Nitric oxide negatively regulates proliferation and promotes neuronal differentiation through N-Myc downregulation

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Summary

Nitric oxide (NO) has been found to act as an important negative regulator of cell proliferation in several systems. We report here that NO negatively regulates proliferation of neuronal cell precursors and promotes their differentiation by downregulating the oncogene N-Myc. We have studied this regulatory function of NO in neuroblastoma cell lines (SK-N-BE) and in primary cerebellar granule cell cultures. In a neuronal NO synthase (nNOS) overexpressing neuroblastoma cell line exposed to the differentiative action of retinoic acid, NO slowed down proliferation and accelerated differentiation towards a neuronal phenotype. This effect was accompanied by a parallel decrease of N-Myc expression. Similar results could be obtained in parental SK-N-BE cells by providing an exogenous source of NO. Pharmacological controls demonstrated that NO's regulatory actions on cell proliferation and N-Myc expression were mediated by cGMP as an intermediate messenger. Furthermore, NO was found to modulate the transcriptional activity of N-Myc gene promoter by acting on the E2F regulatory region, possibly through the control of Rb phosphorylation state, that we found to be negatively regulated by NO. In cerebellar granule cell cultures, NOS inhibition increased the division rate of neuronal precursors, in parallel with augmented N-Myc expression. Because a high N-Myc expression level is essential for neuroblastoma progression as well as for proliferation of neuronal precursors, its negative regulation by NO highlights a novel physiopathological function of this important messenger molecule.

Key words: nNOS, Neuroblastoma cells, Cerebellar granule cells, Development, N-Myc, Neurogenesis

Introduction

New functions played by nitric oxide (NO) as an intercellular and intracellular signaling molecule in the brain are continuously demonstrated and increasing evidence suggests that NO might be a primary player in the program of neurogenesis and neuronal differentiation (Bredt and Snyder, 1994a; Contestabile et al., 2003; Packer et al., 2003). NO synthase (NOS) expression and NO synthesis are strongly induced in cultured cells treated with growth factors, and many of the pleiotropic effects of growth factors might be mediated through NO (Heneka et al., 1998; Obregon et al., 1997; Peunova and Enikolopov, 1995; Poluha et al., 1997). Moreover, neuronal NOS (nNOS), the major NOS isoform in the mammalian brain, is transiently expressed in the developing brain in a pattern suggesting its involvement in neural development (Bredt and Snyder, 1994b). Furthermore, the ability of NO reversibly to suppress cell division (Garg and Hassid, 1990; Lepoivre et al., 1990), coupled with its ability to regulate gene expression (Hemish et al., 2003), has been recently exploited in several neural developmental models (Champlin and Truman, 2000; Enikolopov et al., 1999; Peunova and Enikolopov, 1995; Peunova et al., 2001; Poluha et al., 1997). These researches have highlighted a primary role for NO as a negative regulator of neural cells proliferation and the molecular bases for its antiproliferative action are beginning to be understood. NO can regulate gene expression by modulating transcription factors or the translation or stability of mRNA, or by modifying proteins, mainly acting through the cGMP messenger system and/or direct chemical interactions with macromolecules (Bogdan, 2001; Marshall et al., 2000; Schindler and Bogdan, 2001). In *Drosophila*, for instance, the control of cell division is mediated by direct interaction with the retinoblastoma (Rb) pathway, and it has been proposed that this might occur through *S*-nitrosylation of Rb (Kuzin et al., 2000).

N-Myc is a member of the Myc proto-oncogene family, which also includes c-Myc and L-Myc. Myc proteins are known to promote proliferation and/or transformation in many cell types (Henriksson and Luscher, 1996), and their loss results in severely compromised cell cycle regulation (Mateyak et al., 1997). N-Myc oncogene amplification and/or expression play an important role in neuroblastoma tumor aggressiveness. Amplification of N-Myc expression indeed occurs in about 30% of neuroblastomas, it is associated with rapid tumor progression and survival expectancy less than 15% (Brodeur et al., 1984; Westermann and Schwab, 2002). Furthermore, during retinoic acid (RA) induced differentiation

of neuroblastoma cells in vitro, N-Myc undergoes transcriptionally mediated downregulation, in parallel with decreased cell proliferation, which is felt to be a key event in the differentiation process (Bordow et al., 1998; Chan et al., 1997; Seeger et al., 1985; Thiele and Israel, 1988; Thiele et al., 1985). Thus, Myc proteins are involved in fundamental cellular processes including cell proliferation, survival/apoptosis and differentiation, through activation or repression of specific sets of target genes (Dang, 1999; Eisenman, 2001; Grandori et al., 2000). The role of Myc proteins in the regulation of cell proliferation is consistent with studies in *Drosophila* (Johnston et al., 1999) as well as with the effects of their overexpression in mammalian cells (Beier et al., 2000; Iritani and Eisenman, 1999; Kim et al., 2000; Schuhmacher et al., 1999). Concerning neural cells, a recent study has demonstrated that N-Myc is required for brain development and that targeted loss of N-Myc function specifically disrupts the ability of neuronal progenitor cells to expand, differentiate, and populate the brain (Knoepfler et al., 2002). Regulation of Myc gene expression has been shown to occur at multiple levels, including gene transcription (Park and Wei, 2003), premature termination of translation (Krumm et al., 1992; Perez-Juste et al., 2000) and translocation (Kanda et al., 2000; Ratsch et al., 2002).

On the basis of the literature data surveyed above, negative regulation of N-Myc expression is a plausible mediator of the antiproliferative action of NO. However, no research has yet been performed to address this possible link. We report here for the first time that the negative regulation of proliferation exerted by NO on neuroblastoma cells is paralleled by downregulation of N-Myc expression. Furthermore, we provide evidence that the same holds true also for neuronal precursors in primary cultures of cerebellar granule cells (CGCs). Finally, we suggest that the cellular pathway linking NO to N-Myc downregulation involves transcriptional regulation of N-Myc expression by the Rb-E2F system.

Materials and Methods

Cell culture

The human neuroblastoma SK-N-BE cells (Biedler et al., 1978) were seeded at a density of 10^5 cells cm^{-2} on plastic culture plates and were grown to confluence in RPMI 1640 medium (Gibco BRL) containing 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL), 2 mM glutamine, 100 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Sigma) at 37°C in a 5% CO_2 humidified atmosphere. Upon reaching confluence, the cells were dispersed with trypsin, split and subcultured. Cultures were routinely observed under a phase-contrast inverted microscope and under fluorescence microscope after vital staining with fluorescein diacetate.

Primary cultures of CGCs were prepared from the cerebella of 7-day-old Wistar rat pups as previously described (Gallo et al., 1982). Briefly, cerebella were removed and dissected from their meninges in Krebs' buffer (120 mM NaCl, 25 mM $NaHCO_3$, 3.3 mM KH_2PO_4, 0.8 mM K_2HPO_4, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM glucose pH 7.4) containing 0.3% bovine serum albumin (BSA). Tissue was dissociated with trypsin at 37°C for 15 minutes and triturated 15 times using a Pasteur pipette in a DNase/soybean-trypsin-inhibitor solution. The cells were plated at a density of 2.4×10^6 per 35 mm dish previously coated with poly-L-lysine and maintained in basal modified Eagle's (BME) medium (Gibco) containing 10% FCS, 2 mM glutamine, 100 μg ml^{-1} gentamicin and 25 mM KCl.

Treatments

All-trans retinoic acid was dissolved in dimethyl sulfoxide (DMSO) at a 10 mM concentration and stored in the dark at –20°C. This stock was added to culture flasks of neuroblastoma cells to a final 10 μM concentration. Control flasks were treated with an equivalent volume of DMSO solvent alone. The various pharmacological agents tested (L-NAME, DETA NONOate, ODQ, 8Brc-GMP) were added in a volume of 1 μl ml^{-1} in the medium at the concentrations and for the times specified in the figure legends.

Measurements of neurite growth

Cells were fixed in 4% paraformaldehyde, 4% sucrose at 37°C, and were stained using hematoxylin and eosin. Cells possessing one or more neurites longer than the diameter of the cell bodies were considered to be positive for counting. The neurite growth index (NGI) (Image pro plus software) was used to obtain an overall measurement of neurite length. Images of the treated cells were captured with a digital camera. All neurites in each region of interest (ROI) were manually traced and the length was measured using Image Pro Plus (Scanalytics, Fairfax, VA, USA). The NGI was calculated by dividing the sum of neurite length by the number of cells in each ROI. For each experiment, at least 150 cells were measured.

Proliferation assay

Cell proliferation was determined using a bromodeoxyuridine (BrdU) labeling and detection kit III (Roche, Mannheim, Germany) following the instruction manual. Briefly, cells were cultured in a microtiter plate (96 wells) and BrdU was added to the culture medium for 2 hours to be incorporated into newly synthesized DNA. Following fixation of cells, cellular DNA was partially digested by nuclease. Next, a peroxidase (POD) labeled antibody to BrdU (anti-BrdU POD) was added. The absorbance of the sample was determined in a microtiter plate reader at 405 nm with a reference wavelength of 490 nm.

Ornithine-decarboxylase assay

Ornithine decarboxylase (ODC) was measured in supernatants of cell homogenates, centrifuged at 10,000 ***g*** for 15 minutes at 4°C using a radiochemical method (Baudry et al., 1986).

Luciferase measurement

Transient transfection experiments were performed using polyethylenimine (PEI 25K) as DNA carrier according to the previously described method (Bunone et al., 1997). Plasmids used for transfection were: CMVLuc, rsvSP1, N-MycLuc and N-MycΔE2FLuc. 24 hours after transfection, cells were washed twice with ice-cold PBS and lysed by incubation in 50 mM Tris-MES (pH 7.8) (Sigma), 1 mM dithiothreitol (DTT) and 1% Triton X-100 for 5 minutes on ice. The lysate was cleared of cellular debris by centrifugation (de Wet et al., 1987). Luciferase assays were performed with a TD-20/20 luminometer (Promega, Madison, WI).

Determination of nitrite and nitrate concentrations

Nitrite, a stable product of NO oxidation, plus nitrates previously converted to nitrites (Miranda et al., 2001), were determined by measuring accumulation in culture medium through the Griess reaction as described previously (Dong et al., 1994). In brief, culture media (100 μl) were collected, mixed with 100 μl vanadium (III) chloride (400 mg per 50 ml 1 M HCl), added with 50 μl of sulfanilamide (dissolved in 1.2 M HCl) and 50 μl *N*-naphthylethylenediamine dihydrochloride. After 15 minutes at room temperature, samples were measured at a wavelength of 540

nm. Nitrite concentrations were calculated against a $NaNO_2$ standard.

Apoptosis assay

A sandwich enzyme-linked immunosorbent assay (ELISA) method was used to assess apoptosis (Cell Death ELISA; Roche Biochemicals). 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 POD label. Bound anti-DNA-POD is quantified using the POD substrate 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS), whose product is measured by absorbance at 405 nm.

Northern analysis

Approximately 30 μg total RNA per sample were loaded per lane and fractionated on a 1% agarose formaldehyde gel. Following transfer to Hybond N membrane (Amersham International, Wiltshire, UK), N-*Myc* mRNA was detected using an α^{32}P-labeled full-length probe. Cyclophilin probes were used for quantification as described previously (Taubenfeld et al., 2001). Membranes were washed to a maximum stringency of 0.1× sodium chloride-sodium citrate (SSC) buffer, 0.1% sodium dodecyl sulfate (SDS) at 65°C. Autoradiography was performed using Kodak XAR film at –70°C with an intensifying screen. To measure RNA half-life, cells were exposed to actinomycin D at 10 μg ml^{-1} on day 2 of treatment with RA or solvent control. RNA was harvested from treated and control cells after 0 minutes, 45 minutes, 90 minutes and 180 minutes of exposure to actinomycin D. The time course and intensity of such treatment has been shown to inhibit N-*Myc* RNA synthesis by >95% without significantly decreasing viability (data not shown). Once harvested, RNA was analysed by northern blotting as described above. The N-*Myc* message half-life was calculated from densitometric laser scans of autoradiographs using the signal intensity from cyclophilin-encoding mRNA as a standard.

RT-PCR assays

Total RNA, free from chromosomal DNA contamination, was isolated using Trizol according to the supplier's instructions and reverse transcribed with SUPERSCRIPT II RNase H-Reverse transcriptase (Life Technologies) using customer-synthesized (Life Technologies) oligo-$dT_{12\text{-}18}$ primers. Reverse-transcription polymerase chain reaction (RT-PCR) primers for glyceraldehyde-3-phosphate dehydrogenase (GADPH) and N-Myc are as follows. GADPH (451 bp) sense, 5′-ACCACAGTCCATGCCATCAC-3′, antisense, 5′-TCCACCACCCTGTTGCTGTA-3′; N-Myc (500 bp) sense, 5′-GTCACCACATTCACCATCACTGT-3′, antisense 5′-AGCGTG-TTCAATTTTCTTTAGCA-3′. Briefly, 2 μg of total RNA was incubated for 45 minutes at 42°C in 20 μl containing 25 μM of the primer, 10 mM DTT, 0.5 mM dATP, dCTP, dGTP and dTTP, 200 units reverse transcriptase and 1× First Strand Buffer (Life Technologies). A 1 μl aliquot of reverse-transcribed cDNA was subjected to PCR in a 50 μl mixture containing 3′ and 5′ primers, each 2 μM. PCR amplification was conducted for 21 cycles (GADPH) or 28 cycles (N-Myc) using the following conditions: denaturation, 40 seconds at 94°C; annealing, 30 seconds at 55°C (N-Myc) or 56°C (GADPH); primer extension, 2 minutes at 72°C. PCR products were electrophoresed on agarose gels containing ethidium bromide and visualized under ultraviolet light. Band intensities (arbitrary optical density units) were evaluated using a Bio-Imaging Analyzer system (Amersham) combined with densitometry. Intensities for amplified Bcl-2 were normalized against those obtained for GADPH in the same samples. The linearity of PCR amplification was verified by amplifying several dilutions of reverse-transcribed cDNA.

Western blotting

The following antibodies were used for analysis of nNOS (polyclonal antibody at 1:500), PCNA (polyclonal antibody at 1:500), N-Myc (monoclonal antibody at 1:250), Rb (polyclonal antibody at 1:500), phosphorylated Rb (P-Rb) (polyclonal antibody at 1:500) all from Santa Cruz Biotechnology and β-actin (polyclonal antibody at 1:1000; Sigma). Western blots were performed on 2×10^6 cells per experimental point. Cell pellets were lysed by homogenization at 4°C with a lysis buffer containing 1% deoxycholate, 1 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin, 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate for 10 minutes. Lysates were immediately processed by western blot or kept frozen until assayed. Protein concentrations of samples were estimated by the Lowry method (Lowry et al., 1951). Equivalent (50 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 a 4% suspension of dried skimmed milk in PBS and incubated overnight at 4°C with: primary antibodies. Membranes were washed and incubated for 1 hour at room temperature with peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (dilution 1:1000). Specific reactions were revealed with the ECL western blotting detection reagent (Amersham, Piscataway, NJ.).

Immunoprecipitation studies

To prepare cell extracts, cells were washed three times with PBS and then lysed in RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate, 1× protease inhibitor mixture (Roche Applied Science), 1 mM sodium vanadate] for 15 minutes on ice. The lysates were centrifuged in an Eppendorf centrifuge at 10,000 ***g*** and 4°C for 15 minutes. Cell lysates containing an equal amount of protein (500 μg) were incubated with prewashed protein-G/Sepharose beads for 1 hour before incubation with primary antibody overnight at 4°C under gentle shaking. Prewashed Sepharose beads were further incubated with the lysate-antibody mixture for 1-2 hours. Beads were washed twice in 200 mM NaCl, 1× RIPA, followed by a final wash in 1× RIPA. Proteins were resolved by 10% SDS polyacrylamide-gel electrophoresis (SDS-PAGE) and subsequent western blot analysis.

Immunocytochemistry

To label cells in S phase in vitro, purified CGCs were incubated in 10 μM BrdU (Sigma) for 2 hours, 24 hours after plating. Then the cells were fixed in 70% ethanol in 50 mM glycine buffer, pH 2.0, at –20°C for 20 minutes. The cells were then incubated with an anti-BrdU monoclonal antibody (diluted 1:100; Chemicon), followed by an avidin-biotin amplified immunohistochemical method as described previously (Ciani and Contestabile, 1994). For double-immunofluorescence cytochemistry, cells were grown on coverslips, washed twice with PBS and fixed for 10 minutes with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (0.1 M NaH_2PO_4/Na_2HPO_4) at pH 7.4. After washing with PBS, cells were permeabilized and treated with blocking solution (5% normal goat serum and 0.4% Triton X-100 in PBS) for 30 minutes. Cells were incubated overnight with anti-BrdU monoclonal antibody (diluted 1:100; Chemicon) and anti-glial-fibrillar-acidic-protein (GFAP) polyclonal antibody (dilution 1:800; Sigma). Coverslips were washed four times for 5 minutes each, with PBS/Triton X-100 solution (0.1% Triton X-100 in 0.1 M phosphate buffer 0.9% NaCl). Cells were incubated with Cy5-conjugated goat anti-rabbit secondary antibody (dilution 1:500; Sigma) and fluorescein-isothiocyanate-conjugated goat anti-mouse secondary antibody (dilution 1:250; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. Coverslips were washed

twice with PBS, mounted on glass slides, observed and photographed with a Zeiss fluorescence microscope.

Statistics

Data were expressed as means±s.e.m. and statistical significance was assessed by using one-way analysis of variance (ANOVA) followed by Bonferroni's test (SigmaStat software). Differences were considered to be significant starting from $P<0.05$.

Results

Overexpression of nNOS or exogenous NO speed up differentiation of neuroblastoma cells

Human cytomegalovirus (HCMV) can opportunistically use retinoic acid (RA), an essential control molecule for cellular differentiation, to promote its own replication (Angulo and Ghazal, 1995). We used this knowledge to create an inducible expression system in SK-N-BE neuroblastoma cells, in which exposure of HCMV/nNOS-infected cells to RA concomitantly activates the transcription of HCMV promoter and enhances the expression of the transfected nNOS-encoding gene. We have previously studied the effect of nNOS overexpression on cell survival using SK-N-BE cells stably transfected with a HCMV-promoter-driven vector, harboring a full-length human nNOS-encoding cDNA (Ciani et al., 2002). Immunoblotting analysis on cell lysates (Fig. 1A) confirmed the previously reported overexpression of nNOS derived from the transgene in one of the selected clones (clone 4) and also demonstrated a sizeable increase of basal nNOS expression in a pool of all the selected clones. When cells were treated with RA, the level of nNOS protein was further enhanced in clone 4 and, to a lower extent, in pooled clones (Fig. 1B) owing to the presence of RA response elements in the HCMV promoter. The importance of nNOS for the action of RA on neuroblastoma cells was confirmed by the fact that parental cells also started to increase their nNOS expression after longer RA treatment (Fig. 1B, top). The action of RA on the HCMV promoter is complex. Because HCMV has been shown to contain several SP1 sites that can be the target of activated RA receptors (Husmann et al., 2000), we evaluated whether this type of regulation was relevant to the effect of RA in nNOS-overexpressing neuroblastoma cells. In transient transfection experiments with an HCMV-luciferase (HCMV-Luc) reporter construct, the transcriptional activity promoted by RA (6.8-times increase over control) was only slightly enhanced by co-transfection with a SP1 expression vector (7.9-times increase over control). Therefore, it can be concluded that the effect of RA on HCMV promoter in our lines of neuroblastoma cells is only marginally modulated through the interaction of RA receptors with SP1 sites. The abundance of nNOS expression in clone-4 cells correlated with its NO-generating catalytic activity, as assessed by the accumulation in the culture medium of oxidized products of the released NO, nitrites and nitrates (Fig. 1C).

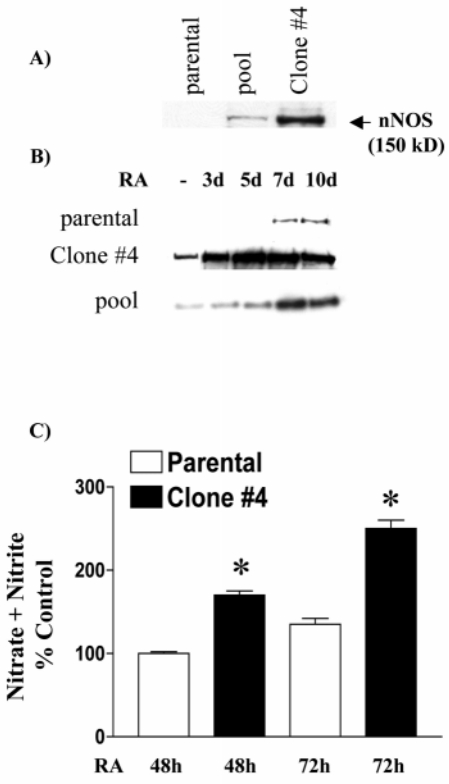

Fig. 1. (A) nNOS expression in parental SK-N-BE, clone-4 and pool of all the selected clone cells by immunoblot analysis. (B) Induction of nNOS expression in RA-treated parental SK-N-BE, clone-4 and pooled clone cells. The expression of nNOS was determined by western-blot analysis. Cells were exposed to 10 μM RA for a total of 10 days. 50 μg protein were subjected to SDS-PAGE and the blot was probed with anti-nNOS antibody. (C) Catalytic NOS activity evaluated through nitrite-plus-nitrate accumulation in the medium, expressed as a proportion of the activity in control cultures without RA (the absolute concentration of the control is ~1.5 μM). Bars are the mean±s.e.m. of three independent experiments performed in duplicate. *, $P<0.01$ (Bonferroni's test after ANOVA).

Retinoic acid has been shown to slow down proliferation of SK-N-BE cells and to induce their differentiation towards a neuronal phenotype (Melino et al., 1997b), a process that starts to become evident in parental cells after several days of RA exposure. We used our nNOS-overexpressing clone to look at the effect of increased NO availability in this differentiation paradigm. Parental and clone-4 cells were exposed to a standard concentration (10 μM) of RA for 2-7 days. Clone-4 cells were morphologically distinguishable from the parent SK-N-BE cells before starting RA treatment. Whereas parental cells had rounded cell bodies and tended to grow in focal clumps (Fig. 2Aa), the clone-4 cells had characteristic spindle-shaped cell bodies (Fig. 2Ab). Treatment of clone-4 cells with RA resulted in a rapid, dramatic change in their morphology compared with the parental cells. Within 24 hours of RA addition, clone-4 cells started to extend processes of visible length and, by 2 days, many of these cells showed a more differentiated morphology than parental cells (Fig. 2Ac,d). The cells extended long branched processes measuring up to five or six times the length of the cell body (Fig. 2Ad,B). A similar degree of neurite extension was only reached by parental cells exposed for 2 days to RA when they were also treated with a slow-releasing NO donor (DETA-NONOate; half-life of release, 20 hours) (Fig. 2B). In the absence of exogenously added NO, the RA-promoted differentiation of parental cells was much slower, comparable levels of neurite extension being reached only after 7 days (Fig. 2B). As expected, the accelerated differentiation promoted by high NO levels was

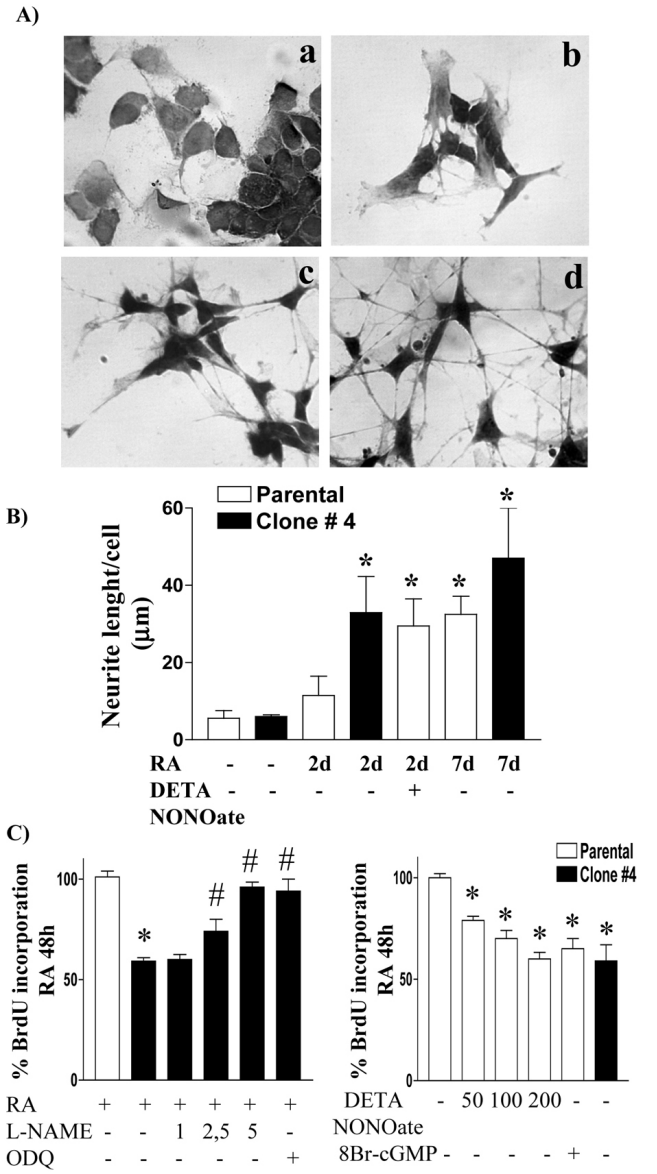

Fig. 2. (A) Parental and clone-4 cells stained with hematoxylin-eosin before (a,b) and after (c,d) 48 hours of treatment with 10 μM RA. Notice the more differentiated neuronal phenotype acquired by clone-4 cells after such a short exposure to RA (d). (B) Effects of RA exposure on neurite length in parental and clone-4 cells. Notice the large increase in neurite length with 2 days of exposure to RA in clone-4 and parental cells co-treated with RA and 200 μM DETA-NONOate. Bars are the mean±s.e.m. from measuring neurite extension of at least 150 cells per dish in three separate dishes. (C, left) In parallel to stimulation of clone-4 differentiation, RA also slowed down cell proliferation in this clone compared with parental cells, an effect completely reversed by blocking NOS activity using L-NAME (1-5 mM) or the guanylate cyclase inhibitor ODQ (50 μM). (C, right) Inhibition of BrdU incorporation in SK-N-BE cells by RA and DETA-NONOate (50-200 μM) or 8Br-cGMP (250 μM). The antiproliferative effects were determined after treating the parental and clone-4 cells for 2 days with 10 μM RA and labeling with BrdU (10 μM) for the last 2 hours. Results are expressed as the percentage BrdU incorporation relative to parental cells treated with RA. Bars are the mean±s.e.m. of four experiments. *, $P<0.01$ compared with control cultures; #, $P<0.01$ compared with clone 4 (Bonferroni's test after ANOVA).

accompanied by a slowing of proliferation, evaluated through the incorporation of the thymidine analog BrdU, which was decreased by almost 50% in clone 4 after 48 hours exposure to RA, compared with parental cells (Fig. 2C, left). This effect was abolished by the concomitant inhibition of NOS activity through nitro-arginine methyl esther (L-NAME), thus confirming its dependence on NO produced in excess by the overexpressed nNOS (Fig. 2C, left). A specific inhibitor of the NO-dependent soluble guanylate cyclase ODQ affected the proliferation rate of clone-4 cells in a way comparable to L-NAME (Fig. 2C, left). To confirm the link between NO and RA in speeding up differentiation of SK-N-BE, DETA-NONOate was tested for its ability to reduce proliferation of parental cells under RA treatment. The NO donor significantly reduced, in a dose-dependent way, proliferation of SK-N-BE cells treated for 48 hours with RA (Fig. 2C, right). The cGMP analog 8Br-cGMP replicated the anti-proliferative effect of the NO donor (Fig. 2C, right).

Other markers of the proliferation/differentiation program were affected in a way coherent with the involvement of NO suggested by the above experiments. Proliferating cell nuclear antigen (PCNA) is a proliferation-induced 36 kDa nuclear protein that is the auxiliary component of DNA polymerase δ and its levels in tissues have been found to correlate positively with proliferative activity (Keim et al., 1993). The high levels of PCNA observed in parental cells in control condition only slightly decreased after 3-5 days of RA treatment, whereas a more substantial decrease in expression was noted in clone-4 cells (Fig. 3A). Ornithine decarboxylase (ODC), the key enzyme of polyamine biosynthesis, decreases its activity during the progressive differentiation of neuronal cells (Sparapani et al., 1998). In agreement with other evidence suggesting an antiproliferative role for NO, ODC activity was significantly decreased upon RA exposure in clone-4 cells, compared with parental cells (Fig. 3B). Under several conditions leading to differentiation, neuroblastoma cells increase their apoptotic rate in addition of slowing down proliferation (Peverali et al., 1996). Although apoptotic rate slowly increased in parental cells exposed to RA, the process was significantly accelerated in clone-4 cells (Fig. 3C).

Overexpression of nNOS or exogenous NO speeds up the differentiation-related suppression of N-Myc expression in neuroblastoma cells

Induction of neuronal differentiation in neuroblastoma cells in vitro is accompanied by N-Myc downregulation (Keim et al., 1993; Melino et al., 1997b). Therefore, we compared the effects of nNOS overexpression on N-Myc levels under treatment with RA. In parental cells, N-Myc protein level slowly decreased under RA action, almost disappearing after 7 days of treatment (Fig. 4A,B). More drastic changes were observed in clone-4 cells, in which basal level of N-Myc was lower, probably because of the higher basal NO production, and RA treatment led to a rapid decrease of N-Myc protein expression with an almost total disappearance after only 2 days (Fig. 4A,B). The pool of selected clones exhibited a response of N-Myc to RA that was intermediate between parental and clone-4 cells (Fig. 4A). The effect of RA treatment for 2 days on N-Myc expression was completely reverted, in a concentration-dependent manner, in clone 4 by adding to the

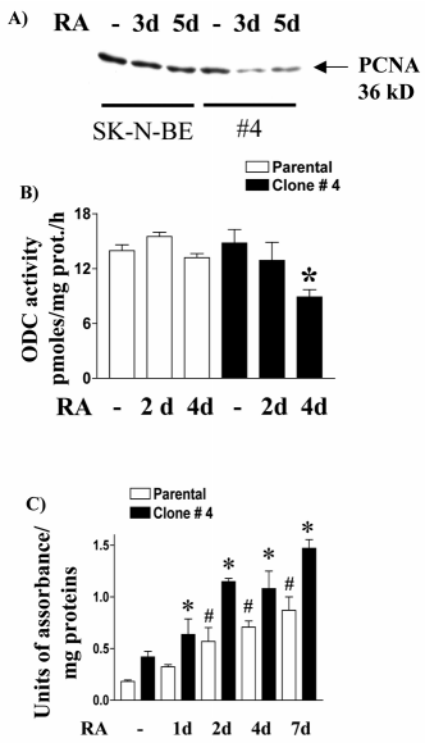

Fig. 3. (A) Effect of RA treatments on PCNA expression in parental and nNOS overexpressing SK-N-BE cells. Levels of PCNA were visualized by western blot. (B) Effect of RA treatments on ODC activity in parental and clone-4 cells. Bars are the mean±s.e.m. of three experiments. *, $P<0.01$ compared with control (Bonferroni's test after ANOVA). (C) Quantification of cell death after 1-7 days RA exposure by a specific ELISA kit. Bars are the mean±s.e.m. of four experiments. *, $P<0.01$ compared with clone 4 without RA; #, $P<0.01$ compared with parental cells without RA (Bonferroni's test after ANOVA).

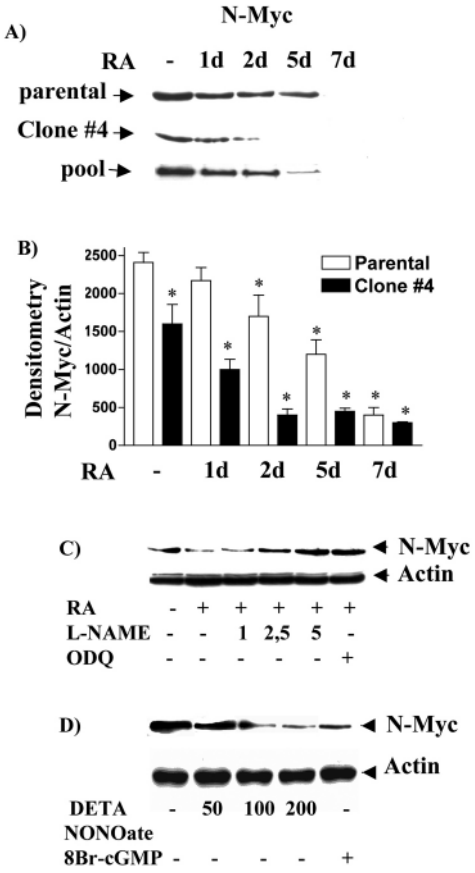

Fig. 4. (A) Effect of RA treatment on N-Myc expression in parental and nNOS overexpressing cells (clone 4 and pooled clones). Levels of N-Myc were evaluated by western blot. (B) N-Myc was quantified through its ratio to actin content of the samples. Bars are the mean±s.e.m. of four experiments. *, $P<0.01$ compared with control (Bonferroni's test after ANOVA). (C) Effect of RA treatment on N-Myc in clone 4 after the addition of the NOS inhibitor L-NAME (1-5 mM) and the guanylate cyclase inhibitor ODQ (50 μM) to the culture medium. (D) The negative regulation of N-Myc protein expression was replicated in parental cells using increasing concentrations of DETA NONOate (50-200 μM) and the cGMP analog 8Br-cGMP (250 μM).

medium the NOS inhibitor L-NAME (Fig. 4C). A similar result was also obtained by adding to the medium the soluble guanylate-cyclase inhibitor ODQ (Fig. 4C). The negative regulation of N-Myc protein expression by NO was replicated in parental cells by treating them with increasing concentrations of DETA NONOate (Fig. 4D). The effect of the NO donor was mimicked by the cGMP analog 8Br-cGMP (Fig. 4D).

Nitric oxide modulation of N-Myc transcriptional activity

Theoretically, lowered expression of N-Myc protein could be due to reduced mRNA stability. We therefore measured the apparent half-lifes of N-*Myc* mRNAs in parental and clone-4 cells treated with RA. After 2 days in culture, transcription was blocked by addition of actinomycin D and the levels of N-*Myc* mRNA were monitored at various intervals. As shown in Fig. 5A, the N-*Myc* mRNA half-life was approximately 60 minutes in both parental and clone-4 cells. Therefore, N-*Myc* mRNA stability was not affected by NO, whereas its protein expression was modified.

To test whether NO was able to inhibit the N-*Myc* promoter activity in neuroblastoma cells, we transiently co-transfected parental and clone-4 cells with a reporter construct in which luciferase expression was controlled by 230 bp of the human N-*Myc* gene, which encompass the major transcription starting site and three closely spaced putative E2F-binding sites (Strieder and Lutz, 2003). N-*Myc* promoter activity was downregulated when transiently transfected in clone-4 cells, but not in parental cells, after 24 hours stimulation with RA, compared with the respective conditions in the absence of RA (Fig. 5B). E2F proteins regulate N-*Myc* transcription and are required for full activity of the N-*Myc* promoter in neuroblastoma cells (Strieder and Lutz, 2003). To determine whether the inhibitory effect of NO was mediated by this regulatory region of the N-*Myc* gene, we took advantage of a previously engineered N-MycLuc reporter construct lacking all three E2F sites (Strieder and Lutz, 2003). As shown in Fig. 5B, the N-MycΔE2F reporter activity was not inhibited by the increased NO production in clone-4 cells. Therefore, the N-*Myc* promoter inhibition by NO in clone 4 cells was largely dependent on intact E2F-binding sites. Moreover, DETA-

NONOate dose-dependently decreased the activity of N-MycLuc reporter transiently transfected in parental cells (Fig. 5C). These combined results demonstrate that NO is indeed able to downregulate N-*Myc* gene expression in neuroblastoma cells and that this action is mediated by the regulatory E2F region of the N-*Myc* gene.

During neuronal differentiation, N-*Myc* expression is suppressed through sequestration of the regulatory E2F proteins in a Rb-E2F complex promoted by Rb dephosphorylation (Stevaux and Dyson, 2002). To verify whether this mechanism could contribute to NO-mediated downregulation of endogenous N-*Myc* expression, we examined the phosphorylation state of the Rb protein by western blot analysis after immunoprecipitation with an antibody specific for Rb. Parental and clone-4 cells were treated with RA and with 8Br-cGMP or L-NAME, respectively, for 48 hours, and whole cell lysates were used for immunoprecipitation. Clone-4 cells treated for 48 hours with RA showed a decrease in Rb phosphorylation (Fig. 6A,B), an effect reversed by concomitant treatment with L-NAME (Fig. 6B). This did not happen in RA-treated parental cells, in which, however, levels of phosphorylated Rb were significantly reduced by the concomitant exposure to DETA-NONOate or 8Br-cGMP (Fig. 6B).

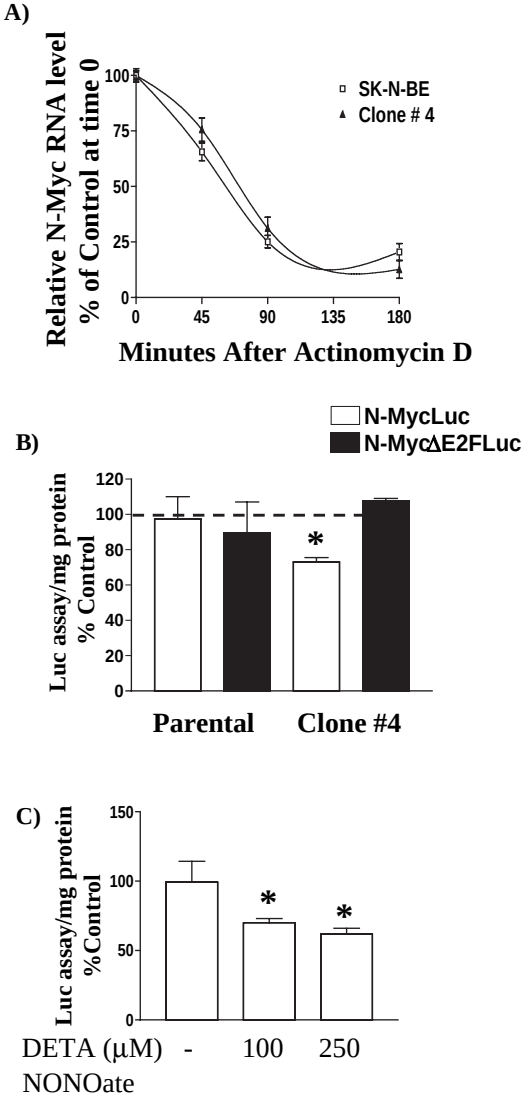

Fig. 5. (A) N-*Myc* mRNA half-life. The intensity of the N-*Myc* mRNA signal was measured by laser densitometry of autoradiographs from northern blots and plotted as a function of time after exposure to actinomycin D. Parental and clone-4 cells treated with RA did not show alteration in the half-life of N-*Myc* mRNA. (B) Effect of RA treatment on luciferase activity in parental and clone-4 cells transfected with N-MycLuc and N-MycΔE2FLuc (3 μg). 12 hours after transfection, cells were exposed to RA for 24 hours. Luciferase activity is given as a percentage of the respective control condition in the absence of RA (hatched line) for each transfection condition. Bars are the mean±s.e.m. of four experiments. *, $P<0.01$ compared with control (Bonferroni's test after ANOVA). (C) DETA-NONOate dose-dependently decreased the activity of N-MycLuc reporter transiently transfected in parental cells. *, $P<0.01$ compared with control (Bonferroni's test after ANOVA).

NO negatively regulates CGC proliferation and N-*Myc* expression

In order to verify whether the negative regulation of proliferation exerted by NO, and its mediation through N-Myc downregulation could be also demonstrated in primary neuronal cells, we treated freshly plated cultures of CGCs from neonatal rats with L-NAME to block NOS activity. In a similar preparation from mice, it has been recently demonstrated that N-Myc plays a crucial role in the proliferation of neuronal

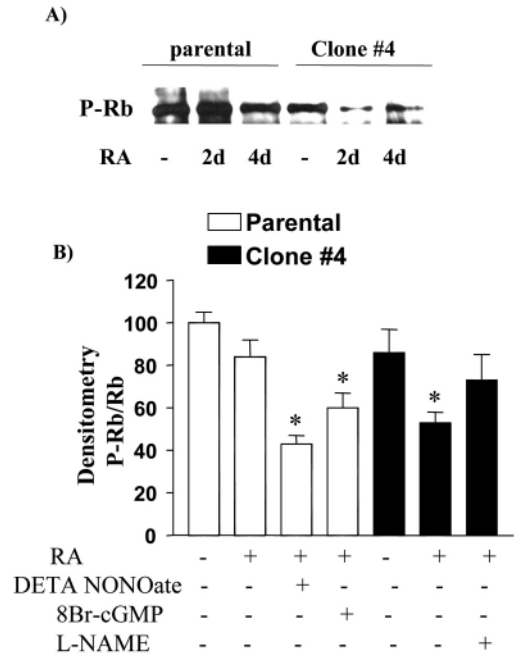

Fig. 6. Alterations in Rb phosphorylation in parental and clone-4 cells exposed to RA. (A) Total cell lysates from parental or clone-4 cells were immunoprecipitated with an anti-Rb antibody and western blotted with an anti-phospho-Rb monoclonal antibody. (B) Immunoprecipitate densitometric analysis of the proportion of P-Rb to total Rb from parental cells treated with RA and the NO donor DETA-NONOate (200 μM) or the cGMP analog 8Br-cGMP (250 μM), and from clone-4 cells treated with RA and L-NAME (5 mM). Bars are means±s.e.m. of four experiments. *, $P<0.01$ compared with control (Bonferroni's test after ANOVA).

precursors under the transcriptional control of sonic hedgehog (Shh) (Oliver et al., 2003). We therefore tested whether L-NAME treatment had the same effect as Shh on cell division and N-Myc expression in CGCs. To do that, we labeled dividing cells with BrdU (Fig. 7A) and counted them in control and treated cultures. Both NOS inhibition and Shh treatment stimulated cell proliferation (Fig. 7B). Similar results were obtained by evaluating cell division through a kit measuring incorporation of BrdU in the DNA extracted from the cells. Under these conditions, both L-NAME and Shh increased BrdU incorporation, whereas DETA NONOate slightly decreased DNA synthesis (data not shown). In parallel with the increased rate of cell division, both L-NAME and Shh robustly stimulated N-*Myc* mRNA expression in CGC cultures (Fig. 7C). Double-labeling experiments with BrdU and GFAP were performed to evaluate the proportion of proliferating cells exhibiting a glial phenotype, with respect to CGCs under conditions of L-NAME exposure (Fig. 7D, left). Cells positive for both BrdU and GFAP appeared to be relatively scanty and cell counting demonstrated that the ratio between the total number of BrdU-labeled cells and the (BrdU+GFAP)-positive cells was 2.5 times higher in cultures treated with L-NAME than in control cultures (Fig. 7D, right). This demonstrates that the largest effect of NO deprivation on cell proliferation is not on the glial cell population and must, therefore, be exerted on the population of CGC precursors present in the culture.

Discussion

With the present data, we provide for the first time evidence that the negative regulation of neuronal precursor proliferation exerted by NO is linked to downregulation of N-Myc expression. This relationship holds true not only in a neuroblastoma cell line but also in a model of primary neuronal cultures of CGCs. Furthermore, we provide evidence that NO-mediated inhibition of N-Myc transcription requires intact E2F sites in the gene promoter region and that high levels of NO negatively regulate the phosphorylation state of Rb. Correlatively, this suggests that NO might downregulate N-Myc expression by promoting the sequestration of E2F proteins from dephosphorylated Rb.

NO negatively regulates neural proliferation

That NO may act on differentiation of neural cells by negatively affecting cell division, relies on several previous in vitro and in vivo observations. In pheochromocytoma (PC12) cells, the growth arrest and differentiative effect of nerve growth factor (NGF) were shown to be mediated by NO (Peunova and Enikolopov, 1995; Phung and Black, 1999). In the human neuroblastoma cell line SK-N-BE, RA-induced differentiation was found to be paralleled by increased NO synthesis (Ghigo et al., 1998). Here, we provide multiple and more-direct evidence linking neuroblastoma cell differentiation to NO, by showing that a nNOS overexpressing clone responds much more quickly than parental cells to the differentiative action of RA. The fact that a similar result is obtained by providing exogenous NO to parental cells makes us confident that what we demonstrate here reflects a true biological action of NO and not a fortuitous property acquired by a given clone through the cloning selection steps. Indeed,

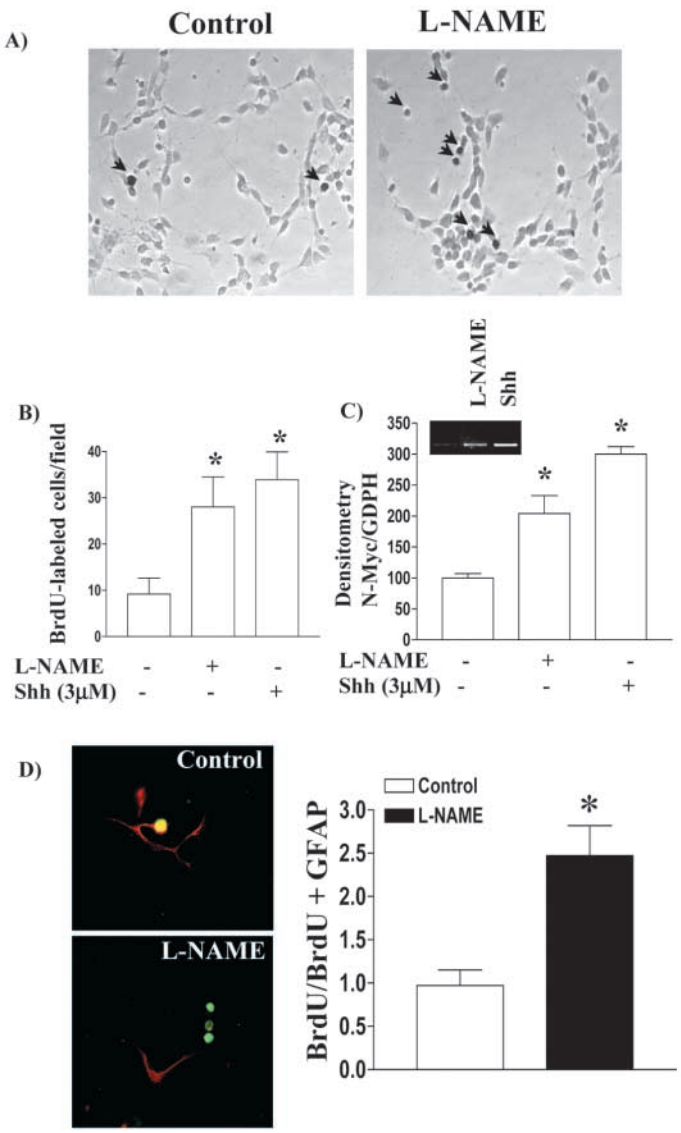

Fig. 7. (A) Phase-contrast photographs of CGCs after BrdU immunocytochemistry. Arrows point to BrdU labeled cells. (B) L-NAME and Shh stimulate BrdU incorporation in CGC cultures: quantification of BrdU labeled cells. Cells from neonatal rat cerebellum were cultured for 1 day in control medium or supplemented with 500 μM L-NAME or 3 μg ml^{-1} Shh. Cultures were pulsed with 10 μM BrdU for the final 4 hours and processed for BrdU immunocytochemistry and subsequent quantification. Bars are the mean±s.e.m. of four experiments. *, P<0.01 compared with control (Bonferroni's test after ANOVA). (C) Semiquantitative RT-PCR analysis was performed with RNA isolated from CGCs treated with L-NAME or Shh for 24 hours. Amplification of the 500 bp PCR product represents N-*Myc* mRNA. Bars are the mean±s.e.m. of four experiments. *, P<0.01 (Bonferroni's test after ANOVA). (D) Double-immunofluorescence cytochemistry with anti-BrdU and anti-GFAP antibodies of CGCs cultured for 1 day in control medium or supplemented with 500 μM L-NAME. Images of immunoreactivity (left) for BrdU (green), GFAP (red) and both markers (yellow) in CGC cultures. The ratio of total BrdU-labeled cells on BrdU+GFAP-positive cells was quantified in control and compared with L-NAME-treated cultures (right). Bars are the mean±s.e.m. of three experiments. *, P<0.01 (Bonferroni's test after ANOVA).

experiments performed on a pool of clones expressing different levels of transfected nNOS (Ciani et al., 2002) demonstrated a similar, if less pronounced, response to RA in terms of enhanced protein expression. Furthermore, we also demonstrate that the process of differentiation towards a neuronal phenotype is accompanied by a sizeable decrease of proliferation, an effect that, on the basis of appropriate pharmacological controls, is clearly dependent on NO production. NO has been reported to inhibit the proliferation of various cell types by either cGMP-dependent or -independent mechanisms (Blachier et al., 1996; Buga et al., 1998; Chambaut-Guerin et al., 2000; Garg et al., 1989; Garg and Hassid, 1989; Garg and Hassid, 1990; Melino et al., 1997a). The use of a specific inhibitor of guanylate cyclase or of a cGMP analog allows us to state that, in SK-N-BE cells, the negative regulation of proliferation exerted by NO is primarily cGMP dependent. The finding that neuroblastoma cells require NO to slow down proliferation and to speed up differentiation suggests that these cells might be especially sensitive to changes in the availability of NO. Thus, loss of NO production within a specific developmental window might contribute to the development of neuroblastomas, and possibly other tumors, by driving precursor cells to maintain their embryonic, short-duration cell cycle and therefore favoring their uncontrolled expansion.

The inhibitory action of NO on neuroblast proliferation has been previously demonstrated in vivo, in the optic tectum of *Xenopus* tadpoles (Peunova et al., 2001) and in the optic lobe of moth (Champlin and Truman, 2000). NO also regulates the balance between cell proliferation and cell differentiation in developing *Drosophila* imaginal disks and embryos (Enikolopov et al., 1999; Kuzin et al., 1996; Wingrove and O'Farrell, 1999). Furthermore, it has recently been reported that NO acts as an important negative regulator of neural precursor cell proliferation in the adult mammalian brain. Specifically, inhibition of NOS activity stimulates the overproduction of neural cells in both the forebrain subventricular zone and the hippocampal dentate gyrus, two regions that generate new neurons throughout adulthood (Cheng et al., 2003; Packer et al., 2003). Our results demonstrate that NO exerts a negative regulation of cerebellar precursor proliferation in vitro and that, in parallel, it downregulates N-Myc expression. The similarity with what has been demonstrated for precursors of granule neurons of the dentate gyrus, whose proliferation rate is increased by NOS inhibitor in adult mice (Cheng et al., 2003; Packer et al., 2003), allow us to hypothesize a role for NO in cerebellar neurogenesis, also on the basis of the essential role played by N-Myc during cerebellar development (Knoepfler et al., 2002). We are currently testing NO involvement in cerebellar neurogenesis and its correlation with N-Myc expression during neonatal rat development.

NO negatively regulates N-Myc expression

The molecular mechanisms by which NO inhibits cell proliferation are poorly characterized, because of the high reactivity of this molecule, which can interact with many potential targets (Contestabile et al., 2003). We demonstrate here that a novel mechanism by which NO interferes with cell proliferation is through inhibition of N-Myc expression. Our results imply that NO represses the N-*Myc* gene, whose activity appears to be crucial for the achievement of full proliferative capacity of neuroblastoma cells and of CCG precursors. Three aberrant forms of nervous system growth (neuroblastomas, medulloblastomas and retinoblastomas) have been shown to contain overexpressed N-*Myc* or otherwise amplifications of the N-*Myc* locus (Badiali et al., 1991; Kohl et al., 1983; Lee et al., 1984; Schwab, 1993). N-Myc belongs to Myc transcription factor family (Cole and McMahon, 1999), which is associated with neuroblastoma progression and is downregulated during the terminal differentiation of neuroblastomas induced by RA or its derivatives (Peverali et al., 1996; Reynolds et al., 2000; Thiele et al., 1985; Wada et al., 1997). Accordingly, RA-induced growth arrest and differentiation of neuroblastoma cells are counteracted by N-Myc overexpression (Peverali et al., 1996).

N-Myc was shown to be expressed in the proliferative zone of the cerebellum, suggesting its potential role in CGC precursor proliferation (Knoepfler et al., 2002). A role for N-Myc in cerebellar development was also suggested by studies in mice in which the N-*Myc* gene had been selectively ablated in neuronal progenitors (Knoepfler et al., 2002). This conditional targeting of N-Myc in the developing central nervous system results in generalized, severe brain hypoplasia, with the CGC population being the most severely affected (Knoepfler et al., 2002). We found that the block of NO production in CGC precursors exposed to L-NAME in culture results in increased cell proliferation accompanied by increased levels of N-Myc mRNA, which identifies N-Myc as a possible target for the antiproliferative action of NO towards cerebellar neuronal precursors. Noticeably, a similar correspondence between stimulation of cell division and N-Myc expression was found when CGC precursors were exposed to Shh, whose activity is required for expansion of neuronal precursor cells in the external granular layer of the cerebellum and is the most potent known mitogen for the same precursors in vitro (Kenney et al., 2003; Kenney and Rowitch, 2000). Taken together, the present results on neuroblastoma cells, as well as on CGC precursors, strongly support the conclusion that N-Myc is a major downstream effector negatively regulated by NO in its antiproliferative action.

Finally, our present data suggest that one of the mechanisms through which NO affects N-Myc expression involves a decrease in Rb phosphorylation, a process that is known to increase the Rb/E2F-1 association (Young et al., 2003). Rb proteins interact with E2F to regulate gene transcription, and several mechanisms have been proposed for Rb-E2F transcriptional regulation (Young et al., 2003). Previous data have highlighted the interaction between NO and Rb in the control of cell division in *Drosophila*, and it was suggested that Rb could be a direct target for *S*-nitrosylation (Kuzin et al., 2000). Our data on neuroblastomas suggest a major role for the cGMP pathway in mediating the interaction between NO and Rb, because both a NO donor and a c-GMP analog replicated the inhibition of Rb phosphorylation in parental cells. A similar picture of the interactions between the cGMP system and Rb has recently emerged from a study in a different cellular model (Hanada et al., 2001).

Conclusions

Although evidence for a role of NO in neuronal proliferation

and differentiation is accumulating, very little is still known about the targets of these functions. Our findings lead to the conclusion that NO regulates a switch in neuronal precursor programming, from proliferation to differentiation, through N-Myc. The observation that the action of NO on N-Myc is similar in different cellular models suggests that this effect might be shared by other neuronal populations and play a significant role during brain development. Finally, the negative regulation of N-Myc expression by NO demonstrated in neuroblastoma cells, provides a potential target for therapeutic intervention, because high expression of N-Myc is associated with rapid growth and fatal progression of these tumors (Badiali et al., 1991; Kohl et al., 1983; Lee et al., 1984; Schwab, 1993).

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