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

Elisabetta Ciani[1], Vincenzo Calvanese[1], Christophe Crochemore[2], Renata Bartesaghi[1] and Antonio Contestabile[2,*]

[1]Department of Human and General Physiology, University of Bologna, Piazza di Porta San Donato 2, 40126 Bologna, Italy
[2]Department of Biology, University of Bologna, via Selmi 3, 40126 Bologna, Italy
*Author for correspondence (e-mail: antonio.contestabile@unibo.it)

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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 resulted, 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 (Brenman 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 temporarily 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

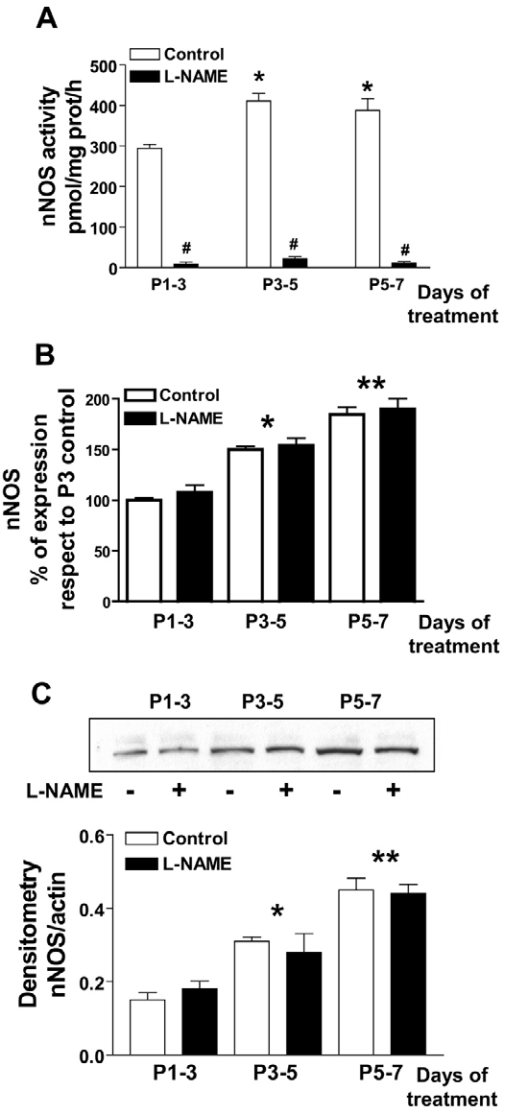

Fig. 1. Activity and expression of nNOS in cerebellar development of control and L-NAME-treated rat pups. Pups were s.c. injected with L-NAME (60 mg/kg/day, divided in two daily administrations) for various neonatal intervals (P1-P3, P3-P5, P5-P7) while control pups received vehicle. Animals were killed on the last day of treatment. (A) Maturation of calcium-dependent NOS activity in developing cerebella (white bars) and almost complete inhibition obtained by L-NAME treatment (black bars). Values are the mean ± s.e.m. of at least three experiments for each time point. *P<0.05, compared with the control at P3, #P<0.001 compared with the corresponding control (Bonferroni's test after ANOVA). (B) Total RNA was extracted from cerebella of control and L-NAME-treated rat pups and equal amounts of RNA were used for real-time PCR performed with specific primers for nNOS RNA as described in the Materials and Methods section. These results, expressed as a percentage of control at P3 are the mean ± s.e.m. of four different experiments performed in triplicate. *P<0.05, **P<0.01 compared to P3 (Bonferroni's test after ANOVA). (C) In western blot experiments, cerebellar extracts from control and L-NAME-treated rat pups were probed with an antibody specific for nNOS. Bands were quantified by optical densitometry and normalized for the amount of β-actin. These results are the mean ± s.e.m. of four different experiments. *P<0.05, **P<0.01 compared with P3 (Bonferroni's test after ANOVA).

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 β_1 subunit was apparent between P3 and P5 or P7, whereas no modifications of subunits α_1 and α_2 were observed. Subunit β_2 was almost undetectable in our samples. Interestingly, the increase in expression of the β_1 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 β_1 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).

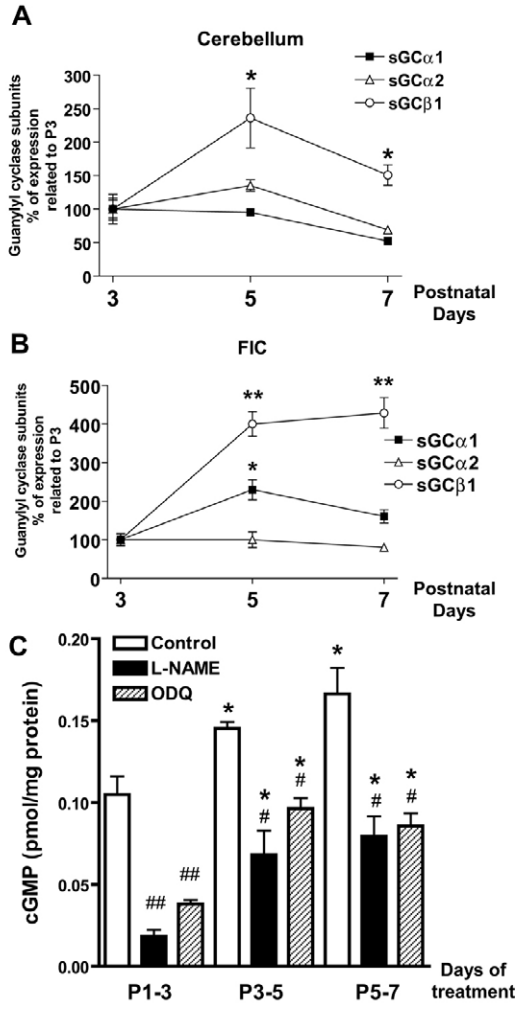

Fig. 2. Expression of soluble guanylyl cyclase (sGC) subunits (α_1, α_2 and β_1) during cerebellar development. Total RNA was extracted from cerebella (A) or from freshly isolated cerebellar granule cells (FIC) (B) of rat pups and equal amounts of RNA were used for real-time PCR performed with specific primers for guanylyl cyclase subunits as described in Materials and Methods section. The results, expressed as % of control at P3, are the mean ± s.e.m. of four different experiments performed in triplicate. **P*<0.01, ***P*<0.001 compared to P3 (Bonferroni's test after ANOVA). (C) Concentration of cerebellar cGMP determined by an enzyme-linked immunoassay. Pups were treated with L-NAME (60 mg/kg/day) for various neonatal intervals (P1-P3, P3-P5, P5-P7) or with the guanylyl cyclase inhibitor ODQ (5 mg/kg/day) in two daily administrations for various neonatal intervals (P1-P3, P3-P5, P5-P7), while control pups received vehicle. Values are the mean ± s.e.m. of three different experiments **P*<0.01 compared with P3, #*P*<0.01, ##*P*<0.001 compared with the corresponding control (Bonferroni's test after ANOVA).

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

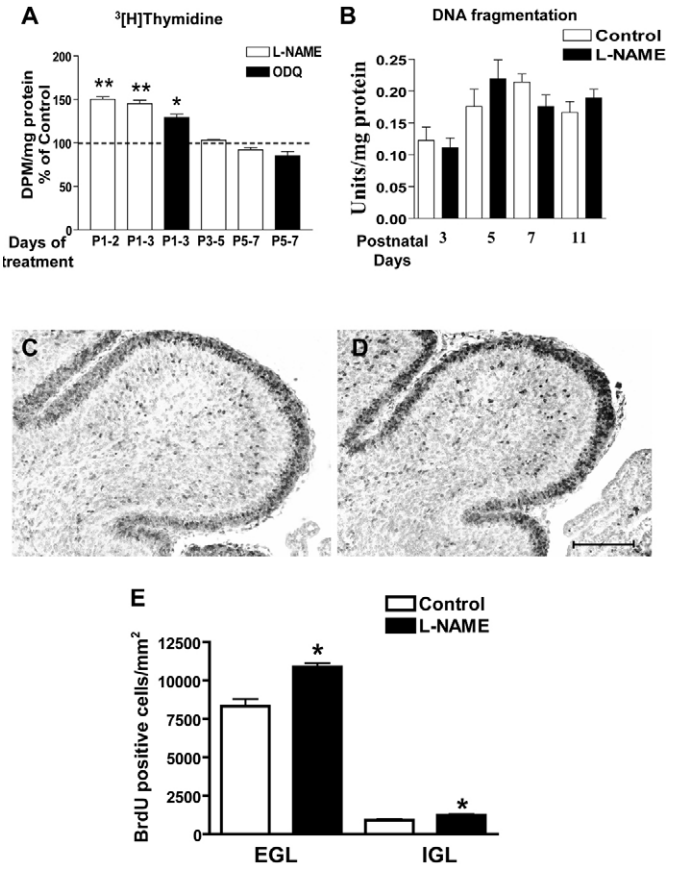

Fig. 3. Effect of blockade of NO production on cell proliferation. (A) Cell proliferation was measured on cerebellar homogenates from rat pups treated with L-NAME (60 mg/kg/day) or with ODQ (5 mg/kg/day) for various neonatal intervals (P1-P2, P1-P3, P3-P5, P5-P7), while control pups received vehicle. Two hours prior to sacrifice, pups were s.c. injected with [^{3}H]thymidine (5 μCi/g body weight). The % of [^{3}H]thymidine incorporation into DNA was expressed as dpm/μg protein. Values are the mean ± s.e.m. of four different experiments **P*<0.05, ***P*<0.01 compared with the corresponding controls of the same age (dotted line) (Bonferroni's test after ANOVA). (B) To assess apoptosis in the developing cerebellum, cerebellar homogenates from pups of various postnatal ages (3, 5, 7 and 11 days) were monitored, using a sandwich ELISA method, for histone-associated DNA fragments in the cytoplasm. L-NAME-treated pups received the usual drug dosage at the following intervals: P1-P3, P3-P5, P5-P7, P9-P11. Values are the mean ± s.e.m. of three independent experiments (Bonferroni's test after ANOVA). (C,D) BrdU-labelled cells in the cerebellum of 3-day-old pups after BrdU administration at P3, in a control rat (C) and a rat treated with L-NAME from P1 to P3 (D). Bar, 100 μm. (E) Density of BrdU-labelled cells (number of cells/mm^2) in the external granular layer (EGL) and internal granular layer (IGL) of the cerebellum of control and L-NAME P1-P3 treated 3-day-old rat pups. Values are the mean ± s.e.m. of three experiments. **P*<0.01, compared with the control (Student's *t*-test).

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

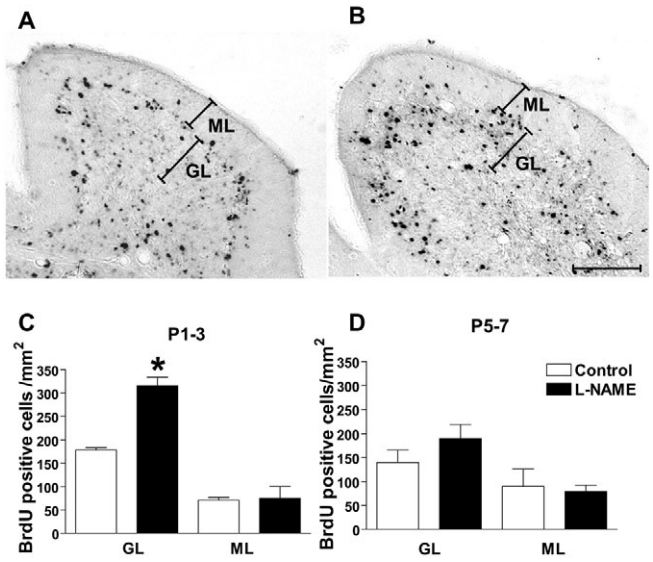

Fig. 4. Survival of the postnatally generated cells. (A,B) BrdU-labelled cells in the molecular layer (ML) or the granular layer (GL) of the cerebellum of 60-day-old rats after BrdU administration at P3, in a control rat (A) and a rat treated with L-NAME from P1 to P3 (B). Bar, 200 μm. (C) Density of BrdU-labelled cells (number of cells/mm^2) in the molecular layer (ML) and granular layer (GL) of the cerebellum of control rats and rats treated with L-NAME from P1 to P3 rats. (D) Density of labelled cells in 60-day-old animals treated with L-NAME or saline from P5 to P7 and injected with BrdU at P7. For C and D, bars are the mean ± s.e.m. of three experiments. **P*<0.01, compared to control (Student's *t*-test).

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).

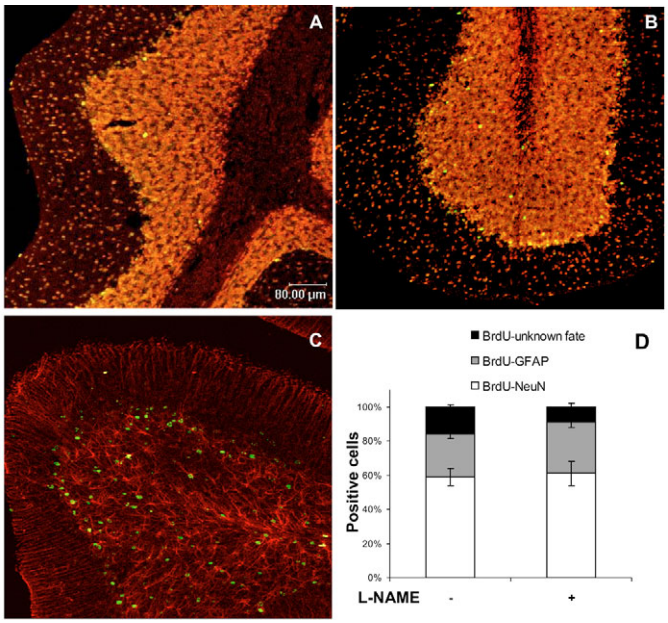

Fig. 5. Phenotype of neonatally BrdU-labelled cells in 60-day-old rats. Merged confocal microscope images of immunoreactivity for BrdU (in green), and NeuN (in red) (A,B) and for BrdU (in green), and GFAP (in red) (C) in 40 μm thick sections of cerebellum of 60-day-old animals; colocalization appears yellow. (A) Control, (B,C) P1-P3 L-NAME-treated rats. These animals received on P3 a BrdU injection (100 μg/g body weight) after 3 days administration of L-NAME or vehicle and were sacrificed at 60 days of age. (D) Number of BrdU and NeuN (white) or BrdU and GFAP (hatched) labelled cells expressed as a percentage of total number of BrdU-labelled cells. The black part of the bars represents the percentage of cells that were of indeterminate phenotype. Values are the mean ± s.e.m. of three experiments.

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).

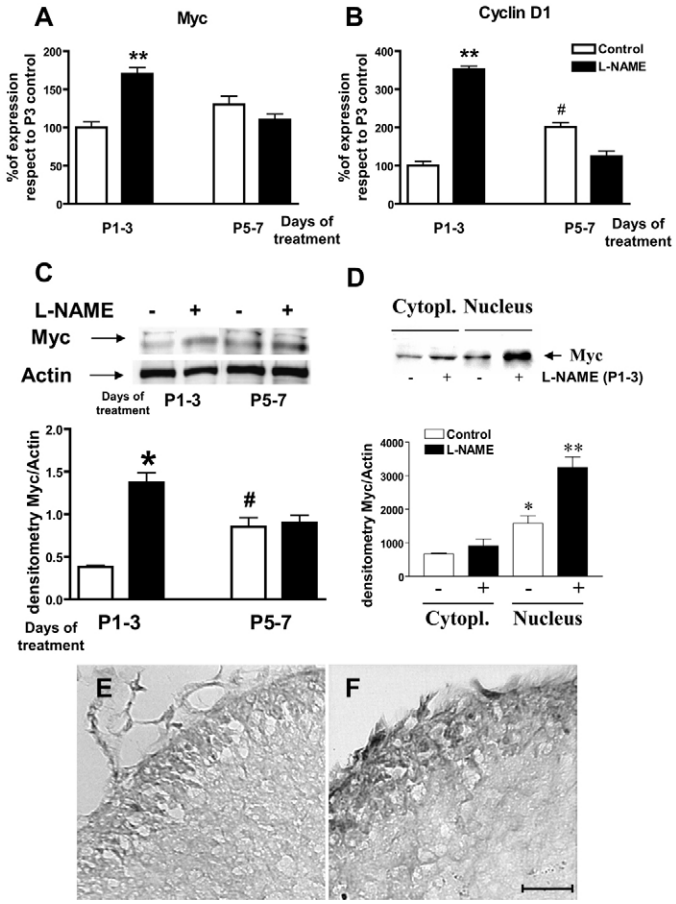

Fig. 6. Expression of Myc (A) and cyclin D1 (B) in cerebellar homogenates of rat pups treated with L-NAME (60 mg/kg/day) for various neonatal intervals (P1-P3, P5-P7) and control pups. Total RNA was extracted from cerebella and equal amounts of RNA were used for real-time PCR performed with specific primers for Myc and cyclin D1, as described in the Materials and Methods section. These results, expressed as percentage of control at P3, are the mean ± s.e.m. of three different experiments performed in triplicate. **P*<0.01, ***P*<0.001 compared with control, #*P*<0.01 compared with P3 control (Bonferroni's test after ANOVA). (C) Total cerebellar lysates from L-NAME-treated (P1-P3 and P5-P7) and control rat pups were immunoblotted with an antibody to Myc and quantification of bands was obtained with respect to β-actin content. Values are the mean ± s.e.m. of three experiments. ***P*<0.001 compared to control, #*P*<0.01 compared to P3 control (Bonferroni's test after ANOVA). (D) Nuclear and cytoplasmic cerebellar lysates from L-NAME treated (P1-P3) and control rat pups were separately immunoblotted with an antibody to Myc and quantified respect to β-actin content. Bars are the mean ± s.e.m. of three experiments. **P*<0.05, ***P*<0.001 compared to cytoplasmic extracts, (Bonferroni's test after ANOVA). (E,F) Expression of Myc protein in the cerebellum of control (E) and P1-P3 L-NAME-treated (F) rat pups. Immunohistochemistry with an anti-Myc antibody reveals an increased stain in the external granular layer of L-NAME-treated pup. Bar, 40 μm.

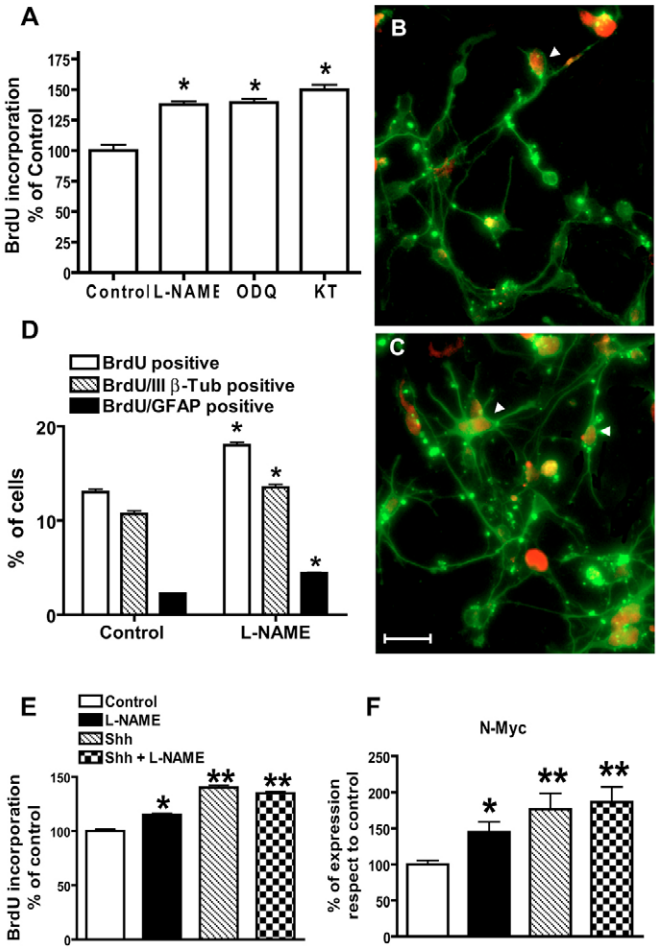

Fig. 7. (A) Freshly plated CGC cultures prepared from P2 cerebella were stimulated with L-NAME (1 mM), ODQ (5 μM) or KT5823 (500 nM), for 20 hours. BrdU (10 μM) was added for the last 6 hours, after which time cells were processed for BrdU incorporation by an ELISA method. Values are the mean ± s.e.m. 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 β_1 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 Koesling, 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 neurogenetic 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

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 bioethical 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 [^{3}H]arginine (specific activity 40 Ci/mmol; NEN Dupont) diluted with cold arginine to a final concentration of 4 μM, and β-NADPH (0.7 mM final concentration). Incubation started by adding $CaCl_2$ 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, [^{3}H]citrulline, was eluted by chromatography on columns filled with Dowex 50Wx8 sodium form resin which retains arginine. Radioactive citrulline was collected in scintillation vials and mixed with an appropriate scintillation cocktail for counting. Enzyme activity was expressed as pmoles citrulline formed/mg of protein/hour. Protein content was determined by the method of Lowry (Lowry et al., 1951).

Apoptosis assay

A sandwich ELISA method was used to assess apoptosis (Cell Death ELISA; Roche Biochemicals). Tissues were homogenized in the lysis buffer provided with the kit. The assay measures the enrichment of histone-associated DNA fragments in apoptotic cells. Detection of bound nucleosomes from the samples is made using a monoclonal anti-DNA antibody with a peroxidase (POD) label. Bound anti-DNA-POD is quantified using the peroxidase substrate 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS), and the product is measured by absorbance at 405 nm.

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% trichloracetic acid (TCA) and centrifuged at 1500 ***g***, for 10 minutes. Trichloroacetic acid 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 plate was developed with Ellman's reagent and the colorimetric reaction was measured reading the absorbance at 415 nm in a photometric plate reader (BioRad). Data from each sample were normalized for protein concentration measured by the Lowry method (Lowry et al., 1951).

[^{3}H]Thymidine incorporation assay

Two hours prior to sacrifice, pups were given a subcutaneous (s.c.) injection of 5 μCi/g body weight of [^{3}H]thymidine (specific activity 25 Ci/mmol; Amersham Pharmacia Biotech, The Netherlands). Pups were killed by decapitation and the cerebella were quickly removed. Samples were homogenized in ten volumes of ice-cold H_2O and an aliquot was precipitated using a standard TCA protocol (Tao et al., 1996; Tao et al., 1997). After two washes in ice-cold 5% TCA, the pellet was solubilized in 0.2 N NaOH/0.1% SDS and radioactivity linked to the DNA present in the pellet was counted and normalized to protein content and to the total radioactivity measured in whole homogenates.

Western blotting

Protein samples were extracted by homogenization in RIPA buffer (Tris-HCl 50 mM, pH 7.4; NP-40 1%, NaCl 150 mM, sodium deoxycholate 0.25%, EDTA 1 mM) containing protease inhibitors (Sigma). Homogenates were incubated on ice for 30 minutes prior to clarification by centrifugation (13,000 ***g***, for 30 minutes at 4°C). Total proteins were quantified (Lowry et al., 1951) and equivalent (30 μg) amounts of proteins per sample were subjected to electrophoresis on a 10% SDS-polyacrylamide gel. The gel was then blotted onto a nitrocellulose membrane and equal loading of protein in each lane was assessed by brief staining of the blot with 0.1% Ponceau S. Blotted membranes were blocked for 1 hour in 5% defatted milk in TBS (Tris-HCl 10 mM, NaCl 150 mM, pH 8.0)-Triton X-100 (0.1%) and incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: anti-β-actin (rabbit polyclonal 1:2000; Sigma); anti-nNOS (rabbit polyclonal 1:500; Santa Cruz); anti-Myc (mouse monoclonal 1:250; Santa Cruz). Membranes were washed and incubated for 1 hour at room temperature with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Amersham, Piscataway, NJ, USA; diluted 1:1000). Specific reactions were revealed by the ECL western blotting detection system (Amersham). On some immunoblots, the optical density was quantified using the image processing and analysis program Scion Image.

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 before sacrifice. Cerebella were quickly removed and fixed for 24 hours in the glyoxal-based fixative Glyofix (Italscientifica spa, Geneva, Italy). After dehydration in a graded ethanol series, the cerebella were embedded in Paraplast plus (Sherwood Medical, St Louis, MO, USA; mp 55-57°C), parasagittally 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 0.3% 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, Amersham) in PBS containing 1% BSA and 0.1% Tween 20. After rinsing in 0.1 M phosphate buffer, pH 7.4, the sections were treated with a diaminobenzidine substrate kit (Vector Laboratories). The sections were then dehydrated in ethanol, cleared in xylene and mounted in Permount (Fisher Scientific, Pittsburgh, PA) with coverslips. Sections from cerebella of pups injected with BrdU (three controls and three L-NAME treated) were denatured in 2 N HCl for 20 minutes at 37°C, 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). Bright-field images where taken on 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 of area (mm^2).

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), on 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 parasagitally in 40 μm-thick slices with a freezing microtome and serially collected in PBS. For BrdU immunohistochemistry, free-floating sections were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. The sections were treated with 0.3% hydrogen peroxide in methanol for 30 minutes to quench endogenous peroxidase activity and thereafter rinsed three times for 5 minutes in 0.1% Triton X-100 in PBS. The sections were denatured in 2 N HCl for 20 minutes at 37°C, 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, and BrdU was detected using a DAB kit (Vector Laboratories). BrdU-positive cells in the cerebellum were counted in corresponding three or four lamellae every fourth or sixth section in a series of 40 μm parasagittal sections for each cerebellum in three animals for condition. The count was done separately in the molecular layer (ML) and granular layer (GL) and the number of BrdU-positive cells was related to the area and expressed as number of BrdU-positive cells per unit of area (mm^2).

To characterize the phenotype of the BrdU-positive cells in P60 animals that had received a BrdU injection on P3 or P7, 40 μm-thick slices were double-labelled 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 fluorescent immunostaining, the sections were incubated overnight at 4°C with the following primary antibodies: (1) sheep polyclonal antibody against BrdU (Chemicon; diluted 1:100); (2) mouse anti-GFAP (Sigma; dilution 1:500) or mouse anti-NeuN (Chemicon; diluted 1:250). Sections were incubated with Cy3, conjugated goat anti-mouse secondary antibody (Sigma; dilution 1:200) and fluorescein isothiocyanate-conjugated goat anti-sheep secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; dilution 1:200) for 1 hour, and the double-labelled section were analyzed using a confocal microscope (Leitz).

For immunofluorescence studies in cultures (see below,) cells plated on poly-D-lysine coated coverslips, were fixed for 30 minutes in 4% paraformaldehyde, 4%

Table 1. Primers used for real-time PCR

Accession number	Gene	Sense and antisense primers	Amplicon length	Annealing temperature
NM_001013096	Myc	CCGCCAAGGTGGTCATCTTAA		
		CCGAGCGTGTTCGATCTTCTT	136	60°C
NM_171992	CycD1	GAGTCTTGAGTCTGGCACATTCT		
		ACTTCTTCTCTCACATCCCCTT	149	60°C
NM_017090	sGCα1	TCCGAAGTGAGTGTACCG		
		GCAGAAGAGCGAAGTAGG	131	52°C
NM_023956	sGCα2	ACATGGTGGTCCTTCAACTTGG		
		GGGTGGACAATCGTAGCAAGAC	147	60°C
NM_012769	sGCβ1	ACAGGCACAGAGGAAACAAACC		
		TGGCTTGACGCACATTAGGC	106	58°C
NM_012770	sGCβ2	CACCGAGGATGAGATAATGG		
		GCTGATGGTCTGTGTTCC	116	52°C
XM_219805	PKGI	TGAGGGAAAGAGAGAAGATGAG		
		AGGTAAAGAGCACTGTCGTAG	121	53°C
NM_013012	PKGII	AAGGCGACCCGAGGATTTG		
		AGGCTCCGTGCTTTCAACC	143	58°C
NM_052799	nNOS	AGGCTGAGGGATGACAACC		
		CGATGAAGGCGATGGACTC	102	53°C
NM_017008	GAPDH	GAACATCATCCCTGCATCCA		
		CCAGTGAGCTTCCCG TTCA	77	60°C

sucrose 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 in PBS, pH 7.4. Cells were then incubated with Cy3-conjugated goat anti-mouse secondary antibody or with FITC-conjugated goat anti-rabbit secondary antibody (dilution 1:200; Sigma) for 1-2 hours at room temperature. After all incubations, specimens were extensively washed with PBS containing 0.1% Triton X-100. 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 L-NAME, Quinoxaline-1-one (ODQ), KT5823 (Calbiochem), or Sonic hedgehog (Shh; Sigma) after 1 hour in vitro at the concentrations and for the times specified in the figure legends.

Cell proliferation assay

CGC, plated on poly-D-lysine-coated 96-wells plates (5×10^4 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 and reverse transcribed with M-MLV reverse transcriptase (Invitrogen) using customer-synthesized oligo(dT_{12-18}) primers (Invitrogen). Briefly, 2 μg of total RNA was incubated for 45 minutes at 42°C in 20 μl containing 25 μM primer, 10 mM DTT, 0.5 mM dATP, dCTP, dGTP and dTTP, 200 units of reverse transcriptase, and 1× 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 was inactivated 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.

Statistics

The results are expressed as mean ± s.e.m. of the number of experiments indicated in the figure legends. The data were analyzed using either Student's *t*-test or by ANOVA followed by post-hoc comparison through Bonferroni's test. A probability level of $P<0.05$ was considered to be statistically significant.

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