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Nitric Oxide Signaling in Developing Trigeminal Neurons

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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Original Research Article

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ABSTRACT

Aims: This study aimed to investigate the roles of neuronal (nNOS) and endothelial (eNOS) nitric oxide synthase in the nerve growth factor (NGF) response during the development of the trigeminal ganglion (TG). In addition the roles of these nitric oxide synthases following NGF withdrawal were ascertained. Finally the effects of administration of high doses of exogenous reactive nitrogen species were considered.
Study Design and Methodology: Primary cultures of trigeminal sensory neurons were pharmacologically treated with reagents known to specifically modify the activity of NOS isoforms or were treated with reagents that release exogenous reactive nitrogen species *In vitro*. Cultures were maintained for 24 hours after treatment and the number of surviving neurons determined. *In vivo* determination of NOS isoform expression was carried out using immunohistochemistry.

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Results: eNOS and nNOS are widely expressed during TG neurodevelopment as ascertained immunohistochemically. Pharmacological inhibition of these enzymes reduces the *In vitro* NGF survival response of E16 neurons, but only eNOS inhibition reduces E19 TG neuron survival. Both developmental stages are vulnerable to the neurotoxic effects of high levels of exogenous nitric oxide when grown with NGF but susceptibility is higher at E19.
Conclusion: During the period of naturally occurring neuronal death in the trigeminal ganglion, eNOS and nNOS are required for the survival of a proportion of trigeminal sensory neurons but when this period of cell death has ended there is a switch, and only eNOS is required for the NGF survival response. Furthermore, as these neurons mature beyond the period of naturally occurring cell death, their susceptibility to damage by elevated concentrations of reactive nitrogen species increases. This implies that there may be important mechanisms found within developing neurons that confer protection from nitrosative stress that warrant further investigation.

Keywords: eNOS; neurodevelopment; nitric oxide; NGF; nNOS; peroxynitrite; trigeminal.

ABBREVIATIONS

7-NIO : 7-N5-(1-iminoethyl)-ornithine, dihydrochloride
ATP : adenosine triphosphate
DAF-2DA : 4, 5-diaminofluorescein diacetate
DAPI : 4'6-diamidino-2-phenylindole
DMSO : dimethyl sulfoxide
E(#) : embryonic day (#)
eNOS : endothelial nitric oxide synthase
GFW : growth factor withdrawal
GSNO : S-nitrosoglutathione
iNOS : inducible nitric oxide synthase
L-SMTC : S-methyl–L-thiocitrulline
NGF : nerve growth factor
nNOS : neuronal nitric oxide synthase
NO : nitric oxide
NOC-18 : (z)-1-[2-aminoethyl-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
PBS : phosphate buffered saline
RNS : reactive nitrogen species
sGC : soluble guanylate cyclase
SIN-1 : 3-morpholinosydnonimine
TG : trigeminal ganglion
YC-1 : 3-(5-hydroxy-methyl-2-furyl)-1-benzylindazole

1. INTRODUCTION

Nitric oxide (NO) is a small multi-functional messenger that plays a variety of physiological roles. The molecule has a short half-life but is rapidly diffusing and hydrophobic allowing it to move swiftly to its site of action within tissues [1]. NO generation is enzymatically controlled by three nitric oxide synthase isoforms that generate the molecule from L-arginine [2]. Thus endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) produce sustained low doses of NO [3,4], whilst inducible nitric oxide synthase (iNOS) activation very

quickly releases large amounts of NO [5]. It is well established that following the activation of eNOS or nNOS, low dose NO can trigger a soluble guanyline cyclase (sGC) -mediated second messenger system but it is also clear that NO can signal via direct nitrosylation of cellular components, with both mechanisms working concurrently at times. For instance, NO-mediated modulation of voltage-gated calcium channels in rat hippocampal neurons relies on both sGC signaling and direct protein nitrosylation [6].

Within the nervous system NO is required for long term depression in cerebellar Purkinje neurons [7] as well as promoting the survival of these neurons as they develop [8]. NO can enhance synaptic transmission [9] and plays a role in some forms of long term potentiation [10]. Given that these physiological parameters are studied as the cellular basis of memory formation, it is not surprising that it has been clearly shown that NO is involved in all stages of memory formation in animal models [11]. Further evidence of a role for NO is neurophysiology comes from the demonstration that it can regulate potassium channels, with both adenosine triphosphate (ATP) and calcium-activated channels targeted [12]. Expression of both eNOS and nNOS has been reported in a number of neuronal populations but the main source of iNOS in the nervous system is the microglia and astrocytes. Release of NO following iNOS activation in these cells is a central part of the nervous system's immune defences [2]. Thus iNOS activation prevents the spread of murine cytomegalovirus within the retina [13]. Following iNOS activation the levels of NO produced are far higher than those observed following activation of eNOS and nNOS, and due to the nature of NO, it will rapidly move from the astrocytes and microglia where it is produced into adjacent neurons. There is a plethora of literature describing the maladaptive consequences of high concentrations of NO for the nervous system. To give one example, following trauma, iNOS-deficient animals display a reduction in secondary or delayed damage revealing the important role of iNOS in this type of neuronal loss [14,15].

It is known that low levels of NO, released via eNOS or nNOS, can be neuroprotective thus inhibition of nNOS depletes the survival of developing cerebellar Purkinje neurons [8], and eNOS activation is proposed as an underlying mechanism mediating the neuroprotective consequences of ischemic postconditioning in mice [16]. Downstream of nNOS, activation of a pro-survival PI3-kinase/Akt pathway has been noted [17] together with increased expression of the anti-apoptotic protein Bcl-2 [18] further elucidating the mechanism whereby low dose NO activation protects neurons from cell death. High levels of NO have been shown to be potently neurotoxic [19] and NO can further react with cellular superoxide ions forming peroxynitrite which can also trigger neuronal loss [20]. Finally, peroxynitrite can be converted to another neurotoxin, nitrogen dioxide [21] revealing a further pathway whereby NO and related reactive nitrogen species (RNS) can evoke neuronal death.

The trigeminal sensory ganglion (TG) is the ganglion of cranial nerve V and as such is part of the peripheral nervous system. The neurons of this ganglion have been extensively studied as a model to aid our understanding of the neurotrophic hypothesis [22]. This theory states that developing neurons are generated in excess and thereafter superfluous neurons that fail to obtain sufficient target field trophic support degenerate during the period of naturally occurring neuronal death. A number of neurotrophic factors have been shown to support TG neurons during their development but in the mouse, by embryonic day 16 (E16), the majority depend upon nerve growth factor (NGF) for their survival. During the development of the murine TG naturally occurring neuronal death begins at E12.5 [23], and is completed by the end of the prenatal period.

This investigation aimed to uncover the role of cellular nitric oxide generation in neuronal survival during and after the period of naturally occurring neuronal death using cultures of trigeminal sensory neurons as a model system. Given that iNOS expression in the nervous system is largely confined to non-neuronal populations, only eNOS and nNOS-mediated NO generation were considered. Furthermore, given the negative consequences of elevated levels of RNS for neurons, the survival response of TG neurons to NO and peroxynitrite over the same developmental timescale was studied.

2. MATERIALS AND METHODS

2.1 Primary Neuronal Culture

2.1.1 Establishment of trigeminal neuron cultures

C57/BL6 mice (Charles River, UK) were housed in environmentally enriched conditions with access to food and water *ad libitum* in accordance with the Animal (Scientific Procedures) Act, UK, 1986. Time-mated pregnant females were culled by a Schedule One method (cervical dislocation) in keeping with the guidance of the UK Home Office; and the embryos removed and transferred to sterile phosphate buffered saline (PBS). Trigeminal cultures were prepared from embryonic day 16 and embryonic day 19 embryos as described previously [24,25]. Briefly the embryos were decapitated and the top of the skull removed in a single cut through the level of the eyes and whisker pad. Further cuts were made to extract the trigeminal ganglia which were transferred to fresh Hanks's Blanced Salt Solution containing 0.1% trypsin (Worthington, USA). The ganglia were trypsinised for 12 minutes and then dissociated to a single cell suspension. The neurons were plated onto 13mm glass coverslips coated with 20μg/ml laminin (Sigma, UK), at a density of 100-500 neurons per coverslip. Neurons were grown in Neurobasal medium (Invitrogen, UK) supplemented with 200μM L-glutamine, 2% serum replacement 2 supplement, and 10ng/ml NGF (all Sigma, UK). For the duration of each experiment, coverslips were placed in 24 well plastic dishes and maintained in a 37^{o}C humidified incubator containing 5% CO_2.

2.1.2 Pharmacological treatment of cultures

After 24 hours in culture, the medium was replaced and supplemented with pharmacological reagents as required. For cultures in which trophic factor support was removed, the neurons were washed three times, for five minutes each change, in culture medium without NGF. This washing aimed to remove any residual NGF from the cultures. The reagents used to treat the cultures were: nNOSinhibitor, S-methyl–L-thiocitrulline (10μM, L-SMTC, Calbiochem, UK); eNOS inhibitor, 7-N5-(1-iminoethyl)-ornithine, dihydrochloride (50nM, 7-NIO, Alexis Biochemicals UK), soluble guanylate cyclase (sGC) inhibitor 3-(5-hydroxy-methyl-2-furyl)-1-benzylindazole (160nM, YC-1, Calbiochem, UK); bolus nitric oxide generator S-nitrosoglutathione (500μM, GSNO, Alexis Biochemicals, UK); sustained nitric oxide generator (z)-1-[2-aminoethyl-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (200μM, NOC-18, Alexis Biochemicals, UK); peroxynitrite generator 3-morpholinosydnonimine (250μM, SIN-1, Alexis Biochemicals, UK).

2.1.3 Determination of neuronal survival

After 24 hours of treatment, neurons were assessed to determine cell viability. Briefly the number of surviving neurons was counted under phase contrast microscopy for each

coverslip as described previously [26]. In accordance with other workers, only cells with a large phase bright cell body and visible neurites were counted. The data was expressed as percent survival relative to that attained following treatment with 10ng/ml NGF and this was assumed to be maximal (100%) survival [25]. For each condition investigated, cultures were established on at least 4 separate occasions and in triplicate on each occasion. Further illustration of the neurons was undertaken by fixing the cultures for 15 minutes in neutral buffered formalin followed by repeat washes in PBS to remove the fixative. Non-specific antibody staining was blocked using 10% horse serum in PBS and the cells incubated overnight at 4ºC in 1:100000 dilution of neuron-specific βIII-tubulin antibody (Promega, UK). Thereafter the primary antibody was washed off in PBS and detected using an HRP-conjugated anti-mouse secondary antibody (Sigma, UK) and antibody binding visualized using diaminobenzidine (FastDAB, Sigma, UK). Neurons were imaged in brightfield using a Nikon TS100 microscope and Nikon Coolpix digital camera.

2.1.4 Determination of nitric oxide release in cultured trigeminal neurons

To determine whether trigeminal neurons release nitric oxide *in vitro*, neurons were maintained in 10ng/ml NGF for 24 hours, or had the NGF washed out of the culture medium, and thereafter incubated with 5nM 4,5-diaminofluorescein diacetate in dimethyl sulfoxide as previously (DAF-2DA, Calbiochem, UK) [27] for 15 minutes at 37ºC, 5% CO_2. The neurons were then fixed in neutral buffered formalin for 15 minutes, washed three times in PBS and imaged using a Leica confocal microscope. Relative fluorescent intensity was measured with the associated software.

2.2 Immunohistochemistry

2.2.1 Fixation and wax embedding

The heads of E16 and E19 C57/BL6 murine embryos were fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid) overnight at 4ºC. Thereafter they were dehydrated through a graded alcohol series, cleared in chloroform and embedded in paraffin wax containing dimethyl sulfoxide (DMSO). Serial sections were cut at 8μm and mounted on polysine-coated microscope slides (vwr, UK). Sections containing TG were identified using brightfield microscopy.

2.2.2 Detection of NOS isoforms

To detect NOS isoforms, sections were cleared in xylene, rehydrated through a graded alcohol series and washed in PBS prior to blocking non-specific antibody binding using 10% goat serum in PBS for 20 minutes at room temperature. Primary antibodies were diluted in this blocking solution and sections were incubated in primary antibody solution overnight at 4ºC. Antibody dilutions were 1: 750 for both rabbit anti-nNOS and rabbit anti-eNOS (both from Insight Biotechnologies, UK). Following incubation in the primary antibody, sections were washed repeatedly in PBS and incubated with a FITC-conjugated goat anti-rabbit IgG (1:1000; Sigma, UK) for 1 hour at room temperature. Sections were again washed in PBS and the nuclei of the cells therein stained using 500ng/ml 4'6-diamidino-2-phenylindole (DAPI) for 10 minutes. Following further repeat washing in PBS, sections were mounted in citifluor AF3 (Citifluor Ltd, UK) and imaged using a Leica confocal microscope.

2.3 Statistical Analysis

To ascertain the significance of numerical findings, data were entered into minitab for windows (Minitab Inc, USA) for statistical analyses. As multiple groups were being compared, data were analyzed using one-way ANOVA with Tukey post hoc tests to determine where any statistically significant differences lay. Residuals were plotted to check for normality using Kolmogrov-Smirnov test.

3. RESULTS AND DISCUSSION

3.1 eNOS AND nNOS are Expressed in TG During, and Beyond, the Period of Naturally Occurring Cell Death

It is clear that in other neuronal populations in the central nervous system there are developmental trends in the role of nitric oxide for both neuronal survival and in the susceptibility to the neurotoxic influences of exogenous RNS [27]. Whether this is also true of trigeminal sensory neurons of the peripheral nervous system remains undetermined. Previous work has established that nNOS is expressed in a subset of neurons in adult trigeminal ganglia [28] and that there is also activity of NOS within the ganglia as demonstrated using NADPH-diaphorase histochemistry [29]. To determine whether eNOS and nNOS are expressed during the development of the trigeminal ganglion, histological sections of E16 and E19 ganglia were immunochemically stained (Fig. 1).

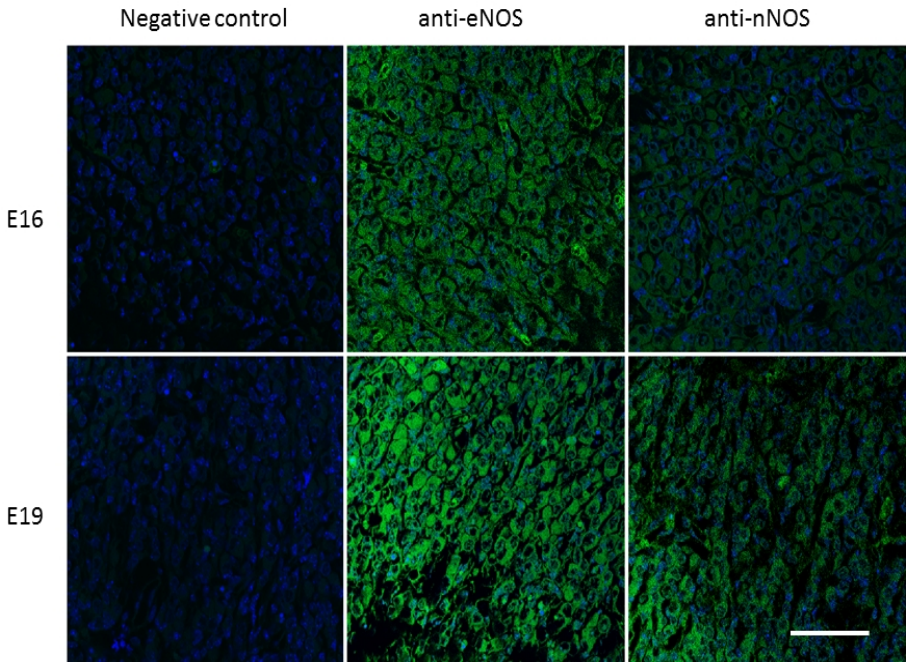

Fig. 1. Expression of NOS isoforms in the developing trigeminal ganglion

Confocal photomicrographs of TG demonstrating eNOS and nNOS staining (green) with DAPI nuclear staining shown in blue. Low levels of background staining were observed in the absence of primary antibody (negative control) with clear staining observed with both eNOS and nNOS antibodies. Scale bar represents 100μm

Sections of TG stained with secondary antibody alone demonstrated low levels of background staining (Fig. 1 negative control). In contrast positive immunoreactivity for both eNOS and nNOS was observed in E16 and E19 ganglia. Sections were counterstained with the nuclear dye DAPI. It is therefore clear that both enzymes are expressed in all trigeminal neurons both during (at E16) and after (at E19) the period of naturally occurring cell death. This raises the possibility that there is a physiological role for NOS activity in either promoting the survival of developing trigeminal neurons, or in promoting the death of neurons that are lost during this developmental period.

3.2 Developmental Switch in the Roles of NOS Isofroms in Trigeminal Neurons

To accurately ascertain the relative roles of the NOS isoforms eNOS and nNOS during this developmental timeframe, primary cultures of TG neurons were established and treated with routinely used pharmacological reagents known to specifically inhibit eNOS or nNOS. 7-NIO has been widely used to inhibit eNOS activity [30] and was used at concentrations that have successfully inhibited eNOS in other neuronal populations [8,27]. Here it was shown that administration of 50nM 7-NIO significantly reduced neuronal viability, in the presence of 10ng/ml NGF, for primary TG neurons derived from either E16 or E19 murine embryos (P<.001, n=6; Figs. 2A, 2D). At E16, specific inhibition of eNOS resulted in a 30.3±5.9% reduction in TG neuronal viability in response to 10ng/ml NGF *In vitro*, rising to 64.3±6.4% reduction in viability in response to saturating doses of NGF by E19. This increase in neuronal death between the two ages in the presence of the eNOS inhibitor was statistically significant (P<.001, n=6). These data reveal that TG neurons become increasingly dependent on eNOS activity for their survival in the presence of NGF *In vitro* as they mature beyond the period of naturally occurring neuronal death.

In contrast to eNOS inhibition, nNOS inhibition only significantly reduced neuronal viability at E16 (Fig. 2B). Thus nNOS activity was specifically inhibited with L-SMTC [31] at concentrations that have reduced nNOS activity in other neuronal populations [8,27]. In NGF-treated E16 TG cultures, inhibition of nNOS resulted in a 28±8.6% decrease in neuronal viability (P=.010; n=6). However, in cultures derived from neurons that have survived the period of naturally occurring cell death, E19, there was no decrease in neuronal viability. These data reveal that TG neurons no longer require nNOS activity to maintain their survival *In vitro* beyond the period of naturally occurring cell death.

Downstream of NOS activation, two intracellular signaling pathways are activated namely the sGC-dependent and sGC-independent pathways. To consider which of these pathways are active in TG neurons in culture, neurons were treated with the sGC inhibitor YC-1 [32] at a concentration that has been used in other neuronal populations (160nM) [8,27]. The presence of YC-1 in the culture medium did not significantly reduce neuronal viability in E16 cultures indicating that sGC-dependent signalling is not required for their *In vitro* survival. In contrast, there was a 52±14.9% decrease in neuronal viability in E19 cultures (P<.001; n=6) revealing that sGC-dependent signaling is required for the NGF response in E19 TG neurons *In vitro*.

These data reveal an interesting developmental switch in the NGF-dependent TG neurons as they come to the end of the period of naturally occurring neuronal death. Thus whilst neuronal death is ongoing, there is a reliance on nNOS signaling in a proportion of these neurons with this phenomenon not observed in E19 neurons (i.e beyond the period of neuronal death). Between E16 and E19 approximately 20% of TG neurons are lost in vivo

[33], and it can be postulated that those that depend upon nNOS activity for survival at E16 are therefore no longer present in the E19 cultures.

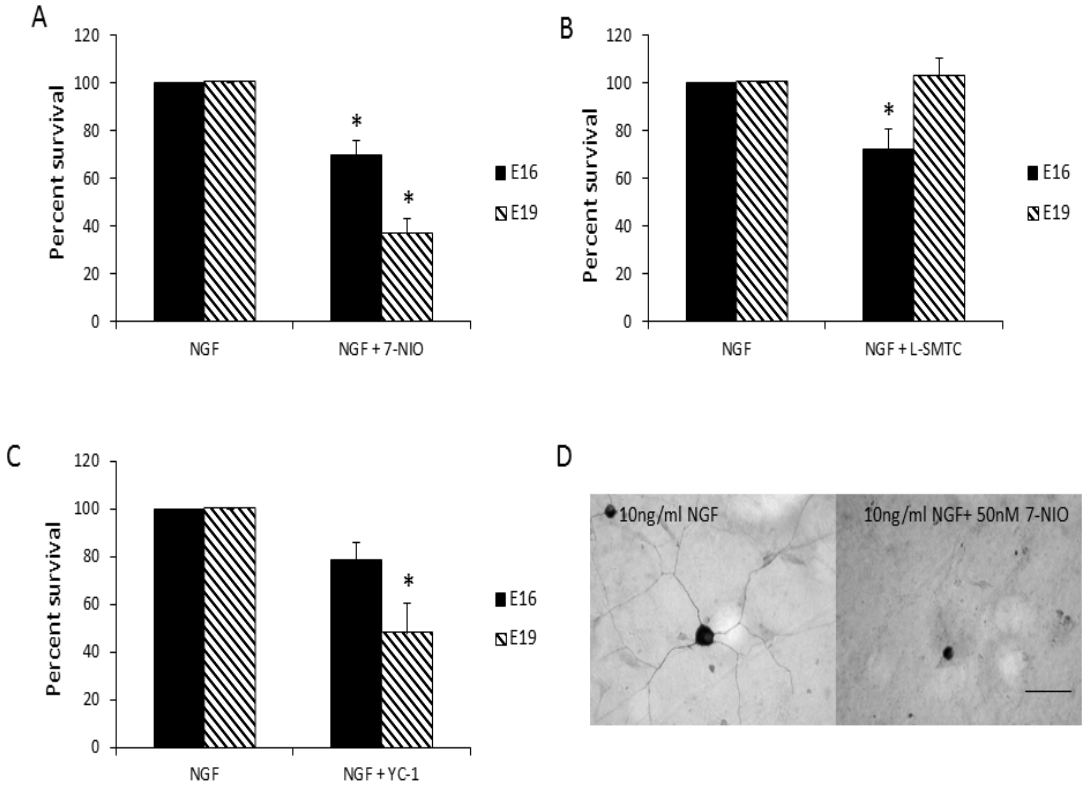

Fig. 2. Response of TG neurons to pharmacological inhibition of eNOS, nNOS and sGC activity *In vitro*

*Histographs depicting the survival response of TG neurons to pharmacological inhibition of eNOS (50nM 7-NIO; A), nNOS (10□M L-SMTC; B) or sGC (160nM YC-1; C). The means and standard error of the mean for 6 separate experiments are shown. * denotes statistical significant differences in neuronal viability relative to the 10ng/ml NGF control. Representative images showing βIII-tubulin stained E19 TG neurons treated with 10ng/ml NGF alone or in combination with 50nM 7-NIO are shown (D). Scale bar represents 40μm*

Furthermore, it is interesting to note the changing role of sGC in the TG NGF survival response during this developmental period. Whilst inhibition of this signaling pathway does not significantly reduce the survival of E16 neurons grown with NGF, it causes a 52% decrease in viability of E19 neurons grown with NGF. Thus whilst NO generated by either eNOS or nNOS is required for the survival of at least a proportion of E16 TG neurons cultured with NGF, this response does not require the activation of an sGC second messenger system. In the adult rat approximately 50% of TG neurons express sGC *In vivo* suggesting that its expression is restricted to TG neurons of a particular phenotype [34]. It is plausible that this population begin to express sGC only after the period of naturally occurring cell death. Certainly there is a clear change in the role of this messenger system during TG neurodevelopment that warrants further investigation.

One final point that deserves mention is that the immunohistochemical data (Fig. 1.) reveals widespread expression of eNOS and nNOS throughout developing TG neurons and yet only a proportion of these neurons die when these enzymes are pharmacologically inhibited. It has been demonstrated that NO is an important mediator of neuronal differentiation in rat primary cortical cultures [35], suggesting that NO may have important developmental roles in the nervous system beyond an involvement in neuronal survival.

3.3 Nos Activity is not involved in Cell Death Following Growth Factor Withdrawal

Following NGF withdrawal, TG neurons undergo apoptotic cell death [36]. Given that high levels of NO are well known to be neurotoxic [14,15], the possibility that NOS activation, leading to NO release, downstream of growth factor withdrawal (GFW) is involved in this cell death was investigated. Thus E16 and E19 TG neurons were grown in culture with NGF for 24 hours and then the NGF washed out of the culture medium and the activity of eNOS and nNOS inhibited pharmacologically as in section 3.2 above.

Following GFW, neither inhibition of eNOS (50nM 7-NIO; Fig. 3A), nor nNOS (10μM L-SMTC; Fig. 3B), prevents GFW-mediated cell death indicating that activity of these enzymes is not required to bring about cell death following GFW. Thus in the case of eNOS at E16, 60±3% of neurons die within 24 hours of GFW with 55.1±7.3% death recorded in cultures treated with 7-NIO at the time of GFW (*P*=.169; n= 4). Similarly, 7-NIO showed no neuroprotective or neurotoxic actions in E19 TG neurons following GFW (GFW: 47±6.5% death; GFW+7-NIO: 44±11% cell death).

Inhibition of nNOS had no significant effects on TG neuron viability at either E16 or E19 following NGF withdrawal. At E16 48±10.5% death occurred in nNOS inhibitor-treated GFW cultures and at E19 40.1±19% cell death occurred. Neither of these values differed significantly from GFW alone (*P*=.231; n=4).

To further verify whether NO plays a role following GFW, the level of NO release within individual TG neurons was ascertained by measuring fluorescent intensity per cell using the NO-sensing dye DAF-2DA (Figs. 3C-D). This dye is a cell permeable analog of DAF-2 that is hydrolyzed to DAF-2 by intracellular esterases. DAF-2 reacts rapidly with NO in the presence of oxygen to form the fluorescent compound triazolofluorescein which can be detected by fluorescent microscopy. There was no significant difference in NO levels between 10ng/ml NGF-treated TG neurons and following GFW at either E16 or E19 (*P*=.238; n= 3 experiments at each age). Thus there is no difference between the levels of NO detected in neurons undergoing cell death following GFW.

Thus neither activity of eNOS nor nNOS is required for TG neuron death following GFW. Given that there is no observed increase in NO release following NGF withdrawal it is clear that TG neuronal death following GFW is NO-independent. Coupled to the data obtained in section 3.2 (above), this implies that eNOS and nNOS generated low levels of NO are neuroprotective for TG neurons, and do not promote cell death that occurs during the period of naturally occurring neuronal loss. In keeping with this hypothesis it has been demonstrated that NO and sGC are required for the upregulation of protective factors in either NGF-deprived dorsal root ganglion (DRG) cultures or in DRG *In vitro* following axotomy [37]. Furthermore, during early development all DRG neurons express nNOS, and

the presence of either NO or NGF is required for their sustained survival *in vitro* further implicating NO are a neuroprotective factor in developing sensory neurons [38].

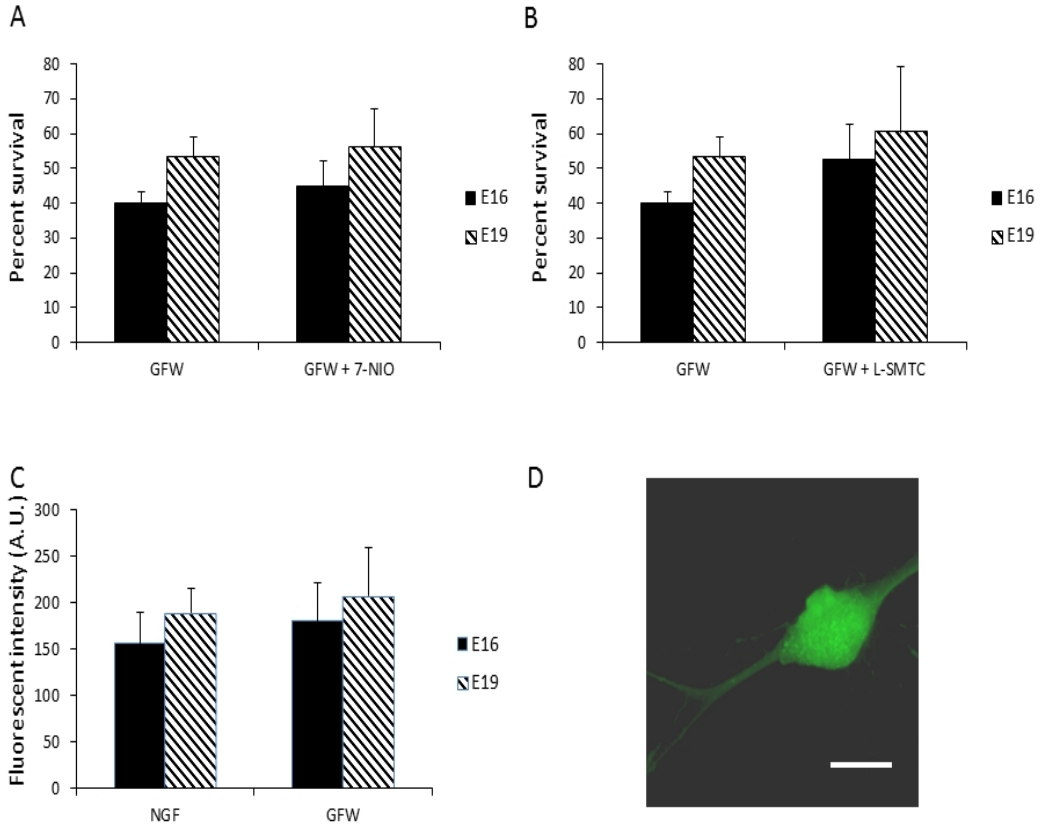

Fig. 3. Inhibition of eNOS or nNOS does not prevent growth factor withdrawal-mediated cell death

Histographs depicting the survival response of TG neurons to pharmacological inhibition of eNOS (50nM 7-NIO; A) or nNOS (10μM L-SMTC; B) following growth factor withdrawal (GFW) are shown. The means and standard error of the mean for 4 separate experiments are shown. The relative release of NO in neurons growth with NGF (10ng/ml) or following GFW was measured using the NO-sensing dye DAF-2DA (C) with a representative image of a DAF-2DA stained E19 trigeminal neuron grown with 10ng/ml NGF shown (D) in which the scale bar represents 12μm. The means and standard error of the mean for 3 separate experiments are shown

3.4 No Donors Trigger Apoptosis in NGF-Treated Trigeminal Cultures

It has long been known that administration of high levels of exogenous NO can be neurotoxic. To ascertain the effects of acute or sustained nitrosative insult, E16 and E19 TG neurons were exposed to potentially toxic levels of NO donors 500μM GSNO [8,27] or 200μM NOC-18 [8,27]. These experiments were carried out both in the presence of NGF (10ng/ml) or following GFW.

In the presence of NGF (10ng/ml), the administration of high levels of exogenous NO decreases neuronal viability whether given as a bolus or a sustained dose. Thus GSNO

reduces neuronal viability by 43.1±5.8% and 44.3±12.4% at E16 and E19 respectively (*P*<.001; n=4; Fig. 4A). There is no developmental change in the vulnerability of NGF-supported TG neurons to a bolus dose of NO *In vitro*. NOC-18 also markedly reduces the NGF survival response with a 60.1±7.2% decrease in cell survival established at E16 and 87.8±3.5% at E19 (*P*<.001; n=4; Fig. 4B). Furthermore, there is a significant difference between the viability decrease at E16 and that at E19 (*P*<.001; n=4) indicating that TG neurons become more susceptible to sustained NO-mediated toxicity with increasing development age.

In contrast following GFW, there is no additional neurotoxic effect of NO following administration of pharmacological donors (Figs. 4C and D). However, it is extremely interesting to note that in the case of sustained release of NO via NOC-18, neurons undergoing GFW survive better than those maintained in the saturating dose of NGF during pharmacological treatment.

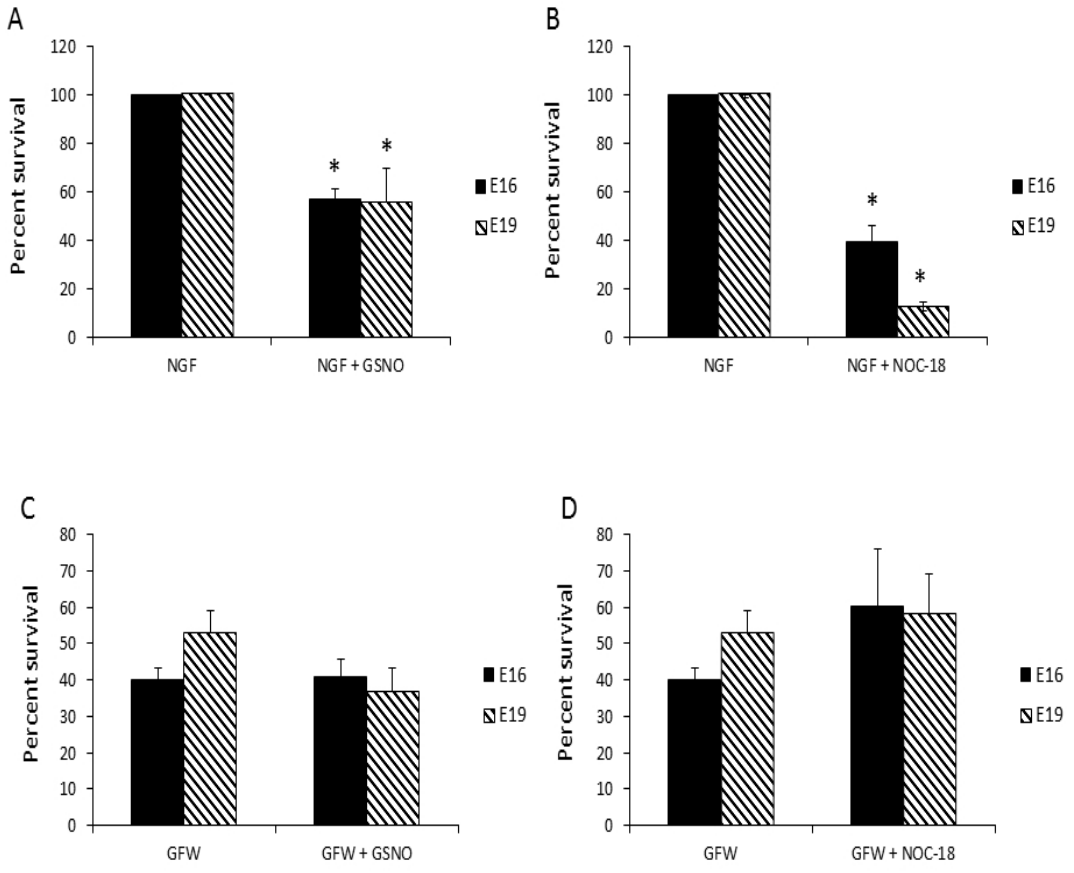

Fig. 4. Effects of exogenous NO on TG neurons in culture

*Histographs depicting the survival response of TG neurons to pharmacological donation of bolus (500μM GSNO; A, C), or sustained (200μM NOC-18; B, D) NO. Data for 10ng/ml NGF treated cultures (A, B) and following GFW (C, D) are shown. The means and standard error of the mean for 4 separate experiments are shown. * denotes statistical significant differences in neuronal viability relative to the 10ng/ml NGF control*

It is clear that the bolus dose of NO released following GSNO administration reduces the viability of both E16 and E19 neurons grown with NGF but a proportion of the neurons (around 60% at each age) is resilient to this insult. Following GFW there is no additive neurotoxic effect of GSNO over GFW alone implying that the GSNO vulnerable population of neurons is also vulnerable to GFW.

Sustained NO insult also reduces survival in response to NGF, with an increased effect at E19. At E16, 40% of neurons are invulnerable to sustained release of high levels of NO whereas at E19 only 12% of TG neurons survive this insult. Indeed it is clear that sustained nitrosative insult is more toxic than acute NO release at both ages implying a vulnerability to sustained NO-mediated damage and a better ability to deal with an acute insult. Interestingly after GFW, NOC-18 does not decrease neuronal survival further. Indeed NOC-18-treated neurons survive better following GFW than they do when NGF, which is neuroprotective, is co-administered. It is plausible that in the presence of NGF, because trigeminal neurons are already producing NO via the activities of eNOS and nNOS, the further addition of NOC-18 means that these NGF-supported cultures are actually exposed to far higher levels of NO than the GFW cultures, enhancing the observed neurotoxicity.

3.5 Trigeminal Neurons Become More Susceptible to Peroxynitrite as They Mature

In addition to the deleterious effects of NO *per se*, its metabolite peroxynitrite is a potent neurotoxin [27]. To test whether TG neurons are vulnerable to peroxynitrite, these cells were treated with the peroxynitrite donor SIN-1 (250μM) [27] either in NGF-maintained cultures or following GFW.

Administration of SIN-1 markedly attenuates the NGF survival response at both E16 and E19 (Fig. 5A) resulting in the loss of 48±4.1% of neurons and 98±1.1% of neurons respectively. The difference in response to SIN-1 between the two ages is significant (P<.001; n=4).

Following GFW, there is no difference in survival for E16 TG neurons when SIN-1 is administered to the cultures. However at E19 there is 47±6% cell death following GFW rising to 97.8±1.2% in the presence of SIN-1 (Fig. 5B). Thus TG neurons *In vitro* showed an increased death response to presence of peroxynitrite with developmental age, both in the presence of NGF and following GFW.

These studies have revealed an interesting insight into a developmental mechanism whereby the significance of NO signaling for neuronal survival undergoes dynamic changes during the development of the TG. These studies suggest that physiological eNOS and/or nNOS-generated low doses of NO are neuroprotective for at least a proportion of developing trigeminal sensory neurons. This is in keeping with other work mentioned above on DRG sensory neurons *In vitro* and *In vivo* [37,38] and also the discovery that nNOS-deficient mice lose a greater proportion of their neurons following axotomy than wild type counterparts [39] further reinforcing an important neuroprotective role for NO signaling in the peripheral nervous system. Therefore, this study expands on the work on DRG neurons to demonstrate an important role for NO signaling in TG neurons.

Many studies, including this one, have demonstrated a need for sGC signaling downstream of NO activity to confer neuroprotection [37]. However, it is clear that for TG neurons this is

only the case at E19 and that at E16, whilst NO is required for a proportion of the survival observed in response to NGF, sGC is not necessary to mediate this response. sGC-independent neuroprotection triggered by NO has been observed in other neuronal populations. For instance, downregulation of the NMDA receptor to prevent excitotoxicity is proposed to be modulated by direct nitrosylation of the NR1 and NR2 subunits of the receptor [40]. Thus there is precedent to the finding that the E16 NGF survival rsponse in NO-dependent and yet sGC-independent.

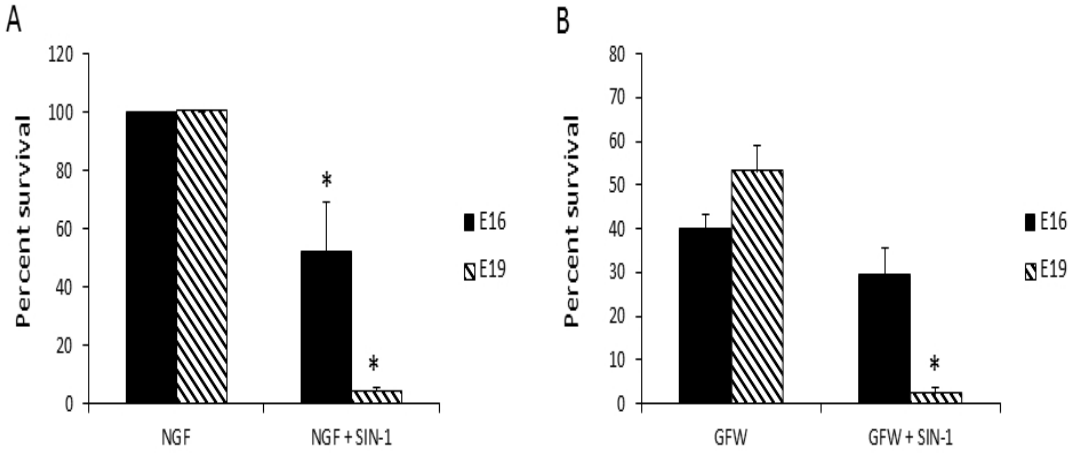

Fig. 5. Effects of peroxynitrite treatment on TG neurons in culture

*Histographs depicting the survival response of TG neurons to pharmacological donation of peroxynitrite (250μM SIN-1), either when cultured with 10ng/ml NGF (A) or following GFW (B). The means and standard error of the mean for 4 separate experiments are shown. * denotes statistical significant differences in neuronal viability relative to the 10ng/ml NGF control (A) and relative to survival following GFW (B)*

It is clear that NO has a role to play in the NGF survival response in TG neurons. However, one paradigm that was not considered in this preliminary study was whether low doses of NO donors were able to promote cell survival as has been demonstrated in other systems. Thus in primary hippocampal neurons, NO donors are neuroprotective and induce signaling mechanisms similar to those triggered by neurotrophins [41]. Therefore an important future consideration, following on from this study, will be to determine whether low dose NO donation is also neuroprotective for TG neurons during development.

The response of TG neurons *In vitro* to high levels of RNS is also interesting with both NO and peroxynitrite showing neurotoxic outcomes in the presence of NGF and only peroxynitrite proving toxic following GFW. Thus whilst low dose NO is neuroprotective for these neurons, it is clear that high doses of NO are toxic revealing the fine balance of NO that must be obtained to maintain normal physiology. What is unique about this study is the observation of a developmental trend in the vulnerability to RNS. Taking this further, the implication is that during the period of naturally occurring neuronal death TG neurons have protective mechanisms that reduce the risk of cell death following nitrosative insult. The maladaptive consequences of high levels of RNS have been extensively studied due to their link to neurodegenerative conditions such as Alzheimer's and Parkinson's diseases [2]. Thus a better understanding of this protective mechanism that supports at least a proportion of

developing TG neurons could confer insight into novel protective strategies to counteract the pathological effects of excessive RNS.

The *In vivo* source of high levels of NO is through activity of iNOS which is widely expressed in non-neuronal cells. In the peripheral nervous system elevated levels of iNOS have been observed in macrophages following sciatic nerve ligature [42], and in both macrophages and Schwann cells in models of neuropathic pain [43]. Thus when an inflammatory response is evoked, the high levels of NO released can contribute to neuropathology. Again a better understanding of why some E16 TG neurons are resistant to nitrosative stress could aid our understanding of peripheral nerve pathologies linked to NO increase and could, in time, aid in the search for treatments for these disorders.

4. CONCLUSION

The role of NO in the developing TG is complex and warrants further, more detailed, investigation following on from this preliminary study. It is clear that there is a shift towards heavier reliance on eNOS and sGC signaling as the neurons mature. Furthermore, vulnerability to nitrosative stress increases with advancing developmental stage. These findings have implications for our better understanding of the endogenous mechanisms that exist to protect neurons from nitrosative stress. It is clear therefore that closer determination of how neurons of the developing nervous system deal with potentially toxic levels of NO offers a number of benefits both in our understanding of neurodevelopment and in our quest to counteract the deleterious effects of nitrosative stress.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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