■ REVIEW

Nitric Oxide and Adult Neurogenesis in Health and Disease

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Adult neurogenesis may be functionally important as a mechanism of brain plasticity in physiological conditions and brain repair after injury. Nitric oxide (NO), a diffusible intracellular and intercellular messenger in the mammalian nervous system, has been shown to affect adult neurogenesis in different ways. In the normal brain, NO, synthesized by the neuronal isoform of NO synthase in nitrergic neurons, is a negative regulator of precursor cell proliferation. However, after brain damage, NO overproduction in different neural and nonneural cell types promotes neurogenesis. Recently reported results on the effects of NO on new neuron generation in the adult brain are reviewed, with special attention to the proposed mechanisms of action and functional consequences in health and disease. NEUROSCIENTIST 11(4):294–307, 2005. DOI: 10.1177/1073858404273850

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Neurogenesis in the Adult Brain

During the past decade, it has been well established that neural stem cells with the ability to produce new neurons and glia remain in the adult central nervous system (CNS) in various mammalian species including humans (Gage 2000; Alvarez-Buylla and García-Verdugo 2002). In physiological conditions, new neurons are generated from these neural progenitors only in two specific regions: the dentate gyrus (DG) of the hippocampus (Eriksson and others 1998) and, in rodents, the subventricular zone (SVZ) in the lateral wall of the lateral ventricles (Lois and Alvarez-Buylla 1993, 1994) (Fig. 1). The proposed lineage from the primary progenitors to the mature neurons in each region is shown in Figure 2. In the DG, precursors divide in the subgranular layer and give rise to mature neurons in the granular cell layer. In the SVZ, the newly generated neuroblasts migrate tangentially through the rostral migratory stream (RMS) (Fig. 3*A*) and become inhibitory interneurons in the granular and periglomerular layers of the olfactory bulb (OB). In humans, progenitors with stem–cell-like properties have been identified in the SVZ, although they do not generate neurons in physiological conditions (Sanai and others 2004).

The functional role of persistent neurogenesis in the mammalian adult brain is not well understood, but it probably represents a new form of CNS plasticity. The continuous neuronal replacement that occurs physiologically in the DG and SVZ may participate in memory acquisition for spatial task performance and olfactory learning, respectively. This is supported by the finding that adult neurogenesis is increased in animals exposed to enriched environmental conditions, which also stimulate their learning abilities (Rochefort and others 2002). In addition, activation of stem cells and neuronal precursors upon injury may contribute to brain repair in pathological situations. Understanding the regulation of adult neurogenesis is of great interest because new therapeutical strategies directed toward improving neuronal replacement may be useful in the treatment of learning problems, as well as in brain recovery after different types of injury or in degenerative diseases.

Why do neural stem cells, at a certain time and in a specific place, become activated and give rise to a differentiated progeny? What kind of signals control proliferation and survival of the neural precursors in the neurogenic areas, their migration, their differentiation in the destination sites, as well as the integration of the newly formed mature neurons in functional circuits? All these questions are far from being solved, although interesting information has been generated during the last few years. In physiological conditions, the neural stem cells, probably quiescent astrocytes (Doetsch and others 1999; Seri and others 2001), become activated and divide in proximity to ependymal (Doetsch and others 1997) or endothelial cells (Palmer and others 2000), thus indicating that both ependyma and endothelium and their basal lamina provide adequate niches for neurogenesis (Alvarez-Buylla and Lim 2004). Many laboratories are currently investigating the messenger molecules and transcription factors that are present in these niches, to elucidate their roles in the cellular events-proliferation, migration, differentiation, and survival-involved in the generation of new neurons.

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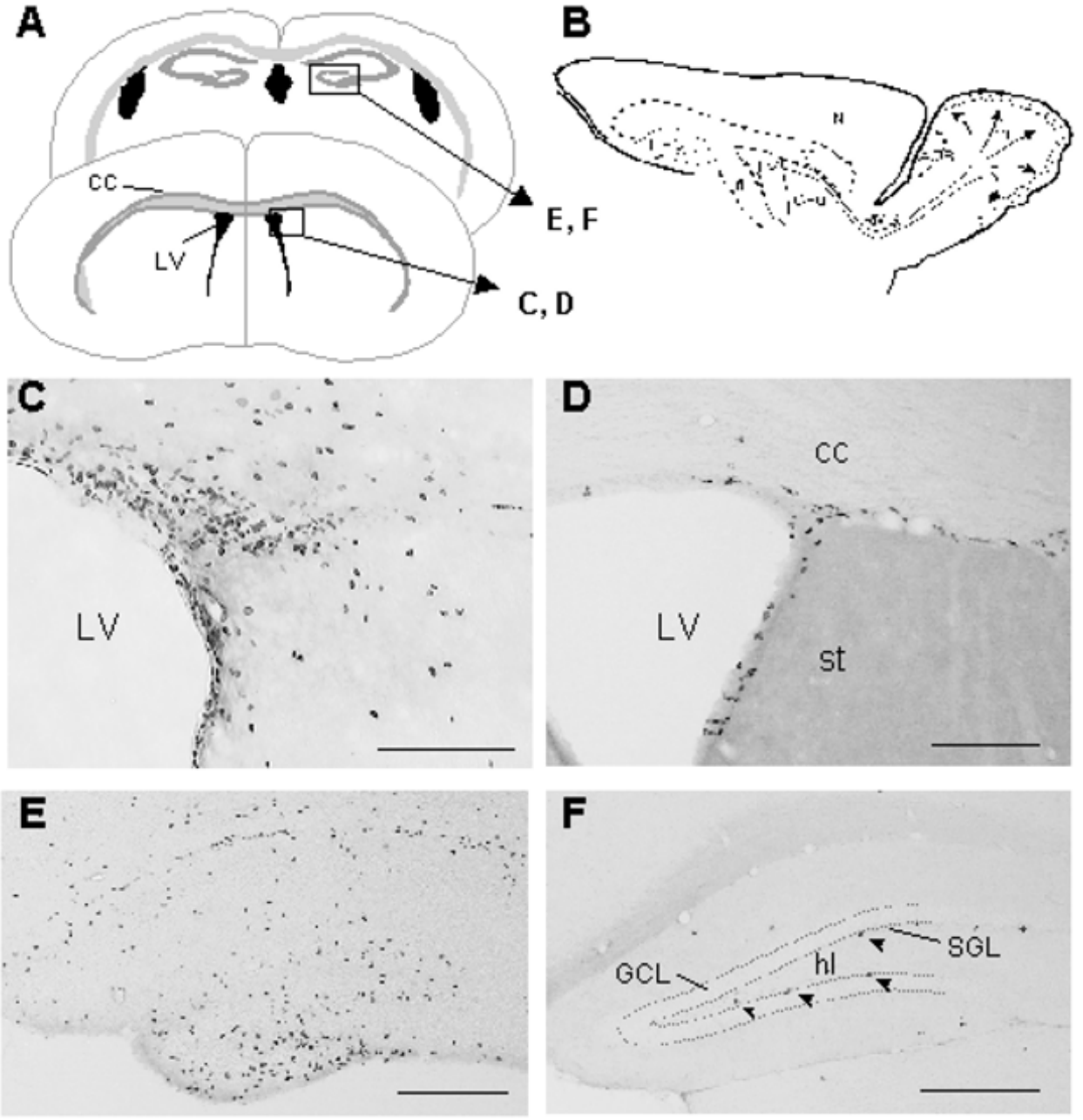


Fig. 1. Neurogenic regions in the mouse brain. *A*, Drawing of two coronal sections through the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus. Open squares indicate the location of the images shown below. *B*, Drawing of a sagittal section indicating the migratory pathway of the neuroblasts from the SVZ to the olfactory bulb (OB). Neuronal precursors in the lateral wall of the lateral ventricles (LV) migrate tangentially through the rostral migratory stream (RMS) to the OB and then migrate radially and differentiate in the granular and glomerular cell layers. *C*, Postnatal (P7) mouse SVZ containing a large number of mitotic cells, identified by positive BrdU staining. *D*, Adult mouse SVZ. $BrdU^+$ nuclei are restricted to a narrow area close to the ventricle wall and extended between the corpus callosum and the striatum. *E*, Postnatal (P7) mouse hippocampus showing a large number of widely distributed $BrdU^+$ cells. *F*, Adult mouse DG. A few mitotic cells (arrowheads) are seen in the subgranular layer (SGL). cc = corpus callosum; GCL = granule cell layer; hl = hilum; st = striatum. Scale bars:100 μm.

Results obtained using either the embryonic or adult brain indicate that neural stem cells divide in response to fibroblast growth factor 2 (FGF-2) (Gritti and others 1999; Palmer and others 1999; Tropepe and others 1999) and give rise to highly proliferative transit-amplifying cells. In the SVZ (Fig. 2*A*), this transit-amplifying cell population, which can be identified by the expression of nestin and the transcription factor Dlx2, together with the lack of neuronal- or glial-specific antigens (Doetsch and others 1997; Doetsch and others 2002),

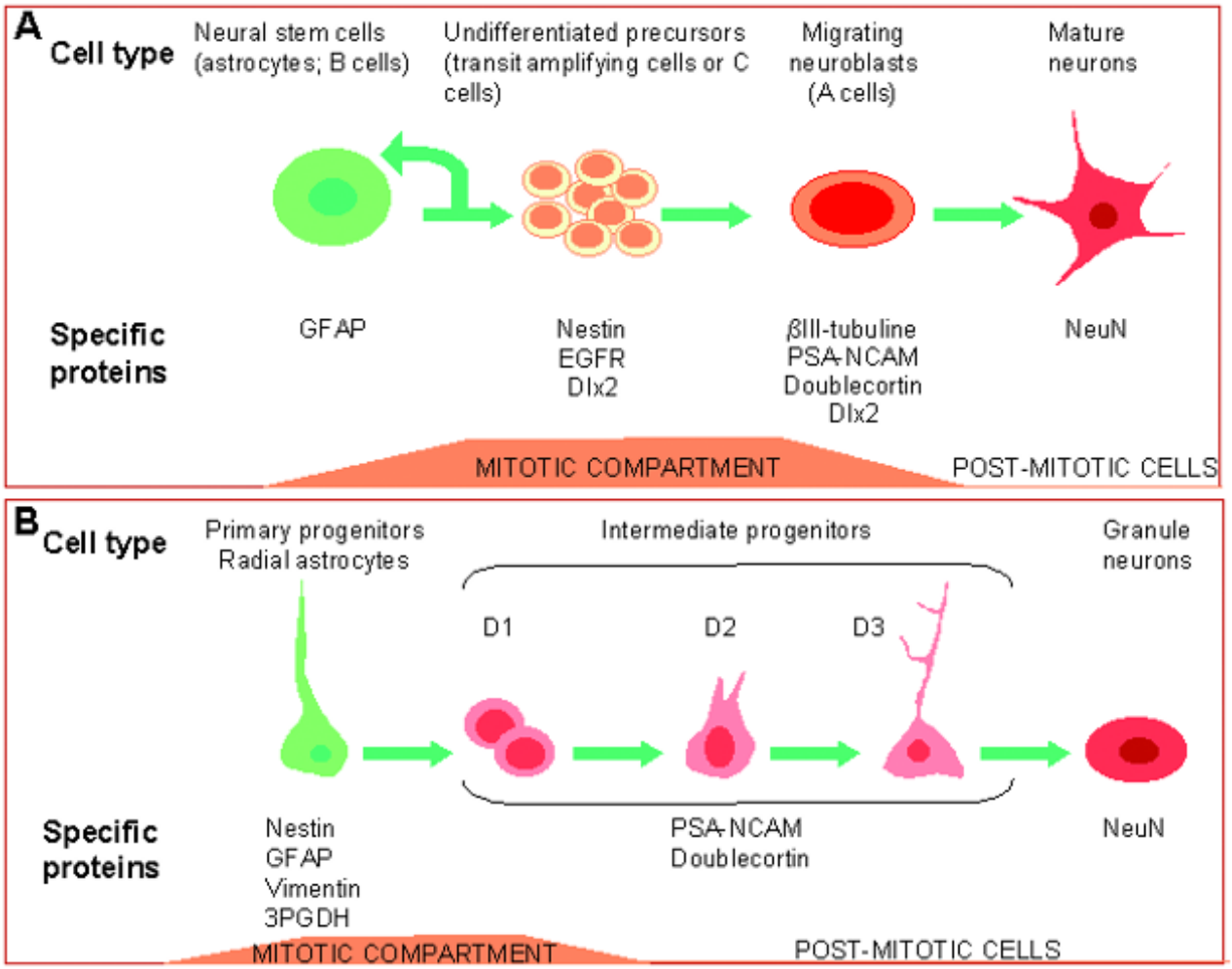


Fig. 2. Proposed cell lineages from neural stem cells to mature neurons in the subventricular zone (SVZ)–olfactory bulb (OB) neurogenic system (*A*) and the dentate gyrus (DG) of the hippocampus (*B*). *A*, In the SVZ, astrocytes (B cells) divide and give rise to highly proliferative transit-amplifying cells (C cells), which, in turn, differentiate as neuroblasts (A cells). Neuroblasts, which are still able to divide but at a slower rate, migrate through the rostral migratory stream and reach the OB, where they become inhibitory interneurons (based on Doetsch and others 1997; Doetsch and others 1999; Doetsch and others, 2002). *B*, In the DG, astrocytes in the subgranular layer function as early progenitors giving rise to intermediate D cells, which finally differentiate as granule neurons. Three subtypes of D cells (D1-3) have been identified based on their morphology, and these probably represent consecutive maturation stages. D1 cells appear to divide only once, whereas D2 and D3 are postmitotic cells (based on Seri and others 2001; Seri and others, 2004). Some specific proteins expressed by each cell subtype and used for phenotype identification are shown. 3PGDH = 3-phosphglycerate dehydrogenase; Dlx2 = homeobox-containing transcription factor implicated in the development of GABA-ergic neurons and oligodendrocytes in the embryo; EGFR = epidermal growth factor receptor; GFAP = glial fibrillar acidic protein; PSA-NCAM = polysialylated neural cell adhesion molecule.

specifically expresses epidermal growth factor receptors (EGFRs); (Doetsch and others 2002). Intracerebroventricular (i.c.v.) administration of EGFR ligands, such as epidermal growth factor (EGF), transforming growth factor α (TGF-α), or heparine-binding EGF (HB-EGF), largely increases proliferation of this cell population and produces profound changes in the fate of SVZ postmitotic cells, which migrate away from the walls of the lateral ventricles into adjacent brain parenchyma, including the striatum, septum, corpus callosum, and cortex (Craig and others 1996; Kuhn and others 1997; Doetsch and others 2002; Jin, Mao, and others 2002). Among these EGFR ligands, TGF-α seems to be the most relevant one in vivo, in that TGF-α knockout mice present reduced cell proliferation in the SVZ and fewer new neurons reaching the OB, indicating that TGF-α is a mitogenic signal necessary for the maintenance of the transit-amplifying cell population (Tropepe and others 1997). In addition to FGF-2 and EGFR ligands, other factors such as vascular endothelial growth factor (VEGF) (Jin, Zhu, and others 2002; Schanzer and others 2004) and brain-derived neurotrophic factor (BDNF) (Pencea and others 2001) increase neurogenesis in several brain regions.

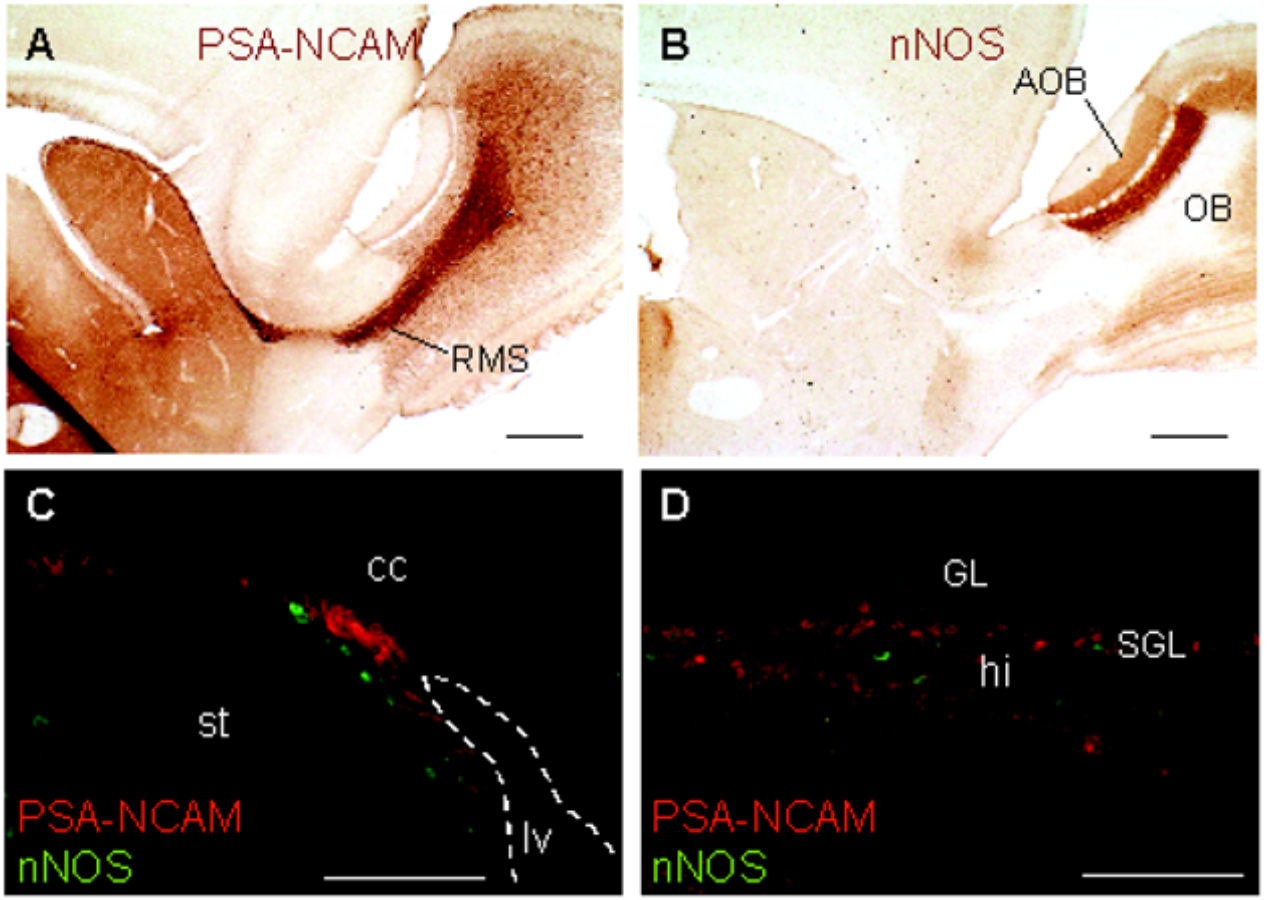


Fig. 3. Anatomical relationship between neural precursors and nitrergic neurons. *A-B*, Sagittal sections of an adult mouse brain stained with specific antibodies against PSA-NCAM, a marker for migrating neuroblasts (*A*), and neuronal isoform nitric oxide synthase (nNOS) (*B*). Observe the complementary distribution of the stainings. Migrating neuroblasts are located along the rostral migratory stream (RMS) and penetrate the core of the olfactory bulb (OB). Nitrergic cell bodies are located close to, but outside, the RMS. Note the dense nNOS-positive neuropil in the accessory olfactory bulb (AOB), devoid of PSA-NCAM staining. *C*, Adult mouse subventricular zone double stained for PSA-NCAM (red) and nNOS (green). Here also, nitrergic cell bodies lie on the boundary of the neurogenic area. *D*, In the dentate gyrus of the hippocampus, nitrergic neurons are observed in the hilum (hi) and precursor cells in the subgranular layer (SGL). PSA-NCAM = polysialylated neural cell adhesion molecule; cc = corpus callosum; GL = granular cell layer; st = striatum; Scale bars: *A* and *B*, 500 μm; *C* and *D*, 100 μm.

Nitric Oxide

Nitric oxide (NO) a gaseous radical synthesized in many cell types, is a diffusible intercellular messenger with multiple functions in the cardiovascular, immunological, and nervous systems. NO is synthesized from L-arginine in a reaction catalyzed by NO synthase (NOS), a heme-containing enzyme that is linked to NADPH-derived electron transport. Three main NOS isoforms have been cloned and well characterized. Endothelial NOS (eNOS or NOS 3; the numeric nomenclature corresponding to the order in which the isoenzymes were cloned), constitutively expressed in endothelial cells from all vessel types, contributes to blood vessel dilation and prevents platelet aggregation and proliferation of vascular smooth muscle cells. The inducible isoform (iNOS or NOS 2) was initially described in macrophages and other cell types involved in inflammation and is permanently active when expressed, leading to the production of large amounts of NO that may be cytotoxic. The neuronal isoform (nNOS or NOS 1) is constitutively expressed by specific neurons widely distributed in the mammalian nervous system. Both constitutive enzymes, eNOS and nNOS, are activated by calmodulin when cytoplasmic calcium concentrations increase, although their activity can also be modulated by phosphorylation and by changes in their subcellular compartmentalization (reviewed in Knowles and Moncada 1994, and Alderton and others 2001).

A well-established pathway for NO signal transduction is the activation of guanylyl cyclase, leading to the intracellular increase of cGMP. However, NO can also produce cGMP-independent effects in living cells through protein S-nitrosylation (Stamler and others 2001). A large number of proteins have been reported to contain cysteine residues susceptible of forming nitrosothiol groups when exposed to NO, and this chemical modification may regulate protein activity.

A dual role of NO has been reported, with beneficial or cytotoxic actions depending on the NO concentration and the cell environment (reviewed in Keynes and Garthwaite 2004). Whereas low amounts of NO serve beneficial functions, such as lowering blood pressure, excessive amounts, or its combination with oxygen free radicals, produce deleterious effects, such as the cell death associated with ischemia or with some inflammatory or degenerative processes. In general terms, it is presently accepted that eNOS and nNOS produce small amounts of NO in discrete locations, in a very controlled way, and with precise functions, whereas excessive NO derived from pathological iNOS expression or from excitotoxic overstimulation of nNOS may result in cell damage and disease progression.

Soon after its discovery as a biological agent, NO emerged as a neural messenger in the CNS, where nNOS is expressed in widely distributed neurons (Bredt and others 1990). In the brain, NO modulates neurotransmitter release, contributes to synaptic formation and remodeling, and participates in the control of cerebral blood flow, among other actions. Alterations of cerebral NO production, either by pharmacological inhibition of NOS activity or in knockout animals, affect a large variety of functions such as specific sensory-motor responses, olfactory learning, pain, or ischemia-induced apoptosis.

During development, there is a transient expression of nNOS in different brain areas (Bredt and Snyder 1994; Santacana and others 1998), which suggests that NO also performs some function in embryonic neural tissue formation. Such an effect has been demonstrated in developing *Drosophila* imaginal disks (Kuzin and others 1996) and *Xenopus* tadpole optic tectum (Peunova and others 2001), in which NO effectively suppresses the division of neural precursor cells.

NO Inhibits Neurogenesis In Vitro

NO modulates the balance between proliferation and differentiation of neural cells in culture. Addition of NO donors to neuroblastoma cells (Murillo-Carretero and others 2002), as well as to neural precursors isolated from the postnatal mouse SVZ (Matarredona and others 2004), significantly decreases the rate of cell proliferation. Furthermore, embryonic and postnatal mouse-derived neural precursor cells, which express NOS and spontaneously produce NO in culture, increase their mitotic index when NO synthesis is inhibited or when exposed to a NO scavenger (Cheng and others 2003; Ciani and others 2004; Matarredona and others 2004).

Both nNOS and iNOS gene expression can be induced in tumoral cells of neural origin by differentiating agents, such as nerve growth factor (Peunova and Enikolopov 1995), tumor necrosis factor α (Obregón and others 1997), or retinoic acid (Ogura and others 1996), and in mouse embryo neural progenitor cells when stimulated with BDNF (Cheng and others 2003). In all cases, NO production was necessary to observe the antiproliferative and differentiating effects of the cytokines.

NO and Physiological Adult Neurogenesis

In the adult brain, nNOS is expressed by discrete neuronal populations. Therefore, a necessary criterion for proposing a role for NO in adult neurogenesis is the identification of possible NO sources in the vicinity of the precursor cells. Taking into account the short half-life and the diffusion coefficient of the NO molecule, it has been estimated that NO can spread from its site of production and reach a volume of parenchymal tissue that corresponds to a sphere with a radius of ~100 μm around the NO point source. In the adult brain of both mice and rats, neurons expressing nNOS are located near the SVZ-OB neurogenic system (Figs. 3*C* and 5). In the SVZ, while nitrergic cell bodies lie on the limits of the neurogenic area, their abundant varicose processes are intermingled with the chains of neuroblasts, recognized by polysialylated neural cell adhesion molecule (PSA-NCAM) staining (Moreno-López and others 2000; Packer and others 2003). Although the number of nitrergic somas is not high, it is very likely that, due to their dense neuropil, the whole proliferation area is under the influence of NO when nitrergic neurons are activated. In the RMS, where migrating neuronal precursors are ensheathed by a layer of glial cells (Lois and Alvarez-Buylla 1994), nitrergic somas are always found outside the stream (Fig. 3 *A*,*B*), with processes forming a loose network surrounding the migrating precursors (Moreno-López and others 2000; Packer and others 2003). Also, large nitrergic neurons can be found in the core of the OB, and small round interneurons expressing nNOS are located in a periglomerular position (Moreno-López and others 2000) (see Fig. 5). Neural precursors in the subgranular layer of the DG are also in proximity with the nitrergic neurons present in the hilum (Fig. 3*D*). Therefore, the anatomical relationship between potential NO sources and neural progenitors allows the hypothesis that NO signaling exerts a role in the control of neurogenesis in the adult brain.

Experimental results from several laboratories indicate that NO, produced by nitrergic neurons, exerts a negative control on neurogenesis in physiological conditions in the SVZ (Fig. 4 and Table 1). Systemic administration of the NOS inhibitor nitro-L-arginine methyl ester (L-NAME), but not of the inactive stereoisomer D-NAME, to adult mice produced a dose- and time-dependent increase in the number of proliferating cells in the SVZ, RMS, and OB (Moreno-López and others 2004). Similar effects were obtained when the same drugs were administered directly in the cerebral ventricles, either as a single injection (Cheng and others 2003) or using osmotic minipumps that allow slow release during seven days (Packer and others 2003). Rates of mitosis were estimated in all cases by quantitative analysis of the cells incorporating Br-deoxyuridine (BrdU), although the time of survival after BrdU administration was different in each report (see Table 1 and Box 1). No effects on cell survival or on migration were found upon L-NAME systemic (Moreno-López and others 2004) or local (Packer and others 2003) administration.

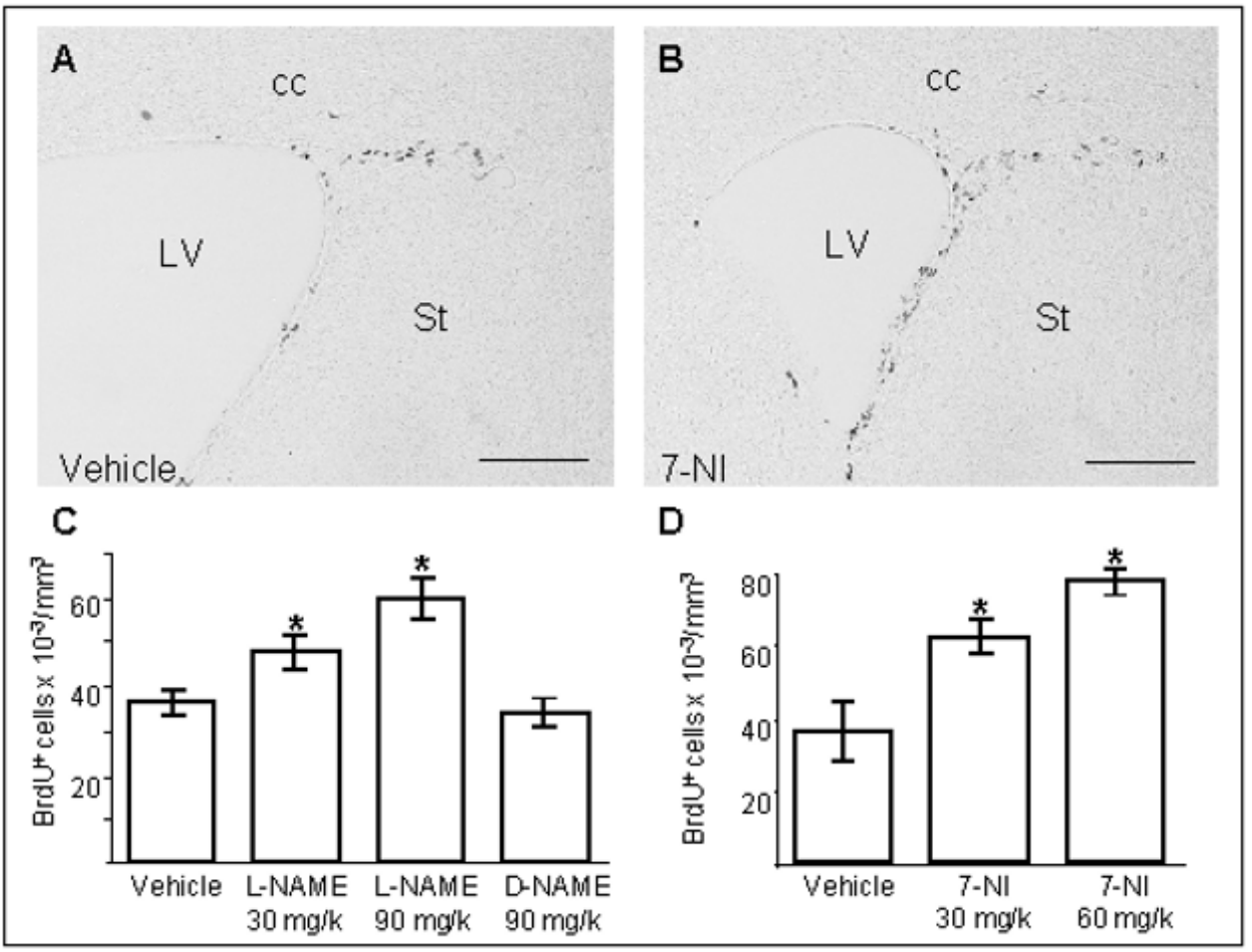


Fig. 4. Effect of NOS inhibition on neural precursor cell proliferation in the mouse subventricular zone (SVZ). *A-B*, Coronal sections through the SVZ of a control mouse (*A*) and a mouse treated with 7-nitroindazol (7-NI), a specific inhibitor of nNOS (*B*). *C-D*, Number of cells that incorporated BrdU in control animals (vehicle) and in animals treated with the indicated doses of NOS inhibitors for 7 (L-NAME) or 4 (7-NI) consecutive days. Results obtained using the inactive enantiomer D-NAME are also shown. In all cases, BrdU was administered at the end of the treatment, and the animals were killed the same day for analysis of BrdU incorporation (see Box 1). NOS = nitric oxide synthase; nNOS = neuronal isoform NO synthase; BrdU = Br-deoxyuridine; L-NAME = nitro-L-arginine methyl ester; 7-NI = 7-nitroindazole; D-NAME = nitro-D-arginine methyl ester; cc = corpus callosum; LV = lateral ventricle; St = striatum. Scale bars: 100 μm.

Two lines of evidence indicate that the endogenous NO controlling neural precursor proliferation is synthesized by nNOS. First, systemic administration of the nNOS specific inhibitor 7-nitroindazole to adult mice increased SVZ neural precursor proliferation to a similar extent as L-NAME (Moreno-López and others 2004). Second, targeted disruption of the nNOS gene enhanced proliferation in neurogenic zones (Packer and others 2003). These findings suggest that local nitrergic neurons, and not capillary endothelial cells, which are also located in the vicinity of neural progenitor cells, are the main source of the endogenous NO controlling neurogenesis.

Whereas all experimental results reported so far support the inhibitory role of endogenous NO in SVZ neurogenesis, data obtained in the hippocampus are controversial. Systemic administration of L-NAME (up to 15 days) or 7-nitroindazole (up to 5 days) to adult rodents did not modify the number of mitotic cells in the DG of the hippocampus (Park and others 2003; Moreno-López and others 2004), although the density of $BrdU^+$ cells increased significantly after longer periods of NOS inhibition (Park and others 2003), or after short-term nNOS inhibition in adrenalectomized rats (Park and others 2001), in which nNOS is overexpressed in the hippocampus (López-Figueroa and others 1998). In addition, a significant increase in the overall size, as well as in the absolute number of $BrdU^+$ cells, was observed in the DG of rats receiving i.c.v. L-NAME and daily BrdU injections for one week (Packer and others 2003). The use of mutant mice lacking functional nNOS also resulted in increased proliferation in the DG (Packer and others 2003), whereas mice lacking eNOS showed a significant reduction in neuronal progenitor cell proliferation in the same structure (Reif and others 2004).

In contradiction to the antiproliferative effect of nNOS-derived NO observed in adult animals (Park and

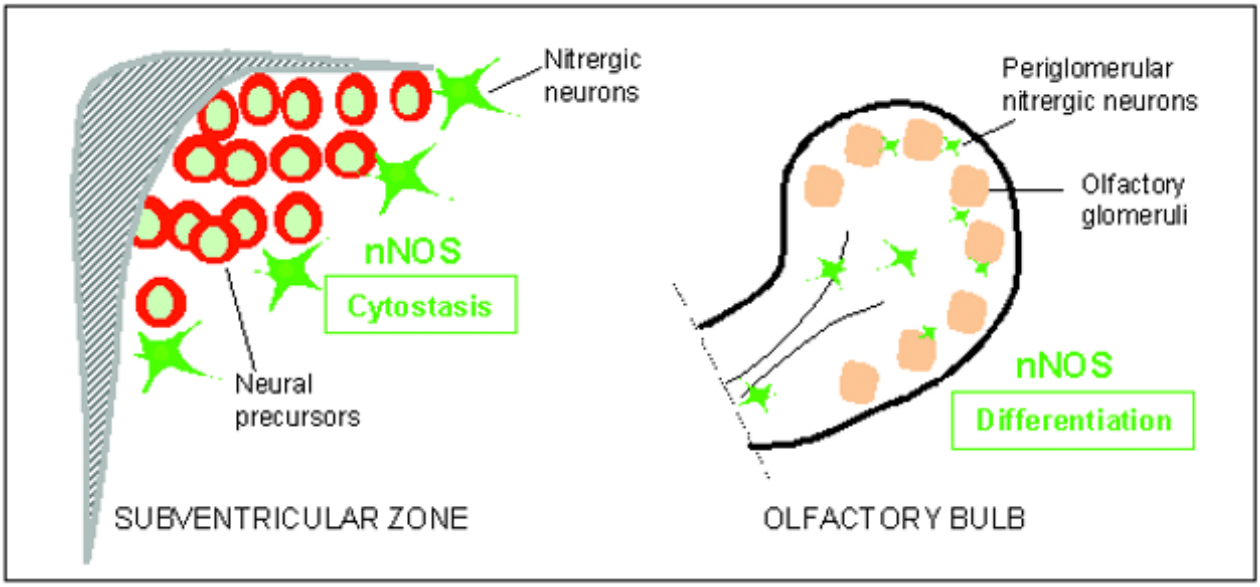


Fig. 5. Image summarizing the distribution of nitrergic neurons and the effects of nNOS-derived nitric oxide in adult rodent subventricular zone and olfactory bulb in physiological conditions. In the SVZ, large, well-differentiated neurons expressing nNOS are distributed on the periphery of the area where neural precursor cells proliferate. Inhibition of NO production increases the proliferation rate of the undifferentiated precursors, indicating that endogenous NO has a cytostatic action and therefore exerts a negative control on the size of the neural precursor pool in the SVZ. In the olfactory bulb, large nitrergic neurons are located in the core of the structure, close to the chains of migrating neuroblasts. In addition, small round nNOS-positive neurons are found around the glomeruli. Some of the recently arrived periglomerular neuroblasts express nNOS. Inhibition of NO production produces a delay in neuroblast differentiation and maturation. nNOS = neuronal isoform nitric oxide synthase; SVZ = subventricular zone.

others 2001; Cheng and others 2003; Packer and others 2003; Moreno-López and others 2004), NO donor administration has been reported to promote neurogenesis (Zhang and others 2001). The reasons for the discrepancy may reside in the experimental protocol because these authors analyzed the brain one to two weeks after drug administration, a time at which not only proliferation but also possible effects on cell migration and survival would affect the $BrdU^+$ cell counting. Furthermore, because NO is a powerful vasodilator, intravenous administration of a NO donor produces significant decreases in blood pressure and changes in cerebral blood flow that may indirectly affect neurogenesis. In addition, the effect of a NO donor is not necessarily the opposite of the effect of a NOS inhibitor in vivo because the former includes actions of NO in cells that may have target molecules and respond to NO but that are not exposed to this mediator in physiological conditions.

A possible effect of NO on neural precursor differentiation has also been investigated in the adult OB, the structure in which neuroblasts, generated in the SVZ, differentiate and become integrated as inhibitory interneurons in olfactory circuits (Carleton and others 2003). In the OB, some neuroblasts, identified by PSA-NCAM staining, express nNOS, a feature that was never observed in the SVZ or RMS (Moreno-López and others 2000). This nNOS expression at the final differentiation site correlates well with the above-mentioned results obtained in cultured cells, in which different NOS isoforms are induced prior to, or concomitantly with, the differentiation process (Peunova and Enikolopov 1995; Ogura and others 1996; Obregón and others 1997; Matarredona and others 2004). The transitory nNOS expression in differentiating neurons suggests that NO production may also participate in the differentiation process. In this respect, analysis of maturation markers in newly generated neurons indicates that chronic NOS inhibition causes a delay in neuronal differentiation in the adult mouse OB (Moreno-López and others 2004).

Neurogenic Response to Brain Injury

It is well known that the adult CNS has a very limited capacity for self-repair or regeneration after excitotoxic, ischemic, or traumatic lesions. However, it has been observed that several lesions, including transient global (Liu and others 1998) or focal (Jin and others 2001; Nakatomi and others 2002; Teramoto and others 2003) ischemia or seizures (Parent and others 1997), stimulate the proliferation of neural progenitors in the DG and/or SVZ, which leads to an increased production of new neurons that eventually migrate toward the lesion area. Nevertheless, the capacity of neuronal replacement in the adult brain is very low, with estimations between 0.2% and 10% of the lost neurons, depending on the area and the type of lesion (Arvidsson and others 2002; Nakatomi and others 2002; Teramoto and others 2003). Although the mechanisms mediating this moderate increase in neurogenesis are not known, it is interesting

Table 1. Summary of the Effects of Genetic or Pharmacological Modifications of NO Concentrations on Adult Neurogenesis

Species	Condition	Method Used to Modify NO Concentration	BrdU Administration/ Survival Time	Cerebral Region	Results: Number of BrdU+ Cells	Reference
Mouse	Physiological	Systemic L-NAME (7–15 days) or 7-NI (4 days)	3 doses at 2.5-h intervals; survival time, 2.5 h after the last injection	SVZ DG	Increased No change	Moreno-López and others (2004)
Rat	Physiological	i.c.v. L-NAME infusion for 7 days	Daily for the 7 days of treatment	SVZ DG	Increased	Packer and others (2003)
Mouse	Physiological	i.c.v. single injection of L-NAME	2 doses with 2-h intervals, 24 h after treatment	SVZ	Increased	Cheng and others (2003)
Rat	Physiological	Systemic L-NAME (25 days) or 7-NI (15 days)	Single administration; survival time, 24 h	DG	Increased	Park and others (2003)
Rat	Physiological Ischemia	Systemic NO donor for 1, 2, or 6 days	Daily for 14 days	SVZ	Increased	Zhang and others (2001)
Rat	Physiological Adrenalec-tomized	Systemic 7-NI for 5 days	Single administration; survival time, 2 h	DG	No change Increased	Park and others (2001)
Mouse		nNOS knockout	Cumulative for 2.5 days	SVZ DG	Increased	Packer and others (2003)
Mouse		eNOS knockout	4 doses at 2-h intervals; survival time, 6 days	DG	Decreased	Reif and others (2004)
Rat	Ischemia	Systemic amino-guanidine for 5 days	2 doses with 8-h intervals	DG	Decreased	Zhu and others (2003)
Rat	Induced epileptic seizures	Systemic 7-NI or aminoguanidine	2 doses with 2-h intervals; survival time, 24 h	DG	Decreased	Jiang and others (2004)

NO = nitric oxide; BrdU = Br-deoxyuridine; L-NAME = nitro-L-arginine methyl ester; 7-NI = 7-nitroindazole; SVZ = subventricular zone; DG = dentate gyrus; i.c.v. = intracerebroventricular; nNOS = neuronal isoform NO synthase; eNOS = endothelial NO synthase.

to note that following injury in the CNS, there is a widespread activation of TGF-α, HB-EGF, and VEGF gene expression in both neuronal and glial cells (Kawahara and others 1999; Zhang and others 2000). Simultaneously, growth factor receptors are up-regulated in reactive astrocytes that migrate toward the lesion from adjacent undamaged parenchyma and also in undifferentiated precursors in the neurogenic areas (Kawahara and others 1999). By conveying survival and/or proliferation signals, these growth factors may help in the generation of new neurons and exert a chemotactic action directing the new cells to the lesion area (Jin, Mao, and others 2002; Jin, Zhu, and others 2002; H. Zhang and others 2003).

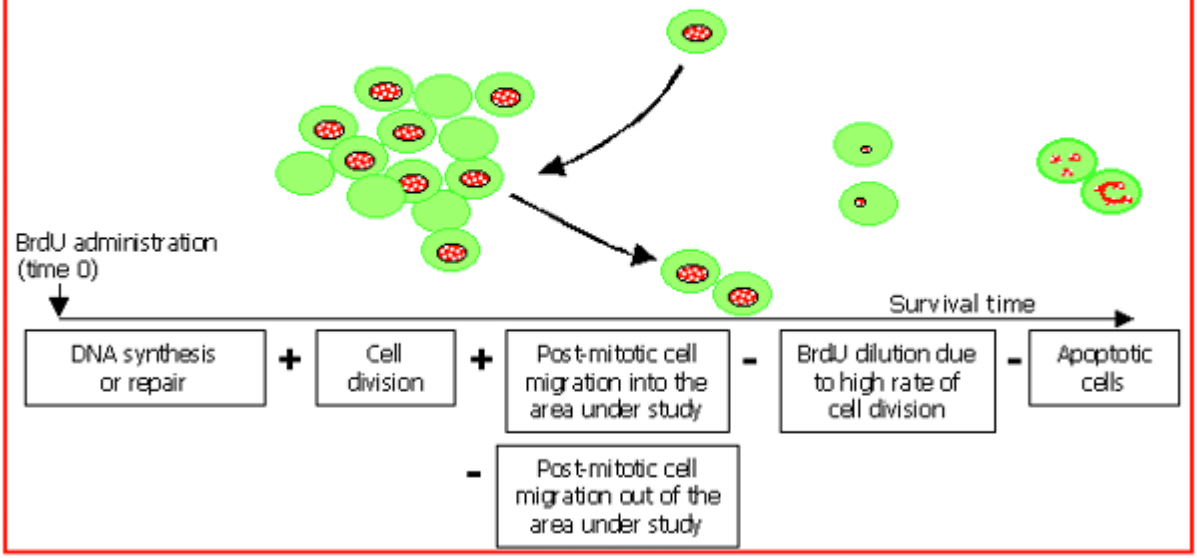
If neural progenitors contribute to the production of new neurons, expansion of their endogenous pool by administration of exogenous growth factors may improve the regenerative capacity of the damaged CNS. In this respect, it has been reported that EGF infusions in animals exposed to cerebral ischemia significantly increased the number of newborn cells that migrated from the SVZ into the infarcted striatum and became mature neurons, as well as the number of astrocytes exhibiting long processes, characteristic of radial glia, which provide a scaffold for neuroblast migration (Teramoto and others 2003). Continuous infusion of EGF and FGF-2 also increased the number of new neurons in the hippocampus after induction of ischemia, resulting in a recovery of approximately 40% of the lost neurons four weeks later (Nakatomi and others 2002). The newly formed neurons acquired the phenotype characteristic of the regenerated region and received synaptic inputs, suggesting their integration in neuronal circuits.

In response to ischemia, there is also a systemic response that includes stem cell mobilization from the bone marrow into the circulation, differentiation as endothelial progenitor cells, and homing to sites of ischemia (Fig. 6), where they contribute to the formation of new blood vessels and tissue repair (Rafii and Lyden 2003). Expression of VEGF in the ischemic site is thought to participate in stem cell mobilization and hom-

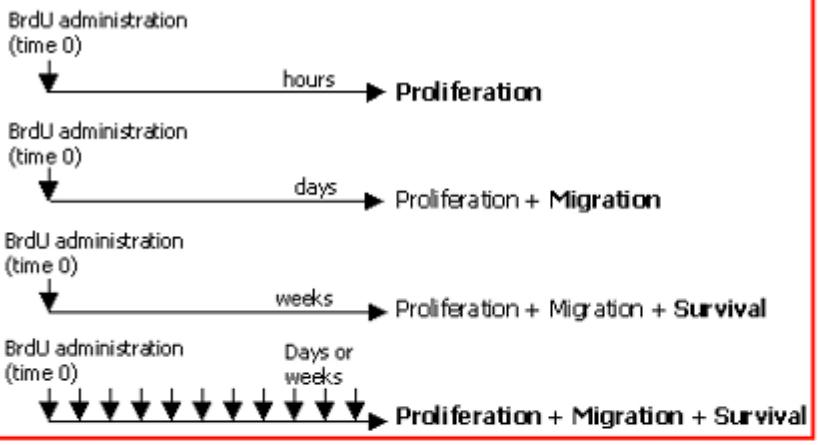
BOX 1.- ADVANTAGES AND PITFALLS OF USING Br-DEOXYURIDINE (BrdU) TO EVALUATE NEUROGENESIS

BrdU is a thymidine analog that is incorporated into the DNA during the S-phase of cell division. Systemic administration of BrdU, followed by its immunohistochemical detection, allows the identification of proliferating cells. However, the protocol used for BrdU administration, the time elapsed until death, and the proliferation rate of the cell population under analysis, modify the interpretation of the results.

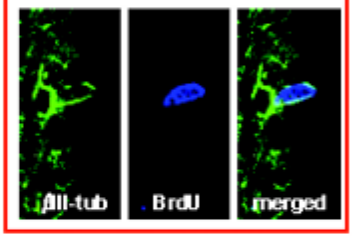
➢ Cell events that determine the number of labeled cells, depending on the time elapsed between BrdU administration and the animal death.



➢BrdU administration protocols for the analysis of different cell events



➢The demonstration of neurogenesis requires the visualization of cells double labeled for BrdU plus a neuron-specific marker, such as βIII-tubuline or doublecortin



Apparently contradictory results concerning the effect of certain drugs or conditions on neurogenesis are due to the use of different protocols to detect BrdU incorporation by DNA synthesizing cells. When animals receive BrdU and are sacrificed within a few hours, the number of BrdU⁺ cells indicates how many cells have been in the S phase of the cell cycle during this time, which is indicative of the mitotic index of the cell population. However, if BrdU is administered over several days or weeks, results may reflect not only cell proliferation, but also survival of the postmitotic cells. Furthermore, if the SVZ is analyzed, cells that divided 7 or 15 days before perfusion in the SVZ will be in the RMS or in the OB at the time of analysis. Therefore, any treatment affecting cell migration may modify the number of BrdU⁺ cells in the SVZ, without necessarily acting on cell proliferation. In addition, the observation of an increased number of BrdU⁺ cells does not mean that neurogenesis is increased; it is necessary to demonstrate that the newly generated cells will indeed become mature neurons. Even if we know that in physiological conditions only neuronal cells are generated in the SVZ, under certain conditions, such as EGF administration or in lesioned brains, glial cells are also produced from the neural progenitors.

SVZ = subventricular zone; RMS = rostral migratory stream; OB = olfactory bulb; EGF = epidermal growth factor.

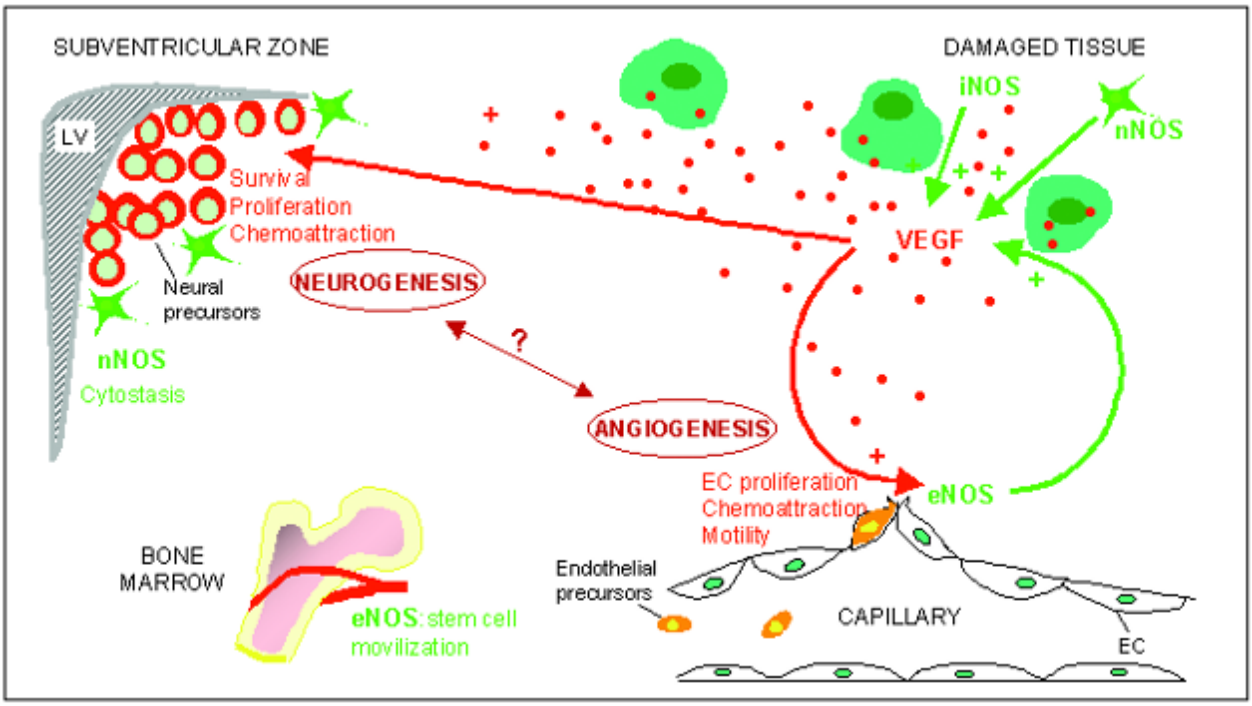


Fig. 6. Proposed model for NO-VEGF action on angiogenesis and neurogenesis in brain lesions. In damaged areas, VEGF, nNOS, and iNOS genes are up-regulated in neural cells. A positive feedback loop is created, in which NO enhances VEGF expression and VEGF increases the expression of eNOS in capillary endothelial cells. VEGF causes migration and proliferation of capillary endothelial cells, leading to angiogenesis, and chemoattraction and homing of hematopoietic stem cell–derived endothelial precursors, leading to vasculogenesis. VEGF also enhances proliferation and survival of neural precursors and acts as a chemoattractant factor for these cells. In addition, NO produced by eNOS in the bone marrow promotes hematopoietic stem cell mobilization and, therefore, contributes to neovascularization. Although it has not been clearly demonstrated so far, it is possible that a positive feedback loop is established between angiogenesis/vasculogenesis and neurogenesis. All these actions may result in the increased neurogenesis and migration of neuroblasts toward the lesion area observed in the damaged brain. NO = nitric oxide; VEGF = vascular endothelial growth factor; nNOS = neuronal isoform NO synthase; iNOS = inducible isoform NO synthase; eNOS = endothelial NO synthase; EC = endothelial cells; LV = lateral ventricle.

ing (Asahara and others 1999). Accordingly, intravenous infusion of bone marrow–derived stem and progenitor cells augments neovascularization at the boundaries of the ischemic zone (Chen and others 2004; Taguchi and others 2004). Taguchi and others (2004) reported that neovascularization is followed by a neurogenic response, with neuronal progenitors migrating to the damaged area, reaching maturity, and surviving for extended periods of time. Suppression of neovascularization by an antiangiogenic agent impaired neurogenesis, thus suggesting a connection between the two regeneration processes.

NO and Brain Injury–Induced Neurogenesis

A common feature in several types of brain injuries, including ischemia, traumas, and seizures, is an overproduction of NO. Several mechanisms contribute to the increased NO production. First, nNOS activity is enhanced owing to the massive release of glutamate and the corresponding stimulation of NMDA receptors. In addition, there is up-regulation of nNOS expression in neurons in and around the lesion area and of iNOS in neurons, vascular cells, and infiltrating blood cells. The overall effects of NO in the ischemic brain remain controversial, with contrary results depending on the amount of NO and the time at which it is suppressed or administered. Thus, in ischemic brains, NO may be neuroprotective, increase blood flow, or promote angiogenesis, but it can also contribute to neuronal cell death (reviewed in Keynes and Garthwaite 2004).

Only a few studies have so far addressed the question of whether NO produced in the injured brain may affect neurogenesis and neuronal replacement in the lesion area. The enhanced proliferation of progenitor cells found in the DG after pharmacologically induced epileptic seizures is diminished when either nNOS or iNOS is selectively inhibited (Jiang and others 2004). iNOS-derived NO also appears to have a positive influence on neurogenesis after the induction of experimental ischemia (Zhu and others 2003). Likewise, administration of NO donors after transient ischemia has been reported to promote neurogenesis (Zhang and others 2001; Chen and others 2004). Taken together, these results suggest that under pathological conditions, when large amounts of NO are generated in the brain, this messenger has an overall effect of promoting neurogenesis.

Mechanism of Action of NO on Neurogenesis

Antiproliferative Effects

NO is a direct inhibitor of proliferation in many cell types. Both cGMP-dependent and cGMP-independent mechanisms seem to be involved in NO-mediated cytostasis (Garg and Hassid 1989, 1990). Several proteins and molecular pathways have been proposed as directly or indirectly responsible for the NO antiproliferative effect. Among these, ribonucleotide reductase and ornithine decarboxylase are inhibited by NO, whereas p38 mitogen-activated protein kinase and the p53 and retinoblastoma pathways are activated (reviewed in Gibbs 2003).

Although the antiproliferative effect of endogenous NO on neural progenitors is probably complex and may involve several of the above-mentioned proteins, the inhibition of the EGFR by NO is of special interest in the case of the SVZ. EGFR activation is required for the proliferation of SVZ neuronal precursors both in vitro (Reynolds and Weiss 1992; Gritti and others 1999) and in vivo (Craig and others 1996; Kuhn and others 1997; Tropepe and others 1997). Furthermore, Doetsch and others (2002) demonstrated that in the SVZ, EGFR is expressed selectively by undifferentiated precursors that do not express glial nor neuronal markers, that is, by the transit-amplifying cell population. Interestingly, this undifferentiated precursor pool is also the selective target of the endogenous NO action (Moreno-López and others 2004). In vitro experiments (Estrada and others 1997; Murillo-Carretero and others 2002) have shown that NO directly inhibits the tyrosine kinase activity of the EGFR in several cell types, including neuroblastoma cells. These cells arrest their proliferation in the presence of NO, and the highest sensitivity to NO is observed in those cultures whose growth is stimulated by EGF.

Pro-Neurogenic Effects

Because the direct action of NO on isolated neural precursors is to inhibit their proliferation rate, it seems reasonable to propose that the pro-neurogenic effects of NO in the injured brain are mediated by indirect mechanisms. After exposure of the nervous tissue to conditions that produce abnormal cell death, some determinants, such as the balance between neurotoxicity and neuroprotection, angiogenesis, and blood flow–dependent nutrient and oxygen availability, all of which are regulated by NO, turn out to be important for neurogenesis and damage repair. Some recent results on these mechanisms of action are summarized below and represented in Figure 6.

Induction of Growth Factors That Promote Neurogenesis

VEGF is a major angiogenic factor induced by hypoxia in the ischemic brain. The VEGF receptor VEGFR2/Flk-1 is expressed by neural stem cells, and i.c.v. administration of VEGF has been shown to significantly increase the number of neural precursors (Jin, Zhu, and others 2002; Schanzer and others 2004) owing to mechanisms that may involve both cell survival and proliferation.

A functional relationship between NO and VEGF has been observed using different approaches. VEGF up-regulates eNOS expression in vascular endothelial cells (Bouloumie and others 1999), and NO mediates part of the effects of VEGF on endothelial cell proliferation and motility (Papapetropoulos and others 1997; Shizukuda and others 1999). On the other hand, after stroke, NO has been shown to increase VEGF levels at the lesion boundaries, and blockade of the growth factor with a neutralizing antibody significantly reduces NO-induced angiogenesis (R. Zhang and others 2003). Up-regulation of VEGF gene expression may also explain the neurogenic action of iNOS-derived NO reported in the ischemic brain (Zhu and others 2003) (Fig. 6).

Angiogenic and Vasculogenic Actions of NO

Formation of new blood vessels in a hypoxic/ischemic tissue is accomplished by two different processes. New endothelial cells, generated by mitosis of preexisting capillary endothelial cells, digest the extracellular matrix, migrate, become vacuolated, and form new capillaries in a process called angiogenesis. In addition, endothelial precursors derived from bone marrow hematopoietic stem cells can reach the ischemic tissue, divide, and give rise to new capillaries, a process known as vasculogenesis. Although there is not a clear relationship between neovascularization and neurogenesis (Peterson 2004), it has been shown that substances that inhibit angiogenesis, such as endostatin, also reduce neurogenesis, whereas erythropoietin, which promotes vasculogenesis, also improves the neurogenic response to ischemia (Chen and others 2003; Taguchi and others 2004; Wang and others 2004).

NO is pro-angiogenic (Cooke 2003), an action mediated in part by the up-regulation of VEGF gene expression. Recently, it has been shown that NO produced by eNOS in bone marrow stromal cells is necessary for stem cell mobilization leading to neovascularization of ischemic tissues (Aicher and others 2004). Thus, eNOS-deficient mice do not mobilize endothelial progenitor cells in response to VEGF, exercise, or estradiol, as do their wild-type littermates, and they present reduced neovasculogenesis in response to ischemia.

The rich vascular environment generated by NO may explain in part the pro-neurogenic actions observed when NO donors are systemically administered (Zhang and others 2001; Chen and others 2004).

Conclusion

A dual action of NO on adult neurogenesis has been reported in the last few years. In physiological conditions, nNOS-derived NO is a negative regulator of adult

neurogenesis. However, exogenously administered NO donors, as well as iNOS-derived NO in pathological situations, when this enzyme is induced in infiltrating, vascular, or neural cells, promote neurogenesis. Because in vitro studies demonstrate that NO is primarily a direct cytostatic agent in many cell types including neuroblasts, the neurogenic action of NO is probably due to indirect mechanisms. Among these, up-regulation of VEGF, which enhances neural precursor proliferation, and mobilization of hematopoietic stem cells, leading to neovasculogenesis, are good candidates for explaining the overall increase in neurogenesis produced by NO in the damaged brain.

Previous observations made in mice with targeted deletions in the genes encoding different NOS isoforms need to be reinterpreted, taking into account the recently known participation of NO in the control of adult neurogenesis in health and disease. It has been reported that nNOS knockout mice present alterations in two types of behaviors: aggression and sexual patterns (Nelson and others 1995). It may be no coincidence that these are two behaviors for which olfactory learning is crucial; it is therefore possible to hypothesize that the alteration of OB cell turnover by long-term modifications of NO production may be responsible for these, and maybe other, abnormal olfactory-guided behaviors. As an example of a pathological situation, it has been assumed that the increased sensitivity to cerebral ischemia observed in eNOS null mice (Lo and others 1996) is due to hemodynamic changes, but it could also be a consequence of impaired angiogenesis and neurogenesis during the recovery period.

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