Proliferation of cerebellar precursor cells is negatively regulated by nitric oxide in newborn rat

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Summary
The diffusible messenger, nitric oxide plays multiple roles in neuroprotection, neurodegeneration and brain plasticity. Its involvement in neurogenesis has been disputed, on the basis of results on models in vivo and in culture. We report here that pharmacological blockade of nitric oxide production in rat pups during a restricted time window of the first three postnatal days, in increased cerebellar proliferation rate, as assessed through tritiated thymidine or BrdU incorporation into DNA. This was accompanied by increased expression of Myc, a transcription factor essential for cerebellar development, and of the cell cycle regulating gene, cyclin D1. These effects were mediated downstream by the nitric oxide-dependent second messenger, cGMP. Schedules of pharmacological NO deprivation targeted to later developmental stages (from postnatal day 3 to 7), no longer increased proliferation, probably because of partial escape of the cGMP level from nitric oxide control. Though limited to a brief temporal window, the proliferative effect of neonatal nitric oxide deprivation could be traced into adulthood. Indeed, the number of BrdU-labeled surviving cells, most of which were of neuronal phenotype, was larger in the cerebellum of 60-day-old rats that had been subjected to NO deprivation during the first three postnatal days than in control rats. Experiments on cell cultures from neonatal cerebellum confirmed that nitric oxide deprivation stimulated proliferation of cerebellar precursor cells and that this effect was not additive with the proliferative action of sonic hedgehog peptide. The finding that nitric oxide deprivation during early cerebellar neurogenesis, stimulates a brief increase in cell proliferation may contribute to a better understanding of the controversial role of nitric oxide in brain development.

Key words: Nitric oxide, cGMP, Myc, Neurogenesis, Cerebellum

Introduction
The diffusible messenger molecule nitric oxide (NO) plays multiple roles in the nervous system. Among its physiological actions, those related to neuronal survival and differentiation, as well as to synaptogenesis and synaptic plasticity, have been well characterized during the last few years (Brennan et al., 1997; Ciani et al., 2002; Contestabile, 2000; Contestabile and Ciani, 2004; Gibbs, 2003; Holscher, 1997; Keynes and Garthwaite, 2004; Thippeswamy et al., 2001). However, ample evidence also exists that NO may be an agent promoting neurodegeneration when uncontrolled production and/or inefficient scavenging results in dangerous chemical reactions with various cellular components (Contestabile et al., 2003; Dawson and Dawson, 1998; Keynes and Garthwaite, 2004; Yun et al., 1997). Most actions of NO are mediated downstream through the guanylate cyclase/cGMP system (Garthwaite and Boulton, 1995).

Nitric oxide is a negative regulator of proliferation in several cell types, including neural cells in culture (Ciani et al., 2004; Maragos et al., 1993; Murillo-Carretero et al., 2002; Nisoli et al., 1998; Peunova and Enikolopov, 1995; Yang et al., 1994). Recently, a role for NO in neurogenesis has also emerged (Cardenas et al., 2005; Chen et al., 2004; Contestabile and Ciani, 2004; Enikolopov et al., 1999; Matarredona et al., 2005; Packer et al., 2003). The actual effect of NO in the regulation of neurogenesis, however, is still poorly understood and has been the source of conflicting results and interpretations. Recent data demonstrate that NO acts, in vivo, as a negative regulator of precursor proliferation in restricted areas of the brain, the dentate gyrus of the hippocampal formation and the subventricular zone of the forebrain, where neurogenesis persists in adult mammals (Cheng et al., 2003a; Matarredona et al., 2005; Moreno-Lopez et al., 2000; Packer et al., 2003; Park and Wei, 2003). In these same areas, however, NO appears to stimulate neurogenesis as a consequence of brain damage caused by ischemia or traumatic injury (Chen and Cheung, 2005; Lu et al., 2003; Zhang et al., 2001; Zhu et al., 2003). The stimulation of neurogenesis under traumatic conditions, has been related to the neuroprotective role of NO (Cardenas et al., 2005; Estrada and Murillo-Carretero, 2005; Keynes et al., 2004). To better understand how NO affects neurogenesis, it is important to study its function during development. However, relatively few studies have addressed this issue so far, and some contrasting results have emerged from those that have. Nitric oxide was found to negatively regulate neurogenesis in the toad optic tectum (Peunova et al., 2001) and to affect the balance between cell cycle progression and apoptotic elimination in the chick neural tube (Plachta et al., 2003). The only study...
performed so far on mammalian developmental neurogenesis, has been carried out in mice knocked down for the neuronal nitric oxide synthase (nNOS) isoform (Chen et al., 2004). In this model, it was found that impaired NO production resulted in temporarily restricted decrease of neuronal precursor proliferation in the olfactory epithelium (Chen et al., 2004). This suggests that, contrary to what has been described for adult neurogenesis (Cheng et al., 2003a; Moreno-Lopez et al., 2004; Packer et al., 2003), NO may stimulate neurogenesis during early brain development. Evidence from studies in culture, however, do not support this suggestion. In a recent report based on cultures of foetal rat neocortex, exogenously provided NO depressed the proliferation of neuronal precursors (Li, 2005). In line with this result, we have recently observed that NOS inhibition increased the division rate of neuronal precursors in cultures from neonatal rat cerebellum (Ciani et al., 2004). Furthermore, under these conditions we noticed that a transcription factor essential to promote cerebellar neurogenesis, Myc (Knoepfler et al., 2002), was upregulated. The data briefly reviewed above, indicate that the conflicting roles attributed to NO in neurogenesis regulation might depend on the species, the developmental stage and the in vivo versus in vitro condition.

Cerebellar development represents a very useful model to explore the role of NO in developmental neurogenesis. Genesis of cerebellar granule neurons, the largest neuronal population of the cerebellum, is an essentially postnatal event in rodents, lasting from birth up to the third week of age and being particularly prominent during the first week of life (Altman, 1982). A major determinant of granule cell neurogenesis has been identified in the Sonic hedgehog (Shh) peptide, which is produced by Purkinje cells and promotes proliferation of granule neuron precursors through positive regulation of the transcription factor N-Myc and factors of the Gli family (Corrales et al., 2004; Kenney et al., 2003; Wechsler-Reya and Scott, 1999). Granule neurons express the neuronal isoform of NOS (Baader et al., 1997) and we have previously exploited schedules of pharmacological NOS inhibition, able to almost completely block the enzymatic production of NO in the rat cerebellum during development (Virgili et al., 1999).

In the present study, we report that neonatal NO deprivation in the rat increases the rate of cell proliferation during cerebellar neurogenesis and upregulates Myc and cyclin D1 expression only during a restricted time window that corresponds to the first three postnatal days. This effect appears to be mediated by the primary downstream effector of NO, cGMP. Its temporally restricted spanning may be attributed to escape of cGMP from NO control with the progression of development.

Results

NO/cGMP system in developing cerebellum

Different schedules of administration of the NOS inhibitor L-NAME to rats during the first postnatal week demonstrated an almost complete inhibition of NO production in cerebellar tissue (Fig. 1A). The inhibition of the catalytic activity, however, did not interfere with the normal developmental expression of nNOS at both the mRNA and protein level (Fig. 1B,C). During development, a moderate, but significant increase in nNOS activity (Fig. 1A) and expression (Fig. 1B,C) was noticed. In order to better clarify the developmental profile
of the NO/cGMP system in the neonatal rat cerebellum, we have quantified, at the mRNA level, by using real-time PCR, the expression of the main subunits of soluble guanylyl cyclase at post-natal day (P) 3, P5 and P7. As shown by Fig. 2A, a substantial increase in the expression of the β1 subunit was apparent between P3 and P5 or P7, whereas no modifications of subunits α1 and α2 were observed. Subunit β2 was almost undetectable in our samples. Interestingly, the increase in expression of the β1 subunit between P3 and P5 or P7 was even larger when examined at the same ages in the freshly isolated population of cerebellar granule neurons (Fig. 2B). We have also quantified, at the mRNA level, the expression of the two main isoforms of cGMP-dependent protein kinases, PKGI and PKGII, without finding any significant change between P3 and P7 (data not shown). The developmental increase of the β1 subunit described here is probably the main cause of the increased level of cGMP observed in the cerebellum of control rats between P3, P5 and P7 (Fig. 2C), as this subunit forms functionally active catalytic dimers with both subunits (Koesling et al., 1991). The pharmacological blockade of NO production resulted in a significant drop of cGMP content in the cerebellum (Fig. 2C). The extent of cGMP decrease in the cerebella of L-NAME-treated pups appeared, however, to vary depending on the various schedules, reaching almost 85% after P1-P3 administration and being limited to about 50% after P3-P5 or P5-P7 administration (Fig. 2C). Administration of the soluble guanylyl cyclase inhibitor ODQ, at a dose similar to that previously reported to be effective in adult rats (Zacharowski et al., 2001), also decreased cGMP content at P3, P5 and P7, though somewhat less efficiently than L-NAME treatment (Fig. 2C).

Effect of NOS inhibition on cerebellar neurogenesis

Cerebellar neurogenesis was evaluated at the various neonatal stages through incorporation of radioactive thymidine into DNA extracted from cerebellar tissue. NOS inhibition resulted in different effects on cerebellar neurogenesis, depending on the time window of the pharmacological schedules adopted. Administration of L-NAME during the first 2 or 3 postnatal days, increased thymidine incorporation by 40% to 50%, whereas administration during P3-P5 or P5-P7 did not change proliferation rate (Fig. 3A). The increased rate of thymidine incorporation observed at P3 following L-NAME administration from P1 to P3, was essentially replicated by a schedule of ODQ treatment covering the same time window (Fig. 3A). A substantial number of newly generated cells in the postnatal rodent cerebellar cortex are eliminated by apoptosis with a process partly overlapping the neurogenetic phase (Monti and Contestabile, 2000; Wood et al., 1993). We analyzed, through a sensitive ELISA method whether L-NAME treatment differentially affected the apoptotic process in the developing cerebellum, but we did not find any quantitative difference in the apoptotic process from P3 to P11 (Fig. 3B). The effect of NOS inhibition from P1 to P3 on cell proliferation in the neonatal cerebellum, was confirmed through BrdU labelling. A significant increase in labelled cells was observed in the external granular layer (EGL), where the dividing precursors of granule neurons are present (Fig. 3C-E). A few dividing cells were observed in the internal granular layer (IGL) and also in this case their number was somewhat higher in the cerebellum of L-NAME-treated animals (Fig. 3C-E). These observations suggested that the excess proliferation occurring during the first 3 postnatal days as a consequence of NOS inhibition could be traced up to adulthood in the rat cerebellum. To verify this, we counted BrdU-positive cells in the granular and the molecular layers of the cerebellum of 60-day-old rats that had received a single BrdU injection on P3 after 3 days administration of L-NAME or vehicle (Fig. 4A,B). A large
increase of labelled cells was observed in the granular layer of L-NAME-treated rats, but no difference was seen in the molecular layer (Fig. 4C). No such an effect was observed in rats treated with L-NAME from P5 to P7 and injected with BrdU at P7 (Fig. 4D). To ascertain whether the pharmacological treatment had differentially influenced the proliferation of either neuronal or glial cells we performed double immunofluorescence for BrdU and the neuronal marker, neuron-specific nuclear antigen (NeuN), or the glial marker, glial fibrillar acidic protein (GFAP) and we analyzed cerebellar sections using confocal microscopy. The results of this analysis showed that about 60% of BrdU-positive cells were neurons, 20% to 30% were glial cells and the remaining cells were of undetermined fate both in control animals and in animals treated with L-NAME from P1 to P3 (Fig. 5A-D). Thus, blocking of neonatal NO production affected the increase in number of both neuronal and glial cells in a proportionally similar way.

Effect of NOS inhibition on Myc expression in the developing cerebellum

Analysis at the mRNA level revealed that Myc expression was significantly increased only at P3 as a consequence of P1-P3 NOS inhibition (Fig. 6A) and that the expression of a cell-cycle regulatory gene which is controlled by Myc, cyclin D1, was also up-regulated at the same stage (Fig. 6B). At the protein level, western blot analysis confirmed that L-NAME treatment from P1 to P3, but not at a later stage, significantly increased Myc expression (Fig. 6C) and that this increase was essentially due to an increased nuclear localization of the transcription factor (Fig. 6D). Immunocytochemistry performed at P3, revealed a more intense stain in the cerebellum of L-NAME-treated pups, specifically localized in the external granular layer, which contains the precursors of granule neurons at this stage (Fig. 6E,F).
Effect of NOS inhibition on cerebellar precursor proliferation in culture

To isolate the effect of NOS inhibition on granule neuron precursors, to ascertain its dependence on cGMP downstream activity and to detect possible interactions with Sonic hedgehog (Shh), the main determinant of granule cell proliferation, we performed experiments on cultures from P2 cerebella, where a population of dividing precursors is present. The results showed that in these cultures proliferation was significantly stimulated by 24-hour treatment with L-NAME as well as by the inhibition of guanylate cyclase through ODQ or by the inhibition of cGMP-dependent protein kinase through KT5823 (Fig. 7A). Immunofluorescent labelling, revealed a remarkable L-NAME-dependent increase in BrdU-labelled cells, and double immunofluorescence demonstrated that several of them also expressed III beta-tubulin, an early marker of neuronal lineage (Fig. 7B,C) (Fanarraga et al., 1999; Ferguson et al., 2002) whereas others were GFAP positive (not shown). Cell counting in these cultures, showed that the increased proliferation caused by L-NAME treatment was significant for precursors of both granule neurons and astrocytes (Fig. 7D). As expected, precursor proliferation was also strongly stimulated by Shh (Fig. 7E). No additive effect was observed on precursor cell proliferation by combining Shh and L-NAME treatment (Fig. 7E). In a parallel experiment, we evaluated the effect of these treatments on Myc at the mRNA level and we observed increased expression in response to L-NAME and Shh without any evidence for additive action (Fig. 7F).
Fig. 7. (A) Freshly plated CGC cultures prepared from P2 cerebella were stimulated with L-NAME (1 mM), ODQ (5 μM) or KT5823 (500 nM), for 20 hours. BrdU (10 μM) was added for the last 6 hours, after which time cells were processed for BrdU incorporation by an ELISA method. Values are the mean ± s.e.m. of three experiments. *P<0.05, compared to control (Bonferroni’s test after ANOVA). (B,C) Colour merged images of double immunofluorescence for III beta-tubulin (green) and BrdU (red) of control (B) and L-NAME-treated (C) CGC cultures. Plated CGC cultures were stimulated with L-NAME (1 mM) for 20 hours. BrdU (10 μM) was added for the last 6 hours, after which time cells were processed for immunofluorescence. Arrowheads point to III beta-tubulin-BrdU labelled cells. Bar, 30 μm. (D) Percentage of BrdU-labelled cells positive for the early neuronal marker, III beta-tubulin, or the glial marker, GFAP in the same cultures as A-C. Counting was done in random fields of control and L-NAME-treated cultures from two independent experiments. *P<0.01, compared to control (Bonferroni’s test after ANOVA). (E) BrdU incorporation during the last 6 hours of culture was measured in CGC by ELISA method after 20 hours in culture in the presence or absence of L-NAME (1 mM) and/or Shh (3 μg/ml). Data are expressed as the mean ± s.e.m. of three experiments. *P<0.05, **P<0.01, compared to control (Bonferroni’s test after ANOVA). (F) Expression of Myc in CGC cultures treated with L-NAME (1 mM) and/or Shh (3 μg/ml) for 12 hours. Total RNA was extracted from CGC cultures and equal amounts were used for real-time PCR reaction performed with specific primers for Myc, as described in the Materials and Methods section. The results, expressed as percentage of control, are the mean ± s.e.m. of two different experiments performed in triplicate. *P<0.05, **P<0.01 (Bonferroni’s test after ANOVA).

Discussion

The novel finding of the present study is that inhibition of NO production in vivo causes a substantial increase in the proliferation rate of cerebellar precursor cells, which clearly demonstrates that NO acts as a negative regulator of granule cell production during early cerebellar development. These data are in line with previous evidence, obtained from cultures of cerebellar granule cells (Ciani et al., 2004). The increased proliferative rate obtained through pharmacological blockade of NOS activity, was accompanied by overexpression of Myc, a transcription factor the lack of which severely impairs cerebellar neurogenesis by depressing precursor proliferation and granule neuron formation (Knoepfler et al., 2002). The time window during which NOS inhibition was effective in promoting proliferation in the cerebellum was, however, restricted to the first 3 days of neonatal life. This was not due to a reduced efficacy of the pharmacological treatment, as the catalytic activity of the enzyme was also efficiently inhibited at later developmental stages by the schedule of L-NAME administration used here. A probably explanation is that some of the mechanisms mediating the downstream action of NO change during development, in such a way that the stimulation of proliferation caused by the lack of NO is overcome with time. The physiological role of the transient NO-mediated negative regulation of proliferation is not understood at present. One can speculate that it may be related to the goal of controlling, during early developmental phases, the size of the pool of precursor cells in order to avoid excessive growth stimulated by factors favouring proliferation, such as Shh. This may be addressed in future studies by using knockout mice for nNOS or eNOS, or both.

Our data indicate that cGMP is involved in the antiproliferative effect of NO and suggest that one of the downstream steps that bypass NO deprivation may be represented by the increased availability of cGMP in the developing cerebellum. Whereas our schedules of L-NAME treatment were able to decrease cerebellar cGMP by about 85% at P3, the decrease was only around 50% at P5 and P7, in the presence of a maintained inhibition of NOS higher than 95%. The reasons why production of cGMP partially escapes NO deprivation at certain developmental stages in the cerebellum are probably multiple. These may include development-related changes in the NO/cGMP system, such as changes in the properties of soluble guanylyl cyclase itself, and/or changes in the mechanisms by which cGMP production is regulated. For instance, the developmental profile of guanylyl cyclase subunits, and in particular the substantial increase in the B1 subunit between P3 and P7, could contribute to a decreasing effect of NO deprivation on the catalytic efficacy of the enzyme and, thus, on cGMP cerebellar levels after the first three postnatal days. Previous reports, however, have pointed to the fact that the catalytic potency of the enzyme cannot be solely explained on the basis of the expression of the various subunits in different brain regions (Nedvetsky et al., 2002) as it is also regulated by several, still not completely understood mechanisms, such as phosphorylation and protein-protein interactions (Friebe and Koelsling, 2003; Nedvetsky et al., 2002; Pyriochou and Papapetropoulos, 2005). Possible developmental alterations in the efficiency of these regulatory mechanisms have not been studied so far in the cerebellum and are surely worthy of future investigation. A further factor...
involved in the uncoupling of NO and cGMP levels under conditions of NO deprivation after the very early postnatal period, may be represented by the fact that NO-independent cGMP synthesis occurs in the brain and could vary during development (Kuhn, 2003; Tanaka et al., 1997).

Most NO-evoked cGMP was found in glial cells in the developing cerebellum (De Vente et al., 1990), and this may suggest the contribution of an indirect regulation of proliferating CGC precursors through Bergman glia. Although this issue was not directly addressed by the experiments reported here, present, as well as previous (Ciani et al., 2004) data in cultures highly enriched in CGC, suggest that these neurons and their precursors may directly respond to the inhibition of the NO/cGMP system. This is consistent with the finding that in early postnatal cerebellum, detectable amounts of nNOS, in addition to capillary localized eNOS, are detected in the external granular layer (Wang et al., 1998).

A major determinant in promoting proliferation of granule neuron precursors in neonatal cerebellum is the peptide Shh, which acts by positively regulating the transcriptional activity of Myc and of factors of the Gli family (Corrales et al., 2004; Kenney et al., 2003; Wechsler-Reya and Scott, 1999). It is, therefore, important to consider potential interactions between NO deprivation and Shh signalling. We addressed this issue by studying cultures prepared from P2 cerebella, enriched in granule neurons and containing a population of dividing precursors. In these cultures, L-NAME and Shh stimulated proliferation of granule cell precursors in increasing order of potency and the maximal effect obtained by Shh was not further potentiated by L-NAME. The two treatments demonstrated corresponding orders of potency also in inducing the expression of Myc by cultured cells. A possible interpretation of these results is that Shh maximally stimulates Myc expression and proliferative potency, and that a concomitant stimulation through NO deprivation cannot further potentiate this effect. Our present results help to better define the role of NO in developmental neurogenesis. The fact that this role appears to be a transitory one, explains why no relevant alterations in the final size and architecture of brain regions occur in the mice knocked out for the various NOS isoforms (Huang et al., 1993; Huang et al., 1996) as well as by rats subjected to chronic NOS inhibition during postnatal development (Prickaerts et al., 1998; Virgili et al., 1999). As the role of NO in the cerebellum appears to be critical only during a temporally restricted neurogenetic period and to be subsequently overcome by other developmentally regulated mechanisms, the impact of deregulation of NO may be too limited to alter in a relevant way the final size of the very large population of cerebellar granule cells. Nevertheless, our results demonstrate that the surplus neurogenesis derived from early neonatal inhibition of NO production is not completely lost during further development, as we were able to see an increased number of labelled cells, most of which were neurons, in the cerebellum of adult animals injected with BrdU, which corresponded to the P1-P3 schedule of L-NAME administration. Although it was not possible from our experiments to evaluate what proportion of supernumerary cells did survive to adulthood, no increased elimination was observed, as a consequence of L-NAME treatment, during the critical period, from P3 to P11, when many newly generated CGC die by apoptosis (Wood et al., 1993). The phenotypical characterization of the surviving cells in the adult cerebellum demonstrated that the neonatal NO deprivation also affected proliferation of glial cells. Our experiments in culture substantiated this finding by directly demonstrating a small, but significant, increase in the proliferation of glial cell precursors with L-NAME treatment. In the present report we have established a correlation between the action of NO on cerebellar neurogenesis and the expression of the transcription factor Myc in vivo. The same relationship was previously demonstrated in cultures of the same cell type (Ciani et al., 2004). Furthermore, a mechanistic relationship between NO and the transcriptional activity of Myc was demonstrated in the same study in a line of neuroblastoma cells (Ciani et al., 2004). Myc is essential for the expansion of neuronal progenitors and its role is particularly important in cerebellar granule cells (Knoepfler et al., 2002; Oliver et al., 2003). In mice with conditional disruption of Myc in neuronal progenitors, the cerebellar volume was decreased by about 80% and granule neuron number were reduced by ~30-fold (Knoepfler et al., 2002). Looking at cell cycle-related genes regulated by Myc as possible candidates for the NO effect on granule cells neurogenesis, we found a significant increase of cyclin D1, a target for the transcriptional activity of Myc (Ciemerych et al., 2002).

Conclusion
The present data clearly show that NO deprivation promotes an increased neurogenesis during a very restricted time window of cerebellar development. This action is mediated downstream by the decreased availability of cGMP and the effect is lost when the levels of this second messenger are partially restored, notwithstanding the maintained inhibition of NO production. Whether a similarly restricted time window for the antiproliferative action of NO during neurogenesis is also present in other brain regions, should be verified by specific experiments targeted at the respective neurogenic periods. The granule neurons of the hippocampal dentate gyrus could be particularly interesting to investigate as their precursors retain the property to respond to NO deprivation by increasing proliferation in the adult animal (Packer et al., 2003). Similar studies should also be extended to adult neurogenesis occurring in the subventricular zone, where the proliferative rate has been found to be positively regulated by NO deprivation (Matarredona et al., 2005; Moreno-Lopez et al., 2004; Packer et al., 2003). Interestingly, different levels of inhibition of NO production seem to be necessary in order to stimulate adult neurogenesis in these two neuronal populations. An inhibition of approximately 60% was sufficient to stimulate neurogenesis in the subventricular zone of adult rats (Moreno-Lopez et al., 2004). A more efficient inhibition of NOS activity, obtained through continuous intraventricular infusion of L-NAME in adult rats or through genetic knockout in mice, also stimulated neurogenesis in the hippocampal dentate gyrus (Packer et al., 2003). The comparison of the molecular mechanisms affecting the regulatory role of NO in adult and developmental neurogenesis should be able to provide a better understanding of the action of the messenger itself as well as of its downstream effectors.

Materials and Methods
Animals and treatments
Wistar rats (Harlan, Italy) were kept in our animal facility under conditions of constant temperature and with 12 hour light:12 hour dark cycle. Pregnant mothers were isolated in single cages when pregnancy became evident. Day of birth was

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considered P0 and treatment started on P1 or at later stages. Pups of various postnatal ages were subcutaneously administered with the broad spectrum NOS inhibitor L-nitroarginine methyl ester, L-NAMe (Sigma, St Louis, MO, USA) dissolved in saline at a dose of 60 mg/kg/day and divided into two daily administrations at 8 a.m. and 8 p.m. Pups were treated for various neonatal intervals (P1-P2, or P1-P3, P3-P5, P5-P7; the last day of treatment the pups received only the morning dose). Control pups were injected with saline vehicle. Some pups were administered the guanylyl cyclase inhibitor, ODQ (Calbiochem, San Diego, CA) at a dose of 5 mg/kg/day, using similar time schedules to those for L-NAMe. For the various experiments described below, animals were routinely used 6 hours after the last L-NAMe or ODQ injection. Pups were killed by decapitation, the cerebellum was rapidly dissected and immediately frozen in dry ice or liquid nitrogen and kept at –80°C until used. Experiments were performed in accordance with the Italian and European Community law for the use of experimental animals and were authorized by a local ethical committee.

Determination of NOS activity
Tissues were homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and centrifuged at 11,000 g for 15 minutes. Aliquots of the supernatant, containing 10-20 μg of protein, were mixed with a solution containing [H]arginine (specific activity 4 Ci/mmol; NEN Dupont) diluted with cold arginine to a final concentration of 4 mM, and β-NADPH (0.7 mM final concentration). Incubation started by adding CaCl2 to a final concentration of 0.4 mM and lasted for 10 minutes at 25°C. At the end of the incubation, the reaction was blocked by dilution with excess of 20 mM Hepes buffer (pH 5.5) containing 2 mM EDTA. The product of the enzymatic reaction, [H]citrulline, was eluted by chromatography on columns filled with Dowex 50Wx8 sodium form resin which retains arginine. Radioactive citrulline was washed in scintillation vials and mixed with an appropriate scintillation cocktail for counting. Enzyme activity was expressed as pmoles [H]citrulline formed/mg of protein/hour. Protein content was determined by the method (Lowry et al., 1951).

Cyclic GMP measurements
Cyclic guanosine monophosphate (cGMP) was determined by enzyme-linked immunoassay using a kit from Cayman Biosciences (Tallinn, Estonia) according to the manufacturer’s specification. Briefly, cerebellar samples were homogenized in ten volumes of cold 10% trichloroacetic acid (TCA) and centrifuged at 1500 g, for 10 minutes at 4°C. The supernatant was removed by four washes with diethyl ether, and the samples were dried under nitrogen flow at 60°C. After appropriate dilution, the samples were subjected to enzyme immunoassay utilizing a standard curve prepared with known amount of cGMP (curve range, 2-512 fmol/well). The colorimetric reaction was measured at 450 nm.

Immunohistochemistry and double immunofluorescent labelling
Pups treated with L-NAMe from P1 to P3 were killed by decapitation six hours after the last treatment. Some of them received a single s.c. injection of the thymidine analogue 5-bromo-2-deoxyuridine (BrDU, 100 μg/g; Sigma) 2 hours after being killed. BrDU-positivity was determined in the glyoxal-based fixative Glyofix (Italscientifica spa, Geneva, Italy). After dehydration in a graded ethanol series, the cerebellum were embedded in Paraplast plus (Sherwood Medical, St Louis, MO, USA; mp 55-57°C), paraffin sectioned (8 μm) with a Leitz 1516 microtome and mounted on poly-L-lysine-coated slides. The sections were dewaxed, rehydrated and the endogenous peroxidases were inactivated with 3% hydrogen peroxide in methanol for 30 minutes and extensively washed. The sections were then incubated with primary antibody (rabbit anti-Myc, 1:100; Santa Cruz) overnight in a moist chamber at 4°C and subsequently incubated for 1.5 hours in the secondary antibody: goat HRP-conjugated anti-rabbit (dilution 1:100, Vector Laboratories). Brightfield images were taken. A sandwich ELISA method was used to assess apoptosis (Cell Death ELISA; Roche Medical, St Louis, MO, USA; mp 55-57°C), parasagittally sectioned (8 μm) with a Leitz Diaplan microscope equipped with a motorized stage and a Coolsnap-Pro Color digital camera (Media Cybernetics, Silver Spring, MD). BrDU-positive cells in the external granular layer (EGL) and internal granular layer (IGL) of the cerebellum were counted separately in 12 sections from each animal. The number of BrDU-positive cells was expressed as the number of BrDU-positive cells per unit area (mm²).

To determine cells undergoing division and surviving up to 60 days of age, rat pups received a single s.c. injection of the marker of proliferating cells and their progeny, BrDU (Nowakowski et al., 1989), in P3 or P7 after 3 days administration of L-NAMe or vehicle. At 60 days of age the animals were deeply anesthetized and perfused through the heart with saline, followed by 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.4. The brains were dissected from the skull and additionally fixed overnight in the same fixative, washed and immersed overnight in 18% sucrose in phosphate buffer. Cerebella were cut parasagittally in 40 μm-thick slices with a freezing microtome and serially collected in PBS. BrDU immunohistochemistry, free-floating sections were mounted on Leitz Diaplan microscope and coverslips. Sections from cerebella of pups injected with BrDU (three controls and three L-NAMe treated) were denaturated in 2 N HCl for 20 minutes and rinsed for 20 minutes in 0.1 M boric acid (pH 8.5) and incubated overnight with an anti-BrDU mouse monoclonal antibody (Chemicon; diluted 1:100). Slices were then incubated with HRP-conjugated anti-mouse (dilution 1:200, Amersham) secondary antibody for 1.5 hours at room temperature. BrDU was detected using a DAB kit (Vector Laboratories). Brightfield images were taken. Immunohistochemistry, free-floating sections were denaturated in 2 N HCl (pH 1.8) for 20 minutes. After rinsing in 0.1 M glycine, for 5 minutes, the sections were blocked with 3% normal horse serum in PBS for 1 hour. Slices were reacted with rabbit anti-Myc (1:400; Chemicon) overnight at 4°C. Sections were rinsed in PBS, and sections were then incubated with primary antibody (rabbit anti-Myc, 1:100). After rinsing the sections were incubated with secondary antibody (Dakopatts) for 1 hour at room temperature. After rinsing in PBS and incubating with anti-rabbit HRP-conjugated antibody (Dakopatts) for 1 hour, the sections were rinsed in PBS and coverslips mounted with Vectorshield mounting medium (Vector Laboratories). Digital images were acquired with a Zeiss Axiosplan microscope equipped with a motorized stage. Brightfield images were subsequently acquired at ×40 magnification. Adjacent sections were then reacted with an antibody for BrdU and an antibody either for a neuronal protein (NeuN, neuron-specific nuclear protein) or the glial protein GFAP (glial fibrillary acidic protein). For double immunofluorescent labelling, the sections were reacted with the following primary antibodies: (1) sheep polyclonal antibody against BrdU (Chemicon; diluted 1:100); (2) mouse anti-GAP-43 (Sigma; dilution 1:500) or mouse anti-NeuN (Chemicon; diluted 1:250). Sections were incubated with Cy3, conjugated goat anti-mouse secondary antibody (Dianova) or FITC conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) at room temperature for 1 hour. After rinsing, the sections were coverslipped with Vectorshield and visualized using an Axioplan fluorescence microscope (Zeiss).

For immunofluorescence studies in cultures (see below), cells plated on poly-Dlysine coated coverslips, were fixed for 30 minutes in 4% paraformaldehyde, 4%
sucreose in 120 mM sodium phosphate buffer, pH 7.4, and then rinsed three times with PBS. For BrdU immunofluorescence, coverslips were treated with 2 N HCl for 30 minutes at 37°C and extensively washed with PBS. The following primary antibodies were used: monoclonal anti-NeuN (Chemicon), anti-GFAP (Sigma) and anti-BrdU (Roche Applied Science). Coverslips were incubated overnight at 4°C with appropriate dilutions of the primary antibody in 1.5% goat serum, 0.1% Triton X-100. Cell pellets were rapidly frozen in liquid nitrogen and kept at –80°C until used. Primary cultures of CGC were prepared from the cerebella of rat pups at P3, P5, P7 as previously described (Gallo et al., 1982). Briefly, cerebella were removed and dissected from their meninges in Krebs buffer containing 0.3% BSA. Tissue was dissociated with trypsin at 37°C for 15 minutes and triturated 15 times using a Pasteur pipette in a DNAase/soybean trypsin inhibitor solution. Cell pellets were then collected using a Pasteur pipette and washed with PBS. For BrdU immunofluorescence, coverslips were mounted on glass slides in PBS containing 70% glycerol and Hoechst 33342 (2 μg/ml). Fluorescence images were taken using an Eclipse TE 2000-S microscope (Nikon, Tokyo, Japan) equipped with an AxioCam MRm (Zeiss, Oberkochen, Germany) digital camera.

Freshly isolated and cultures of cerebellar granule cells (CGC)

Freshly isolated CGC were prepared from the cerebella of rat pups at P3, P5, P7 as previously described (Gallo et al., 1982). Briefly, cerebella were removed and dissected from their meninges in Krebs buffer containing 0.3% BSA. Tissue was dissociated with trypsin at 37°C for 15 minutes and triturated 15 times using a Pasteur pipette in a DNAase/soybean trypsin inhibitor solution. Cell pellets were rapidly frozen in liquid nitrogen and kept at –80°C until used. Primary cultures of CGC were prepared from the cerebella of rat pups at P2 and maintained in basal modified Eagle’s medium (BME) supplemented with 5 mM KCl, 2 mM glutamine and 0.05 mg/ml gentamicin (Sigma). Cultures were treated with 1-NAME, Quinoline-1-one (ODQ), KT5823 (Calbiochem), or Sonic hedgehog (Shh; Sigma) for 24 hours. Cell proliferation assay

CGC, plated on poly-D-lysine-coated 96-wells plates (5x10^4 cells/well), were cultured for 14 hours and then treated with 10 μM BrdU for an additional 6 hours. BrdU incorporation was measured in quadruplicate with the Cell Proliferation ELISA kit (Roche Applied Science) according to the manufacturer’s instructions.

Real-time PCR assays

Total RNA, free from chromosomal DNA contamination, was isolated from tissues or freshly isolated cells (FIC) using TRI REAGENT™ (Sigma) according to the supplier’s instructions. Total RNA was incubated for 45 minutes at 42°C in 20 μl containing 25 μM primers, 10 mM DTT, 0.5 mM dATP, dCTP, dGTP and dTTP to 200 units of reverse transcriptase, and 1 X first strand buffer (Invitrogen). Reactions were performed in a final volume of 20 μl containing 2 μg RNA with an incubation step of 5 minutes at 65°C to disrupt RNA template base pairing structure. The reverse transcription reaction was performed at 37°C for 50 minutes and reverse transcriptase inactivation was performed before the PCR reactions by heating the samples at 70°C for 15 minutes. Real-time PCR was performed using IQ SYBR green Supermix and the iQCycler thermocycler (Bio-Rad). Primers for real-time PCR are listed in Table 1. Quantifications were always normalized using endogenous control GAPDH.

Table 1. Primers used for real-time PCR

| Accession number | Gene | Sense and antisense primers | Amplicon length | Annealing temperature |
|------------------|------|-----------------------------|-----------------|----------------------|
| NM_001031906     | Myc  | CCAGCCAGTGTTGTCATCTTAAA     | 136             | 60°C                 |
| NM_171992        | CycD1| GAGTCTTTAGCTGTCAGACCTCTT   | 149             | 60°C                 |
| NM_017090        | sGcs | ACTCCTCTCTCCTACATCCCCCTT   | 131             | 52°C                 |
| NM_023956        | sGcs2| TTCCAGGATGTTGACCTTCCC     | 147             | 60°C                 |
| NM_012769        | sCGB1| ACCAGGCAACAGGAGAAACGACC   | 106             | 58°C                 |
| NM_012770        | sCGB2| TGCTCTGACCCACATTAGGC       | 116             | 52°C                 |
| XM_219805        | PKGI | TGAAGGGAAGAGGAGAGTGAG      | 121             | 53°C                 |
| NM_013012        | PKGII| AGGGCTCTGTCCTGTTTACC      | 143             | 58°C                 |
| NM_052799        | nNOS | AGGGCTTTAGAGTTCACC        | 102             | 53°C                 |
| NM_017008        | GAPDH| CCATCATCCCTGCATCCA         | 77              | 60°C                 |

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