transition of NPCs from proliferation to differentiation are beginning to be identified, with several growth factors, including basic fibroblast growth factor, epidermal growth factor, brain-derived neurotrophic factor (BDNF), and notch ligands (Vescovi et al., 1993; Zigova et al., 1998; Benraiss et al., 2001; Caldwell et al., 2001), differentially modulating NPC proliferation and differentiation. BDNF and its high-affinity receptor TrkB are widely expressed in the developing and adult nervous system, and data suggest that BDNF plays multiple roles in regulating the differentiation, survival, and plasticity of several different pop-

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ulations of neurons (Lewin and Barde, 1996). The phenotypic abnormalities of CNS neurons in BDNF-deficient mice indicate that BDNF is essential for normal brain development. However, the cellular mechanism whereby BDNF regulates NPC proliferation and differentiation during brain development is largely unknown. Studies of developing and adult neurons suggest that BDNF signaling may require cooperative interactions with nitric oxide (NO): BDNF and NO production appear to be coregulated in rodent neocortical neurons (Xiong et al., 1999), and BDNF regulates synaptic plasticity in the hippocampus (Kang and Schuman, 1995, 1996; Figurov et al., 1996) in a manner similar to that of NO (Shibuki and Okada, 1991; Bohme et al., 1991). NO synthase (NOS) oxidizes the guanidine nitrogen of L-arginine to form citrulline and the short-lived free radical gas NO (Geller and Billiar, 1998). There are three different isoforms of NOS expressed in the brain, and their expression is restricted to specific cell types: neuronal NOS (nNOS; neurons), inducible NOS (iNOS; microglia and astrocytes), and endothelial NOS (eNOS; astrocytes and vascular endothelial cells). NO may perform many functions in the mature nervous system, including modulating synaptic plasticity and cell survival (Holscher, 1997), and may also promote neuronal death in neurodegenerative disorders (Dawson and Dawson, 1998). In nonneural cells, NO acts as an efficient antiproliferative agent by suppressing DNA synthesis (Garg and Hassid, 1989; Kuzin et al., 1996). Recent findings suggest that NO can also regulate cell proliferation and differentiation in neural cells. NO may mediate nerve growth factor-induced growth arrest and differentiation of PC12 cells (Peunova and Enikolopov, 1995; Phung et al., 1999; Rialas et al., 2000). The role of NO in brain development is unclear. Neuronal NO synthase (nNOS) is transiently expressed in the cerebral cortical plate and hippocampus in embryonic rat brain during the period of peak cortical neurogenesis (Bredt and Snyder, 1994). In addition, NO production is regulated in an activity-dependent manner in the brain during the period of developmental synaptogenesis (Ogilvie et al., 1995; Contestabile, 2000). Moreover, it was recently reported that an NO donor can enhance neurogenesis following ischemic brain injury in adult rats (Zhang et al., 2001). The signals that regulate the production of NO during neural development are not known, although the ability of BDNF to upregulate nNOS expression in several populations of CNS neurons in the developing and adult CNS (Cellerino et al., 1999; Klocker et al., 1999) suggests a potential role for this neurotrophic factor. In the present study, we provide evidence that NO acts as a paracrine messenger stimulated by BDNF signaling in newly generated neurons to control the proliferation and differentiation of NPCs, a novel mechanism for the regulation of developmental and adult neurogenesis.

Material and methods

Cell cultures and experimental treatments

Pregnant C57/B1-6J mice were euthanized on gestational day 12 by cervical dislocation, and embryos were harvested and their brains were removed. Meninges were removed from the brain, and the cortical neuroepithelium was dissected, collected in cold HBSS, and then transferred to culture medium (DMEM/F12 supplemented with B27; Gibco BRL). The tissues were gently triturated with a fire-polished glass Pasteur pipette to yield small clusters of cells consisting of 20–50 cells. Cell clusters were diluted with the culture medium and plated onto poly-L-lysine-coated glass coverslips or plastic culture dishes. The clusters were plated at a density of approximately 200 per cm^2. For pure stem/progenitor cells cultures, we isolated fetal multipotent neuroepithelial precursor (NEP) cells from E10.5 rat embryos as described previously (Wu et al., 2002; Cai et al., 2002). Truncated segments of the E10.5 embryos were dissected and incubated in an enzyme solution containing collagenase type I (1 mg/ml; Worthington Biochemical) and dispase II (2 mg/ml; Roche) in Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (Gibco/BRL) at room temperature for approximately 10 min. The enzyme solution was then replaced by NEP basal medium (Kalyani et al., 1997) with 10% chicken extract (CEE). The segments were gently triturated with a pasteur pipette to release neural tubes from surrounding somites and connective tissue. Isolated neural tubes were dissociated by using trypsin–EDTA, and NEP cells were grown in NEP basal medium with 10% CEE and basic fibroblast growth factor (bFGF, 20 ng/ml; Peprotech). Sodium nitroprusside (SNP), N(G)-nitro-L-arginine methyl ester (L-NAME), and hemoglobin were prepared as 200–500× stocks in culture medium immediately prior to use. Recombinant human BDNF was purchased from Boehringer (Indianapolis, IN) and was stored at −80°C as 100-μg/ml stocks in PBS.

Evaluation of NPC proliferation and differentiation

Twenty-four hours after plating, cluster cell cultures were exposed to experimental treatments for designated time periods. Bromodeoxyuridine (BrdU) was then added to cultures at a final concentration of 10 μM, and 4 h later, the cells were fixed in 4% paraformaldehyde in PBS, processed for immunocytochemistry, and counterstained with PI to label all the nuclei. To determine the proliferation index, we acquired confocal images of 10–15 cell clusters per coverslip by using dual channels for BrdU immunostaining (488 nm excitation and 510 nm emission) and propidium iodide (543 nm excitation and 590 nm emission). The BrdU- and propidium iodide-positive cells are counted simultaneously in each image. The proliferation index was calculated as the percentage of BrdU-positive cells divided by the total number of the cells (propidium iodide-positive). A minimum of

3 coverslips for each condition were used in each experiment, and data presented are the results of 3–4 separate experiments. To evaluate neuronal differentiation, cells were immunostained with MAP2 and NeuN antibodies, and immunoblot analysis of MAP2 protein levels was also performed.

Evaluation of cell survival

After exposure to experimental treatments, cells were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature washed with PBS, and then stained with the DNA-binding dye Hoechst 33258. Coverslips were mounted onto glass slides and examined under epifluorescence illumination by using a 40× objective lens. Cells were considered "apoptotic" if their nuclear chromatin was condensed or fragmented, whereas cells were considered viable if their chromatin was diffusely and evenly distributed throughout the nucleus. To further confirm and detect apoptotic DNA fragmentation, a TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick-end labeling] kit was used (R&D Systems). Briefly, the cells were fixed with 4% paraformaldehyde in PBS for 30 min and washed with PBS (pH 7.4). After blocking of endogenous peroxidases, the cells were incubated at 37°C for 1 h in a reaction mixture containing terminal transferase, biotinylated nucleotide (dNTP), or PBS as control. Cells were then incubated in the presence of streptavidin-conjugated horseradish peroxidase (HRP) for 20 min at room temperature. After rinsing with PBS, DNA strand breakage was visualized in the presence of HRP substrate. The apoptotic cells exhibited a dark blue nuclear staining. Approximately 10–15 clusters on each coverslip were scored, 3 separate coverslips were assessed for each condition in each experiment, and 3 separate experiments were performed for both Hoechst 33258 staining and TUNEL staining.

Histology and immunochemistry

Timed pregnant C57BI/6J mice at embryonic day (E) 12, E16 were sacrificed by cervical dislocation, embryos were removed and decapitated, and heads of fetal mice were fixed in 4% paraformaldehyde in PBS overnight. Brains were cryoprotected by immersing in 25% sucrose in PBS and embedded in Tissue-Tek (Sakura, Torrance, CA), and cryostat sections were cut in the coronal plane at a thickness of 10–40 μm. Sections were transferred to Superfrost Plus slides (Fisher Scientific, Springfield, NJ), dried, and stored at −20°C or collected in PBS for staining. Brain sections or coverslips containing cultured cells were processed for immunocytochemistry. The primary antibodies used in this study and their dilutions were: mouse anti-nestin IgG1 (1:200, Chemicon); mouse ant-E-NCAM IgM (1:50); rabbit anti-Sox2 IgG (1:2000); mouse anti MAP2 IgG1 (1:200, Sigma); mouse anti-NeuN IgG1 (1:200, Chemicon), rabbit anti-GFAP IgG (1:1000, Chemicon); rabbit anti-nNOS IgG (1:1000, BD Transduction Laboratories); mouse anti-BrdU IgG1 (1:100, Becton-Dickson); rabbit anti-TrkB IgG (1:1000, Santa Cruz); and rabbit anti-BDNF IgG (1:1000, Santa Cruz). Secondary antibodies used and their dilutions were: fluorescein isothiocyanate (FITC)-conjugated goat anti- mouse IgG1 (1:100, South Biotechnology Associate, AL) (nestin, MAP2, and BrdU); rhodamine- conjugated goat anti-mouse IgG1 (1:100, South Biotechnology Associate, AL) (MAP2); FITC-conjugated goat anti-mouse IgM (1:100, Jackson Immunoresearch, PA) (E-NCAM); FITC-conjugated donkey anti-rabbit IgG (1:100, Jackson Immunoresearch, PA) (GFAP, nNOS, TrkB, BDNF).

For immunofluorescence cytochemistry, coverslips from cultures that had been fixed in 4% paraformaldehyde in PBS for 30 min or sections were washed in PBS, permeabilized, and preincubated with the blocking solution (0.2% Triton X-100, 10% normal goat serum) in PBS for 30 min, and then incubated overnight with a primary antibody diluted in the same blocking solution at 4°C. Cells were washed with PBS and incubated with appropriate secondary antibodies diluted in same blocking solutions for 2 h at room temperature. Sections or coverslips were counterstained with propidium iodide (PI) (0.02% PI and 1% Rnase in PBS) for 10 min; they were then washed with PBS and mounted on microscope slides by using a fluorescence anti- fade medium (Vector Lab, CA).

For the avidin-biotin-HRP methods (ABC Kit, Vector Lab, CA), BrdU, nNOS, TrkB, and BDNF immunohistochemistry were performed on free-floating sections. Instead of mounting on slides, frozen sections (40 μm) were collected in PBS and washed several times in PBS. Sections were then incubated in 1% H_2O_2 for 30 min to quench endogenous peroxidase activity. After rinsing with PBS, sections were incubated overnight with primary antibodies at 4°C, then washed in PBS and further processed by using a Vector ABC Elite Kit (Vector Labs). The sections were further processed by incubation in a solution containing 0.035% diaminobenzidine and 0.01% H_2O_2. The developed sections were mounted on Superfrost Plus slides and dehydrated in a graded ethanol series, and immersed in 100% Xylene for 15 min. Sections were then coverslipped in Permount medium (Fisher Scientific, NJ). For BrdU immunohistochemistry, similar procedures were followed, except that the sections were denatured by incubating in a solution of 2 N HCl for 45 min before the primary antibody was added.

RT-PCR analysis

Total RNA was isolated directly from freshly dissected mouse E9 neural tubes and E12, E15, and E17 cerebral cortex with Trizol (Invitrogen), a modification of the guanine isothiocyanate-phenol-chloroform extraction method. cDNA was synthesized by using 100 ng total RNA in a 20-μl reaction with superscript II (Invitrogen) and oligo (dT)

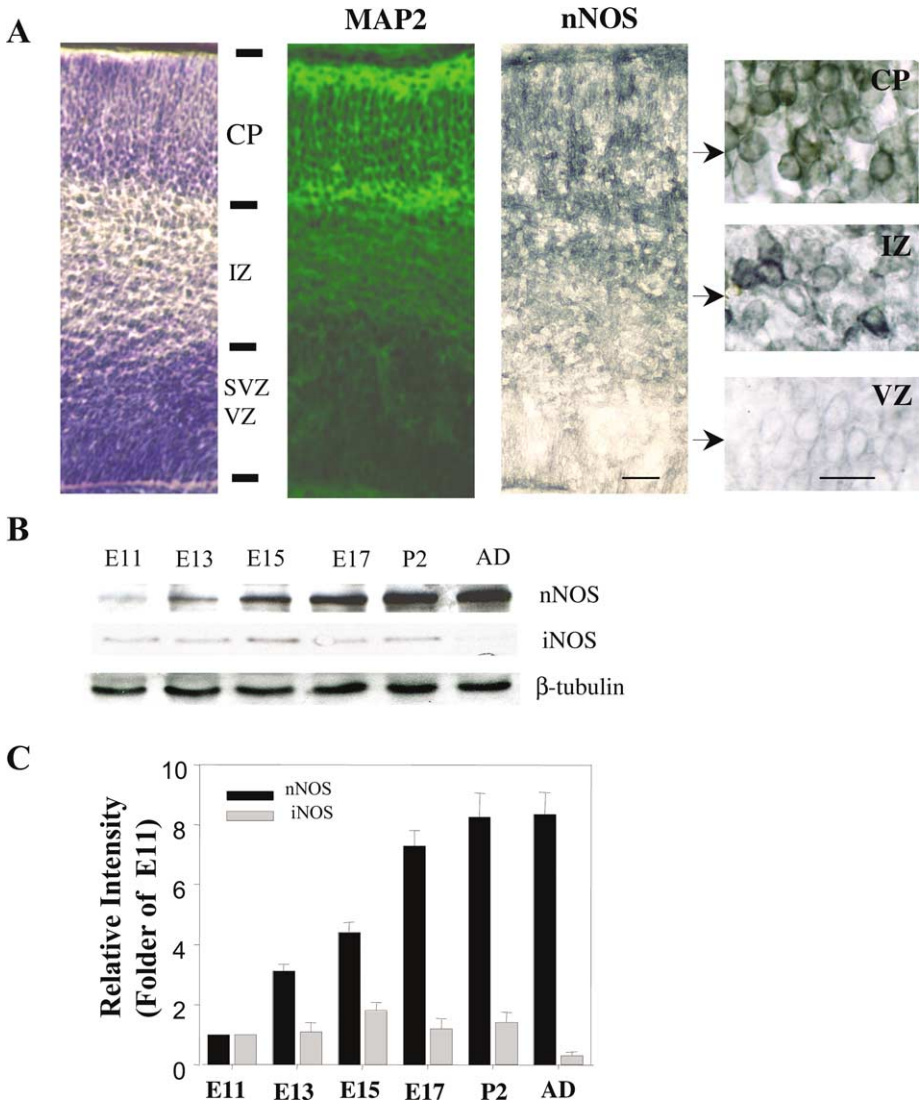


Fig. 1. Neuronal nitric oxide synthase (nNOS) is absent from proliferating neural stem cells and is progressively upregulated in differentiating neuronal progeny during embryonic and postnatal development. (A) nNOS immunoreactivity overlaps with that of MAP2, a marker of differentiated neurons. Both nNOS and MAP2 immunoreactivities are absent in NPC of the ventricular zone (VZ), present in the postmitotic cells of developing zones including cortical plate (CP) neurons and the presumptive migrating neuronal cells in the intermediate zone (IZ) at embryonic day 16 (E16). The section shown at the left is stained with cresyl violet, the middle panel shows an adjacent section stained with nNOS antibody, and the panels at the right show high magnification views of cells in the corresponding cortical regions. Scale bar, 100 μm. (B) Immunoblot analysis showing a progressive increase of nNOS protein levels in the developing cerebral cortex. In contrast, iNOS protein level shows a low and constant expression pattern during development. Proteins in cortical homogenates (50 μg/lane) were subjected to immunoblot analysis by using antibodies against nNOS or iNOS. The blots were stripped and reprobed by using an antibody against β-tubulin, which demonstrated equal protein loading. (C) Results of densitometric analysis of blots of samples from four different mice of each age (mean and SD).

12–18 (Invitrogen). One microliter of RNase H (Invitrogen) was added to each reaction tube, and the tubes were incubated for 20 min at 37°C before proceeding to the RT-PCR analysis.

For PCR amplification 0.5 μl cDNA template was used in a 50-μl reaction volume with the RedTaq DNA polymerase (Sigma). The cycling parameters were: 94°C, 1 min; 55°C, 1 min; 72°C, 1 min, for 30 cycles. The PCR cycles were preceded by an initial denaturation of 3 min at 94°C and followed by a final extension of 10 min at 72°C. Primer sequences were as follows: TrkB forward: 5′-TGA AAC AAG CCA CAC ACA G-3′; TrkB reverse: 5′-AAT CAC CAC CAC GGC ATA G-3′; the length of the PCR product was 592 bp. G3PDH forward: 5′-TGA TGG GTG TGA ACC ACG AG-3′; G3PDH reverse: 5′-CTC CTG TTG TTA TGG GGT CTG- 3′; the length of the PCR product was 758 bp.

Immunoblot analysis

After experimental treatments, cells were solubilized in SDS-PAGE sample buffer, and proteins in cell lysates were

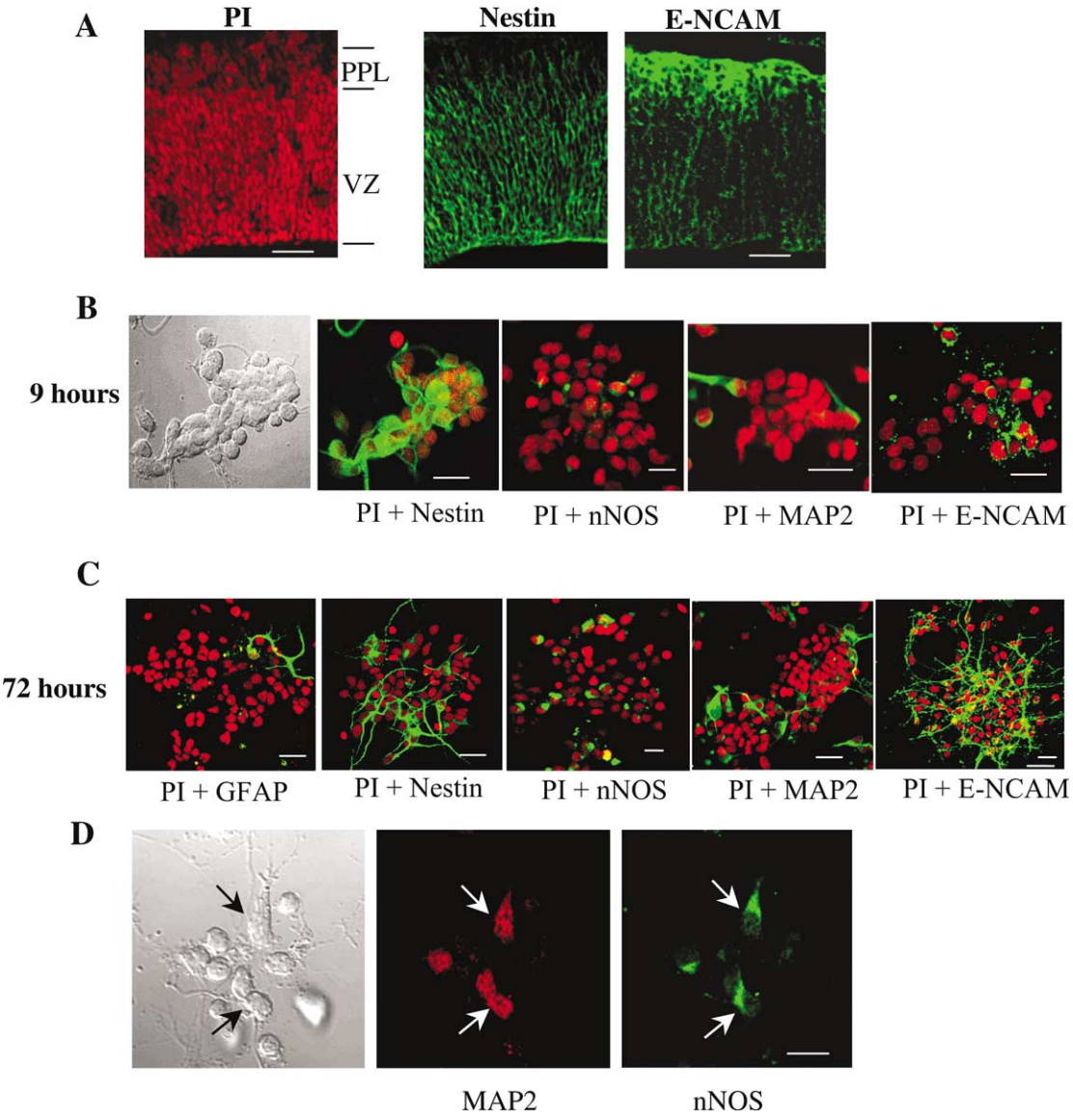


Fig. 2. Characterization of cultured E12 cortical neural clusters growing in absence of exogenous growth factors. (A) Sections of E12 mouse cortex stained with propidium iodide (PI) to label the nuclei of all cells, or with antibodies against nestin and E-NCAM. Scale bar, 50 μm. (B, C). Cells were stained with nestin, MAP2, E-NCAM, GFAP, and nNOS antibodies after 9 h (B) or 72 h (C) in culture. Cells were counterstained with propidium iodide to label nuclei (red). Note the increase in numbers of cells immunoreactive with antibodies against neuronal antigens (nNOS, E-NCAM and MAP-2) with increasing time in culture. (D) Double immunostaining of MAP2 (red) and nNOS (green) after 72 h in culture. Scale bar, 20 μm.

resolved in a SDS-polyacrylamide gel (7.5–12% acrylamide; 50 μg protein per lane) and electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked with 4% nonfat milk in TBST and then incubated for 2 h in the presence of primary antibody. Membranes were washed and then incubated for 1 h in the presence of a 1:5000 dilution of secondary antibody (anti-mouse or anti-rabbit IgG) conjugated to horseradish peroxidase. Reaction products were visualized by using an enhanced chemiluminescence (ECL) Western Blot Detection Kit (Amersham Pharmacia Biotech). The primary antibodies included a rabbit polyclonal antibody against nNOS (1:1000, BD Transduction Laboratories), a rabbit polyclonal antibody against iNOS (1:1000, BD Transduction Laboratories), a rabbit polyclonal antibody against beta-tubulin (1:5000, Sigma), mouse monoclonal antibody against MAP2 (1:500, Sigma), and a rabbit polyclonal antibody against TrkB (1:2000, Santa Cruz).

In vivo studies

Mice were anesthetized by using isofluorane and mounted in a stereotaxic apparatus. A sagittal incision was made through the skin along the midline of the head, and a hole was drilled in the skull at a position 0.2 mm posterior and 1.2 mm lateral (right) to bregma. A Hamilton syringe containing L-NAME (50 mM solution in PBS) or PBS was positioned over the hole, and the needle tip was lowered into

the lateral ventrical (2.0 mm below the brain surface). One microliter of solution was injected (5 mice received L-NAME and 5 mice received PBS). In order to determine whether the proliferation potential of NPC in VZ was affected by the nNOS inhibitor, 24 h after stereotaxic injection of L-NAME or PBS, mice were given two intraperitoneal injections of BrdU (50 mg/kg) separated by 2 h. This protocol was used to ensure that BrdU-labeled cells did not have time to migrate away from the SVZ, which would complicate the quantification. Using this protocol, all BrdU-labeled cells are in S phase and are located in SVZ. Assuming that L-NAME and PBS injections did not change the total number of NPC in the SVZ, the number of BrdU-labeled cells in SVZ provides a reliable estimate of the proliferation potential of the NPC. Two hours after the second BrdU injection, the mice were anesthetized by inhalation of isoflurane and were transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde in phosphate- buffered saline (pH 7.4). Brains were postfixed overnight, cryoprotected, frozen, and microtome-sectioned coronally at 20 μm thickness. Every fourth section in the forebrain was processed for BrdU immunohistochemistry, and all BrdU-positive cells in the SVZ surrounding the third ventricle in each section were counted in a total of 10 sections for each brain. The values presented represent the total number of BrdU-positive cells in 10 sections for each brain.

Statistics

All data are presented as mean ± SD. Comparisons between control and treatment groups were performed by using Student's unpaired *t* test or ANOVA when appropriate. A value of $P < 0.05$ was considered to be statistically significant.

Results

nNOS production progressively increases during cortical development in vivo and in cell culture

In the mammalian cortex, the majority of neurons are generated from the ventricular zone (VZ) adjacent to the lateral ventricles. The newly generated neurons migrate outward through the intermediate zone and into the cortical plate, where they form the six layers of the adult cortex. Cresyl violet staining of coronal sections of E16 mouse somatosensory cortex reveals the typical developing zones (Fig. 1A), and nNOS immunostaining reveals the localization of nNOS protein in postmitotic neurons in the CP and in the intermediate zone (IZ), with little or no nNOS being present in proliferative cells of the VZ (Fig. 1A). The nNOS immunoreactivity is similar to that of MAP2, a marker of differentiated neurons, suggesting that nNOS expression occurs as the neurons differentiate. Immunoblot analysis of cortical homogenates showed that the level of nNOS was very low at E11, and then progressively increased from E13 through postnatal day 2 (Fig. 1B and C). These results are consistent with the previously described pattern of nNOS gene expression in the developing rat cerebral cortex (Bredt and Snyder, 1994). In contrast to the dramatic developmental increase in nNOS levels, iNOS protein levels remained low and constant during cortical development (Fig. 1B and C).

Table 1

	Percentage ± SD				
	Nestin	E-NCAM	MAP2	nNOS	GFAP
9 h	82.8 ± 7.2	50.3 ± 7.5	8.7 ± 3.0	5.4 ± 2.5	0
24 h	70.2 ± 1.4	68.4 ± 2.0	18.6 ± 5.8	14.4 ± 3.8	0
72 h	62.0 ± 8.5	91.2 ± 2.5	32.0 ± 6.4	25.2 ± 4.3	1.9 ± 1.1

Because NO is a diffusible intercellular messenger, we sought to determine whether NO produced by differentiating neurons might influence the behavior of neighboring cortical neural stem cells. To study the effects of NO on the process of NPC differentiation under well-defined experimental conditions, we employed neural cluster cell cultures established from the cortical epithelium of E12 mice, a time when the first postmitotic neurons of the cortex are beginning to differentiate (Ghosh and Greenberg, 1995). At E12, the cerebral wall consists mainly of the VZ and a very thin layer of differentiating cells (preplate layer) (Luskin and Shatz, 1985), located adjacent to the VZ as indicated by propidium iodide (PI) staining in Fig. 2A. In order to characterize the phenotypes of the cells at this stage of cortical development, we employed antibodies against the intermediate filament protein nestin (Lendahl et al., 1990) and the transcription factor sox2 (Uwanogho et al., 1995; Zappone et al., 2000), which are markers of NPC, and an antibody against E-NCAM, a cell surface protein expressed by neuron-restricted progenitor cells and differentiated neurons (Mayer-Proschel et al., 1997; Mujtaba et al., 1999). The vast majority of cells in the VZ express nestin and sox2 and some of them also express E-NCAM (Fig. 2A and data not shown). Cells in the differentiated preplate layer were heavily labeled with the E-NCAM antibody, but were not immunoreactive with nestin (Fig. 2A). Therefore, E12 is a time when the first postmitotic neurons of the cortex are beginning to differentiate, and cells in VZ at this stage are a combination of restricted neuronal progenitors and NPC.

We next characterized the phenotypes of cells in the cluster cell cultures using antibodies against nestin, E-NCAM, MAP2 (a neuronal microtubule-associated protein), GFAP (an astrocytic intermediate filament protein), and nNOS. Nine hours after plating, more than 80% of the cells were nestin-positive, only 9% were MAP2-positive, and none expressed GFAP (Fig. 2B; Table 1). More than 90% of the cells exhibited an undifferentiated morphology, although a few cells in most clusters elaborated short processes and were MAP2-positive. There was a progressive

decrease in the percentage of cells that were nestin-positive and a corresponding increase in the percentage of cells that were MAP2-positive through 72 h in culture (Fig. 2B and C; Table 1). The percentage of cells expressing E-NCAM increased from 50.3% at 9 h to 91% at the 72-h time point (Table 1). By 72 h in culture, many cells exhibited long processes and expressed both MAP2 and E-NCAM (Fig. 2C). GFAP-positive cells were extremely rare, comprising less than 2% of the cells at 72 h in culture (Fig. 2C; Table 1). These results indicate that the majority of NPC in these cortical cluster cell cultures differentiate into neurons.

We next characterized the developmental regulation of nNOS expression in cortical cluster cell cultures. The number of nNOS imunoreactive cells and the level of nNOS protein was very low at the time of plating and then increased through culture day 3 (Figs. 2 and 4; Table 1). Double immunostaining of cluster cell cultures with MAP2 and nNOS revealed that nNOS immunoreactivity is present at high levels in cells with a neuronal phenotype (MAP2-positive cells with neurites) and at undetectable or very low levels in cells that lacked MAP2 (Fig. 2D). The latter results are consistent with the immunostaining pattern in vivo (Fig. 1A) and demonstrate that nNOS levels increase in neurons as they differentiate. Neither iNOS nor eNOS was detected by immunostaining or immunoblotting in the cluster cell cultures (data not shown), consistent with lack of microglia and vascular endothelial cells in these cultures.

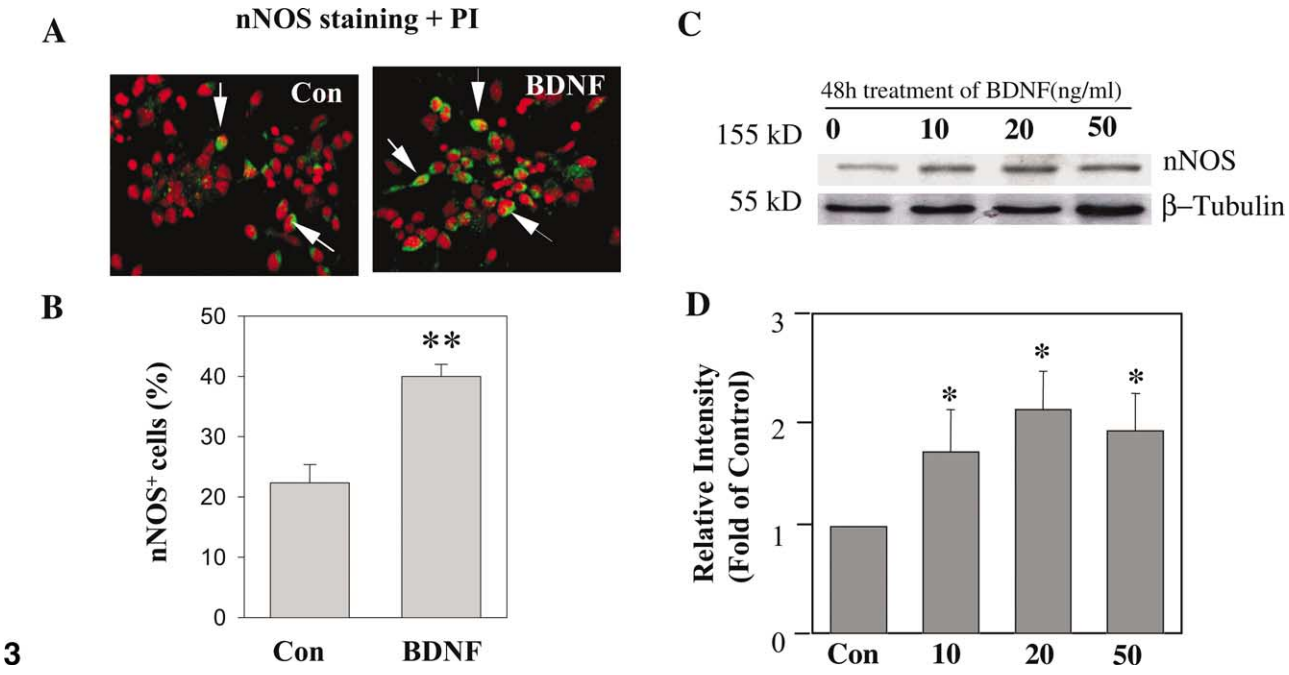
BDNF enhances nNOS production during differentiation of embryonic neural progenitor cells

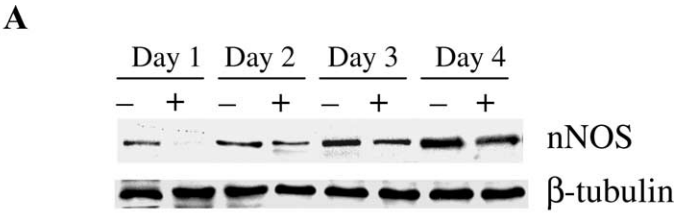
Although BDNF and NO have been shown to exert similar effects on synaptic plasticity and neuronal differentiation (Xiong et al., 1999; Kang and Schuman, 1995, 1996; Figurov et al., 1996; Shibuki and Okada, 1991; Bohme et al., 1991), it is not known if they act in the same or different signaling pathways. To determine whether BDNF can modulate the production of NO during neurogenesis, NPC cluster cultures were treated with BDNF for 2 days, and the percentages of nNOS-positive cells and nNOS level were quantified. There was a two-fold increase in the percentage of nNOS immunoreactive cells (Fig. 3A and B). Nearly all of the nNOS-positive cells were MAP2-positive (data not shown). Immunoblot analysis confirmed that levels of nNOS were greatly increased in cluster cell cultures treated with BDNF, with physiologically relevant concentrations of 10–50 ng/ml being effective (Fig. 3C and D). In order to determine whether endogenous BDNF signaling contributed to differentiation of NPC into neurons and nitric oxide signaling, we treated NPC cultures with a BDNF blocking antibody previously shown to neutralize endogenous BDNF signaling (Duan et al., 2001). Levels of nNOS were significantly decreased in NPC cultures at each culture time point examined from 1 to 4 days in culture (Fig. 4), demonstrating a role for endogenous BDNF in nNOS production.

Nitric oxide promotes the switch of NPC from proliferation to differentiation

BDNF was reported to induce differentiation and maturation of hippocampal and cortical NPC into a neuronal phenotype (Maisonpierre et al., 1990; Ghosh and Greenberg, 1995) and can also enhance adult neurogenesis (Benraiss et al., 2001; Pencea et al., 2001). The ability of BDNF to upregulate the expression of nNOS in NPC suggested a role for nitric oxide signaling in the regulation of neurogenesis. We first determined whether NO can directly affect the switch of NPC from proliferation to differentiation by quantifying the proliferation rate of NPC following manipulations that either induce or inhibit NO production. Cultures were exposed to the nitric oxide donor sodium nitroprusside (SNP), the NOS inhibitor, N-ω-nitro-L-arginine methylester (L-NAME), or the nitric oxide scavenger reduced hemoglobin, and cell proliferation rate was determined. Twenty-four hours after plating, the cells were treated with SNP, L-NAME, or hemoglobin for 24 h, pulse-labeled with BrdU for 4 h, and then fixed and immunostained with BrdU antibody. In control cultures, the proliferation index for 4 h BrdU incorporation is approximately 35%. In order to more clearly show the effect of each treatment, and at the same time reduce variability, we presented proliferation index after different treatments as a percentage of that of untreated control cultures. Exposure of cluster cell cultures to SNP significantly decreased the number of BrdU-positive cells in a concentration-dependent manner (Fig. 5A and B). Because it had been previously suggested that BDNF can promote neuronal differentiation (Benraiss et al., 2001; Lee et al., 2002), we compared the effects of SNP and BDNF on NPC proliferation and neuronal differentiation. At a concentration of 40 μM, the proliferation index was approximately 20%, and SNP inhibited cell proliferation by 40%, a magnitude of inhibition similar to that of cells exposed to BDNF (Fig. 5A and B). Because nNOS is not expressed in proliferating cells in the VZ, but is present in differentiating neurons (Fig. 1), we designed experiments to establish whether NO produced by differentiating neurons acted in a paracrine manner to regulate the proliferation and differentiation of NPC. First, we quantified NPC proliferation in cultures in which the NO scavenger hemoglobin was added to the medium. Hemoglobin abolished the inhibitory effect of SNP on NPC proliferation (Fig. 5C). Moreover, hemoglobin itself significantly increased NPCs proliferation, suggesting a role for intercellular NO signaling in controlling the basal rate of NPC proliferation (Fig. 5C). A role for endogenous NO production in regulating NPC proliferation was further supported by data showing that exposure of cells to the NOS inhibitor L-NAME significantly increases NPC proliferation (Fig. 5C).

A decrease in the proliferation index is usually accompanied by a corresponding increase in cell differentiation. We therefore determined the percentages of cells expressing MAP2 and NeuN, markers of neurons. After 48 h of treat-





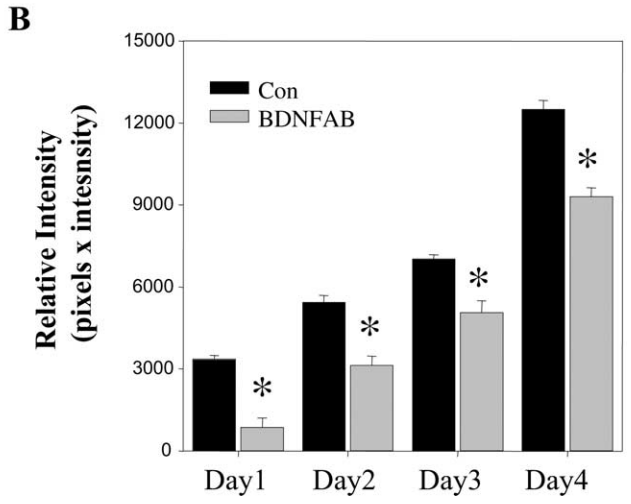
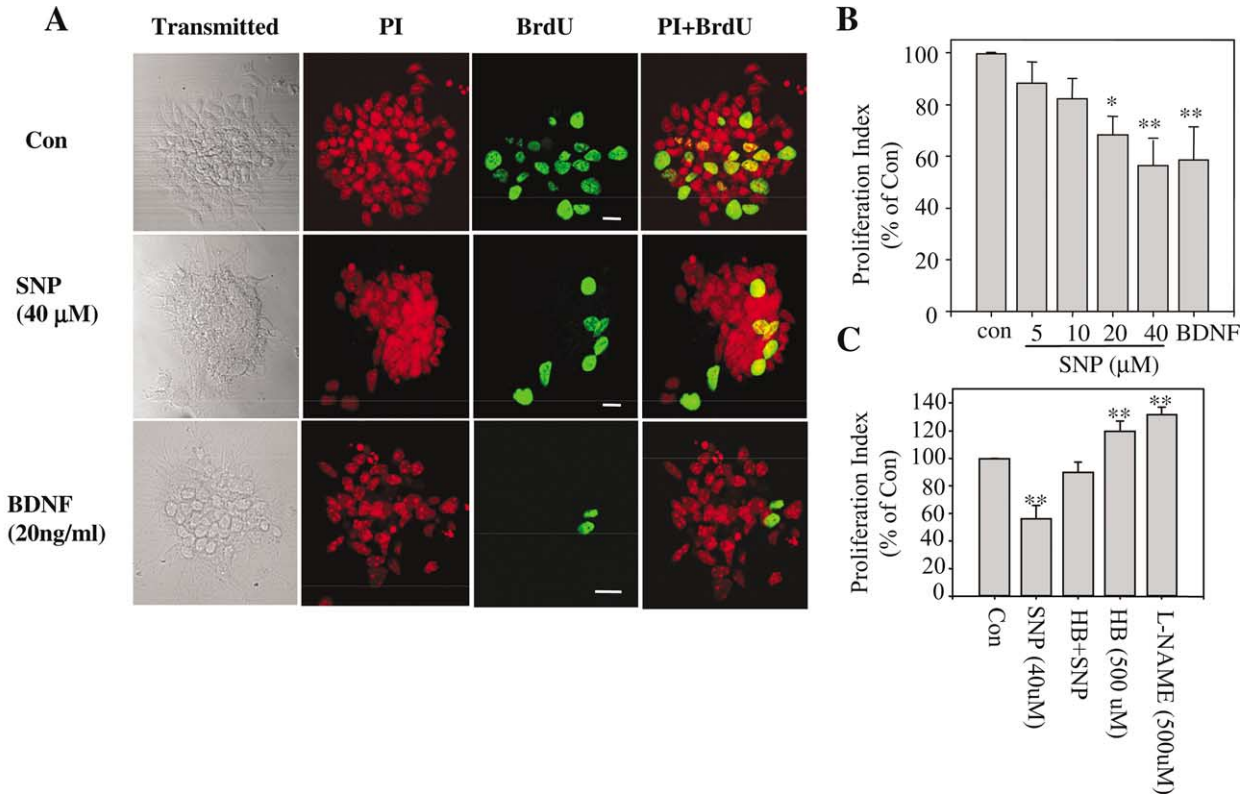


Fig. 3. BDNF increases nNOS levels in neural progenitor cells. (A) Confocal images of cultured neuroepithelial cell clusters showing nNOS-positive cells after 48 h of treatment with either BDNF (30 ng/ml) or saline control (Con). (B) The percentage of nNOS-positive cells was increased significantly with BDNF treatment. Values are the mean and SD from three separate experiments (**, $P < 0.01$; paired t test). (C) Immunoblots of proteins (50 μg/lane) probed with an nNOS antibody documenting a marked increase in the level of nNOS protein in BDNF-treated cultures and the blots were reprobed with β-tubulin to show relative amounts of total protein in each lane. (D) Results of densitometric analyis of immunoblots. Values of nNOS protein were normalized to the β-tubulin in the same lane. Values are expressed as fold change compared with the nNOS protein level in untreated control cultures. Values are the mean and SD of at least three different cultures per condition. *, $P < 0.01$ compared with the value for control cultures.

Fig. 4. A BDNF neutralizing antibody (BDNF AB) reduces the expression of nNOS in neural progenitor cells. (A) NPC were cultured in absence or presence of BDNF neutralizing antibody (1 μg/ml) for the indicated number of days and nNOS protein levels were determined by immunoblot analysis (50 μg protein/lane). Densitometric analysis of samples from three different experiments (B) revealed significant decreases in nNOS protein levels at each time point (mean and SD; *, $P < 0.01$).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20
40
BDNF
SNP (μM)
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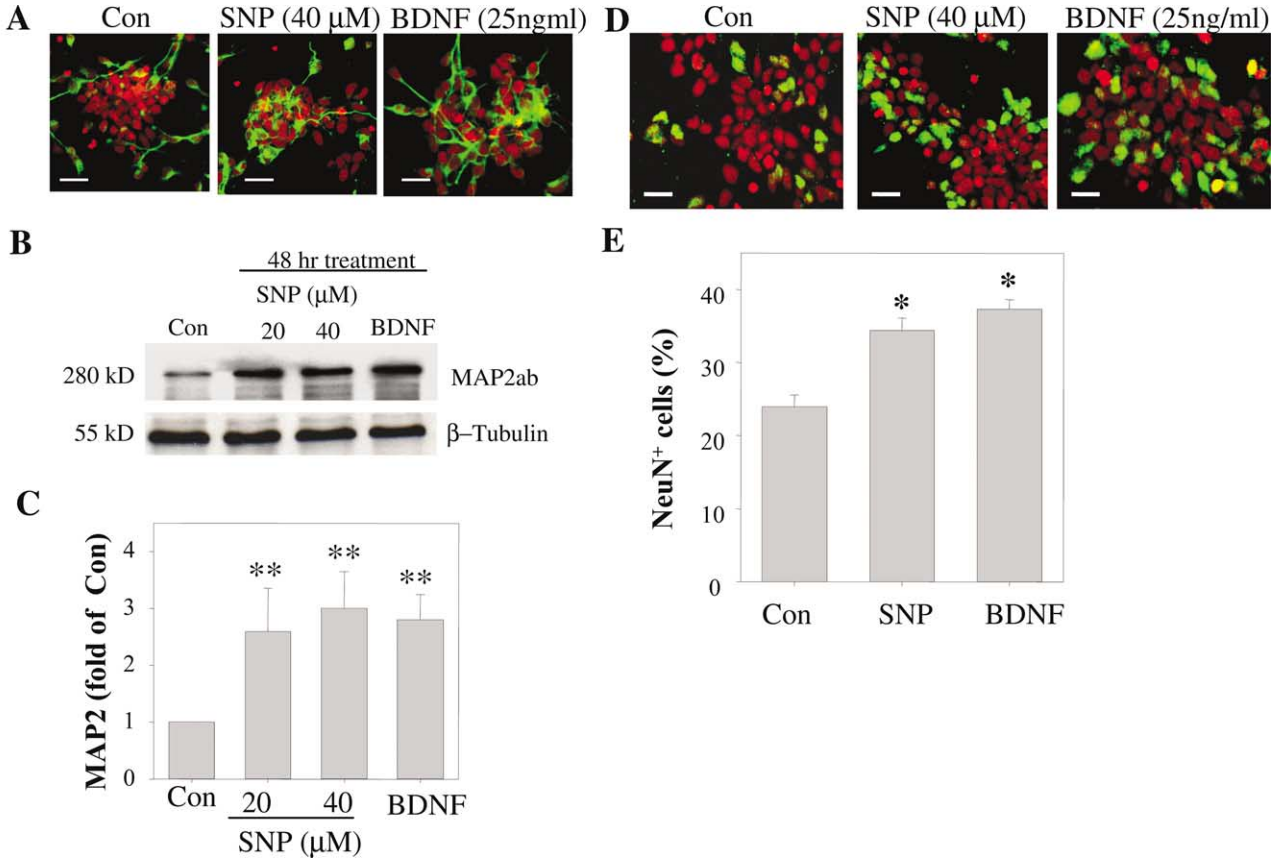
HB+SNP
HB (500 uM)
L-NAME (500uM)

5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B
48 hr treatment
SNP (μM)
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20
40
BDNF
280 kD
MAP2ab
55 kD
β–Tubulin
E
NeuN+ cells (%)
Con
SNP
BDNF
C
MAP2 (fold of Con)
Con
20
40
BDNF
SNP (μM)

6

ment with SNP or BDNF, the cells were fixed and immunostained MAP2 or NeuN antibodies and counterstained with propidium iodide to label all nuclei. Cell lysates from additional cultures were subjected to immunoblot analysis by using a MAP2 antibody. There was a marked increase in the number of MAP2- and NeuN-positive cells (Fig. 6A, D, and E) and in the level of MAP2 protein (Fig. 6B and C) in BDNF- and SNP-treated cells compared with vehicle-treated control cells. These findings indicate that both NO and BDNF promote a developmental switch of NPCs from a proliferative state to differentiation into neurons.

BDNF is a well-known neurotrophic factor promoting neuronal survival (Lewin and Barde, 1996), and SNP was previously reported to induce NPC cell death in a dose-dependent manner (Cheng et al., 2001). To test whether a BDNF blocking antibody and 40 μM SNP (the maximal dosage we used in this study) can cause cell death, we exposed E12 neural cluster cultures to BDNF antibody or SNP and quantified the percentage of cells exhibiting apoptotic nuclear changes (nuclear chromatin condensation/fragmentation and DNA strand breaks) using Hoechst 33258 and TUNEL staining (Fig. 7). The percentages of cell death quantified using each method were essentially identical. The percentage of cells with apoptotic nuclei in control cultures (5%) was similar to that in cultures treated with BDNF antibody or SNP, indicating that BDNF antibody and SNP did not kill cells. The latter results are consistent with our previous studies showing that essentially no cell death occurred in cultures of a NPC line exposed to less than 100 μM SNP (Cheng et al., 2001).

Nitric oxide mediates the neurogenic action of BDNF

To determine whether BDNF signaling and NO production affect the transition of NPCs from proliferation to differentiation in vivo, we first evaluated the cellular localization of BDNF and its high-affinity receptor TrkB in the developing cerebral cortex. TrkB immunoreactivity was present in cells throughout the developing cortex, including NPC in the VZ, which exhibited TrkB immunoreactivity in their periphery, consistent with localization in the plasma membrane (Fig. 8A). TrkB protein can exist as a full-length functional receptor or as a truncated protein; the truncated trkB receptor can be soluble or membrane-associated (Escandon et al., 1994). Immunoblot analysis of cortical homogenates from mice of increasing ages from E11 to adult revealed that levels of full-length TrkB were already high at E11, were further increased at P2, and then remained at a similar level in the adult cortex (Fig. 8C). Levels of truncated TrkB were very low at E11, were increased markedly by P2, and remained elevated in the adult cortex (Fig. 8C), suggesting that NPC in the developing neocortex contain functional TrkB. The presence of TrkB in NPC in developing brains was further confirmed by RT PCR, showing that TrkB mRNA is present in cells of the E12 mice cerebral wall. In contrast, cells located in the IZ and CP exhibited BDNF immunoreactivity, whereas NPC in the VZ do not (Fig. 8B). These findings suggest that VZ NPC are capable of responding to BDNF, but do not themselves produce BDNF.

Because nNOS and BDNF have similar expression patterns in the developing brain, and because NO and BDNF have similar effects on NPC proliferation and differentiation, we sought an understanding of how these two molecules might interact to regulate the progression of NPC from proliferation to differentiation. We tested the hypothesis that upregulation of nNOS expression and nitric oxide signaling in newly generated neurons mediates the stimulation of the switch from proliferation to differentiation by BDNF. We found that the ability of BDNF to suppress NPC proliferation was abolished in cultures treated with either the NOS inhibitor L- NAME and the NO scavenger hemoglobin (Fig. 9). Moreover, the ability of BDNF to induce differentiation of NPC into neurons was blocked by treatment of cultures with L-NAME or hemoglobin (data not shown). These data identified a novel positive feedback loop in which BDNF enhances nNOS expression in differentiating neurons, and the differentiated neurons, in turn, produce NO which diffuses to NPCs wherein it inhibits their proliferation and enhances their differentiation into neurons. To further support the existence of such a positive feedback loop, we isolated fetal neuroepithelial progenitor (NEP)

Fig. 5. Nitric oxide mediates suppression of NPC proliferation and stimulation of neurogenesis by BDNF. (A, B). Incorporation of BrdU into neural progenitor cells is attenuated by the NO donor sodium nitroprusside (SNP) and by BDNF. (A) After 48 h of treatment with SNP (40 μM) or BDNF (20 ng/ml) beginning on culture day 1, the cells were pulsed with BrdU for 4 h and then immunostained with a BrdU antibody (green) and counterstained with propidium iodide to label all the nuclei (red). The micrographs at the left are transmitted light images of the same microscope fields. Scale bar, 20 μm. (B) The proliferation index was calculated by counting BrdU-positive cells and total cells, and is expressed as a percentage of the proliferation index in control cultures. Values are the mean and SD of determinations made in three separate experiments (30 clusters assessed in each experiment). *, $P < 0.05$; **, $P < 0.01$ compared with the control (con) value (ANOVA with Scheffe post-hoc tests). (C) The NO scavenger hemoglobin (HB) abolishes the inhibitory effects of SNP (40 μM) on NPC proliferation, and both hemoglobin and the NOS inhibitor L-NAME enhance NPC proliferation. Values are the means and SD of four separate experiments. *, $P < 0.05$, **, $P < 0.01$ compared with the control value (ANOVA with Scheffe post-hoc tests).

Fig. 6. Nitric oxide and BDNF stimulate the transition of NPC from proliferation to differentiation in embryonic cortical cell cultures. E12 mouse neuroepithelial cells were grown in presence of 25 ng/ml BDNF or SNP (20 or 40 μM) or were left untreated (Con). After 48 h of treatment with SNP or BDNF, the cells were fixed and immunostained with propidium iodide to label all the nuclei (red), and with antibodies against either MAP2 (A) or NeuN (D). (B, C) Cell lysates from additional cultures were subjected to immunoblot analysis using a MAP2 antibody. (E) Quantification of NeuN-positive cells (mean and SD of 4 separate cultures). There was a marked increase in the number of MAP2 positive cells (A) and in the level of MAP2 protein in BDNF- and SNP-treated cells compared with vehicle-treated control cells. *, $P < 0.05$; *, $P < 0.01$ compared with the control value.

cells from E10.5 rat embryos as described previously (Wu et al., 2002; Cai et al., 2002). These NEP cells were previously characterized to be multipotent neural stem cells that do not express early differentiation markers (Cai et al., 2002); therefore, they are pure neural stem cell cultures without $MAP2^+/nNOS^+$ postmitotic neurons. We found that NEP cell proliferation ability is not affected by BDNF (Fig. 9). Collectively, these findings suggested essential roles for NO production and paracrine signaling in BDNF-mediated NPC transition from proliferation to differentiation.

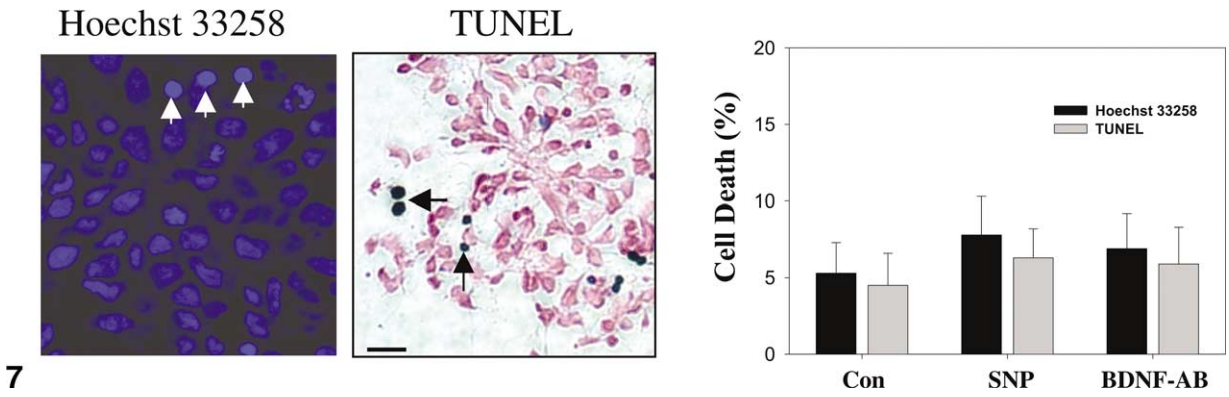
Nitric oxide regulates neural progenitor cell proliferation and differentiation of adult NPCs in vivo

To determine whether NO has a similar function in regulating NPC proliferation and differentiation in adult brains in vivo, we administered L-NAME into the right lateral ventricle of adult mice and then labeled proliferating cells with BrdU. Numbers of BrdU-positive cells were significantly increased in the subventricular zone of the right cerebral hemisphere of mice given L-NAME compared with the contralateral hemisphere, and to both hemispheres of control mice receiving a saline infusion in the right lateral ventricle (Fig. 10). It indicated that NO plays a similar role in arresting cell cycle, promoting a transition from proliferative precursors to postmitotic cells. Thus, as was the case in embryonic cortical development, NO plays an important signaling role in the progression of adult neurogenesis.

Discussion

In the present study, we observed that the nitric oxide donor SNP and BDNF can each trigger a switch of cerebral NPC from proliferation to differentiation. BDNF enhanced the expression of nNOS in newly generated neurons. A similar effect of BDNF on mature cortical neurons in culture was reported by Xiong et al. (1999), suggesting maintenance of this BDNF signaling mechanism in the mature nervous system. Moreover, we showed that NO is an essential mediator of BDNF's ability to regulate NPC proliferation and differentiation. Collectively, these findings suggest a novel role for neuron-derived NO in triggering a switch of cerebral neural progenitor cells from proliferation to differentiation in the developing and adult mammalian brain. A paracrine action of NO produced by newly generated neurons that express nNOS and acting on neighboring NPC is suggested by the ability of the extracellular nitric oxide scavenger hemoglobin and an nNOS inhibitor to maintain NPCs in a proliferative state, thereby suppressing neuronal differentiation. Importantly, our data show that paracrine NO sigaling is an essential mediator of the BDNF-induced transition of NPC from proliferation to differentiation. The effect of endogenous NO in suppressing NPC proliferation and promoting neuronal differentiation of NPC progeny was robust, suggesting that, although other molecules may also regulate these processes, our data suggest that the BDNF-NO-positive feedback loop is an important pathway regulating embryonic and adult neurogenesis. These findings provide a basis for the formulation of a model for regulation of NPC proliferation and differentiation by NO in which NPC in the ventricular zone lack nNOS, but are responsive to NO produced by neighboring neuronal progeny. In this way, nitric oxide produced by newly generated neurons provides a feedback signal to NPC that inhibits their proliferation and promotes differentiation along neuronal lineages. This model fits the temporal changes and cellular localization of nNOS during brain development in that nNOS is expressed in newly generated neurons in the embryonic cortical plate, but is absent from proliferating NPC in the subventricular zone. Consistent with our analysis of the developing mouse cerebral cortex, it was previously shown that nNOS is expressed in migrating cells with a neuronal phenotype in the marginal and intermediate zones of the developing rat cerebral cortex and is absent from glial cells (Santacana et al., 1998). When taken together with our observation that nNOS expression is rapidly upregulated in neuronal progeny of NPC during in vitroneurogenesis, the available data suggest an important role for NO produced by differentiating neuronal neighbors in regulating the proliferation and differentiation of NPC.

We found that BDNF enhances nNOS expression in developing cortical NPC. Previous studies have shown that BDNF and nitric oxide share other biological actions on neurons. For example, both BDNF and nitric oxide enhance synaptic plasticity in the adult hippocampus and promote neuronal survival during development (Bredt and Snyder, 1994; Schuman and Madison, 1994; Garthwaite and Boulton, 1995; Lo, 1995; Stoop and Poo, 1996). Endogenous nitric oxide regulates BDNF production, and conversely, BDNF induces nNOS gene expression and nitric oxide production in cultured neocortical neurons (Xiong et al., 1999). However, a role for nitric oxide in mediating physiological actions of BDNF had not previously been established. Our data suggest that BDNF may facilitate NPC transition from proliferation to differentiation by upregulating the expression of nNOS in differentiating neurons, thereby providing them with the ability to produce nitric oxide. NO is a diffusible gas with an effective signaling range of approximately 150–300 μm. We found that nitric oxide generated by nNOS-expressing neurons can affect the fate of neighboring NPC, causing NPC to exit the cell cycle, consistent with an antiproliferative effect of nitric oxide on in nonneuronal cells (Garg and Hassid, 1989; Lepoivre et al., 1990). Therefore, NO and BDNF appear to function in a positive feedback loop to stimulate the progress of neurogenesis. Moreover, our data show that, in pure neural stem cell cultures (E10.5 NEP cells) that do not express early differentiation markers (Cai et al., 2002), BDNF does not affect cell proliferation even though Trk B is expressed in the cells. Because we found that an extracellular nitric oxide scavenger can block the effect of BDNF in inhibiting the



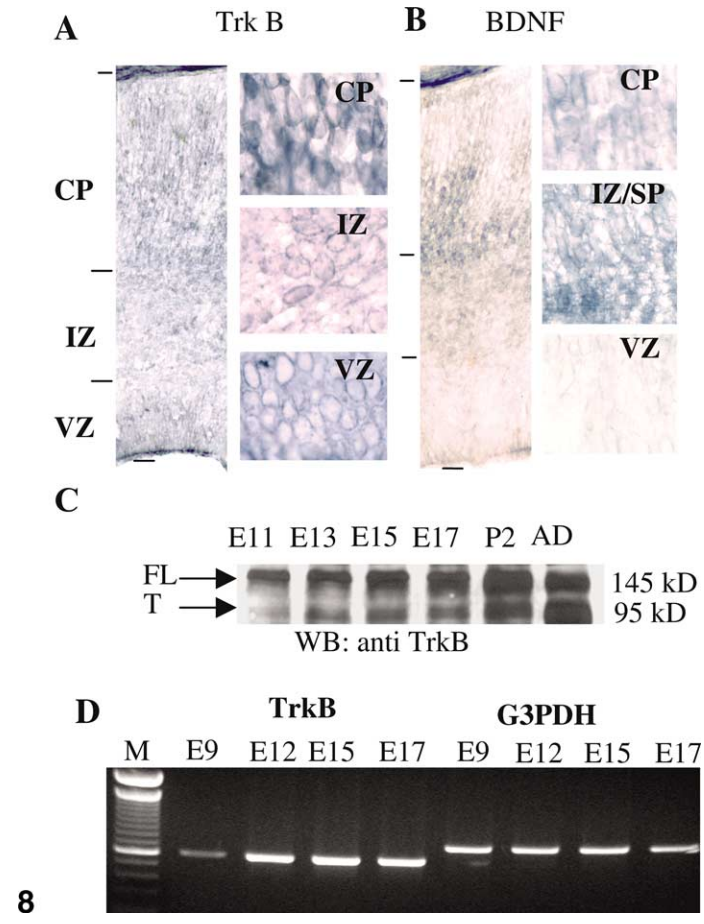
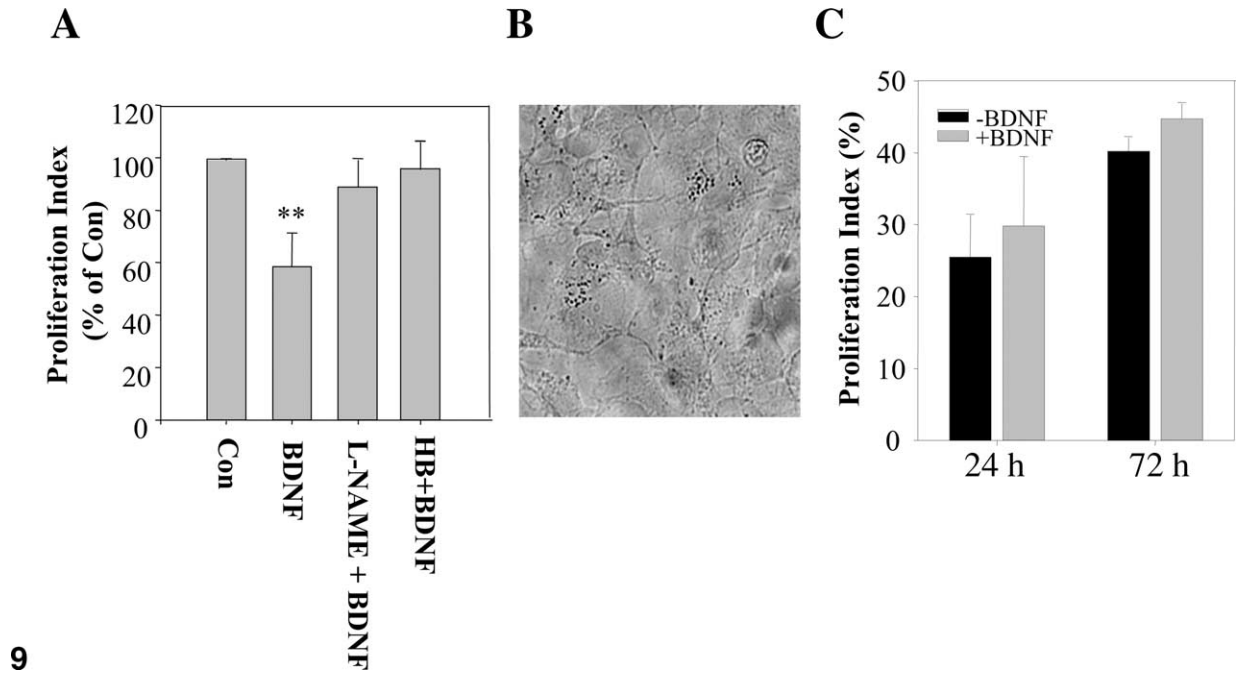


Fig. 7. SNP and a BDNF neutralizing antibody do not cause cell death in NPC cultures. E12 mouse cortical NPC were grown in presence of a BDNF neutralizing antibody (BDNFAB) or SNP (40 μM) or were left untreated (Con). After 48 h of treatment with SNP or BDNFAB, the cells were fixed. And stained with either Hoechst 33258 or TUNEL to identify apoptotic cells. Hoechst-stained cells were considered apoptotic if their nuclear chromatin was condensed or fragmented (A, arrows), whereas cells were considered viable if their chromatin was diffusely and evenly distributed throughout the nucleus. TUNEL-stained cells exhibited dark blue nuclear staining (B, arrows). There was no significant increase in apoptotic cells in cultures treated with either SNP or BDNFAB ($n = 3$).

Fig. 8. Cellular localization and developmental expression of TrkB and BDNF during cortical development. (A, B) Immunostaining of E16 mouse cortex with antibodies against TrkB (A) and BDNF (B). Scale bar, 100 μm. (C) Immunoblot analysis of full-length (FL) and truncated (T) forms of TrkB in homogenates of cerebral cortex from mice of the indicated ages.

NPC proliferation in E12 neural cluster cultures, nitric oxide appears to be an essential mediator of BDNF-induced switch of neural progenitors from proliferation to differentiation. Our immunostaining results show that Trk B is present in NPC in the ventricular zone and in neurons in the postmitotic zones, whereas both BDNF and nNOS are present in the postmitotic neurons, but absent from NPC. Thus, it appears that both BDNF and NO exert their effects on NPC indirectly. BDNF and nitric oxide may act at multiple stages of cerebral cortical development, affecting



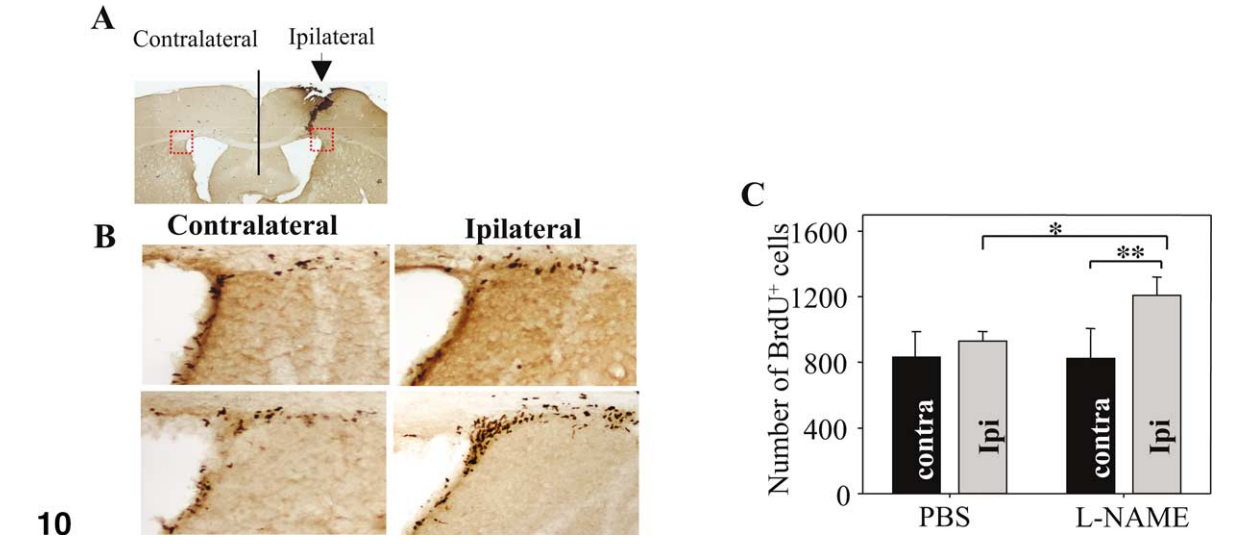


Fig. 9. (A) Hemoglobin and L-NAME abolish the inhibitory effect of BDNF (30 ng/ml) on NPC proliferation (cells were treated for 48 h). Values are the means and SD of four separate experiments. **, $P < 0.01$ compared with each of the other values. (B) Photomicrograph of E10.5 multipotent NEP cells. (C) BDNF did not promote the transition of E10.5 NEP cells from proliferation to differentiation. Cultured NEP cells were administrated 10 μM BrdU for 4 h after 24 or 72 h in the absence or presence of BDNF (25 ng/ml), and then fixed and processed for BrdU immunocytochemistry.

Fig. 10. Intraventricular administration of a NOS inhibitor promotes neural stem cell proliferation in the sub-ventricular zone of adult mice. L-NAME or PBS was infused into the right lateral ventricle. The injected mice were kept alive for 24 h, then BrdU (50 μg/g body weight) was injected intraperitoneally twice with a 2-h interval between injections; mice were killed 2 h after the second injection. (A) Brain sections at the level of frontal cortex immunostained with BrdU antibody showing the injection needle track (arrow). (B) High-magnification images showing BrdU-labeled cells in the subventricular zone of representative L- NAME- and PBS-treated mice. (C) Numbers of BrdU-positive cells in the subventricular zone were quantified (see Materials and methods). Values are the means and SD (5 PBS- and 5 L-NAME-treated mice). *, $P < 0.05$; **, $P < 0.01$ (paired t tests).

the proliferation of NPC, and the differentiation/migration and survival of their neuronal progeny. The specific effect of nitric oxide may depend on its concentration and therefore the location of the cells within the presumptive gradient of nitric oxide concentrations established by the spatial gradient of cells expressing nNOS.

BDNF levels and neurogenesis are increased in adult rats and mice maintained in complex environments (Young et al., 1999) or subjected to dietary restriction (Lee et al., 2002), two environmental factors that also enhance learning and memory. Previous findings suggest that nitric oxide signaling plays important roles in regulating neurite outgrowth and synaptogenesis in the developing brain, and in long-lasting changes in the structure and function of neuro-

nal circuits in the adult brain. For example, nNOS expression and nitric oxide signaling are required for NGF-induced neurite outgrowth in PC12 cells (Phung et al., 1999), the complexity of dendrites in developing motor neurons is decreased in mice lacking nNOS (Inglis et al., 1998), and nNOS expression and localization changes in developing mouse and rat brains in a manner consistent with a role for nitric oxide in synaptogenesis (Ogilvie et al., 1995). A role for nitric oxide in long-term potentiation of synaptic transmission in the hippocampus, a presumptive cellular mechanism of learning and memory, has been established in a series of studies (Hawkins et al., 1998). The results of behavioral studies in which nitric oxide production was manipulated are also consistent with a role for nitric oxide in learning and memory (Holcher et al., 1996). Learning increases BDNF expression in the hippocampus (Hall et al., 2000) and increases the numbers of nNOS immunoreactive neurons in the dentate gyrus and frontal cortex of adult rats (Zhang et al., 1998). In light of recent studies linking neurogenesis to learning and memory (Shors et al., 2001), our data suggest a possible role for nitric oxide-induced neurogenesis in enhanced learning and memory in response to environmental signals, such as complex environments, that upregulate BDNF and nitric oxide signaling in the adult brain.

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References

Benraiss, A., Chmielnicki, E., Lerner, K., Roh, D., Goldman, S.A., 2001. Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. J. Neurosci. 21, 6718–6731.

Bohme, G.A., Bon, C., Stutzmann, J.M., Doble, A., Blanchard, J.C., 1991. Possible involvement of nitric oxide in long-term potentiation. Eur. J. Pharmacol. 199, 379–381.

Bredt, D.S., Snyder, S.H., 1994. Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia, and olfactory epithelium. Neuron 13, 301–313.

Cai, J.L., Wu, Y.Y., Mirua, T., Pierce, J.L., Lucero, M.T., Albertine, K.H., Spangrude, G.J., Rao, M.S., 2002. Properties of a fetal multipotent neural stem cell (NEP cell). Dev. Biol. 251, 221–240.

Caldwell, M.A., He, X., Wilkie, N., Pollack, S., Marshall, G., Wafford, K.A., Svendsen, C.N., 2001. Growth factors regulate the survival and fate of cells derived from human neurospheres. Nat. Biotechnol. 19, 475–479.

Cellerino, A., Arango-Gonzalez, B.A., Kohler, K., 1999. Effects of brain-derived neurotrophic factor on the development of NADPH-diaphorase/nitric oxide synthase-positive amacrine cells in the rodent retina. Eur. J. Neurosci. 11, 2824–2834.

Cheng, A., Chan, S.L., Milhavet, O., Wang, S., Mattson, M.P., 2001. p38 MAP kinase mediates nitric oxide-induced apoptosis of neural progenitor cells. J. Biol. Chem. 276, 43320–43327.

Contestabile, A., 2000. Roles of NMDA receptor activity and nitric oxide production in brain development. Brain Res. Rev. 32, 476–509.

Dawson, V.L., Dawson, T.M., 1998. Nitric oxide in neurodegeneration. Prog. Brain Res. 118, 215–229.

Duan, W., Guo, Z., Mattson, M.P., 2001. Brain-derived neurotrophic factor mediates an excitoprotective effect of dietary restriction in mice. J. Neurochem. 76, 619–626.

Escandon, E., Soppet, D., Rosenthal, A., Mendoza-Ramirez, J.L., Szonyi, E., Burton, L.E., Henderson, C.E., Parada, L.F., Nikolics, K., 1994. Regulation of neurotrophin receptor expression during embryonic and postnatal development. J. Neurosci. 14, 2054–2068.

Figurov, A., Pozzo-Miller, L.D., Olafsson, P., Wang, T., Lu, B., 1996. Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381, 706–709.

Fukumitsu, H., Furukawa, Y., Tsusaka, M., Kinukawa, H., Nitta, A., Nomoto, H., Mima, T., Furukawa, S., 1998. Simultaneous expression of brain-derived neurotrophic factor and neurotrophin-3 in Cajal-Retzius, subplate and ventricular progenitor cells during early development stages of the rat cerebral cortex. Neuroscience 84, 115–127.

Gage, F.H., 2000. Mammalian neural stem cells. Science 287, 1433–1438.

Garcia-Verdugo, J.M., Doetsch, F., Wichterle, H., Lim, D.A., Alvarez-Buylla, A., 1998. Architecture and cell types of the adult subventricular zone: in search of the stem cells. J. Neurobiol. 36, 234–248.

Garg, U.C., Hassid, A., 1989. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J. Clin. Invest. 83, 1774–1777.

Garthwaite, J., Boulton, C.L., 1995. Nitric oxide signaling in the central nervous system. Annu. Rev. Physiol. 57, 683–706.

Geller, D.A., Billiar, T.R., 1998. Molecular biology of nitric oxide synthases. Cancer Metastasis Rev. 17, 7–23.

Ghosh, A., Greenberg, M.E., 1995. Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron 15, 89–103.

Hall, J., Thomas, K.L., Everitt, B.J., 2000. Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. Nat. Neurosci. 3, 533–535.

Hawkins, R.D., Son, H., Arancio, O., 1998. Nitric oxide as a retrograde messenger during long-term potentiation in hippocampus. Prog. Brain Res. 118, 155–172.

Holscher, C., McGlinchey, L., Anwyl, R., Rowan, M.J., 1996. 7-Nitro indazole, a selective neuronal nitric oxide synthase inhibitor in vivo, impairs spatial learning in the rat. Learn. Mem. 2, 267–278.

Holscher, C., 1997. Nitric oxide, the enigmatic neuronal messenger: its role in synaptic plasticity. Trends Neurosci. 20, 298–303.

Inglis, F.M., Furia, F., Zuckerman, K.E., Strittmatter, S.M., Kalb, R.G., 1998. The role of nitric oxide and NMDA receptors in the development of motor neuron dendrites. J. Neurosci. 18, 10493–10501.

Kang, H., Schuman, E.M., 1995. Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science 267, 1658–1662.

Kang, H., Schuman, E.M., 1996. A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. Science 273, 1402–1406.

Klocker, N., Kermer, P., Gleichmann, M., Weller, M., Bahr, M., 1999. Both the neuronal and inducible isoforms contribute to upregulation of retinal nitric oxide synthase activity by brain-derived neurotrophic factor. J. Neurosci. 19, 8517–8527.

Kuzin, B., Roberts, I., Peunova, N., Enikolopov, G., 1996. Nitric oxide regulates cell proliferation during Drosophila development. Cell 87, 639–649.

Lachyankar, M.B., Condon, P.J., Quesenberry, P.J., Litofsky, N.S., Recht, L.D., Ross, A.H., 1997. Embryonic precursor cells that express Trk receptors: induction of different cell fates by NGF, BDNF, NT-3, and CNTF. Exp. Neurol. 144, 350–360.

Lee, J., Seroogy, K, Mattson, M.P., 2002. Dietary restriction enhances neurotrophin signaling and neurogenesis in the hippocampus of adult mice. J. Neurochem. 80, 539–547.

Lendahl, U., Zimmerman, L.B., McKay, R.D., 1990. CNS stem cells express a new class of intermediate filament protein. Cell 60, 585–595.

Lepoivre, M., Chenais, B., Yapo, A., Lemaire, G., Thelander, L., Tenu, J.P., 1990. Alterations of ribonucleotide reductase activity following induction of the nitrite-generating pathway in adenocarcinoma cells. J. Biol. Chem. 265, 14143–14149.

Lewin, G.R., Barde, Y.A., 1996. Physiology of the neurotrophins. Annu. Rev. Neurosci. 19, 289–317.

Lo, D.C., 1995. Neurotrophic factors and synaptic plasticity. Neuron 15, 979–981.

Luskin, M.B., Shatz, C.J., 1985. Studies of the earliest generated cells of the cat's visual cortex: cogeneration of subplate and marginal zones. J. Neurosci. 5, 1062–1075.

Maisonpierre, P.C., Belluscio, L., Friedman, B., Alderson, R.F., Wiegand, S.J., Furth, M.E., Lindsay, R.M., Yancopoulos, G.D., 1990. NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. Neuron 5, 501–509.

Mayer-Proschel, M., Kalyani, A.J., Mujtaba, T., Rao, M.S., 1997. Isolation of lineage-restricted neuronal precursors from multipotent neuroepithelial stem cells. Neuron 19, 773–785.

McConnell, S.K., 1991. The generation of neuronal diversity in the central nervous system. Annu. Rev. Neurosci. 14, 269–300.

McConnell, S.K., 1995. Strategies for the generation of neuronal diversity in the developing central nervous system. J. Neurosci. 15, 6987–6998.

Mujtaba, T., Piper, D.R., Kalyani, A., Groves, A.K., Lucero, M.T., Rao, M.S., 1999. Lineage-restricted neural precursors can be isolated from both the mouse neural tube and cultured ES cells. Dev. Biol. 214, 113–127.

Ogilvie, P., Schilling, K., Billingsley, M.L., Schmidt, H.H., 1995. Induction and variants of neuronal nitric oxide synthase type I during synaptogenesis. FASEB J. 9, 799–806.

Pencea, V., Bingaman, K.D., Wiegand, S.J., Luskin, M.B., 2001. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. J. Neurosci. 21, 6706–6717.

Peunova, N., Enikolopov, G., 1995. Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells. Nature 375, 68–73.

Phung, Y.T., Bekker, J.M., Hallmark, O.G., Black, S.M., 1999. Both neuronal NO synthase and nitric oxide are required for PC12 cell differentiation: a cGMP independent pathway. Mol. Brain Res. 64, 165–178.

Rialas, C.M., Nomizu, M., Patterson, M., Kleinman, H.K., Weston, C.A., Weeks, B.S., 2000. Nitric oxide mediates laminin-induced neurite outgrowth in PC12 cells. Exp. Cell Res. 260, 268–276.

Santacana, M., Uttenthal, L.O., Bentura, M.L., Fernandez, A.P., Serrano, J., Martinez de Velasco, J., Alonso, D., Martinez-Murillo, R., Rodrigo, J., 1998. Expression of neuronal nitric oxide synthase during embryonic development of the rat cerebral cortex. Dev. Brain Res. 111, 205–222.

Schuman, E.M., Madison, D.V., 1994. Nitric oxide and synaptic function. Annu. Rev. Neurosci. 17, 153–183.

Shibuki, K., Okada, D., 1991. Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. Nature 349, 326–328.

Shors, T.J., Miesegaes, G., Beylin, A., Zhao, M., Rydel, T., Gould, E., 2001. Neurogenesis in the adult is involved in the formation of trace memories. Nature 410, 372–376.

Stoop, R., Poo, M.M., 1996. Synaptic modulation by neurotrophic factors. Prog. Brain Res. 109, 359–364.

Takahashi, T., Nowakowski, R.S., Caviness Jr., V.S., 1995. The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. J. Neurosci. 15, 6046–6057.

Takahashi, T., Nowakowski, R.S., Caviness, V.S., 1993. Cell cycle parameters and patterns of nuclear movement in the neocortical proliferative zone of the fetal mouse. J. Neurosci. 13, 820–833.

Takahashi, T., Nowakowski, R.S., Caviness, V.S., 1994. Mode of cell proliferation in the developing mouse neocortex. Proc. Natl. Acad. Sci. USA 91, 375–379.

Uwanogho, D., Rex, M., Cartwright, E.J., Pearl, G., Healy, C., Scotting, P.J., Sharpe, P.T., 1995. Embryonic expression of the chicken Sox2, Sox3 and Sox11 genes suggests an interactive role in neuronal development. Mech. Dev. 49, 23–36.

Vescovi, A.L., Reynolds, B.A., Fraser, D.D., Weiss, S., 1993. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. Neuron 11, 951–966.

Wu, Y.Y., Mujtaba, T., Rao, M.S., 2002. Ioslation of stem and precursor cells from fetal tissue. Methods Mol. Biol. 198, 29–40.

Xiong, H., Yamada, K., Han, D., Nabeshima, T., Enikolopov, G., Carnahan, J., Nawa, H., 1999. Mutual regulation between the intercellular messengers nitric oxide and brain-derived neurotrophic factor in rodent neocortical neurons. Eur. J. Neurosci. 11, 1567–1576.

Young, D., Lawlor, P.A., Leone, P., Dragunow, M., During, M.J., 1999. Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat. Med. 5, 448–453.

Zappone, M.V., Galli, R., Catena, R., Meani, N., De Biasi, S., Mattei, E., Tiveron, C., Vescovi, A.L., Lovell-Badge, R., Ottolenghi, S., Nicolis, S.K., 2000. Sox2 regulatory sequences direct expression of a (beta)-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127, 2367–2382.

Zhang, S., Chen, J., Wang, S., 1998. Spatial learning and memory induce up-regulation of nitric oxide-producing neurons in rat brain. Brain Res. 801, 101–106.

Zhang, R., Zhang, L., Zhang, Z., Wang, Y., Lu, M., Lapointe, M., Chopp, M., 2001. A nitric oxide donor induces neurogenesis and reduces functional deficits after stroke in rats. Ann. Neurol. 50, 602–611.

Zigova, T., Pencea, V., Wiegand, S.J., Luskin, M.B., 1998. Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb. Mol. Cell. Neurosci. 11, 234–245.