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Nitric oxide regulates neurogenesis in adult olfactory epithelium *in vitro*

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ABSTRACT

Nitric oxide regulates neurogenesis in the developing and adult brain. The olfactory epithelium is a site of neurogenesis in the adult and previous studies suggest a role for nitric oxide in this tissue during development. We investigated whether neuronal precursor proliferation and differentiation is regulated by nitric oxide using primary cultures of olfactory epithelial cells and an immortalized, clonal, neuronal precursor cell line derived from adult olfactory epithelium. In these cultures NOS inhibition reduced cell proliferation and stimulated neuronal differentiation, including expression of a voltage-dependent potassium conductance of the delayed rectifier type. In the neuronal precursor cell line, differentiation was associated with a significant decrease in nitric oxide release. In contrast, addition of nitric oxide stimulated proliferation and reduced neuronal differentiation. Nitric oxide regulated olfactory neurogenesis independently of added growth factors. Taken together these results indicate that nitric oxide levels can regulate cell proliferation and neuronal differentiation of olfactory precursor cells.

Introduction

Nitric oxide is a highly diffusible molecule generated by the enzyme nitric oxide synthase (NOS). Three different enzyme isoforms have been identified in mammalian cells: endothelial and neuronal NOS (eNOS and nNOS), which are constitutively expressed in specific tissues and are calcium-stimulated [1,2]; and inducible NOS (iNOS), which is already activated at normal basal levels of intracellular calcium [3,4].

The transient expression of nNOS in different neural structures during brain development suggests that nitric oxide participates in embryonic neurogenesis [5–8]. In the developing nervous system nitric oxide may regulate cell proliferation [9–13], and plays a role in the organization of axonal projection patterns [14,15]. Nitric oxide may also participate in neurogenesis in the adult brain where it usually reduces neuronal precursor proliferation, except after stroke, when it increases proliferation [16]. Nitric oxide donors can assist in recovery after brain injury, partly by increasing neurogenesis in the dentate gyrus and subventricular zone [17–20].

Neurogenesis continues throughout the adult life span in the mammalian olfactory epithelium. In this process olfactory neurons are continually produced from mitotically active basal cells [21,22], giving origin to immature neurons which differentiate into mature sensory neurons [23]. Its accessibility to experimental manipulation makes this tissue a convenient model system for studying the molecular factors that regulate neurogenesis [24–27].

The presence of nNOS in the developing olfactory epithelium and in the regenerating adult olfactory epithelium suggested that nitric oxide may regulate olfactory neurogenesis [7]. This was further confirmed by targeted deletion of nNOS which reduced neuronal precursor proliferation, reducing the pool of immature neurons in the developing olfactory epithelium [28]. In adults lacking nNOS, the number of immature neurons was increased [28]. These observations suggest that nitric oxide may stimulate neuronal precursor proliferation but inhibit their differentiation into immature neurons.

Here, we investigated this hypothesis directly by altering NOS activity in two olfactory culture systems *in vitro*: primary cultures of olfactory epithelium and a neuroblast cell line immortalized from the globose basal cell, the neuronal precursor. Primary cultures of rat olfactory epithelium contain the horizontal basal cell (the putative stem cell [23,29]), the globose basal cell, a population containing a stem-like cell [30] and the immediate neuronal precursor [25,27,30], and the supporting cells which are derived from the globose basal cell [30]. NOS activity was manipulated in these serum-free cultures in the presence and absence of either basic fibroblast growth factor (FGF-2), which stimulates proliferation of neuronal precursors, and transforming growth factor β2

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(TGF-β2), which induces differentiation of the neuronal precursors into neurons *in vitro* [27,31]. A mouse olfactory neuronal precursor cell line [32] was also investigated because it allowed quantification of nitric oxide levels during proliferation and differentiation.

Materials and methods

Overview

Experiments were carried out on primary cultures of rat olfactory epithelial cells [27] and on OLF442, a cell line immortalized from mouse olfactory neuronal precursors [32]. Initial experiments examined the expression of NOS isoforms in these cell cultures. Subsequently nitric oxide levels were altered by NOS inhibition and treatment of the cultures with a nitric oxide donor. In primary cultures, cell proliferation was quantified by counting nuclei that incorporated bromodeoxyuridine (BrdU); differentiation was quantified by counting the numbers of cells expressing neurofilament protein. The effects of alterations in nitric oxide were assessed in the presence and absence of growth factors that induce cell proliferation or differentiation of neuronal precursors. In the immortalized precursor cell line, cell proliferation and viability were quantified using fluorescence activated cell sorting and by counting BrdU-labeled nuclei *in vitro.* Differentiation was quantified by measuring morphological changes. Differentiation of both primary cells and immortalized cells was also assessed using patch-clamp electrophysiology to investigate ion currents induced by NOS inhibition.

Olfactory epithelial cell cultures

In the culture system used here, 5 days after culturing in a serum-free medium containing EGF, the remaining cells comprise the neuronal precursors, the globose basal cells, and other non-neuronal cells of the olfactory epithelium, the horizontal basal cells and the supporting cells [27,31]. EGF stimulates proliferation of the horizontal basal cells [27,33] whereas FGF-2 induces proliferation of the neuronal precursors in olfactory epithelial cultures [25,27,34]. Both growth factors were used to enhance cell proliferation in the present experiments. TGF-β2 induces neuronal differentiation in olfactory epithelial cultures [27,35]. TGF-β2 was used to enhance differentiation in the present experiments. Cultures grown for 5 days in EGF, provided the baseline from which the effects of NOS regulators were observed after 24 h of treatment, in the presence and absence of the growth factors EGF, FGF-2 and TGF-β2.

Animals, tissue dissection and primary cell culture

Adult, outbred Sprague–Dawley rats weighing approximately 300 g were obtained from the Animal House (Faculty of Biological Science, Catholic University, Santiago, Chile) and the Central Animal Breeding House (University of Queensland, Brisbane, Australia). Animals were sacrificed by decapitation after being deeply anaesthetized with sodium pentobarbital (100 mg/kg), according to the guidelines and approval of Animal Ethics Committees at Pontificia Universidad Católica de Chile, Santiago, Chile and Griffith University, Brisbane, Australia. Olfactory epithelial cell suspensions were prepared as described in detail elsewhere [27,31]. Briefly, olfactory mucosae were removed from the septum and immediately placed in Hanks' balanced salt solution (HBSS, Gibco-BRL). The mucosae were incubated for 45 min at 37 °C in a 2.4 U/ml Dispase II (Boehringer, Mannheim), after which the olfactory epithelium was carefully separated from the underlying lamina propria by dissection. Olfactory epithelia were gently triturated by passing them about 20 times through a fire-polished Pasteur pipette, to dissociate the cells.

The resulting cell suspension was transferred to a 15 ml conical tube containing HBSS and centrifuged at 200*g* for 5 min. The supernatant was aspirated and the pellet containing the cells was resuspended in 10 ml of a serum-free medium composed of DMEM (low-glucose, with L-glutamine, Gibco-BRL), with ITS supplement medium (insulin–transferrin–selenium, Gibco-BRL) and with Penicillin–Streptomycin (100 U/ml–0.1 g/ml, Sigma Chemicals Co.). Cells were plated on plastic 4×1.9 cm^2 well culture dishes (Nunc), previously coated with 5 μg/cm^2 human collagen IV (Sigma Chemical Co.) and dried overnight at room temperature under sterile conditions. Cells were plated at a density of approximately 350,000 cells per well in 500 μL of serum-free medium. To purify non-neuronal cell types, the cultures were treated with human recombinant epidermal growth factor (EGF; 25 ng/ml; Sigma Chemical Co.) for 5 days, with a change of medium after 3 days. The cultures were maintained at 37 °C and 10% CO_2.

After 5 days in EGF, the cultures were grown in the presence or absence of growth factors (see below) and examined for cell proliferation and neuronal differentiation after treatments that modulate nitric oxide production (see below). After 24 h of these treatments, the cells were fixed in paraformaldehyde 4% in PBS for immunocytochemistry to analyze cell proliferation and differentiation.

Growth factor treatments

After 5 days in EGF, the cultures were treated for a further 24 h with EGF and FGF-2 (25 and 50 ng/ml, respectively) alone, and with or without nitric oxide modulators. In another set of experiments, after 5 days growing in EGF, the cultures were treated for a further 24 h with TGF-β2 (10 ng/ml) with or without nitric oxide modulators. Some cultures were grown for 5 days in EGF followed by 24 h in a single growth factor: EGF (25 ng/ml), FGF-2 (50 ng/ml) or TGF-β2 (10 ng/ml) and fixed for immunochemistry to identify expression of NOS isoforms.

Analysis of cell proliferation and neuronal differentiation

Cell proliferation was quantified using the thymidine analog, bromodeoxyuridine (BrdU, 2 mM; Sigma Chemical Co.). BrdU was added to the cultures for the last 3 h *in vitro* before fixation and immunochemistry, as described below. Neuronal differentiation was assessed from the number of cells expressing neuronal proteins determined by immunochemistry (see below). Functional differentiation was assessed by measuring voltage-dependent whole cell currents (see below).

Nitric oxide pharmacology

Two NOS inhibitors were used to reduce nitric oxide levels in the cultures: TRIM (1-(2-trifluoromethylphenyl) imidazole) (Calbiochem), which inhibits nNOS and iNOS with a similar efficiency (IC_{50}: 28.2 and 27 μM, respectively) and L-NIL (L-N^6-(1-iminoethyl)lysine, DiHCl), (Calbiochem), which inhibits iNOS at low concentrations (IC_{50}: 3.3 μM) and nNOS at high concentrations (IC_{50}: 92 μM). Neither TRIM nor L-NIL inhibits completely eNOS at the concentrations used here. Nitric oxide levels in the cultures were raised with the nitric oxide donor SNAP (*S*-nitroso-*N*-acetylpenicillamine). The primary cultures were grown for 5 days in EGF and then treated with 10 or 100 μM TRIM, L-NIL or SNAP, in the presence or absence of additional factors.

Immortalized precursor cell cultures

OLF442 can be switched between proliferation and differentiation by altering the serum content of the culture medium. When grown in serum-containing medium, OLF442 cells proliferate and when grown in serum-free medium they differentiate, extending long processes, increasing the expression of neurofilaments and

B50/GAP43 protein and reducing the expression of glial fibrillary acidic protein (GFAP) [32]. In the present experiments, OLF442 cultures were grown until confluent in serum-containing medium and then passaged, providing the baseline from which the effects of NOS regulators were observed after 24 h of treatment, in the presence and absence of serum.

OLF442 cells were grown in Dulbecco modified Eagle's medium (DMEM, low-glucose, with L-glutamine, Gibco-BRL), supplemented with 10% fetal calf serum (FCS, Gibco) and 100 U/ml (0.1 g) of Penicillin–Streptomycin (Sigma). The cultures were maintained at 37 °C in a humid atmosphere of 5% CO_2. For differentiation induced by serum-depletion, cells were grown in the DMEM medium (above), but with the serum replaced with 1% bovine serum albumin (BSA, Sigma). For nitric oxide manipulation, OLF442 cells were grown with or without serum and then treated with 10 or 100 μM TRIM, L-NIL or SNAP.

RT-PCR for NOS isoforms

OLF442 cells from one confluent 75 cm^2 flask were harvested by scraping, washed once in PBS. Total RNA was isolated by using the RNeasy mini Kit. Contamination with genomic DNA was eliminated by a DNase1 treatment (Qiagen, Valencia, CA). mRNA was reverse-transcribed, and cDNA underwent 40 rounds of amplification (2027 thermo cycler) using Taq DNA polymerase kit (Invitrogen). The primers used were: for nNOS, forward primer: CC TGTGATGTGTTCTGTGTGG and reverse primer: CGGCTGGATTTAGG ACTTTG, for eNOS forward primer: TGCCTGTGAAACCTTCTGTG and GGTGGATTTGCTGCTCTGT and for iNOS forward primer: TTGT CCTACACCACACCAAA and reverse primer: GCCTCCAATCTCTGCCT ATC. The expression of the housekeeping gene HPRT (Hypoxanthine phosphoribosyl transferase-encoding gene) was used as internal quantitative control. All cDNA reactions were subjected to an initial denaturing cycle of 5 min. at 95 °C, an annealing temperature of 53 °C and a final elongation step of 10 min. at 72 °C. The final concentrations of the PCR reagents used were as follows: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 μM of both forward and reverse primers for nNOS, eNOS and iNOS, plus 1 U Taq DNA polymerase (Invitrogen). PCR products were electrophoresed through a 2% agarose gel and visualized by ethidium bromide staining.

Analysis of cell proliferation, cell viability and differentiation

Prior to inhibitor treatment, the culture surface was washed once with 5 ml of HBSS which was then aspirated and replaced with DMEM-F12, supplemented with either L-NIL or TRIM at final concentrations of 1 mM, 100 μM and 10 μM. Cells were then incubated for 24 h in 5% CO_2 at 37 °C. Following incubation, cells were harvested by trypsinisation, washed once in PBS and centrifuged at 300*g* for 5 min. The supernatant was aspirated and the pellet was resuspended in 1 ml PBS. Controls included cells incubated for 24 h in the presence of serum-containing and serum-free culture media supplemented with DMSO at the concentration used as diluent for the NOS inhibitors.

Analysis of cell viability

Cell viability was assessed with the membrane impermeable dye, 7AAD (Sigma). Approximately 5×10^5cells/ml were incubated with 10 μg/ml of 7AAD for 30 min at 4 °C, protected from light. Cells were washed three times in 5 ml PBS, centrifuged at 300*g* for 5 min. Following the final wash, the pellet was resuspended in 200 μL PBS and viability analyzed via flow cytometry (see below). The proportion of viable cells was determined as the percentage of cells which had not incorporated 7AAD.

Analysis of cell proliferation

Cell proliferation in OLF442 was assessed via flow cytometry after treatment for 24 h with NOS inhibitors. Two assays were used: counting the number of cells in *S*-phase and counting the number of cells labeled after 8 h incubation in ethynyldeoxyuridine (EdU). EdU is a thymidine analog with an acetylene group to which a fluorescent azide binds covalently for detection [36]. Untreated OLF442 cells acted as controls: cells in FCS-medium were the baseline for NOS treatments while cells in serum-free medium were a positive control, because of the known anti-proliferative effect of this medium on these cells [32]. For EdU analysis of cell proliferation a commercially available kit was used (Click-iT EdU 488 system, Invitrogen). All components were used according to manufacturer's instructions. Cells were grown in serum-containing or serum-free medium for 24 h in the NOS inhibitors at 5% CO_2 at 37 °C. EdU (10 μM) was added to the culture medium for the final 8 h. Cells were then harvested by trypsinisation and washed once in 5 ml PBS/1% BSA, centrifuged at 300*g* for 5 min and resuspended in 100 μL PBS/1% BSA, to which 100 μL of Click-iT fixative (Component D) was added, taking care to mix thoroughly. Cells were incubated for 15 min at room temperature, protected from the light. Following incubation cells were washed once with 3 ml PBS/1% BSA, centrifuged at 300*g* for 5 min. The supernatant was aspirated and the pellet thoroughly resuspended in 100 μL of 1 × saponin-based permeabilisation buffer and mixed well. 0.5 ml of Click-iT reaction cocktail was then added to each tube and mixed well. The presence of EdU was detected using Alexa-488 azide, present in the Click-iT reaction cocktail. Cells were incubated for 30 min at room temperature, protected from light. Cells were then washed once at 300*g* for 5 min with 3 ml of 1× saponin-based permeabilisation reagent. The pellet was then dislodged and resuspended. Cell cycle analysis was undertaken on the same cells by adding 5 μL of RNAse A (Component L) to each tube followed by 2 μL 7AAD (in preference to PI which quenches the fluorescent signal when using Alexa-488 azide [37]. Following thorough mixing the cell were incubated at room temperature for 30 min, protected from light. Proliferation and cell cycle were assessed via flow cytometry (see below). Controls included cells not treated with NOS inhibitors and flow cytometry controls included cells not treated with EdU.

Flow cytometry

Cell viability, *S*-phase and EdU-labeling were assessed using a BD FACSAria™ Cell Sorter (Becton Dickinson, CA) equipped with 488, 633 and 405 nm lasers. Cell viability via 7AAD was assessed by excitation at 633 with a red emission filter (660/20 nm) and logarithmic amplification. EdU was detected via Alexa Fluor 488 azide by excitation at 488 nm with a green emission filter (530/30 nm) and logarithmic amplification. For cell cycle analysis in conjunction with EdU, 7AAD was detected by excitation at 633 with a red emission filter (660/20 nm) and analyzed via linear amplification. All data were analyzed using FACSDiva™ software (Becton Dickinson). All data represent the average of three independent experiments performed independently on three different aliquots of cells. The number of cells counted for each aliquot and treatment averaged 9308 (±295). Data were subject to one-way analysis of variance, two-tailed, with $\alpha = 0.01$ (GraphPad Prism 4.0, GraphPad Software, San Diego, CA, USA).

Differentiation

Differentiation of OLF442 cells was assessed by quantifying morphology *in vitro* using Image J software to measure the length of neurites to give a total cell length (see below).

Nitric oxide production

OLF442 cells were cultured for 24 h in 24-well plates in BSA or in FCS media, in the presence or absence of 100 and 1000 μM TRIM and L-NIL. In the last 30 min of treatment, cells were incubated with PBS containing 100 μM L-arginine and 6 μCi/ml of L-[^{3}H]

arginine (30 min, 37 °C) in the absence or presence of same inhibitors, as above. The cells were then washed in sterile PBS, digested in 95% formic acid and passed through a cation ion-exchange resin Dowex-50W (50 × 8–200) to bind tritiated and non-tritiated L-arginine. The production of nitric oxide was measured from the H_2O eluate containing L-[^{3}H] citrulline. An aliquot of 800 μL of this eluate was added to 4 ml of scintillation liquid for counting of radiolabeled products (Ecoscint, National Diagnostics). Background activity obtained from tubes with no supernatant added was subtracted from all samples [38].

Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde, washed with phosphate-buffered saline (PBS, pH 7.2) and stored in PBS at 4 °C. Immunocytochemistry was performed with monoclonal antibodies to: neuronal proteins (anti-β-tubulin type III, TβIII, 1:100, Sigma Chemical Co.; anti-neurofilament, NF, 1:500, Sigma Chemical Co.; globose basal cell proteins (anti-GBC-1, 1:100, gift of J. Schwob, Tufts University, Boston, USA); epithelial cell proteins (anti-cytokeratin CK14, 1:200, Sigma Chemical Co.); supporting cells (SUS1, gift of F. Margolis, 1:50); and BrdU (Sigma Chemical Co.; 1:200) and the polyclonal rabbit antibodies nNOS, eNOS and iNOS (1:500, Sigma Chemical Co.);. Cells were incubated with the primary antibody overnight at 4 °C in blocking solution, followed by three 5 min washes and a blocking solution for 60 min at room temperature in PBS containing the appropriate secondary antibody conjugated with horseradish peroxidase (1:50), alkaline phosphatase, (1:50, Sigma Chemical Co.) or the fluorescent agents Alexa 486 or 555 (1:500, molecular probes), followed by three 5 min PBS washes. The endogenous peroxidase activity was quenched with 0.6% H_2O_2 added to the methanol or PBS. Non-specific staining was blocked by incubation with non-immune serum, appropriate for the secondary antibody, at a dilution of 1:10 in PBS containing 5% non-fat powder milk, 4% bovine serum albumin (Sigma Chemical Co.) and 0.1% Triton X-100, for 60 min at room temperature. Finally, the cells were incubated in Fast DAB (Sigma Chemical Co.) for the peroxidase staining or Fast Red (Sigma Chemical Co.) for the alkaline phosphatase staining, followed by three 5-min PBS washes. For immunofluorescence the nuclei were stained with Hoechst 333242 (1:1000) for 10 min. Immunofluorescence and immunoperoxidase were used in all the experiments, but mainly immunofluorescence was used for the double marking experiments.

Quantitative analysis of cell cultures

All cell counting and length measurements were performed after the cultures were fixed and stained, with the observer blind to the experimental condition. Multiple images (each 200 μm^2) of cells were photographed with a CCD video camera (Sensicam, Cooke Corporation, Michigan, USA), coupled to an inverted microscope (Olympus IX70, 10× objective). In each primary culture, all cells in the well were considered in each culture well (5–20 images per well, average 12). In each OLF442 culture, a transect of optical fields through the midline of the well was photographed (9–12 images per well). For each data point experiments were repeated at least three times. In every image, all cells were counted manually using the software Scion Image (Scion Corporation, USA). In addition, OLF442 cell length was measured from the images using Image J (NIH, Bethesda MD, USA). Data are expressed as percentage (mean ± SEM) of immuno-positive cells expressed as a proportion of the total cells and were compared to the control group. Statistical comparisons of three or more groups were done with Kruskal–Wallis one-way analysis of variance and Dunn's post-test. Comparisons between two groups were done with Mann–Whitney U-test (GraphPad Prism 4.0, GraphPad Software, San Diego, CA, USA). For illustrations, digital images of cells were adjusted for brightness and contrast, if appropriate, using Adobe PhotoShop 6.0 (Adobe Systems Incorporated). Immunoblot films were scanned to 600 dpi resolution and the optical density of the bands was quantified using the Image J software.

Electrophysiology

Ionic currents were studied using the whole cell patch-clamp technique. Primary cultures were plated onto 12 mm round coverslips coated with 5 μg/cm^2 human collagen IV (Sigma Chemical Co.), and treated for 24 h with 100 and 1000 μM TRIM or L-NIL in serum-free medium with and without growth factors. OLF442 cells were plated onto 12 mm round coverslips and treated with 100 and 1000 μM TRIM or L-NIL for 24 h in FCS-medium. All experiments were performed at 22–23 °C.

Electrical recordings were made with an Axopatch-1D amplifier (Axon instruments, Union City, CA). Experimental protocols and data analysis were conducted using pClamp 6.0 (Axon Instruments). Current signals from the amplifier were filtered at 10 kHz. Recording pipettes were made of untreated soda lime glass micro-hematocrit capillary tubes, (Globe Scientific Inc.). Capillaries were drawn in a vertical pipette puller (model 700C, David Kopf instruments Tujunga, California) and filled with internal solution (in mM): 140 KCl, 10 Hepes, 2 EGTA, pH 7.2. Tip resistances were 2–4 MΩ. Cells were transferred from the culture media to a recording chamber filled with extracellular solution (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 Glucose, pH 7.2. Changes in the extracellular K^+ concentration were made by equimolar substitution of NaCl for KCl, thus the high K^+ solution contained (in mM): 105 NaCl, 40 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 Glucose, pH 7.2. In the TEA solution, Na^+ was replaced equimolarly with TEA (in mM): 120 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 Glucose, 20 TEA–Cl, pH 7.2. TEA and high K^+ solutions were applied by pressure from an extracellular puffer pipette (1–2 μm tip diameter) positioned at 10 μm from the cell. Cells were clamped at a holding potential of −70 mV and stimulated with depolarizing voltage pulses between −70 and 70 mV, in increments of 10 mV. Tail currents were obtained with depolarizing pulses between −120 and +20 mV, in increments of 10 mV from a prepulse of +30 mV. Current signals from the amplifier were filtered at 10 kHz.

Results

All NOS isoforms were expressed by neuronal precursors in primary cultures

Neuronal precursor cells in the primary cultures were identified with anti-GBC-1, a globose basal cell marker [39]. All NOS isoforms were expressed by these cells (Fig. 1). There was no non-specific labeling in the no primary controls (data not shown). All NOS isoforms were also observed in other cell types in the cultures (data not shown) identified with cell type-specific antibodies: anti-cytokeratin-14 (CK-14), a horizontal basal cell marker [40,41], anti-neuron specific tubulin (TβIII), a marker for immature olfactory neurons [42]; and anti-SUS-1, a supporting cell marker, [41,43].

NOS expression was studied in OLF442 cells grown under proliferative (10% FCS) or differentiating conditions (1% BSA, serum-free) using immunofluorescence and RT-PCR, for determining the expression of the NOS isoforms. In both conditions all the cells expressed the three NOS isoforms, as indicated by immunofluorescence (Fig. 2A–F) and RT-PCR (Fig. 2G). The sequencing of the PCR products confirmed the identities of the NOS isoforms.

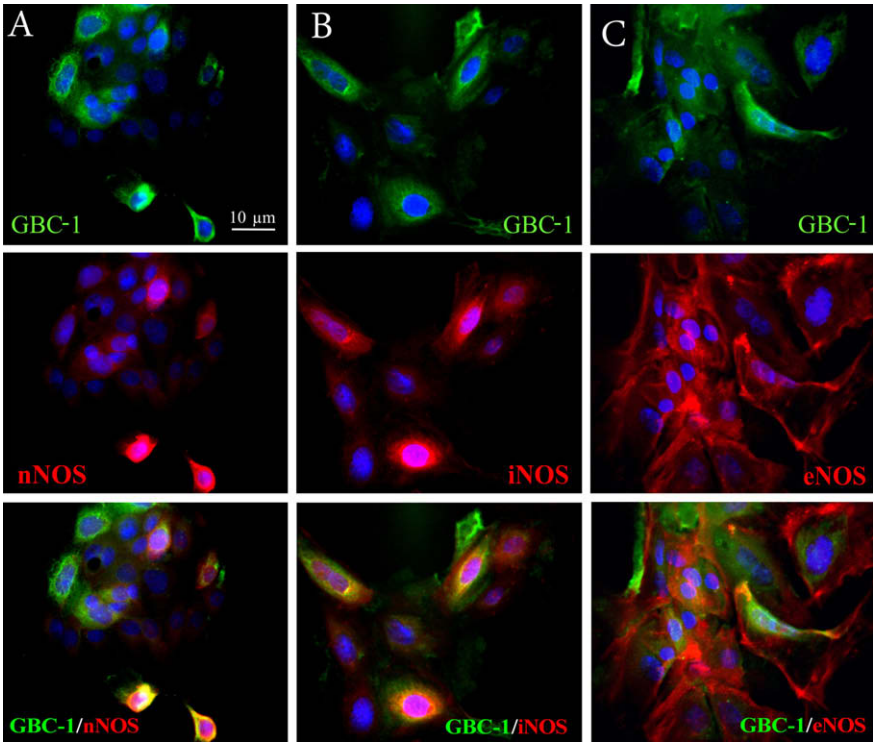

Fig. 1. Neuronal precursors express all NOS isoforms. Double immunofluorescence labelling of the neuronal precursors in green (globose basal cells, GBC-1) and the NOS isoforms in red. (A) GBC-1 and nNOS. (B) GBC-1 and iNOS. (C) GBC-1 and eNOS. Nuclei are shown in blue. Bar: 10 μm.

NOS inhibition reduced cell proliferation and increased neuronal precursor differentiation

In the primary cultures treated with EGF and FGF-2, NOS inhibition reduced cell proliferation, decreasing the proportion of BrdU-positive cells (Fig. 3A and C). At 10 and 100 μM, L-NIL reduced the percentage of proliferating cells from 15.7 ± 1.7% (56.3 ± 0.3 cells per well, 3 wells; 1086 cells in total) to 2.1 ± 0.6% (4.3 ± 0.05 cells per well, 3 wells; 756 cells in total) and 0.7 ± 0.2% of the cells (9.0 ± 0.1 cells per well, 4 wells;3351 cells in total), respectively. TRIM at the same concentrations reduced proliferation from 34.5 ± 3.9% (18.1 ± 3.7 cells per well, 4 wells; 656 cells in total) to 22.0 ± 2.7% (17.4 ± 1.9 cells per well, 3 wells; 1359 cells in total) and 13.5 ± 1.5% (11 ± 1.3 cells per well, 3 wells; 874 cells in total).

In primary cultures treated with TGF-β2, TRIM increased neuronal differentiation. At 10 μM and at 100 μM TRIM, the proportion of neurofilament-positive cells increased from 37.05 ± 3.2% (19.3 ± 3.5 cells per well, 4 wells; 653 cells in total) to 51.4 ± 5.2% (60.3 ± 8.8 cells per well, 3 wells; 858 cells in total) and 69.5 ± 3.1% (120.2 ± 22.8 cells per well, 4 wells; 1952 cells in total), respectively, ($p < 0.05$). L-NIL also significantly induced differentiation at 10 μM and 100 μM ($p < 0.05$), increasing the proportion of neurofilament-positive cells from 49.3 ± 3.4 (30.2 ± 5.0 cells per well, 4 wells; 1415 cells in total) to 75.6 ± 3.5% (32.3 ± 9.8 cells per well, 3 wells; 290 cells in total) and 67.9 ± 2.7% (72.9 ± 13.0 cells per well, 4 wells; 2024 cells in total) (Fig. 3 B and D).

Cell proliferation and differentiation were quantified in OLF442 cell cultures by counting the number of BrdU-labeled cells and measuring cell length in different culture media (Fig. 4). Both TRIM and L-NIL decreased the proportion of BrdU-positive cells in a dose-dependent manner. In control cultures without NOS inhibitors, the percentage of BrdU-positive cells was 78.0 ± 1.9% (of a total of 478 cells). The largest effect was observed with 1000 μM TRIM (19.6 ± 1.9%, of a total of 1190 cells), a concentration that inhibits all three NOS isoforms (Fig. 4I). As with the flow cytometric analysis, L-NIL was less effective at inhibiting proliferation. TRIM, but not L-NIL, also induced morphological differentiation of OLF442 cells (Fig. 4). The mean length of OLF442 cells grown in control condition was 24.5 ± 0.8 μm ($n = 719$ cells). TRIM increased the cell length at all concentrations ($p < 0.05$). These effects of NOS inhibition were observed when cells were grown in serum-containing medium, conditions that do not usually induce differentiation in these cells.

Flow cytometric analysis demonstrated that cell proliferation in OLF442 was reduced, as predicted, when OLF442 cells were grown in serum-free medium, indicated by fewer cells in *S*-phase and fewer cells labeled with EdU (Fig. 5A and B), confirming the utility of the method. NOS inhibition also reduced proliferation, as demonstrated in primary cultures. Cells treated with TRIM showed a dose-dependent reduction in the proportion of cells in *S*-phase (Fig. 5C; $F_{3,11} = 17.11$; $p = 0.0008$). Similarly, the proportion of EdU-labeled cells was also reduced (Fig. 5D; $F_{3,11} = 16.03$; $p = 0.001$). L-NIL had a smaller effect on proliferation with a smaller dose-dependent reduction of cells in *S*-phase (Fig. 5E; $F_{3,11} = 7.829$; $p = 0.009$) and no significant reduction in EdU-labeled cells (Fig. 5F; $F_{3,11} = 2.62$). The FCS controls in A and B are the same data as the 0 μM controls in Fig. 5C–F, because all experiments were performed at the same time.

NOS inhibition and cell viability

OLF 442 cells were grown in serum-containing medium and treated with NOS inhibitors over a concentration range from 0 to 1000 μM. TRIM reduced the number of viable cells at high concentration (Fig. 5G; $F_{3,35} = 5.79$; $p = 0.003$). Post-hoc tests (Bonferroni's multiple comparison test, $p < 0.05$) indicated this difference was due to treatment with 1000 μM TRIM, which was significantly different from all other concentrations, which were not different from each other. L-NIL also reduced viability (Fig. 5H; $F_{3,35} = 4.06$; $p = 0.01$). Post-hoc tests indicated significant differences between

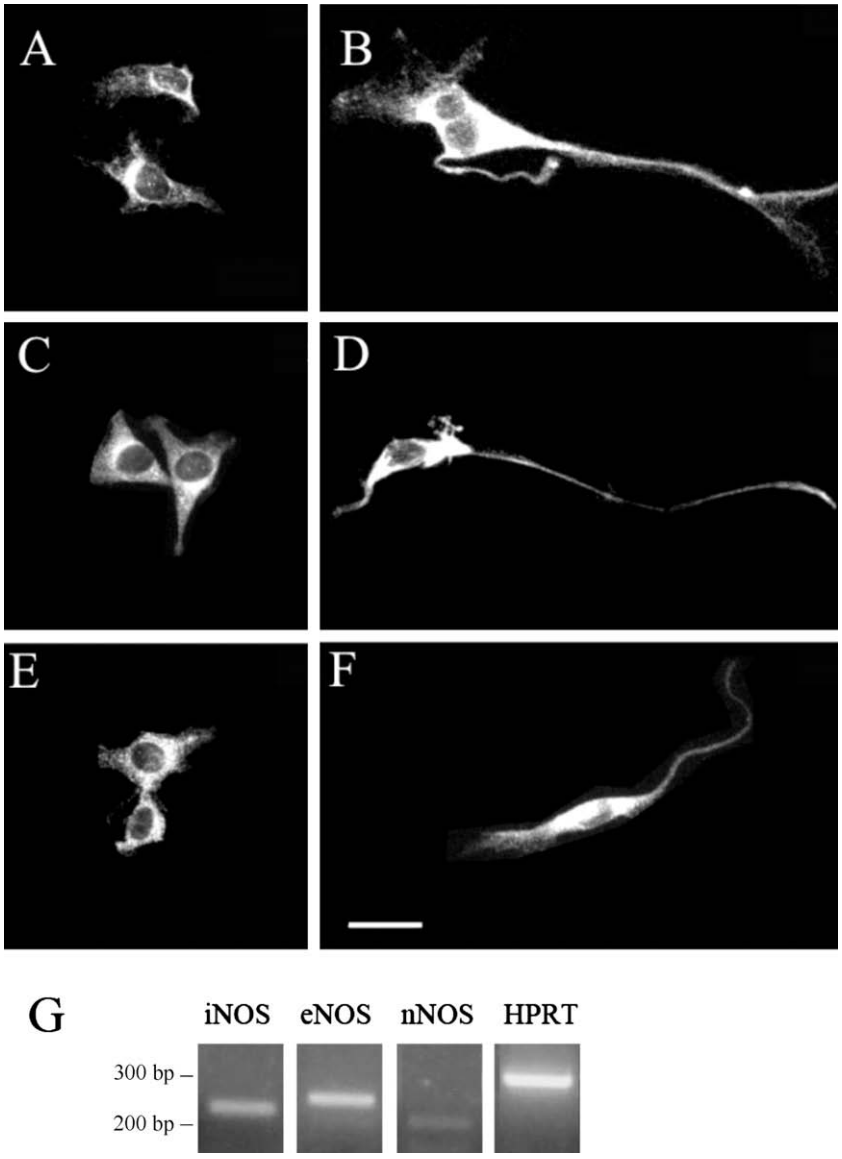

Fig. 2. Immortalized neuroblasts (OLF442) express all NOS isoforms during proliferation and differentiation. (A–C) OLF442 cells grown for 36 h in serum-containing medium (10% FCS). (D–F) OLF442 cells grown for 24 h in serum-free medium (1% BSA). Cells were immunolabeled for nNOS (A and B), iNOS (C and D), and eNOS (E and F) Bar: 10 μm. (G) RT-PCR for the three NOS isoforms and the loading control (HPRT) of OLF442 cells grown in 10% FCS.

100 and 1000 μM and the lower concentrations. These effects were consistent, although relatively small. Cell viability in control cultures (0 μM) was 88%; 1000 μM TRIM further reduced viability by an additional 8% (Fig. 4G) with similar effects of L-NIL (8–11%; Fig. 5H).

Nitric oxide release increased cell proliferation and reduced neuronal differentiation

NOS inhibitors reduced nitric oxide availability in cells. To increase intracellular nitric oxide in primary cell cultures, they were treated with SNAP, a nitric oxide donor that leads to nitric oxide release independently of NOS. SNAP treatment increased cell proliferation in the presence of EGF and FGF-2 and reduced neuronal differentiation in the presence of TGF-β2 (Fig. 6). In control cultures treated with EGF/FGF-2, 42.6 ± 2.6% of the cells (9.0 ± 0.8 cells per well, 4 wells) were BrdU-positive. SNAP increased significantly the number and proportion of dividing cells: 28.3 ± 4.5 cells per well, 4 wells (61.6 ± 2.9%), 10 μM; and 21.8 ± 3.6 cells per well, 3 wells (57.1 ± 3.3%), 100 μM ($p < 0.05$). In control cultures treated with TGF-β2, 36.1 ± 6.9% of the cells (12.6 ± 3.3 cells per well, 3 wells) were neurofilament-positive. 100 μM SNAP reduced significantly the number and proportion of differentiated neurons: 3.0 ± 0.4 cells per well, 3 wells (20.3 ± 1.5%, $p < 0.05$).

Nitric oxide regulates cell proliferation and differentiation in the absence of growth factors

The initial experiments asked whether nitric oxide would modulate cell proliferation and neuronal differentiation in primary cell cultures from baselines set by concomitant growth factor stimulation. The experiments were repeated in the absence of growth factors (i.e. in serum- and growth factor-free medium). NOS inhibition had no effect on cell proliferation. In control cultures the number and proportion of BrdU-positive cells were very low (2.9 ± 1.2 cells per well, 3 wells, 1.65 ± 0.5%; 1832 cells in total). With this already low rate of proliferation, NOS inhibition had no further effect ($p > 0.05$; Fig. 7A). NOS inhibition significantly increased the

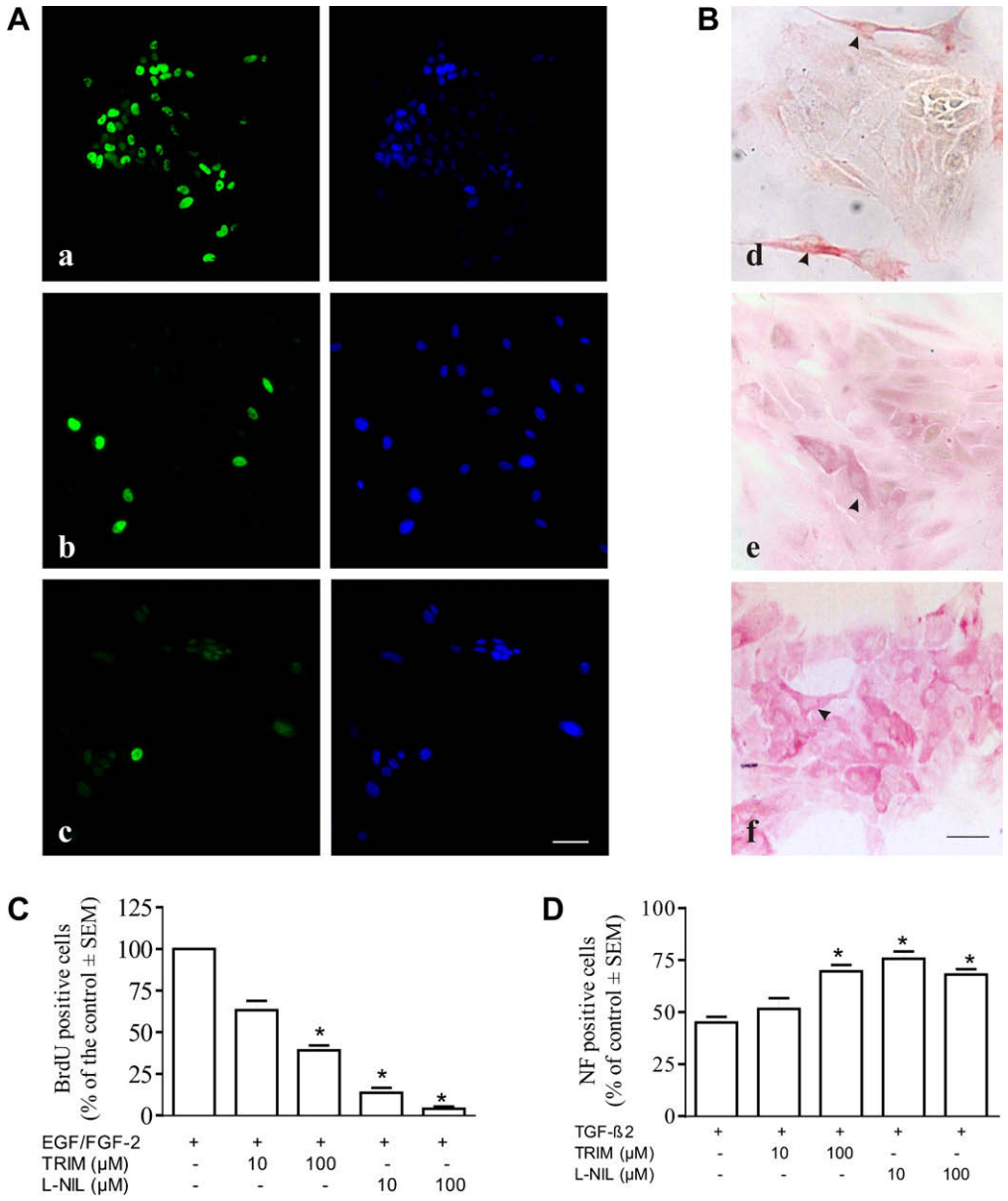

Fig. 3. NOS inhibition decreased cell proliferation and increased neuronal differentiation in primary cultures of olfactory epithelial cells. (A): Dividing cells are labeled with bromodeoxyuridine (BrdU, green in panel on left). All nuclei are labeled with Hoechst 333242 (blue in panel on right). Cells were treated with the NOS inhibitor at a, 0 μM; b, 10 μM or c, 100 μM. Bar: 10 μm. Cells were treated with the NOS inhibitor, TRIM, at a, 0 μM; b, 10 μM or c, 100 μM. Examples of positive cells are indicated by the green nuclei. Bar: 10 μm. (B) Differentiated neurons are labeled with neurofilament (NF, pink). Cells were treated with the NOS inhibitor, TRIM, at d, 0 μM, e, 10 μM or f, 100 μM. Positive cells are shown with a black arrow. (C) Quantification of BrdU-positive cells treated with the NOS inhibitors, TRIM and L-NIL, expressed as percentage of the control ± SEM. (D) Quantification of NF-positive cells treated with the NOS inhibitors TRIM and L-NIL, expressed as percentage of control ± SEM. $^{*}p < 0.05$, statistically different from the control group (Kruskal–Wallis test). All quantification was performed with observer blind to treatment.

number and proportion of differentiated cells (Fig. 7B). In control cultures, the proportion of differentiated cells expressing neurofilament was 33.7 ± 2.4% (10.25 ± 1.6 cells per well, $n = 4$ wells; 293 cells in total). This was increased significantly by treatment with TRIM (100 μM: 52.6 ± 4.4%, 28.0 ± 2.9 cells per well, $n = 4$; 853 cells in total, $p < 0.05$), and L-NIL (10 μM: 49.4 ± 43.1%, 14.5 ± 2.0 cells per well, $n = 4$; 414 cells in total, $p < 0.05$; 100 μM: 55.4 ± 2.7%, 17.4 ± 4.2 cells per well, $n = 4$; 314 cells in total, $p < 0.05$). Nitric oxide donation increased proliferation and decreased differentiation (Fig. 7C and D). Treatment with 10 and 100 μM SNAP increased the proportions of BrdU-positive cells from 5.2 ± 1.3% (9.5 ± 1.8 cells per well, $n = 3$ wells; 3756 cells in total) to 21.7 ± 2.8% (19.0 ± 2.6 cells per well, $n = 4$ wells; 2725 cells in total) and 21.9 ± 4.7% (22.0 ± 3.5 cells per well, $n = 3$ wells; 1779 cells in total), respectively, ($p < 0.05$), while significantly reducing the proportion of neurofilament-positive cells from 39.4 ± 2.6% (31.6 ± 5.3 cells per well, $n = 4$ wells; 1792 cells in total) to 21.8 ± 2.7% (14.0 ± 3.0 cells per well, $n = 4$; 1176 cells in total) and 22.3 ± 2.7% (42.8 ± 10.3 cells per well, $n = 3$ wells; 3822 cells in total), respectively, $p < 0.05$).

NOS inhibition induced an outwardly rectifying, voltage-dependent, potassium current

Whole cell recordings in voltage clamp mode were performed on primary cultures and OLF442 cells. The baseline conditions for the primary cultures were cells grown for 5d in EGF, followed by 24 h in TGF-β2, to induce differentiation. The baseline

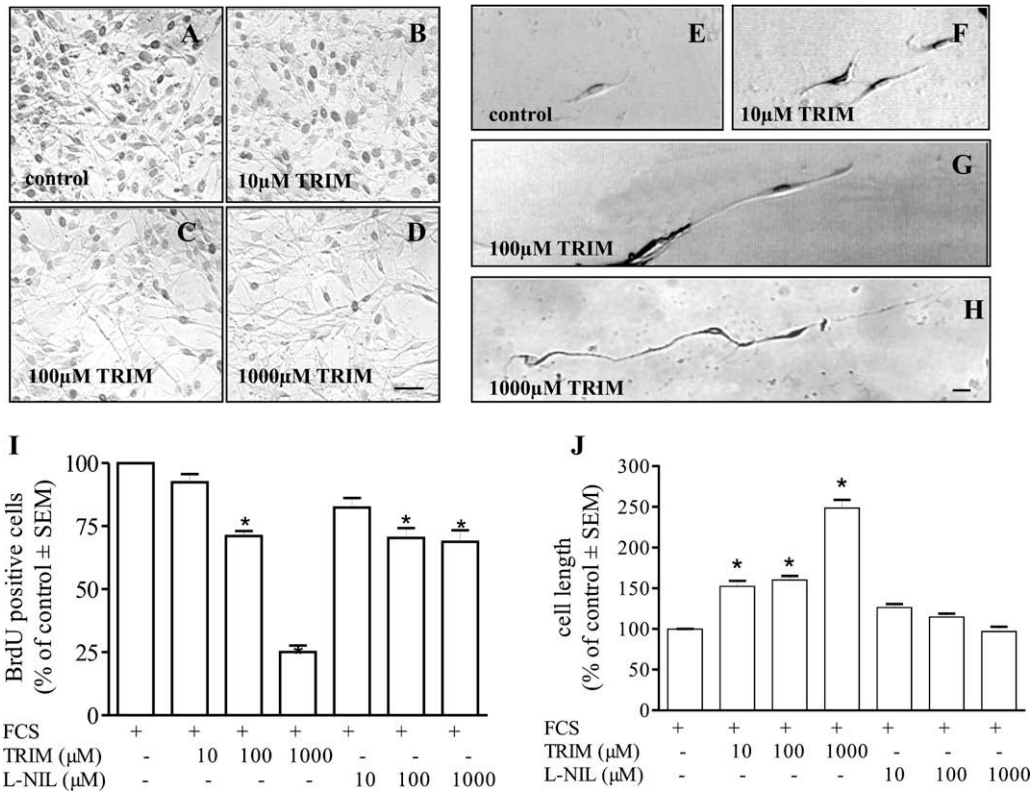

Fig. 4. NOS inhibition decreased cell proliferation and induced morphological differentiation in OLF442 cells. OLF442 cells were grown in serum-containing medium (10% FCS) and treated with TRIM at (A), 0 μM; (B), 10 μM; (C), 100 μM; (D), 1000 μM. BrdU-labeled nuclei show as dark dots. Bar 20 μm. (E–H) Morphological differentiation is demonstrated in differential interference contrast images of cells grown as in (A–D). Bar: 10 μm. (I) Quantification of BrdU-positive cells treated with the NOS inhibitors, TRIM and L-NIL expressed as percentage of control ± SEM. (J) Quantification of the total cell length under different concentrations of TRIM or L-NIL, expressed as percentage of control ± SEM. $^{*}p < 0.05$ statistically different from control group (Dunn's test, after Kruskal–Wallis test, $p < 0.001$). All quantification was performed with observer blind to treatment.

conditions for OLF442 cells were cells grown in serum-containing medium, followed by 24 h in serum-free medium, to induce differentiation.

In primary cultures, most of the cells grown in TGF-β2 medium in the absence of NOS inhibitors presented a flat-epithelioid morphology (Fig. 8A), but a small fraction (2%) of the cells were bipolar. After treatment with the inhibitors TRIM and L-NIL at 10 and 100 μM for 24 h, a larger fraction (approximately 20%) of the cells adopted a bipolar shape (Fig. 8B). Bipolar cells in the presence of TRIM expressed a voltage-dependent outward current (Fig. 8D), whereas flat-epithelioid cells and bipolar cells without TRIM were devoid of currents (Fig. 8C). In cells treated with 10 μM L-NIL the mean current in response to a pulse from −70 (holding potential) to 70 mV was 664 ± 67.6 pA (range 350–990 pA, $n = 8$), whereas with 100 μM TRIM or L-NIL the mean current was 2290 ± 350 pA (range 1033–4200 pA, n = 10) and 1559 ± 308 pA (range 750–3200 pA, $n = 10$), respectively, (Fig. 8E). The shapes of the outward currents and the I–V curve (Fig. 8D and F) suggest that NOS inhibition induced an outward rectifier K^+ conductance. This was confirmed by testing the effect of the K^+ channel blocker, TEA (20 mM), applied externally, which inhibited the maximal current by approximately 80% ($n = 5$) (Fig. 9A). To determine whether the channel was selective for K^+, a high K^+ solution (40 mM) was externally applied to cells treated with TRIM or L-NIL ($n = 5$). The current evoked by a pulse from −70 to 40 mV was reduced by 60% under 40 mM K^+ (Fig. 9B). For examining the possibility of an inward current being masked by the large K^+ current, all K^+ of the internal solution was replaced by Cs^+ (140 mM; $n = 6$). No inward currents could be resolved under these conditions (Fig. 9C). We investigated the tail currents of the cells treated with 100 μM TRIM. The I–V curve built with the tail currents exhibited a reversal potential of −65 mV under control external solution. Under high K^+ external solution the reversal potential shifted to −45 mV, indicating K^+-selectivity of the channel (Fig. 9D–F). Taken together, the data indicate that NOS inhibition induced the expression of a TEA-sensitive K^+ current of the delayed rectifier type that is absent in cells treated with TGF-β2 alone.

In OLF 442 cells grown in control conditions (FCS), whole cell current recordings showed either no current at all or small voltage-dependent outward currents (Fig. 10A, left panel). In bipolar cells differentiated with serum-free medium (1% BSA), a large voltage-dependent outward current was induced (Fig. 10A, middle panel). The treatment with NOS inhibitors for 24–36 h induced a similar voltage-dependent outward current in bipolar cells (Fig. 10A, right panel). Under control conditions (FCS alone), the mean current was 436 ± 213 pA (range 0–900 pA, Vh = −70 mV, $n = 50$) Fig. 10D). In cells treated with 100 and 1000 μM TRIM, a depolarizing pulse from −70 to 70 mV activated a mean current of 2122 ± 701 pA (range 1300–3900 pA, $n = 10$) and 3855 ± 985 pA (range 1300–9400 pA, $n = 30$), respectively, (Fig. 10D). Treatment with 100 and 1000 μM L-NIL had similar effects than TRIM, the mean currents being 1425 ± 625 pA (range 900–2100 pA, $n = 10$) and 2025 ± 785 pA (range 1200–4700 pA, $n = 10$), respectively, (Fig. 10D). The currents and the I–V curves suggested that NOS inhibition induced an outward rectifier K^+ current. This was verified when results similar to those shown in Fig. 9 were obtained when similar experiments were performed on these cells (data not shown). 20 mM TEA inhibited the current induced by a pulse from −70 to 40 mV by approximately 50% ($n = 7$). A higher concentration of TEA (60 mM) had a more pronounced inhibitory

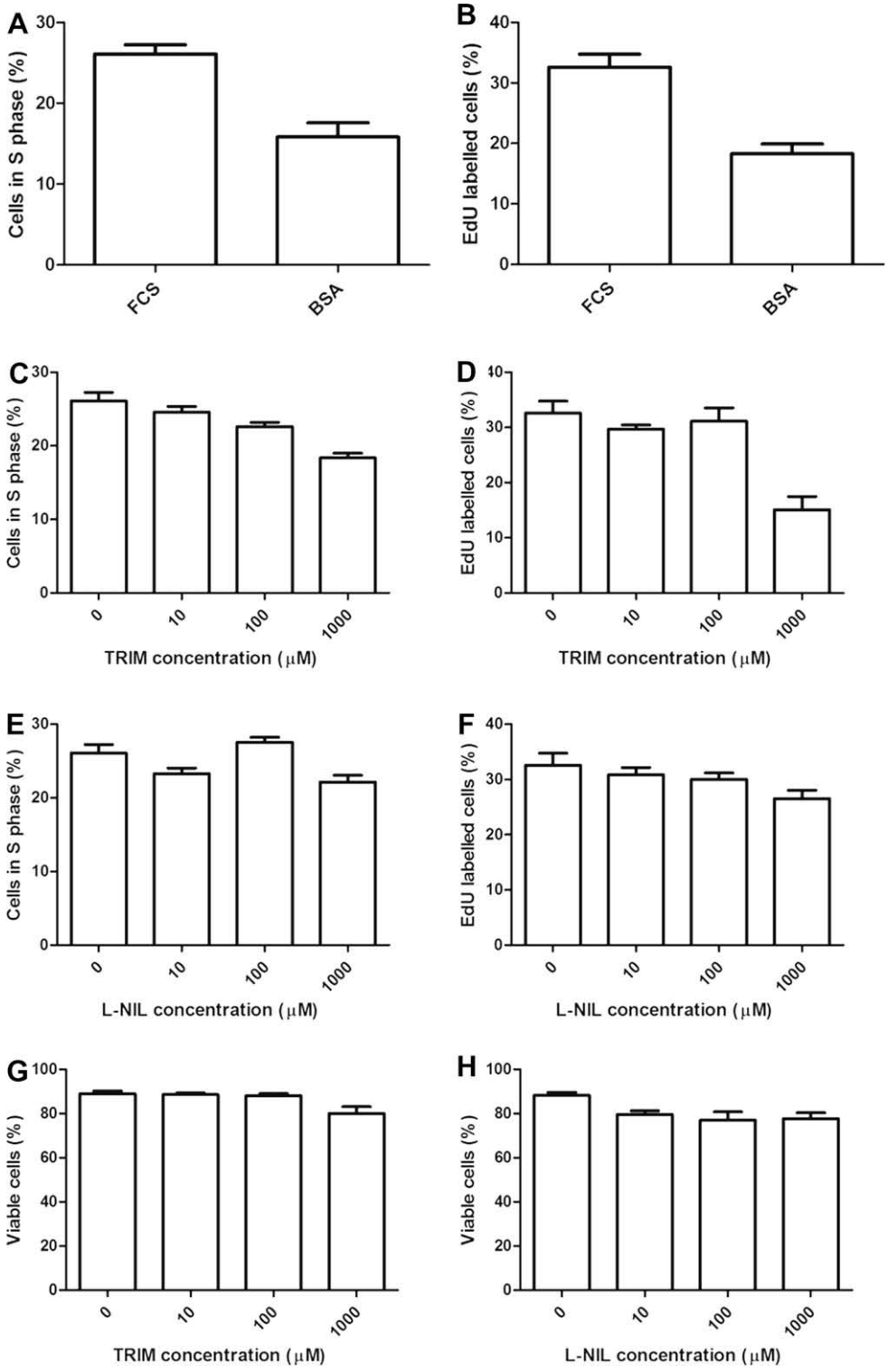

Fig. 5. Flow cytometric analysis of cell proliferation and viability in OLF442 cells. (A and B) Control cells grown in serum-containing medium (Control) and serum-free medium (Serum-free). As expected, serum-free medium reduced both the numbers of cells in *S*-phase (A) and the numbers of EdU-labeled cells (B). (C and D) TRIM induced a significant, dose-dependent reduction in the numbers of cells in *S*-phase (C, $p = 0.0008$) and the numbers of EdU-labeled cells (D, $p = 0.001$). (E and F) NOS inhibition with L-NIL significantly reduced the numbers of cells in *S*-phase (E, $p = 0.009$) but not the number of EdU-labeled cells (F, $p = 0.12$). (G and H) Compared to the controls (0 μM), NOS inhibition had a small effect on cell viability. The highest concentration of TRIM, reduced cell viability by 8% (G, $p = 0.003$). L-NIL reduced cell viability by 9–11% (H, $p = 0.015$).

effect (90%), but only a small recovery was seen after wash. The conductance was selective for K^+, since a high K^+ solution (40 mM) applied to cells treated with 1000 μM TRIM ($n = 5$) reduced by 45% the current evoked by a pulse from −70 to 70 mV. No inward current could be resolved when K^+ of the internal solution was replaced by Cs^+ (140 mM). The I–V curve built from the tail currents of the cells treated with 1000 μM TRIM exhibited a reversal potential of −40 mV. Under high K^+ external solution the reversal potential shifted to −20 mV, in agreement with a K^+-selective channel.

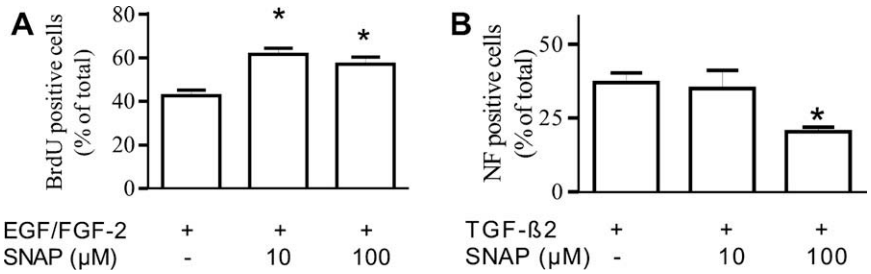

Fig. 6. Nitric oxide release increased cell proliferation and decreased neuronal differentiation in primary cultures of olfactory epithelial cells. (A) Quantification of dividing (BrdU-positive) cells treated with the nitric oxide donor, SNAP, expressed as percentage of total cells ± SEM. (B) Quantification of differentiated (NF-positive) cells, treated with SNAP. $^{*}p < 0.05$, statistically different from control (Kruskal–Wallis test). All quantification was performed with observer blind to treatment.

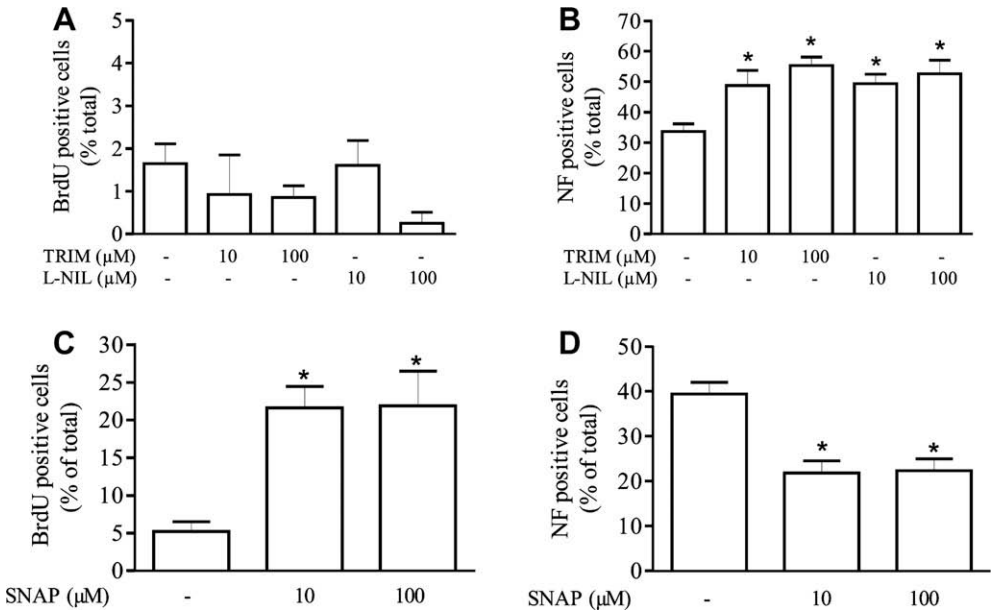

Fig. 7. Nitric oxide regulated cell proliferation and neuronal differentiation in primary cultures of olfactory epithelial cells grown in the absence of growth factors. (A) Quantification of BrdU-positive cells treated with L-NIL or TRIM. (B) Quantification of NF-positive cells treated with L-NIL or TRIM. (C) Quantification of BrdU-positive cells, treated with SNAP. (D) Quantification of NF-positive cells, treated with SNAP. Data are expressed as percentage of total cells ± SEM $^{*}p < 0.05$, statistically different from control (Kruskal–Wallis test).

Nitric oxide levels were reduced during differentiation of neuronal precursors

The ability to grow large numbers of OLF442 cells allowed us to determine nitric oxide levels, which was not possible in primary cultures because of low cell numbers. In control cultures (FCS), nitric oxide was 30.6 ± 8.5 pmol/μg protein (Fig. 11). When cells were differentiated in serum-free medium (1% BSA) there was a significant reduction of nitric oxide, to 6.17 ± 1.6 pmol/μg protein ($p < 0.001$, Fig. 11). When cells were differentiated with NOS inhibitors in serum-containing medium, nitric oxide production was also reduced. NOS inhibition with 100 and 1000 μM TRIM reduced nitric oxide to 9.67 ± 0.9 and 6.68 ± 0.6 pmol/μg protein, respectively, ($p < 0.001$, compared to control; Fig. 11). Similarly, 100 and 1000 μM L-NIL reduced nitric oxide to 8.87 ± 2.2 and 8.68 ± 1.7 pmol/μg protein, respectively, ($p < 0.001$, Fig. 11).

Discussion

This study provides direct evidence that nitric oxide levels regulate cell proliferation and neuronal differentiation in the adult olfactory epithelial cultures. This was demonstrated by experiments to reduce and elevate nitric oxide levels in primary cultures from rat olfactory epithelium and in an immortalized mouse neuronal precursor cell line, OLF442. All three isoforms of nitric oxide synthase (NOS) were present in the primary cultures, expressed by the neuronal precursors (globose basal cells) and the other cells present (horizontal basal cells, supporting cells). All NOS isoforms were expressed by the immortalized neuronal precursor cell line. This demonstrated that both cultures could be susceptible to NOS inhibition. In both primary and immortalized cell cultures, NOS inhibition reduced the numbers of proliferating cells and increased the numbers of differentiated cells. In contrast, nitric oxide release had the opposite effect, increasing cell proliferation and decreasing neuronal differentiation in both primary cultures and immortalized cells. These effects of NOS inhibition and nitric oxide release were repeated in primary cultures in the presence and absence of growth factors in the medium. Electrophysiological analysis of primary cultures and immortalized precursors demonstrated that NOS inhibition induced an outwardly rectifying, voltage-dependent K^+ current in bipolar cells in both cultures. Nitric oxide levels were quantified and shown to be reduced during differentiation of immortalized neuronal precursors. Taken together these results strongly indicate that nitric oxide levels can regulate cell proliferation and neuronal differentiation in olfactory cell cultures.

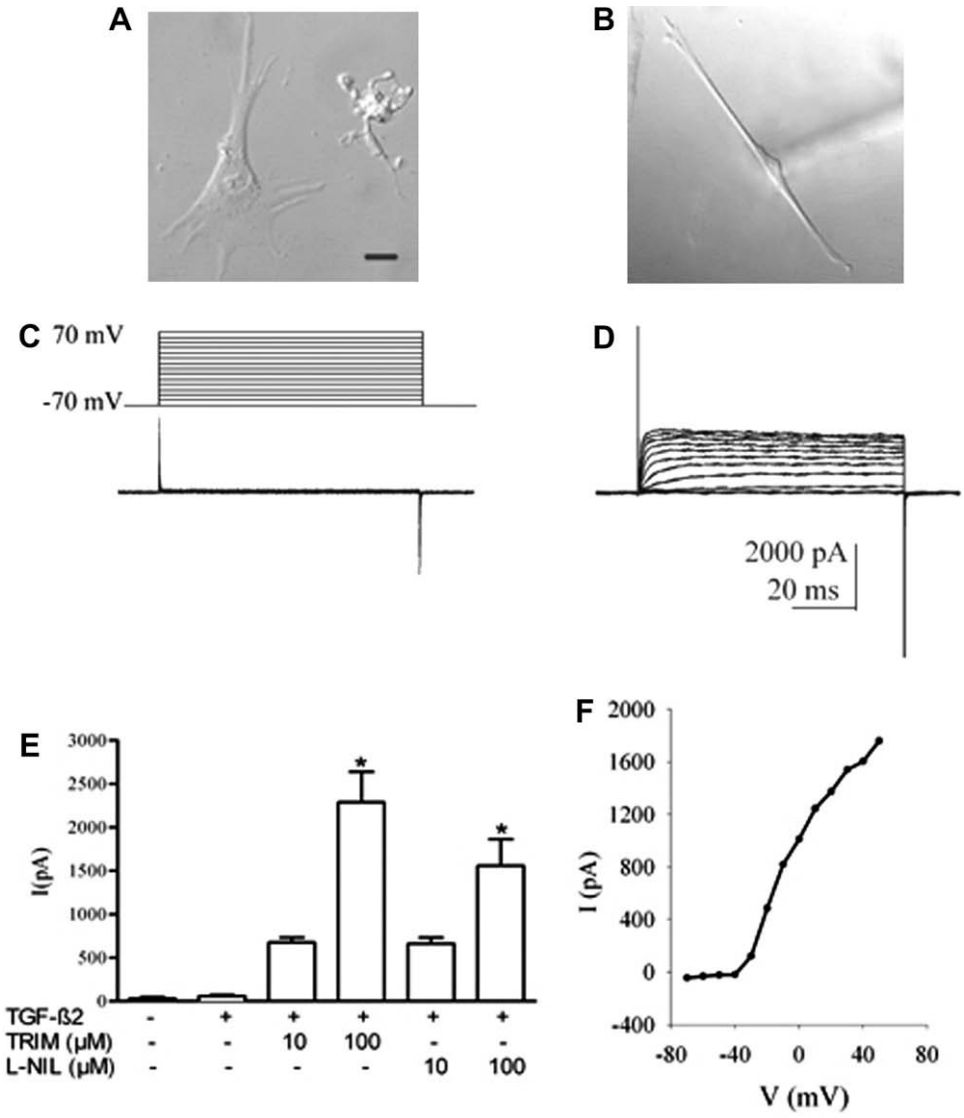

Fig. 8. NOS inhibition induced an outwardly rectifying, voltage-dependent current in bipolar cells in primary cultures of olfactory epithelial cells. (A) A typical cell grown for 24 h in media containing TGF-β2. Bar: 5 μm. (B) A bipolar cell grown in medium containing 100 μM TRIM. (C) Whole cell current profiles for the cell in A with the respective voltage pulse protocol. (D) Whole cell current profiles for the cell in B. (E) Histogram of the peak values of currents evoked by pulses from −70 to 70 mV in cells treated with the NOS inhibitors TRIM and L-NIL and the respective controls. $^{*}p < 0.05$, statistically different from the control group (Kruskal–Wallis test). (F) Current-voltage (I–V) curve for the cell shown in B, built with the data in D. $V_{holding} = -70$ mV.

At present the exact roles of the different NOS isoforms are uncertain given that they are all present in the primary and immortalized neuronal precursors. The important point here is the role of NO in regulating the switch between proliferation and differentiation, demonstrated in two cell systems, with multiple NOS inhibitors and in the presence and absence of other growth factors that also regulate proliferation and differentiation.

Nitric oxide and olfactory neurogenesis in vitro

The primary cell cultures used here contain globose basal cells, horizontal basal cells and supporting cells, all of which may be dividing [27]. They are all candidates as targets for nitric oxide regulation of proliferation. As the neuronal precursors, the globose basal cells are the likely target for nitric oxide regulation of differentiation. These cells are stimulated to proliferate by FGF-2 [25,27] and stimulated to differentiate by TGF-β2 [27,35]. This study indicates that differentiation is regulated additionally by nitric oxide and NOS activity, This is supported by the evidence that nitric oxide production was reduced in the neuronal precursor cell line, OLF442, when it differentiated in serum-free medium. Similarly, NOS inhibition induced differentiation and reduced cell proliferation in these cells even in the presence of serum. Differentiation induced by NOS inhibition was similar in primary cultures and immortalized precursor cell cultures: there was morphological differentiation into a bipolar cell that expressed an outwardly rectifying potassium current. No currents, or only small currents, were evoked in other cells in these cultures or in cultures not treated with NOS inhibitors.

Evidence from cell culture and knock-out animals indicates that nitric oxide is neuroprotective [44]. A similar role for nitric oxide is indicated in the present experiment. NOS inhibition had a small but significant effect, reducing the numbers of viable cells by about 10%, depending on the choice of inhibitor and concentration. Similarly, when the immortalized olfactory neuronal precursor cell line, OLF442, was deprived of serum there was a reduction of nitric oxide production with a parallel increase in both differentiation and cell death and a decrease in cell proliferation. These effects

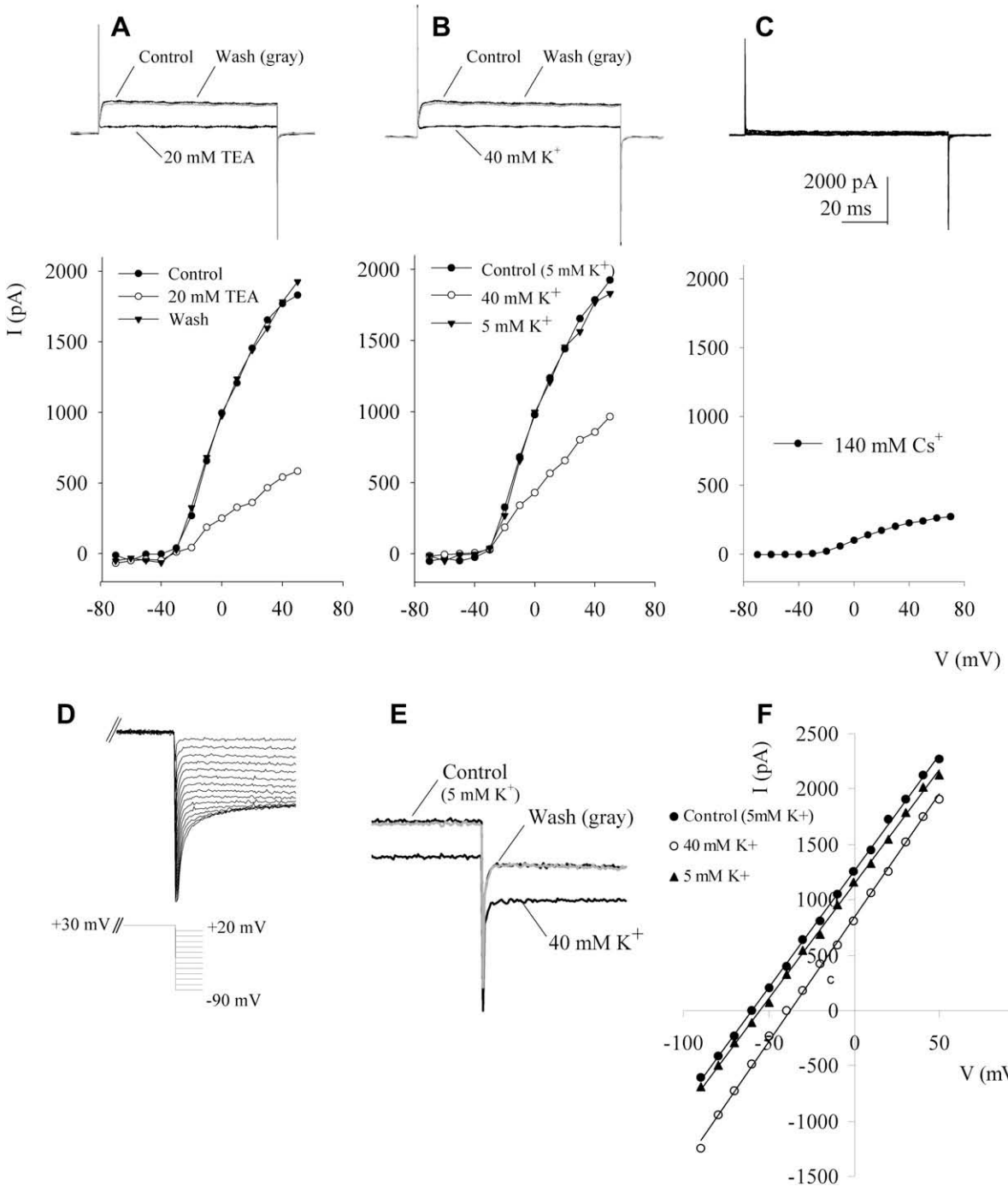

Fig. 9. NOS inhibition induced current in bipolar cells is a K^+ current. (A) Whole cell currents evoked by pulses from −70 to 70 mV from a cell incubated for 24 h in TGF-β2 plus 100 μM TRIM in the presence or absence of 20 mM TEA and after washout. The respective I–V curves are shown below. In A and B the trace shown is the current at 20 mV. (B) Whole cell currents from another cell grown as in A, with 5 mM external K^+ (control), with 40 mM K^+ and after returning back to 5 mM K^+. The I–V curves are shown below. (C) Whole cell currents from another cell grown as in A, but where Cs^+ replaced K^+ in the pipette solution (internal). Currents were evoked by voltage pulses from −70 to 70 mV. The I–V curve is shown below. $V_{Holding}$ = −70 mV in (A–D) family of tail currents obtained with the voltage pulse protocol shown below, stepping from +30 mV to different values. (E) Tail currents evoked by voltage steps from +30 to −20 mV with 5 mM external K^+ (control), with 40 mM K^+ and after returning back to 5 mM K^+. (F) I–V curve for the same conditions as in E.

were observed in the absence of NOS inhibitors, supporting the hypothesis that the cell death is due to loss of nitric oxide rather than an indirect effect of NOS inhibition. This switching between cell proliferation, differentiation and cell death when nitric oxide levels are reduced is similar to the effects of dopamine on olfactory neurogenesis. Dopamine and dopamine receptor agonists induced differentiation of olfactory neuronal precursors in rat primary cultures [45], reduced cell proliferation and induced cell death in human olfactory explant cultures [46] and induced differentiation and apoptosis in an immortalized olfactory cell line [47].

It is also apparent from other studies that nitric oxide can act as a switch between proliferation and differentiation in many cell types. An interesting paradox is that inhibiting NOS in some cell types induces differentiation and in others it induces proliferation [44]. In the nervous system, as in other tissues, there is evidence for both effects. NOS inhibition increased proliferation of mouse

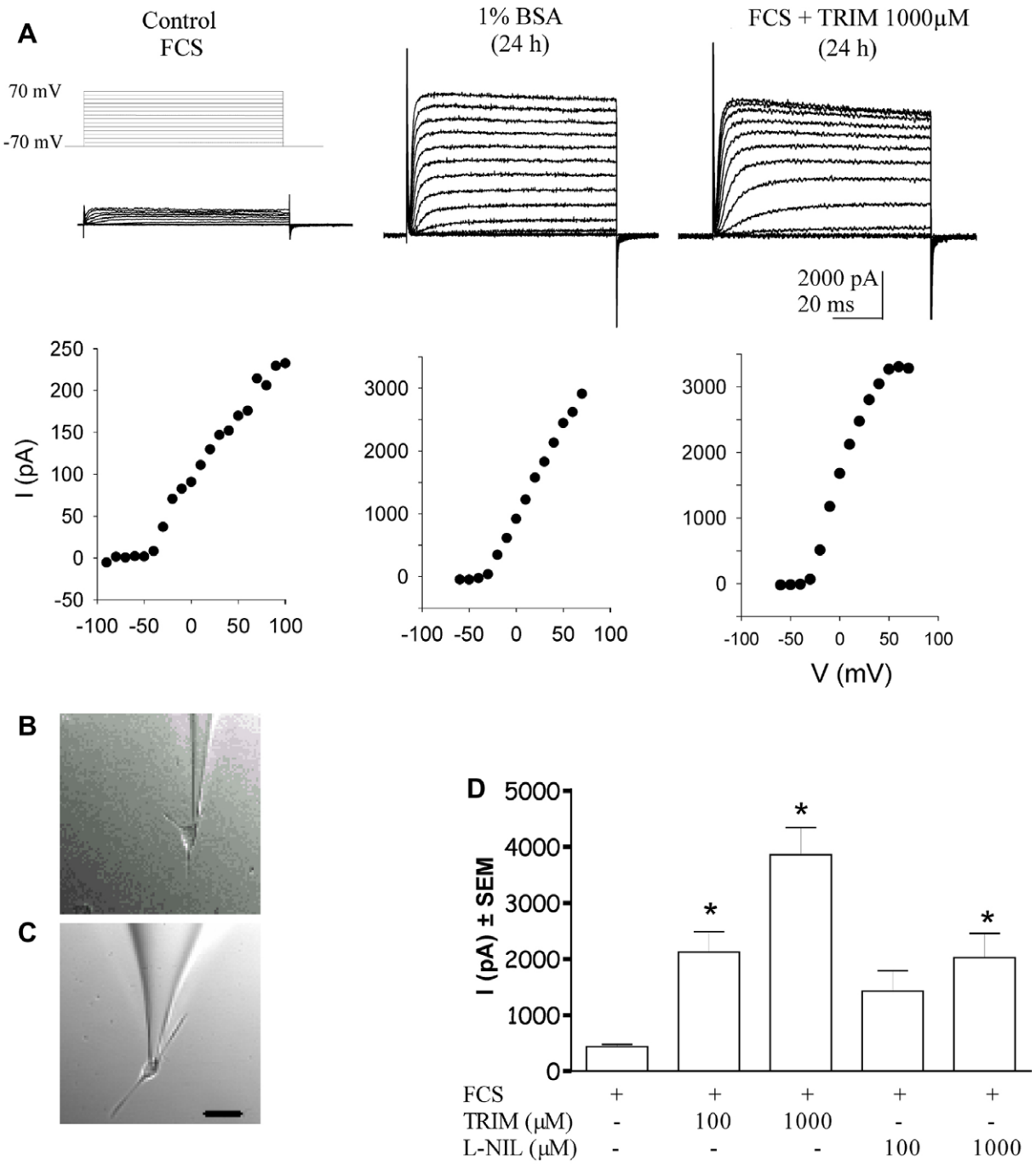

Fig. 10. NOS inhibition induced an outwardly rectifying, voltage-dependent current in bipolar cells in OLF442 cells. (A) left panel: whole cell currents from a cell grown for 24 h in serum-containing medium (FCS) with the voltage step protocol used for all the recorded cells shown above. The I–V curve is shown below. An example of such a cell is shown in (B). Middle panel: whole cell currents with its respective I–V curve from a cell grown in serum-free medium (1% BSA). Right panel: whole cell currents with its respective I–V curve from a cell grown in FCS-containing medium plus 1000 µM TRIM. An example of such a cell is shown in (C). Bar:10 µm. (D) Quantification of the peak values of the currents evoked by pulses from −70 to 70 mV from cells treated with TRIM or L-NIL, at different concentrations. $^{*}p < 0.05$ respect to control, Dunn's test after Kruskall–Wallis test. $p < 0.001$. $V_{holding} = -70$ mV.

subventricular zone precursors *in vitro* [48] and *in vivo* [49,50] whereas exogenous nitric oxide decreased the number of proliferating cells in the *Xenopus* tadpole brain [51]. In contrast to these observations and in agreement with our study, a nitric oxide donor increased cell proliferation in the dentate gyrus and subventricular zone in young adult rats [20] increasing further during recovery from ischemic stroke [20]. Similarly in the cricket, neuroblast proliferation was increased by a nitric oxide donor and decreased by NOS inhibition [52].

Nitric oxide and olfactory neurogenesis in vivo

The cellular role of nitric oxide can vary with species, tissue and developmental state. There can be bi-phasic, dose-dependent effects such as in keratinocytes where low concentrations of nitric oxide donors increased proliferation while high concentrations induced differentiation [53]. There can be effects specific for NOS isoforms: nNOS gene null mutation increased proliferation in subventricular zone and dentate gyrus [54], whereas

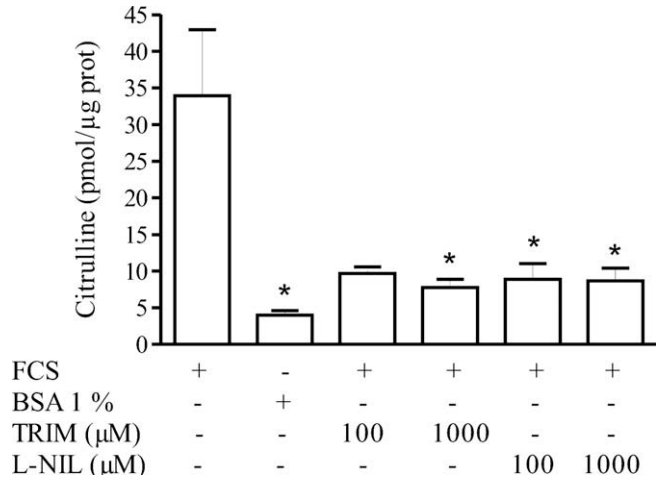

Fig. 11. Differentiation of OLF442, by NOS inhibition and serum-depletion, was associated with reduced nitric oxide levels. Nitric oxide production was determined by arginine-citrulline technique from OLF442 cells grown in the indicated media. $^{*}p < 0.001$ with respect to the control FCS group. (Dunnet's test, after one-way ANOVA test).

eliminating eNOS significantly reduced neuronal progenitor proliferation in dentate gyrus [55]. There can be effects specific for cell type. Nitric oxide donors increased cell proliferation in bladder and bladder cancer cell lines [56]. In breast cancer cell lines high NOS activity inhibited proliferation [57] whereas in mammary epithelium, nitric oxide enhanced prolactin-stimulated cell proliferation [58]. It is possible that nitric oxide also plays different roles in the olfactory epithelium during development and during adulthood.

Nitric oxide was initially implicated in olfactory neurogenesis because nNOS is expressed during embryonic development and adult regeneration of olfactory epithelium [7]. Interestingly, targeted deletion of nNOS alters olfactory neurogenesis during development without preventing the formation of a near normal epithelium in the adult [28]. Specifically, in the mice lacking nNOS, there were fewer proliferating cells and fewer postmitotic neurons at 1 week of age. In contrast, at 6 weeks of age there were more proliferating cells and more postmitotic neurons, associated with a reduced number of apoptotic cells [28]. These data clearly indicate that nNOS is involved in neurogenesis in the olfactory epithelium during development and into adulthood but the mechanism of this regulation remains unidentified because the phenotype of a tissue after gene deletion will include loss of function and compensatory mechanisms that act in time and in space, over development and over different cell types. The regulation of olfactory neurogenesis is complex, with numerous growth factors acting at different points in the lineage pathway [26]. Therefore differences in nNOS expression between young and adult olfactory epithelium [7] may reflect a changing role for nitric oxide as a result of alterations in other regulatory pathways as the epithelium matures [28]. These differences in regulation of neurogenesis may result from changes from "growth" to "maintenance". When the adult epithelium undergoes "repair", this role changes again and the expression of nNOS is greatly increased [7]. The present results *in vitro,* probably reflect mechanisms operating during "repair", rather than "maintenance" *in vivo*, but nevertheless a cellular mechanism is clearly elucidated.

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References

[1] O.W. Griffith, D.J. Stuehr, Nitric oxide synthases: properties and catalytic mechanism, Annu. Rev. Physiol. 57 (1995) 707–736.
[2] R.G. Knowles, S. Moncada, Nitric oxide synthases in mammals, Biochem. J. 298 (Pt 2) (1994) 249–258.
[3] D.J. Stuehr, C.F. Nathan, Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells, J. Exp. Med. 169 (1989) 1543–1555.
[4] Q.W. Xie, H.J. Cho, J. Calaycay, R.A. Mumford, K.M. Swiderek, T.D. Lee, A. Ding, T. Troso, C. Nathan, Cloning and characterization of inducible nitric oxide synthase from mouse macrophages, Science 256 (1992) 225–258.
[5] D.S. Bredt, S.H. Snyder, Transient nitric oxide synthase neurons in embryonic cerebral cortical plate. Sensory ganglia, and olfactory epithelium, Neuron 13 (1994) 301–313.
[6] G. Keilhoff, B. Seidel, H. Noack, W. Tischmeyer, D. Stanek, G. Wolf, Patterns of nitric oxide synthase at the messenger RNA and protein levels during early rat brain development, Neuroscience 75 (1996) 1193–1201.
[7] A.J. Roskams, D.S. Bredt, T.M. Dawson, G.V. Ronnett, Nitric oxide mediates the formation of synaptic connections in developing and regenerating olfactory receptor neurons, Neuron 13 (1994) 289–299.
[8] M. Santacana, L.O. Uttenthal, M.L. Bentura, A.P. Fernandez, J. Serrano, J. Martinez de Velasco, D. Alonso, R. Martinez-Murillo, J. Rodrigo, Expression of neuronal nitric oxide synthase during embryonic development of the rat cerebral cortex, Brain Res. Dev. Brain Res. 111 (1998) 205–222.
[9] B. Kuzin, I. Roberts, N. Peunova, G. Enikolopov, Nitric oxide regulates cell proliferation during *Drosophila* development, Cell 87 (1996) 639–649.
[10] N. Peunova, G. Enikolopov, Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells, Nature 375 (1995) 68–73.
[11] A.G. Estevez, N. Spear, S.M. Manuel, R. Radi, C.E. Henderson, L. Barbeito, J.S. Beckman, Nitric oxide and superoxide contribute to motor neuron apoptosis induced by trophic factor deprivation, J. Neurosci. 18 (1998) 923–931.
[12] M. Tamatani, S. Ogawa, Y. Niitsu, M. Tohyama, Involvement of Bcl-2 family and caspase-3-like protease in NO-mediated neuronal apoptosis, J. Neurochem. 71 (1998) 1588–1596.
[13] A.M. Vincent, K. Maiese, Nitric oxide induction of neuronal endonuclease activity in programmed cell death, Exp. Cell Res. 246 (1999) 290–300.
[14] P. Ogilvie, K. Schilling, M.L. Billingsley, H.H. Schmidt, Induction and variants of neuronal nitric oxide synthase type I during synaptogenesis, FASEB J. 9 (1995) 799–806.
[15] H.H. Wu, C.V. Williams, S.C. McLoon, Involvement of nitric oxide in the elimination of a transient retinotectal projection in development, Science 265 (1994) 1593–1596.
[16] Y. Sun, K. Jin, J.T. Childs, L. Xie, X.O. Mao, D.A. Greenberg, Neuronal nitric oxide synthase and ischemia-induced neurogenesis, J. Cereb. Blood Flow Metab. 25 (2005) 485–492.
[17] J. Chen, Y. Li, R. Zhang, M. Katakowski, S.C. Gautam, Y. Xu, M. Lu, Z. Zhang, M. Chopp, Combination therapy of stroke in rats with a nitric oxide donor and human bone marrow stromal cells enhances angiogenesis and neurogenesis, Brain Res. 1005 (2004) 21–28.
[18] R.G. Keynes, J. Garthwaite, Nitric oxide and its role in ischaemic brain injury, Curr. Mol. Med. 4 (2004) 179–191.
[19] D. Lu, A. Mahmood, R. Zhang, M. Copp, Upregulation of neurogenesis and reduction in functional deficits following administration of DEtA/NONOate, a nitric oxide donor, after traumatic brain injury in rats, J. Neurosurg. 99 (2003) 351–361.
[20] R. Zhang, L. Zhang, Z. Zhang, Y. Wang, M. Lu, M. Lapointe, M. Chopp, A nitric oxide donor induces neurogenesis and reduces functional deficits after stroke in rats, Ann. Neurol. 50 (2001) 602–611.
[21] P.P. Graziadei, G.A. Monti Graziadei, Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons, J. Neurocytol. 8 (1979) 1–18.
[22] D.G. Moulton, Dynamics of cell populations in the olfactory epithelium, Ann. NY Acad. Sci. 237 (1974) 52–61.
[23] A. Mackay-Sim, P. Kittel, Cell dynamics in the adult mouse olfactory epithelium: a quantitative autoradiographic study, J. Neurosci. 11 (1991) 979–984.
[24] A. Calof, J. Mumm, P. Rim, J. Shou, The neuronal stem cell of the olfactory epithelium, J. Neurobiol. 36 (1998) 190–205.
[25] M.K. DeHamer, J.L. Guevara, K. Hannon, B.B. Olwin, A.L. Calof, Genesis of olfactory receptor neurons in vitro: regulation of progenitor cell divisions by fibroblast growth factors, Neuron 13 (1994) 1083–1097.
[26] A. Mackay-Sim, M.I. Chuah, Neurotrophic factors in the primary olfactory pathway, Prog. Neurobiol. 62 (2000) 527–559.

[27] M.P. Newman, F. Feron, A. Mackay-Sim, Growth factor regulation of neurogenesis in adult olfactory epithelium, Neuroscience 99 (2000) 343–350.
[28] J. Chen, Y. Tu, C. Moon, V. Matarazzo, A.M. Palmer, G.V. Ronnett, The localization of neuronal nitric oxide synthase may influence its role in neuronal precursor proliferation and synaptic maintenance, Dev. Biol. 269 (2004) 165–182.
[29] C.T. Leung, P.A. Coulombe, R.R. Reed, Contribution of olfactory neural stem cells to tissue maintenance and regeneration, Nat. Neurosci. 10 (2007) 720–726.
[30] X. Chen, H. Fang, J.E. Schwob, Multipotency of purified, transplanted globose basal cells in olfactory epithelium, J. Comp. Neurol. 469 (2004) 457–474.
[31] F. Feron, A. Mackay-Sim, J.L. Andrieu, K.I. Matthaei, A. Holley, G. Sicard, Stress induces neurogenesis in non-neuronal cell cultures of adult olfactory epithelium, Neuroscience 88 (1999) 571–583.
[32] K.P. MacDonald, A. Mackay-Sim, G.R. Bushell, P.F. Bartlett, Olfactory neuronal cell lines generated by retroviral insertion of the n-myc oncogene display different developmental phenotypes, J. Neurosci Res. 45 (1996) 237–247.
[33] A.I. Farbman, J.A. Buchholz, Transforming growth factor-alpha and other growth factors stimulate cell division in olfactory epithelium in vitro, J. Neurobiol. 30 (1996) 267–280.
[34] A. Calof, A. Bonnin, C. Crocker, S. Kawauchi, R. Murray, J. Shou, H. Wu, Progenitor cells of the olfactory receptor neuron lineage, Microsc. Res. Tech. 58 (2002) 176–188.
[35] N.K. Mahanthappa, G.A. Schwarting, Peptide growth factor control of olfactory neurogenesis and neuron survival in vitro: roles of EGF and TGF-beta s, Neuron 10 (1993) 293–305.
[36] A. Salic, T.J. Mitchison, A chemical method for fast and sensitive detection of DNA synthesis in vivo, Proc. Natl. Acad. Sci. USA 105 (2008) 2415–2420.
[37] P. Cappella, F. Gasparri, M. Pulici, J. Moll, A novel method based on click chemistry, which overcomes limitations of cell cycle analysis by classical determination of BrdU incorporation, allowing multiplex antibody staining, Cytometry A 73 (2008) 626–636.
[38] L. Sobrevia, D.L. Yudilevich, G.E. Mann, Elevated D-glucose induces insulin insensitivity in human umbilical endothelial cells isolated from gestational diabetic pregnancies, J. Physiol. 506 (Pt 1) (1998) 219–230.
[39] B.J. Goldstein, J.E. Schwob, Analysis of the globose basal cell compartment in rat olfactory epithelium using GBC-1, a new monoclonal antibody against globose basal cells, J. Neurosci. 16 (1996) 4005–4016.
[40] E.H. Holbrook, K.E. Szumowski, J.E. Schwob, An immunochemical, ultrastructural, and developmental characterization of the horizontal basal cells of rat olfactory epithelium, J. Comp. Neurol. 363 (1995) 129–146.
[41] M. Satoh, T. Yoshida, Expression of neural properties in olfactory cytokeratin-positive basal cell line, Brain Res. Dev. Brain Res. 121 (2000) 219–222.
[42] V.M. Lee, S.K. Pixley, Age and differentiation-related differences in neuron-specific tubulin immunostaining of olfactory sensory neurons, Brain Res. Dev. Brain Res. 83 (1994) 209–215.
[43] J.L. Hempstead, J.I. Morgan, Monoclonal antibodies to the rat olfactory sustentacular cell, Brain Res. 288 (1983) 289–295.
[44] A. Contestabile, E. Ciani, Role of nitric oxide in the regulation of neuronal proliferation, survival and differentiation, Neurochem. Int. 45 (2004) 903–914.
[45] F. Feron, A. Vincent, A. Mackay-Sim, Dopamine promotes differentiation of olfactory neuron in vitro, Brain Res. 845 (1999) 252–259.
[46] F. Feron, C. Perry, M.H. Hirning, J. McGrath, A. Mackay-Sim, Altered adhesion, proliferation and death in neural cultures from adults with schizophrenia, Schizophr. Res. 40 (1999) 211–218.
[47] V. Coronas, F. Feron, R. Hen, G. Sicard, F. Jourdan, E. Moyse, In vitro induction of apoptosis or differentiation by dopamine in an immortalized olfactory neuronal cell line, J. Neurochem. 69 (1997) 1870–1881.
[48] E.R. Matarredona, M. Murillo-Carretero, B. Moreno-Lopez, C. Estrada, Nitric oxide synthesis inhibition increases proliferation of neural precursors isolated from the postnatal mouse subventricular zone, Brain Res. 995 (2004) 274–284.
[49] B. Moreno-Lopez, C. Romero-Grimaldi, J.A. Noval, M. Murillo-Carretero, E.R. Matarredona, C. Estrada, Nitric oxide is a physiological inhibitor of neurogenesis in the adult mouse subventricular zone and olfactory bulb, J. Neurosci. 24 (2004) 85–95.
[50] C. Park, M. Kang, Y.K. Kwon, J.H. Chung, H. Ahn, Y. Huh, Inhibition of neuronal nitric oxide synthase enhances cell proliferation in the dentate gyrus of the adrenalectomized rat, Neurosci. Lett. 309 (2001) 9–12.
[51] N. Peunova, V. Scheinker, H. Cline, G. Enikolopov, Nitric oxide is an essential negative regulator of cell proliferation in *Xenopus* brain, J. Neurosci. 21 (2001) 8809–8818.
[52] M. Cayre, J. Malaterre, S. Scotto-Lomassese, G.R. Holstein, G.P. Martinelli, C. Forni, S. Nicolas, A. Aouane, C. Strambi, A. Strambi, A role for nitric oxide in sensory-induced neurogenesis in an adult insect brain, Eur. J. Neurosci. 21 (2005) 2893–2902.
[53] V. Krischel, D. Bruch-Gerharz, C. Suschek, K.D. Kroncke, T. Ruzicka, V. Kolb-Bachofen, Biphasic effect of exogenous nitric oxide on proliferation and differentiation in skin derived keratinocytes but not fibroblasts, J. Invest. Dermatol. 111 (1998) 286–291.
[54] M.A. Packer, Y. Stasiv, A. Benraiss, E. Chmielnicki, A. Grinberg, H. Westphal, S.A. Goldman, G. Enikolopov, Nitric oxide negatively regulates mammalian adult neurogenesis, Proc. Natl. Acad. Sci. USA 100 (2003) 9566–9571.
[55] A. Reif, A. Schmitt, S. Fritzen, S. Chourbaji, C. Bartsch, A. Urani, M. Wycislo, R. Mossner, C. Sommer, P. Gass, K.P. Lesch, Differential effect of endothelial nitric oxide synthase (NOS-III) on the regulation of adult neurogenesis and behaviour, Eur. J. Neurosci. 20 (2004) 885–895.
[56] E. Morcos, O.T. Jansson, J. Adolfsson, G. Kratz, N.P. Wiklund, Endogenously formed nitric oxide modulates cell growth in bladder cancer cell lines, Urology 53 (1999) 1252–1257.
[57] S. Reveneau, L. Arnould, G. Jolimoy, S. Hilpert, P. Lejeune, V. Saint-Giorgio, C. Belichard, J.F. Jeannin, Nitric oxide synthase in human breast cancer is associated with tumor grade, proliferation rate, and expression of progesterone receptors, Lab. Invest. 79 (1999) 1215–1225.
[58] F.F. Bolander Jr., The mechanisms by which nitric oxide affects mammary epithelial growth and differentiation, Biochem. Biophys. Res. Commun. 304 (2003) 425–430.