J Mol Neurosci (2007) 32:97–107
DOI 10.1007/s12031-007-0014-7

Evidence of Postnatal Neurogenesis in Dorsal Root Ganglion: Role of Nitric Oxide and Neuronal Restrictive Silencer Transcription Factor

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Received: 5 February 2007 / Accepted: 6 February 2007 / Published online: 23 March 2007
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Abstract The various mechanisms underlying postnatal neurogenesis from discrete CNS regions have emerged recently. However, little is known about postnatal neurogenesis in dorsal root ganglion (DRG). BrdU incorporation and subsequent immunostaining for BrdU, neural stem cell marker, nestin and neuronal marker, PGP 9.5 have provided evidence for postnatal neurogenesis in DRG. We further demonstrate, in vivo and in vitro, that nitric oxide (NO) regulates neural stem cells (nestin$^+$) proliferation and, possibly, differentiation into neurons. Surprisingly, nerve growth factor (NGF) had no effect on nestin$^+$ cells proliferation. Axotomy or NGF-deprivation of DRG neurons-satellite glia co-culture increases NO production by neurons and treating with a NO synthase (NOS) inhibitor, N^G-nitro-L-arginine methylester (L-NAME) in vitro or 7-nitroindazole (7NI) in vivo, causes a significant increase in nestin$^+$ cell numbers. However, a soluble guanylyl cyclase (sGC) blocker, 1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ) treatment of NGF-deprived DRG neurons-satellite glia co-culture had no significant effect on nestin$^+$ cell numbers. This implies NO regulates nestin$^+$ cell proliferation independent of cGMP. We hypothesised that the neuronal-restrictive silencer transcription factor (NRSF, also termed REST), a master regulator of neuronal genes in non-neuronal cells, may be modulated by NO in satellite glia cultures. A NO donor, dimethyl-triamino-benzidine (DETA)-NO treatment of satellite glia cell cultures results in a significant increase in the NRSF/REST mRNA expression. The majority of cultured satellite glia cells express nestin, and also show increased levels of NOS, thus L-NAME treatment of these cultures causes a dramatic reduction in NRSF/REST mRNA. Overall these results suggest that NO inhibits neurogenesis in DRG and this is correlated with modulation of NRSF, a known modulator of differentiation.

Keywords Nestin · Axotomy · NOS inhibitors · BrdU · Neurogenesis · Neural stem cells

Abbreviations

BrdU	5-bromo-2′-deoxyuridine
BMP	bone morphogenic protein
BSA	bovine serum albumin
CNS	central nervous system
cGMP	cyclic guanosine monophosphate
DETA-NO	dimethyl-triamino-benzidine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
FCS	foetal calf serum
FGF	fibroblast growth factor
HBSS	Hank's balanced salt solution
iNOS	inducible nitric oxide synthase

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L-NAME	N^G-nitro-L-arginine methyl ester
NBM	Neurobasal medium
NGF	nerve growth factor
7NI	7 Nitroindazole
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NRSF	neuronal-restrictive silencer transcription factor
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
ODQ	1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one
PBS	phosphate buffer saline
PFA	paraformaldehyde
PGP 9.5	protein gene product 9.5
PNS	peripheral nervous system
RE-1	repressor element-1
REST	RE 1 silencer transcription factor
RT	room temperature
sGC	soluble guanylyl cyclase
trkA	tyrosine kinase A
T_4	thyroxine
T_3	tri-iodothyronine

Introduction

Neurogenesis, the birth of neurons, is an active process during prenatal development which is responsible for populating the developing nervous system. For many years, it has been believed that neurogenesis does not occur during postnatal development. Although the vast majority of neurons are differentiated before birth, there are a few special areas in the brain region, such as subventricular zone of the lateral ventricles and subgranular zone of the dentate gyrus, which generate large numbers of neurons during postnatal development (Alvarez-Buylla et al. 2001; Gage 2000; Goldman and Luskin 1998; Zhu et al. 2006). Postnatal neurogenesis in the peripheral nervous system (PNS) such as dorsal root ganglion (DRG) has been reported, but not as well defined as in the CNS (Devor et al. 1985; Devor and Govrin-Lippmann 1991; Farel 2002, 2003; Groves et al. 2003; La Forte et al. 1991; Namaka et al. 2001; Popken and Farel 1997).

Postnatal neurogenesis in DRG occurs at various time points depending on the age and injury to their axons. A few studies have shown that there are neural stem cells present in the mouse postnatal DRG (Namaka et al. 2001) and the adult rat (Ciaroni et al. 2000; Groves et al. 2003; Singh and Zhou 2002). Unfortunately, there are very few methods to identify the birth of new neurons during postnatal life, the most widely used being 5-bromo-2-deoxyuridine (BrdU) incorporation to identify dividing cells, which has been used in DRG following nerve injury (Ciaroni et al. 2000; Groves et al. 2003). The BrdU technique has its own limitations, hence, nestin, which is an intermediate filament protein expressed by neural stem cells in CNS and PNS neurons and glia has been used in the present study as a marker for neural stem cells in DRG (Hockfield and McKay 1985; Lendahl et al. 1990; Mujtaba et al. 1998). The neural stem cells down-regulate nestin as they differentiate into either neurons or glia (Kato et al. 1999; Lendahl et al. 1990; Rice et al. 2003; Woodbury et al. 2000). Surprisingly, nestin is also expressed by myelinating adult Schwann cells (Friedman et al. 1990), but its expression in either normal or injured DRGs in vivo or in culture and the mechanism underlying the proliferation of these nestin$^+$ cells and their differentiation into neurons is not well understood.

Nitric oxide (NO), a gaseous messenger molecule produced from l-arginine in various cell types by different isoforms of NO synthases, plays distinct roles in the nervous system. NO produced in response to trauma/nerve injury or ischemia, in vivo and growth factor deprivation in vitro, appears to play multiple roles including neuro-protection and/or neurogenesis (Cardenas et al. 2005; Estrada and Murillo-Carretero 2005; Thippeswamy and Morris 1997a,b; Thippeswamy et al. 2001a, 2005b, 2006). We and others have shown that following peripheral nerve injury increased NO in DRG is protective to neurons by up regulating galanin in neurons and/or neurotrophins in glia (Shi et al. 1998; Thippeswamy et al. 2001a, 2006; Zhou et al. 1999). In recent years, NO has been demonstrated to act as a negative regulator of neural stem cell proliferation and differentiation in CNS (Cardenas et al. 2005; Ciani et al. 2004, 2006; Matarredona et al. 2004; Packer et al. 2003). In view of this, we tested the hypothesis that in absence of NO in axotomized DRG or NGF-deprivation of DRG neurons-glia co-cultures in vitro the neural stem cells may proliferate and differentiate into neurons. NO production was blocked using a general NOS inhibitor, N^G-nitro-L-arginine methylester (L-NAME) in vitro or 7-nitroindazole (7NI) in vivo. In order to understand whether cGMP, the downstream of NO pathway, is involved in regulation of nestin$^+$ cell numbers, DRG neurons-glia co-cultures were treated with a soluble guanylyl cyclase (sGC) blocker, 1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ). Further to address the action of NO we correlated modulation of the transcription factor, neuron-restrictive or repressor element-1 (RE-1) silencer transcription factor (NRSF, also termed REST). Indeed NRSF/REST was originally termed a master regulator of neuronal specific genes (Chong et al. 1995; Conaco et al. 2006; Mori et al.

1992; Palm et al. 1998; Roopra et al. 2000). We correlate NRSF/REST modulation with NO-mediated suppression of nestin^{+} cell proliferation and, possibly, differentiation.

Methods and Materials

Dissociated DRG cultures from normal Wistar rats (post-natal day 28–30) and lumbar segments 4, 5 and 6 (L4–L6) DRG sections from normal and sciatic nerve sectioned rats, treated with nNOS inhibitors and BrdU were used. These animals were purchased from Biomedical Unit of the Liverpool University. All efforts were made to minimize animal suffering by deeply anesthetizing animals with halothane followed by decapitation. All experimental procedures were performed according to UK Home Office regulations.

DRG Neurons-satellite Glia Co-cultures

DRGs were collected using a standard procedure (Thippeswamy and Morris 1997a) and dissociated by treating with 0.125% (w/v) collagenase and 0.25% (w/v) trypsin (both from Sigma) in sterile Hank's balanced salt solution (HBSS, Ca^{++} and Mg^{++} free; Gibco, UK) at 37°C with 5% CO_2 in humidified air for 45 min. To obtain uniform suspension of cells, culture medium (2 ml) was added to these ganglia and was then dissociated further by passing this solution 10–12 times through flame-polished Pasteur pipettes of decreasing diameters. To minimize the number of fibroblasts in cultures, the cell suspension was initially plated onto non-coated plastic tissue culture flasks and left for 3 h at room temperature. The supernatant containing DRG neurons with satellite glia cells was then removed carefully and divided into two equal aliquots of 1 ml each, which were then diluted further, by adding a known volume of medium containing 20 ng/ml NGF or without NGF, to achieve a final cell density of $2.5–3.5\times10^{5}$ cells/ml. Then 0.4 ml aliquots of this suspension was plated on 8-chambered slides previously coated with poly-D-lysine (10 μg/ml in ice cold PBS for 2 h at 37°C; Sigma, UK) followed by laminin [10 μg/ml in Dulbecco's modified Eagle's medium (DMEM), overnight at 37°C; Invitrogen, UK]. Four chambers per slide were plated with cell suspension with NGF (200 ng/ml; Alomone labs, Israel) to investigate the effect of NGF on neural stem cells proliferation, and the other four on the same slide without NGF served as control. These cultures were then maintained by incubating them at 37°C with 5% CO_2 in humidified air. The medium (per 100 ml) consisted of Neurobasal Media (NBM, Gibco, UK) (88 ml), foetal calf serum (FCS) 10 ml, T_4 (thyroxin, Sigma) 80 μl, T_3 (tri-iodothyronine, Sigma) 25 μl, N_2-supplement (Gibco, UK) 1 ml, bovine serum albumin (BSA) (5%) 2 ml, Bovine pituitary extract (Sigma) 175 μl, antibiotics-antimycotics (Gibco, UK) 500 μl. Fibroblast Growth Factor (βFGF, Gibco, UK) 20 ng/ml of medium was added for first two days of incubation. NGF was added initially at a concentration of 50 ng/ml, which was then increased to 200 ng/ml on the third day of incubation. 5-bromo-2-deoxyuridine (BrdU; Molecular Probes, UK) was added at a concentration of 30 μM on 0 day and 3rd day of cell culture to mark dividing cells.

Drug Treatment

On the third day after establishment, the cultures were washed and the following drugs or their vehicles were added. The drugs used were: the general NOS blocker, N^{G}-nitro-L-arginine methyl ester (L-NAME; Tocris, UK) and an inhibitor of soluble guanylate cyclase, [(1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one] (ODQ; Tocris, UK) (Thippeswamy and Morris 1997b; Thippeswamy et al. 2001a). Sterile distilled water (DW) or 10% DMSO were used as a vehicle control for L-NAME and ODQ, respectively. The stock solutions of L-NAME and ODQ were added to cultures to achieve final concentrations of 100 and 50 μM in the media, respectively. Each drug or control treatment was repeated on a minimum of three cultures from three different animals and all conditions were kept constant for each set of cultures. The cultures were fixed using 4% paraformaldehyde (PFA) after 48 h of drug treatment. After fixing, they were processed for immunocytochemistry.

Quantitative PCR

Pure glia cultures were prepared as described previously (Thippeswamy et al. 2005a) and total RNA was extracted from these cultures at the end of three hour drug treatments using the acid-phenol extraction method (TRIzol reagent, Gibco-BRL, UK). Subsequently RNA was purified and quantitative real-time PCR (qPCR) was performed in an Opticon qPCR machine [Genetic Research Instrumentation (GRI), UK] using the Dynamo SYBR Green qPCR Kit (Finnzymes). For each experiment a standard curve for each primer set was generated and used to derive the relative amounts in the unknown samples. The content of unknown samples was calculated from the amount of the target gene, normalized to the amount of a housekeeping gene (GAPDH), with each derived from separate standard curves. The primer sequences were, GAPDH, forward primer (For) 5′-accacagtccatgccatcac-3′ and reverse primer

(Rev) 5′-tccaccaccctgttgctgta-3′; rat NRSF, (For) 5′-agcgag-taccactggaggaaaca-3′ and (Rev) 5′-aattaagaggtttaggcccgttg-3′. BLASTN searches confirmed the mRNA gene specificity of the primer sequences chosen. Results were analyzed using software supplied with the Opticon machine (GRI, UK). The default settings of the program were used to define both the threshold value and baseline for analysis of the raw data. The expression of each target gene was normalized with respect to 1,000 copies of GAPDH and was calculated for all samples. Each experiment was performed in triplicate.

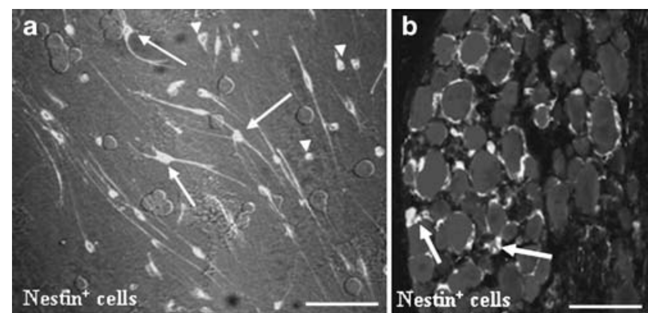


Figure 1 Photomicrographs of DRG neurons-satellite glia co-culture (**a**) and in vivo DRG section (**b**) immunostained for nestin. Brightly stained cells are nestin$^+$ cells. Unstained round cells in the *background* are DRG neurons. In co-culture, two types of nestin$^+$ cells were identified: either small round nestin$^+$ cells without processes (examples are shown by *arrow heads*) or neuron-like phenotype with neurite-like processes (examples are indicated by *arrows*). In DRG section (**b**), nestin$^+$ cells were found in clusters of 3–4 cells and/or solitary cells in between DRG neurons. A large number of satellite glia cells that surround the periphery of neuronal soma were also nestin$^+$. Scale bars, 100 μm

Axotomy and Tissue Sectioning

Wistar albino rats (30-day-old) were used for this study. All experimental procedures were according to UK Home Office regulations. Nerve sectioning on the left side was performed on these animals and a piece of sciatic nerve (4–5 mm) was removed under general anesthesia consisting of a mixture of nitrous oxide (60%) and halothane (5%) for inducing anaesthesia, maintained with Hypnorm (0.3 ml/kg i.m) and diazepam (2.5 mg/kg i.p). Bruphenorphin (0.5 mg/kg i.m) analgesic was given to prevent post-operative pain and discomfort to animals.

After nerve sectioning, these animals were injected intraperitonially (i.p) with BrdU (dissolved in sterile DW; 100 mg/kg) or vehicle (DW) twice daily for nine days. On day 8 and 9, 50% of animal were injected with neuronal NOS inhibitor, 7-nitroindazole (7NI; dissolved in sterile 10% DSMO, 50 mg/kg) and the rest received the same volume of vehicle (sterile 10% DSMO), twice daily at an interval of at least 8 h. Animals were observed closely during the drug treatment. On day 10, all animals were deeply anaesthetized using pentobarbitone (80 mg/kg) and were fixed by vascular perfusion of 4% PFA in 0.1 M phosphate buffered saline (PBS).

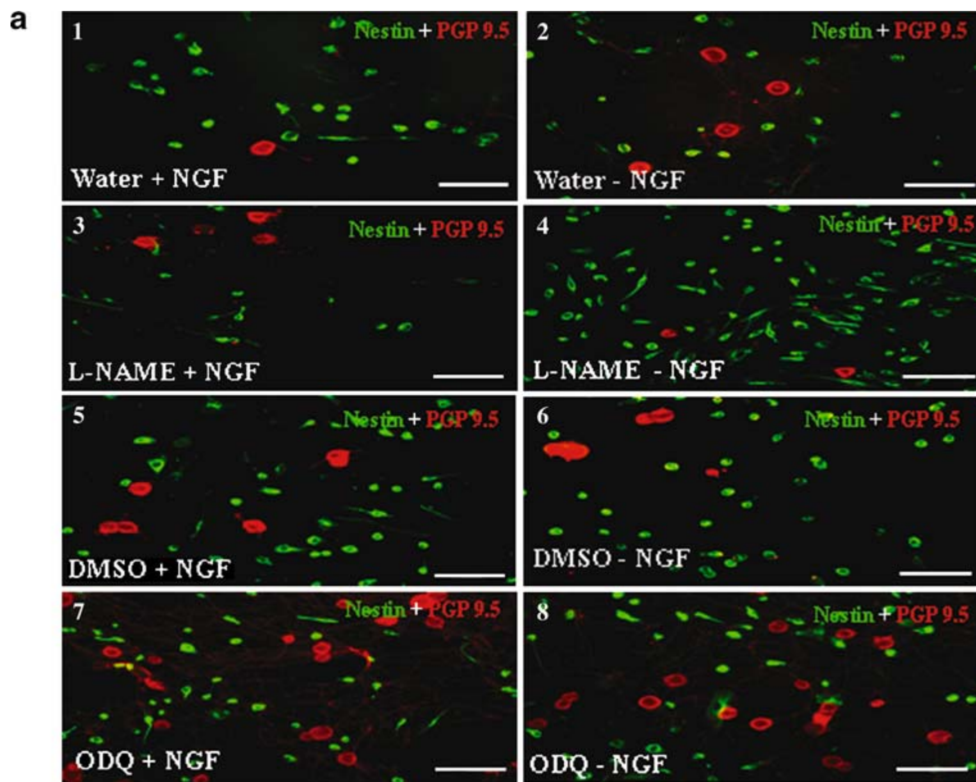
After perfusing animals, L4–L6 DRGs both ipsilateral and contralateral to the nerve section were dissected, post-fixed in 4% PFA for 4 h at 4°C, and then cryoprotected with 30% sucrose in PBS at 4°C overnight. The following day, DRGs were gelatine embedded and serial 10 μm thick sections were cut using a cryostat and thaw-mounted on a set of ten slides so that each slide consisted of every 10th section at 100 μm apart (assuming the diameter of a largest neuron as ~100 μm) so that each slide represents the whole ganglia. The sections were stored at −40°C until they were processed for immunostaining.

Immunocytochemistry

DRG sections and PFA fixed DRG neurons-glia co-cultures were washed with 0.1 M PBS and processed for double/triple immunostaining after blocking non-specific binding by incubating them with 10% donkey serum in PBS for 1 h at room temperature (RT). The primary antibodies used were; anti-BrdU raised in mouse (1:40; Sigma), anti-nestin raised in mouse (1:1,000, Chemicon), and general neuronal marker anti-human PGP 9.5 raised in rabbit (1:4,000; UltraClone Ltd., UK). For double-immunostaining, primary antibodies raised in different species were mixed one with the other without changing the final concentration and were incubated at 4°C overnight. This was followed by appropriate biotinylated anti-species antibodies [for example, anti-mouse 1:500, and anti-rabbit 1:200 (Jackson)] and/or flurochrome labelling such as Cy3-conjugated donkey anti-rabbit (1:300; Jackson) and FITC-conjugated donkey anti-mouse (1:200; Jackson, USA). These antibodies were employed for 1 h at RT. The biotin was then detected, where appropriate, using streptavidin-FITC (1:80; Vector, USA). Between each step the cultures and sections were washed thoroughly with 0.1 M PBS several times. Finally, cultures or sections were mounted with Vectashield mounting medium (Vector Laboratories, Inc.) and were stored at

Figure 2 DRG neurons-satellite glia co-cultures maintained in ▶ presence or absence of NGF and treated with L-NAME (100 μM, 2 days) or ODQ (50 μM, 2 days) and appropriate vehicle (DW, distilled water for L-NAME and 10% DMSO for ODQ). Cultures were subsequently double immunostained (**a**) for neural stem cell marker nestin, and neuronal marker PGP 9.5. *Red* stained cells are PGP$^+$ (neurons) and *green* cells are nestin$^+$. NGF and ODQ (**c**) had no effect on nestin$^+$ cell numbers, but L-NAME treatment of cultures causes a significant increase in the number of nestin$^+$ cells in NGF-deprived cultures when compared to vehicle control (**b**, $^{**}p<0.01$). However, L-NAME treatment of cultures in presence of NGF reduced the number of nestin$^+$ cells compared to vehicle control ($^*p<0.05$). Scale bars, 100 μm

a



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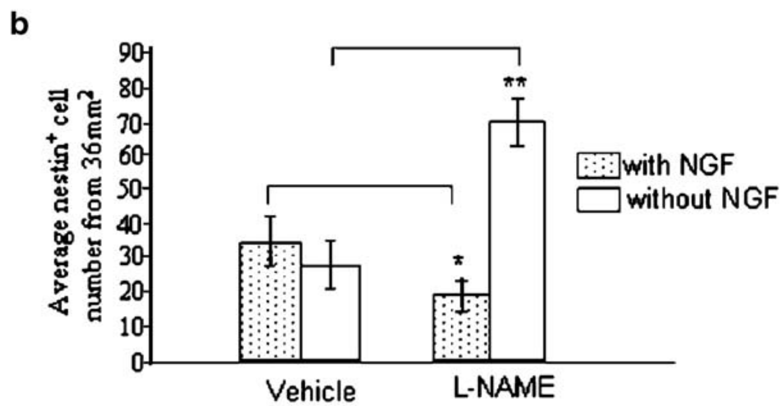

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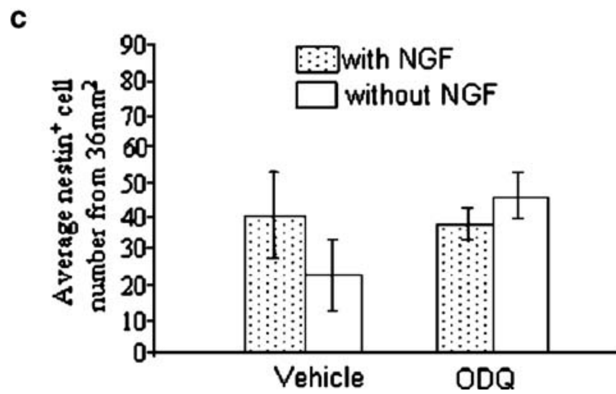

4°C. The immunostaining was visualized using an epifluorescence microscope (Nikon, UK) and images were captured with Hamamatsu digital camera (C4742-95) using IP Lab 3.7 software (Nikon, UK).

Data Analysis

Nestin$^+$ cell counts were taken from all sections of a slide that contained 10 μm thick sections of L5 DRG. A minimum of three slides were counted and the average calculated. In DRG neurons-glia co-cultures, the nestin$^+$ cell counts, from control and drug treated samples represent the average number of nestin$^+$ cells in 36 mm^2, counted from at least three fields/culture well. Such counts were made from at least six cultures. Parallel cultures maintained under similar conditions either treated with drugs or vehicles were used for all counts. Parameters were compared between control and drug treated samples using Student's t test and a significance level of 0.05 was used throughout and data was expressed as mean±SEM. Microsoft Excel was used to prepare the histograms.

Results

Identification of Nestin$^+$ Cells In Vitro and In Vivo

A number of nestin$^+$ cells were found in both DRG neurons-glia co-cultures as well as DRG sections in vivo. In cultures, two types of nestin$^+$ cells were identified (Fig. 1a), some cells with round morphology and the other type with long neurites-like processes showing early stages of differentiation (Fig. 1a). In DRG sections, nestin$^+$ cells were present in clusters in between the neurons (Fig. 1b).

Effect of NOS Inhibition by L-NAME on Nestin$^+$ Cell Numbers in DRG Neurons-glia Co-cultures

NGF-supplemented DRG neurons-glia co-cultures treated with a general NOS inhibitor, L-NAME caused a decrease ($p<0.05$) in the number of nestin$^+$ cells (Fig. 2b) when compared with the vehicle control. This implies that a basal level of NO is required for survival of some of the nestin$^+$ cells. Surprisingly, L-NAME treatment of parallel cultures in absence of NGF caused a significant increase ($p<0.01$) in the number of nestin$^+$ cells compared with appropriate vehicle control (Fig. 2b). However, there was no significant difference in the number of nestin$^+$ cells in presence or absence of NGF alone (Fig. 2b) implying that the difference in nestin$^+$ cell numbers is influenced by NO but not NGF.

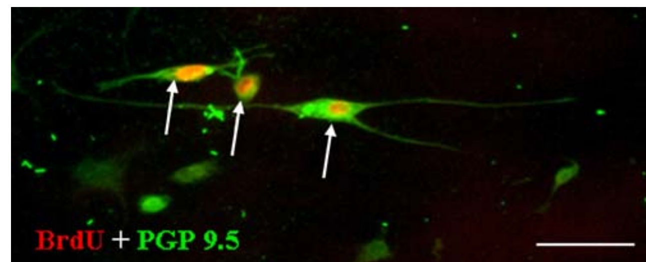

Figure 3 Photomicrograph of a sample DRG neurons-glia co-culture double immunostained for BrdU (*red*) and PGP 9.5 (*green*). Co-localization of BrdU and PGP in the same cell [examples are indicated by *arrows*] suggests that there are newly differentiated neurons in culture. Scale bar: 100 μm

Effect of sGC Inhibition on Nestin$^+$ Cell Numbers in DRG Neurons-glia Co-cultures

To determine whether NO actions are mediated by cGMP, cultures were treated with the sGC blocker, ODQ. There was no significant difference in the number of nestin$^+$ cells in DRG cultures upon treatment with ODQ compared to vehicle control (DMSO) either in presence or absence of NGF (Fig. 2a,c) suggesting that NO regulates nestin$^+$ cell numbers, independent of cGMP.

Do Nestin$^+$ Cells in DRG Neurons-glia Co-cultures Differentiate into Neurons?

A large number of nestin$^+$ cells were found to be immunopositive for BrdU by day 7 in culture. Although the vast majority of nestin$^+$ cells showed BrdU staining, only a very few appear to have differentiated into neurons as revealed by BrdU co-localization with neuronal specific marker, PGP 9.5 (Fig. 3).

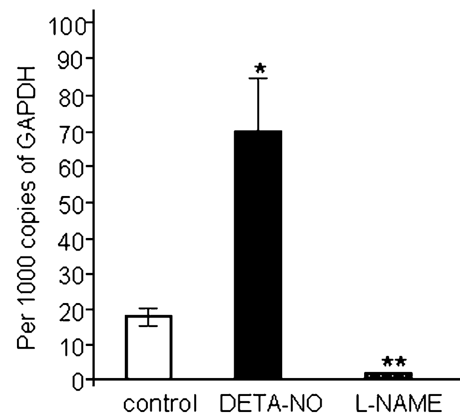

Figure 4 QPCR analysis of NRSF/REST mRNA expression in satellite glia culture. DETA-NO treatment of glia cultures caused 3–4 fold increase in NRSF/REST mRNA expression (*$p<0.001$), while L-NAME treatment reduced its expression significantly (*$p<0.001$) suggesting that NRSF/REST expression is mediated by NO in satellite glia cells

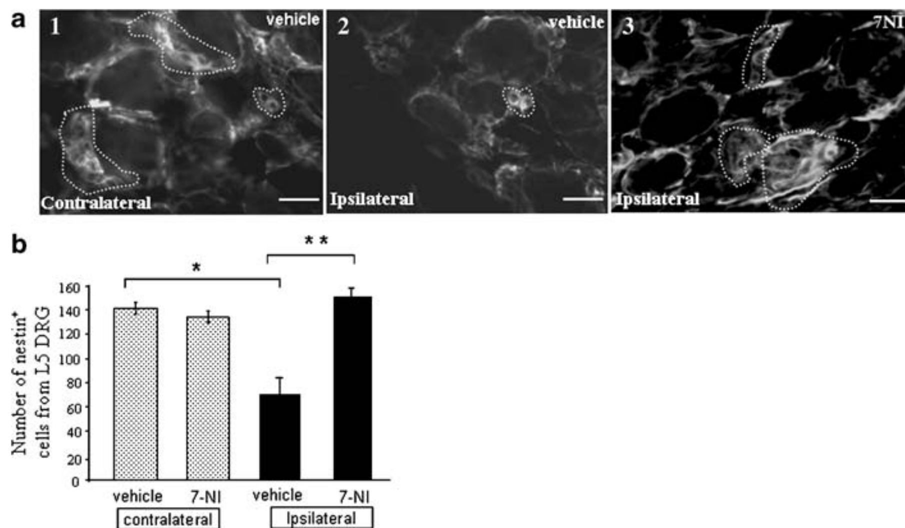

Figure 5 Nestin immunostaining of L5 DRG (**a**) and nestin$^+$ cell quantification (**b**) from contralateral and ipsilateral to the nerve section after previous treatment with neuronal NOS inhibitor, 7NI or vehicle for 2 days, prior to perfusion. Clusters of nestin cells (indicated by *dotted boundaries*) are marked on the photomicrographs. In axotomized DRG (2, vehicle, ipsilateral) the number of nestin$^+$ cells was dramatically reduced compared to contralateral vehicle treated L5 DRG (*$p<0.01$), this could be due to increased neuronal NOS expression in DRG within 8 days post-axotomy. Treatment of axotomized rats with 7NI (3) restores nestin$^+$ expression to almost basal levels (**$p<0.01$, compared between ipsilateral vehicle treated with 7NI treated L5 DRG) suggesting that endogenous NO inhibits nestin cell proliferation. Since less than 3% neurons express nNOS in contralateral L5 DRG (Thippeswamy et al. 2001b), 7NI had no effect on nestin$^+$ cell numbers. Scale bars, 100 µm

NO Mediates NRSF/REST mRNA Expression in Satellite Glia Cells

Down regulation of NRSF has been demonstrated in many cells and stem cell models of differentiation to a neuronal phenotype. We therefore addressed NRSF/REST expression in our model. NGF deprived DRG neurons-glia co-culture experiments suggested that nestin$^+$ proliferation and differentiation appears to be regulated by NO, therefore, we tested the effects of a NO donor, DETA-NO and a NOS inhibitor, L-NAME on NRSF/REST mRNA expression in pure glia cultures. DETA-NO treated glia cells showed a four fold increase in NRSF/REST mRNA was compared with control, but treating with L-NAME caused a dramatic reduction in NRSF/REST expression (Fig. 4) suggesting that NRSF/REST expression is mediated by NO in satellite glia cells.

Effect of Axotomy and nNOS Inhibition on Nestin$^+$ Cells in DRGs

To extend our in vitro findings, we investigated the effect of nNOS inhibition in axotomy model in which DRG neurons show increased nNOS expression. There was a significant decrease in the number of nestin$^+$ cells in ipsilateral DRG compared to the contralateral DRG of vehicle control treated animals. In 7NI (neuronal specific NOS inhibitor) treated animals there was a significant increase in the number of nestin$^+$ cells in ipsilateral DRG sections compared to DRG sections from ipsilateral ganglia of animal treated with vehicle control (DMSO) (Fig. 5a,b).

Discussion

Postnatal neurogenesis has been explored extensively in the CNS (Alvarez-Buylla et al. 2001; Merkle and Alvarez-Buylla 2006; Zhu et al. 2006). However, there has been little investigation of neurogenesis and some is still controversial in DRG of PNS (Devor et al. 1985; Devor and Govrin-Lippmann 1991; Farel 2002, 2003; Groves et al. 2003; La Forte et al. 1991; Namaka et al. 2001; Popken and Farel 1997). We have demonstrated here, that inhibition of NO produced in DRG neurons-satellite glia (homologous to Schwann cells) co-cultures, in absence of NGF, has a direct effect on nestin$^+$ cell numbers in vitro and axotomized DRG in vivo. Soluble GC blocking experiments with ODQ suggest a cGMP-independent mechanism of NO in regulating nestin$^+$ cell numbers. We have also demonstrated a master regulator of neuronal genes, NRSF/REST transcription factor expression is regulated by NO in glia cultures.

We have used nestin as a marker for neural stem cells as this intermediate filament protein is highly expressed in these cells and down-regulated as the cells differentiate into neuron or glia cells (Kato et al. 1999; Lendahl et al. 1990; Rice et al. 2003; Woodbury et al. 2000). NO concentrations were altered in glia cultures using a NO donor, DETA-NO

and a NOS inhibitor, L-NAME. In DRG neurons-glia co-cultures and glia cultures we have tested the effect of general NOS blocker L-NAME, which inactivates all three known isoforms of NOS. In vivo, we have used the neuronal NOS blocker; 7NI since nNOS is up regulated specifically in DRG neurons by 7–8 days post-axotomy and 7NI effectively reduces the level of NO with minimum toxic effects (Escott et al. 1998; Holscher 1999). Systemic administration of L-NAME increases mortality due to hypertension and vascular dysfunction (Leung et al. 2003), and 7NI does not block iNOS-mediated NO production by glia cells in DRG neurons-glia co-cultures (Lee and Shin 2002; own observation). We have also used BrdU incorporation and immunocytochemistry to identify neural stem cell proliferation and newly differentiated neurons in vitro and in vivo.

DRG neurons-glia co-cultures were supplemented with fibroblast growth factor (bFGF) as a mitogen to support nestin$^+$ cell proliferation and survival (Namaka et al. 2001) in both control and drug treated samples. In addition, co-cultures were maintained with NGF and some without NGF. NGF was added to the cultures for two reasons: firstly, NGF absence in DRG cultures mimics axotomy (Thippeswamy et al. 2005b, 2006) and increases neuronal NO activity (Thippeswamy et al. 2001a); secondly, it is assumed that newly differentiated neurons may require NGF for their survival (Namaka et al. 2001, Thippeswamy et al. 2005a). NGF presence or absence in the medium had no effect on nestin$^+$ cell numbers in vitro (Fig. 2b). Whether newly differentiated neurons require NGF and/or other factors for survival or not is yet to be investigated. Surprisingly, L-NAME treatment of NGF-supplemented DRG neurons-glia co-cultures decreased the number of nestin$^+$ cells (Fig. 2). This suggests that a basal level of NO in presence of NGF could be protective to newly differentiated neurons or in absence NO (L-NAME treatment), neurons might activate NGF-p75 neurotrophin receptor mediated apoptosis cascade as in forebrain neurons (Volosin et al. 2006). In addition, newly differentiated neurons may be dependent on specific instructive growth factor/s and/or receptor expression for survival, for example, *N*-methyl-D-aspartate (NMDA) glutamate receptor (NMDAR) (Kuo et al. 2005; Tashiro et al. 2006).

Absence of NGF in DRG neurons-glia co-cultures did not change nestin$^+$ cell numbers. NGF-deprivation of DRG neurons in culture mimics axotomy with respect to increase in endogenous NO production by neurons (Thippeswamy et al. 2001b, 2005b, 2006). However, axotomy decreases the number of nestin$^+$ cells in DRG ipsilateral to the nerve section in vivo compared to contralateral intact DRG (Fig. 5). Axotomy deprives DRG neurons of NGF, which is retrogradely transported to DRG neurons from the skin (Nagata et al. 1987), increases nNOS expression in neurons (Zhang et al. 1993; Thippeswamy et al. 2001b, 2005b). So it is plausible that the decrease in the number of nestin$^+$ cells in axotomized DRG could be due to close proximity of these cells with nNOS$^+$ neurons (data not shown). It has been demonstrated that NO inhibits cell proliferation and differentiation into neurons (Cardenas et al. 2005; Ciani et al. 2004, 2006; Matarredona et al. 2004; Packer et al. 2003; Peunova and Enikolopov 1995). Removing NO, by treating NGF-deprived DRG neurons-glia co-cultures with L-NAME or by treating axotomized rats with 7NI, significantly increases nestin$^+$ cell numbers (Figs. 2b, 5) suggesting that NO inhibits neural stem cell proliferation and possibly differentiation into neurons. This is possibly a compensatory mechanism to replace dead neurons since NO inhibition in NGF-depleted DRG cultures or axotomized DRG causes neuronal apoptosis (Thippeswamy et al. 2001a,b).

BrdU incorporation and subsequent double immunostaining for BrdU and neuronal specific marker, PGP 9.5 suggested that some of these nestin$^+$ cells differentiate into neurons or neuron-like phenotypes (Fig. 3). Kuo et al. (2005) have shown that nestin$^+$ cells also express neuronal markers such as neurofilament 200, beta III-tubulin, PGP 9.5, growth associated protein 43, trkA and calcitonin gene-related peptide in vivo over a period of one month following axotomy, suggesting the positive identification of newly differentiated neurons from precursor cells. In the present study, although there was a significant difference in nestin$^+$ cell numbers between control and L-NAME treated cultures, there were a few fully differentiated PGP$^+$ neurons with BrdU nuclear staining (Fig. 3) and several neuron-like phenotypes that were also BrdU$^+$. This demonstrates that postnatal neurogenesis occurs in DRG. Long-term culture experiments would be useful to further explore the different stages of neurogenesis and various regulatory molecules involved in postnatal neurogenesis in DRG.

We explored the mechanism of action of NO on nestin$^+$ cell proliferation and differentiation. There are two major NO pathways; cGMP-dependent and -independent mechanisms. To determine whether cGMP is involved in nestin$^+$ cells proliferation and/or differentiation, we tested the sGC blocker, ODQ. We have shown previously that ODQ treatment of DRG in vitro and in vivo completely suppresses cGMP expression (Thippeswamy and Morris 1997b, 2001; Thippeswamy et al. 2005a). In the present study, ODQ treatment of DRG neurons-glia co-cultures, either in presence or absence of NGF, did not change the nestin$^+$ cell numbers (Fig. 2a,c). This suggests that NO directly regulates their proliferation in a cGMP-independent mechanism, either via S-nitrosylation of intracellular proteins or surface receptor/s activation. In recent years, S-nitrosylation has emerged as an important NO mechanism of post-translational modification of protein function

(Hess et al. 2005). It has been demonstrated that endogenous NO could directly activate Ras and its downstream effectors, the Erk pathway, in response to NMDA receptor activation (Yun et al. 1998).

To further delineate the mechanism of NO action in postnatal neurogenesis, we hypothesized that NRSF/REST, a master regulator of neuronal genes in non-neuronal cells, may be modulated by NO in satellite glia cultures. It has been suggested that differentiation of neural stem (nestin$^+$) cells into neurons occurs in four steps which is characterized by the expression of genes such as those that encode SCG10, sodium channel type II, synapsin I, glutamate receptor/s, and the acetylcholine receptor/s. These genes are the major targets for the NRSF/REST modulation (Chong et al. 1995; Immaneni et al. 2000). NRSF/REST is a zinc finger transcription factor that represses these genes in non-neural tissues and undifferentiated neural precursors during early embryogenesis (Palm et al. 1998). Initially, we analyzed NRSF/REST expression in both DRG neurons-glia co-culture and pure glia cultures. However, due to the complexity and consequences of endogenous NO-NGF interaction in neuron-glial-neuronal communication, and the various cell types of DRG neurons (at least 20 different subtypes have been identified so far) in co-culture, NRSF mRNA expression was investigated only in pure satellite glial cultures. These cultures contained ~70–80% of nestin$^+$ cells (data not shown). Treating them with a NO donor, DETA-NO increases NRSF/REST mRNA 3–4 fold compared to vehicle control (Fig. 4). Cultured glia cells express iNOS and nNOS (data not shown) and L-NAME treatment of these cultures caused a dramatic reduction in NRSF/REST mRNA expression suggesting that NO promotes NRSF/REST expression in satellite glia cultures (nestin$^+$ cells), thus NO inhibits nestin$^+$ cells proliferation and/or differentiation. Our results also support the findings from other groups that NO inhibits neurogenesis (Cardenas et al. 2005; Ciani et al. 2004, 2006; Matarredona et al. 2004; Packer et al. 2003).

In summary, the neural stem cells (nestin$^+$) in DRG normally remain dormant in vivo and in vitro and presence or absence of NGF has no effect on their proliferation in vitro. However, in vivo it appears that axotomy suppresses nestin$^+$ cells during first few days of post-axotomy which could be due to increased NO activity in DRG and the close proximity of nestin$^+$ cells to nNOS$^+$ neurons. Inhibition of NO production in vitro and in vivo promotes nestin$^+$ cells proliferation and possibly early differentiation, which may be mediated in part by NRSF/REST as revealed by qPCR analysis of NRSF/REST mRNA expression in NO-donor or L-NAME treated satellite glia cell culture.

Acknowledgments We wish to thank the University of Liverpool (RDF 4352); the Wellcome Trust (JPQ) for partly-funding this project.

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