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Nicotine is neuroprotective to neonatal neurons of sympathetic ganglion in Rat

Mahadevappa P. Badanavalu¹ and Malathi Srivatsan² [Professor]

²Department of Biological Sciences, Arkansas State University, Jonesboro, AR 72401, United States

Abstract

Sympathetic neurons of SCG are dependent on availability of nerve growth factor (NGF) for their survival. SCG neurons express nicotinic receptors (nAChR) whose expression levels are modulated by nicotine. Nicotine exerts multiple effects on neurons, including neuroprotection, through nAChR binding. Although sympathetic neurons express robust levels of nAChR, a possible neuroprotective role for nicotine in these neurons is not well-understood. Therefore we determined the effect of nicotine exposure on survival of SCG neurons during NGF withdrawal in a well-established cell culture system. NGF was withdrawn in rat neonatal SCG neuron cultures which were then treated with either 10 μ M nicotine alone or with nAChR antagonists 0.1 μ M α -bungarotoxin (antagonist for α 7 subunit bearing nAChR) and 10 μ M mecamylamine (non-specific antagonist for ganglionic nAChR) for 48 hours. Apoptotic death was determined by TUNEL staining. Cell survival was also determined by MTS assay. Western blot analysis of ERK1/2 was also performed. Our results showed that exposure to 10 μ M nicotine significantly reduced apoptotic cell death in SCG neurons resulting from NGF withdrawal as shown by fewer TUNEL positive cells. The MTS assay results also revealed that 10 μ M nicotine concentration significantly increased cell survival thus indicating neuroprotective effect of nicotine against cell death resulting from NGF withdrawal. Nicotinic receptor antagonists (bungarotoxin & mecamylamine) attenuated the effect of nicotine's action of neuroprotection. Western blot analysis showed an increased expression of ERK1/2 in nicotine treated cultures suggesting nicotine provided neuroprotection in SCG neurons by increasing the expression of ERK1/2 through nicotinic receptor dependent mechanisms.

Keywords

Superior Cervical Ganglion; nicotinic receptor; nerve growth factor; apoptosis; cell survival; TRK receptor

Corresponding Author: Dr. Malathi Srivatsan, Professor, Department of Biological Sciences, Mailing Address: 3712 Ridgeway Circle, Jonesboro, AR 72404, msrivatsan@astate.edu.

¹Present Address: Department of Life Science, Bangalore University, Bengaluru, India 560056, prabhubm1@yahoo.co.in

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1. Introduction

Neurons of the superior cervical sympathetic ganglion (SCG) belonging to the peripheral autonomic nervous system require nerve growth factor (NGF) for their growth and survival during development and early postnatal life both *in vivo* and *in vitro* (Kristiansen and Ham, 2014; Levi-Montalcini, 1987; Levi-Montalcini and Booker, 1960). NGF mediates its trophic effects by binding to tyrosine kinase receptors (TrkA), a transmembrane protein leading to activation of signaling pathways downstream such as Phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinases (ERK1/2) (Anderson and Tolkovsky, 1999; Belliveau et al., 1997; Kuruvilla, 2004; Xue et al., 2000). Absence of Trk receptors in developing mice (Smeyne, 1994) or injection of antibody against Trk receptor to pregnant rat results in death of SCG neurons by apoptosis (Johnson et al., 1989; Smeyne, 1994). Cell culture studies show that the SCG neurons undergo death by apoptosis after NGF withdrawal (Edwards and Tolkovsky, 1994; Levi-Montalcini and Angeletti, 1968) and hence can serve as a good model to investigate mechanisms underlying survival, growth and cell death in sympathetic neurons in the presence and absence of NGF (Anderson and Tolkovsky, 1999; Deckwerth and Johnson, 1993; Xue, et al., 2000).

In addition to Trk receptors, SCG neurons also express significant levels of nicotinic acetylcholine receptors (nAChR) and thus respond to nicotine. nAChRs consist of several subtypes and are members of the neurotransmitter-gated super family of cation channels (Albuquerque et al., 1997; 2009; Lindstrom, 1997). nAChR subtypes are assembled as pentameric complexes of subunits such as $\alpha 1$ – $\alpha 10$, $\beta 1$ – $\beta 4$, γ , δ , and ϵ of which the neurons express α and β subunits. Due to the complexity of nAChR subtypes and their distribution in the nervous system nicotine seems to have protective, modulatory or toxic effects (review by Ferrea and Winterer, 2009) of which its neuroprotective role is getting more attention as it can be useful to treat neurodegeneration. Evidence is accumulating for a potential neuroprotective role for nicotine in different areas of the central nervous system. Both *in vitro* and *in vivo* studies using nicotinic agonists have provided direct support for a role of nicotinic receptors in providing neuroprotection (Mudo et al., 2007; Roncarati et al., 2009; Takeuchi et al., 2009). Increased intracellular calcium (Ca^{2+}) through the activation of homomeric $\alpha 7$ and the heteromeric $\alpha 4\beta 2$ isoforms of nAChRs appears to play a role in nicotine-induced neuroprotection. The increased intracellular Ca^{2+} can subsequently activate a variety of interrelated signal transduction pathways involving calcium-dependent kinases which may lead to activation of survival pathway (Akaike et al., 2010; Dajas-Bailador et al., 2000; Ferchmin et al., 2005; Kaneko et al., 1997). Nicotine at concentrations of 1–10 μM results in the activation of pro-survival signaling pathways. For example, nicotine activates extracellular signal-regulated kinase 1 and 2 (ERK1/2) which belong to family of mitogen-activated protein kinase (MAPK) in primary cortical neurons (Dajas-Bailador et al., 2002; Steiner et al., 2007) and spinal cord neurons in culture (Toborek et al., 2007). Similar effect of nicotine on induction of phosphorylation of ERK is observed in hippocampal slices (Bell et al., 2004), in PC12 cell line (Nakayama et al., 2001) and in different areas of the mouse brain *in vivo* (Valjent et al., 2004). Since SCG neurons express nicotinic receptors and undergo apoptosis in the absence of NGF it is an ideal model to study nicotine's possible neuroprotective effects on sympathetic neurons. Nicotine-mediated

neuroprotection and such neuroprotection involving ERK1/2 in SCG neurons when NGF is withdrawn is not known. Further, there are reports indicating that perinatal exposure to nicotine affects nicotinic receptor function in the sympathoadrenal system (Cohen et al., 2005). Infants exposed to nicotine during intrauterine development as well as neonatal life have more abnormalities related to autonomic function such as respiration (Cohen et al., 2002; Grove et al., 2001; Sharma and Vijayaraghavan, 2002; Sekhon et al., 2001; Slotkin, 1998; WHO report, 2011). Our earlier study showed that exposure to nicotine influences the pattern of expression of nicotinic receptors in neonatal SCG neurons (Srivatsan et al., 2006). Considering the significance of pre and perinatal nicotine exposure on autonomic neurons and possible effects of nicotine on apoptosis, we focused on the neuroprotective effect of nicotine on the cultured SCG neurons against apoptosis in response to NGF withdrawal in this study. We demonstrate that exposure to nicotine at 10 μ M concentration can protect SCG neurons when challenged with NGF withdrawal.

2. Materials and Methods

2.1 Cell culture

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the Arkansas State University (Protocol Number: 462323–1). Primary cultures of neonatal SCG neurons were set up by following the methods described previously by Srivatsan et al. (2006) with minor modifications. One-day-old Sprague-Dawley rat pups were anesthetized by hypothermia and decapitated. The SCG's from right and left sides were dissected out under the microscope using sterile techniques. The dissected ganglia with connective tissue were enzymatically digested by incubating in 0.5% collagenase (Invitrogen) followed by TrypLE (Invitrogen) for 15–20 min at 37°C in each. The ganglia were then washed with Dulbecco's modified essential medium (DMEM, GIBCO) containing 10% fetal bovine serum (FBS, Invitrogen) and deoxyribonuclease (75 μ g/ml DNase, Sigma-Aldrich) and then re-suspended in DMEM containing N2 supplement (Invitrogen), nerve growth factor (50 ng/ml NGF 2.5S, Invitrogen) and DNase. The ganglia were triturated through a 200 μ L sterile pipette tip until the cell clumps dissociated into single cell suspension. The cell suspension was then centrifuged for 3 min at 250 $\times$ g at 4°C to gently pellet the cells. The cell pellet was re-suspended in fresh DMEM containing 1mM glutamax (Gibco) and 1mM glutamine (Invitrogen), antibiotic solution (100U/ml Penicillin, 100 mg/ml Streptomycin, 250ng/ml Amphotericin B, Sigma-Aldrich), uridine (70 μ M/ml, Sigma-Aldrich), 5-fluoro-2-deoxyuridine (30 μ M, Sigma-Aldrich), NGF (50ng/ml), and N2 supplement and pre-plated onto uncoated 100mm culture dishes for 45 minutes of incubation at 37°C to allow the adhesive satellite glial cells to attach to the dish. The unattached neurons in the medium were collected by gently swirling the dish and this pre-plating step was repeated with the collected neurons once more. The medium containing neurons in suspension were then collected, centrifuged to pellet the cells as before, and the pellet was re-suspended in serum free neurobasal™ medium (GIBCO) consisting of the same mixture of constituents as above with one exception of B27 supplement (GIBCO) replacing N2 supplement. The cell density was then determined using a hemocytometer and cells were plated onto dishes coated with

poly-D lysine (80µg/mL, Sigma-Aldrich) and collagen (100µg/mL, Sigma-Aldrich). In the first set of experiment the cells were plated at a density of 3000 cells/ 35 mm glass bottom dishes (Mat Tek) for TUNEL(Terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labeling). In the second set of experiments cells were plated at a density of 1500 cells/ well in 96-well plate (BioFlex) to perform MTS [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays. In the third set of experiments $2.5-3 \times 10^4$ cells were plated in 60mm plastic dishes (Corning) to collect protein for western analysis. After plating, pre-warmed neurobasal™ medium with appropriate supplements as mentioned before was added to the dishes and the wells. The dishes were incubated at 37°C in 5% CO₂. The medium was changed after 24 hours of plating and every second day thereafter.

2.2 Nicotine Treatment

10mM stock solution of (–) Nicotine hydrogen tartrate (Sigma-Aldrich) was prepared in 0.01M phosphate buffered saline (PBS) pH7.4. The appropriate required dilution was made using culture medium and at the desired time, added to the cultures established in 96 well plates, 35 and 60mm dishes. Initially various concentrations of nicotine hydrogen tartrate such as 0.1µM, 1µM, 10 µM and 100 µM were tested and 10 µM concentration was chosen for all experiments described here based on the reliable effects of nicotine at that concentration in our experiments (data not shown) and also based on report that show nAChRs in cultured SCG neurons of rat responds to nicotine at 10 to 100 µM concentrations (Kristufec et al., 1999) as somatic nAChRs vary in their sensitivity from synaptic nAChRs. Four days after plating, in experimental group I, the SCG neuron cultures were exposed to 10µM nicotine in the absence of NGF for a period of 48 hours. In these sets some cultures (experimental group II and III) were pre-treated with nAChR antagonists and then with nicotine. The rat SCG neurons express $\alpha 3/\beta 2$ or $\alpha 3/\beta 4$ and $\alpha 7$ subunits of nAChRs (Signore et al., 2004; Srivatsan et al., 2006) of which $\alpha 7$ as well as $\alpha 3$ receptors bind to mecamylamine (Meca, a nonspecific nAChR antagonist, well known as ganglionic blocker) at 10 µM concentration while $\alpha 7$ receptors bind to α -bungarotoxin (BTX, a $\alpha 7$ nAChR antagonist) at nanomolar affinity (Dwoskin and Crooks, 2001; Papke et al., 2001 and our own dose-response experiments). Hence some of the cultures were treated for 20 minutes prior to nicotine treatment with either 0.1µM of BTX (experimental group II) or with 10 µM of Meca (experimental group III). In control groups, the medium in four days old SCG neuron cultures were replaced with fresh culture medium either with (control I) or without NGF (control II). After 48 hours of treatment, cell lysates collected from 60mm dishes were used for western blot analysis of total ERK1/2. From the cultures that were set up in 96 well plates, MTS assay was performed to determine cell viability and TUNEL staining was performed on cultures set up in glass bottom 35 mm dishes to evaluate cell death by apoptosis.

2.3 TUNEL Staining

TUNEL staining was performed using the *In Situ Death Detection Kit* (Roche) following the manufacturer's instructions. Briefly, after completion of the treatment SCG neuron cultures were fixed with 3% paraformaldehyde for 30 min at room temperature. After washing the cells three times with PBS they were permeabilized using freshly prepared 0.1% Triton X-100 (Sigma-Aldrich) in 0.1% sodium citrate (Sigma-Aldrich) for 2–3 minutes on ice.

Then the cells were incubated in humidified chamber at 37°C with a TUNEL reaction mixture for one hour in dark. The solution over the cells was removed carefully and the cells were washed three times with PBS. Following washing, the cells were incubated with a nuclear stain, DAPI (4', 6-diamidino-2-phenylindole) solution for 5 minutes and washed three times with PBS. The TUNEL positive (green fluorescent) cells were then counted from microscopic images obtained using an Olympus IX71 microscope, Dage camera and NIH image J software. The TUNEL positive and TUNEL negative cells were counted in approximately 20 fields in each dish with different treatment which were selected randomly without overlapping between the selected fields. The TUNEL positive cells were expressed as a percentage of the total number of cells counted in 20 fields.

2.4 MTS assay for the determination of cell viability based on metabolic function

Cell viability was determined by performing MTS assay using a reagent consisting of a tetrazolium compound [(3-(4, 5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H tetrazolium] and an electron coupling reagent (phenazine ethosulphate), the CellTiter 96 Aqueous One Solution (Promega). MTS is chemically reduced by reductase enzymes present in mitochondria of metabolically active, viable, healthy cells into formazan, which is soluble in tissue culture medium. Thus the values from MTS assay indicate the mitochondrial metabolic activity and the healthy status or viability of the cells. This assay was performed using live cells in culture. 20µL of CellTiter 96 Aqueous One solution reagent was directly added to the control and experimental culture wells in the 96-well plate and cells were incubated in the humidified chamber at 37°C and 5% CO₂ for 3 hours. Afterwards, the absorbance was measured at a wavelength of 490 nm using the Bio-Rad microplate reader. The cell viability of the experimental cultures was calculated as a percentage of cell viability in control cultures.

2.5 Western blot analysis

During the withdrawal of NGF, culture medium containing NGF was removed, cells were rinsed with fresh medium without NGF and new culture medium without NGF was added. After such NGF withdrawal, the SCG neuron cultures were treated with 10µM nicotine alone or in the presence of 0.1µM α-bungarotoxin and 10µM mecamylamine (nicotinic receptor antagonists) for 48 hours. The cells were then washed once in 2mL of prewarmed 0.01M PBS at pH7.4 and lysed using ice cold M-Per mammalian protein extraction reagent (Thermo Scientific) with added protease inhibitor cocktail (Pierce) and 0.5% Triton X-100. The lysates were centrifuged at 13,200xg for 10 minutes to remove cell debris. The supernatant were collected and protein concentrations were determined by performing protein assay using Bradford reagent (Sigma-Aldrich). 15 µg of total protein from each sample was prepared in sample buffer containing 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 5% of 2%(w/v) bromophenol blue in 0.5M Tris-HCl pH6.8 and boiled for 3 minutes. The prepared samples were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel at a constant voltage of 150 V for separation of proteins. The electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) for 2 hours at 0.2 amperes. Blots were blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich) in Tris-buffered saline with 0.05% (v/v) Tween-20 (TBS-T) for 60 minutes at room temperature and then incubated

at 4°C overnight in primary antibody diluted in TBS-T [anti-ERK1/2 (mouse) 1: 1000; Millipore]. Blots were washed three times in TBS-T and incubated for 60 minutes at room temperature in secondary antibody (Goat anti mouse IgG; 1:10000, Pierce). After washing for 3 times in TBS-T, the blots were developed using enhanced chemiluminescence (ECL) detection reagents and visualized using Kodak Image station 440CF. Later the densities of the stained bands were quantified using Kodak Image software 3.6.

2.7 Statistical analysis

To determine significance of differences, results were compared by one way *ANOVA* followed by the *Tukey's post-hoc* test. Data from MTS assays and TUNEL quantification are presented as means $\pm$ SEM from 3–6 independent experiments. Data of western blot analysis of total ERK1/2 expression are from four independent experiments. Differences are considered significant at $p < 0.05$.

3. Results

3.1 Exposure to Nicotine on morphology of SCG neurons deprived of NGF

The pre-plating steps to eliminate adhesive satellite glial cells, the addition of uridine (70 μ M) and 5-fluoro 2-deoxyuridine (30 μ M) to remove any remaining dividing satellite glial cells and also the use of serum-free culture medium all helped in obtaining neuron enriched cultures. By day four, cultures containing NGF (Fig. 1A) exhibited neurons with phase-bright soma and long smooth neurites, profusely branched to form a network among the neurons although there is a limited amount of cell death as cultures progressed in age as it does in all primary cultures of neurons.

Such healthy four day old SCG cultures were used for the experiments. Within 24 hours of NGF deprivation, SCG neurons began showing signs of membrane disruption, cellular shrinkage and vacuolization. By 48 hours, NGF-deprived cultures (Fig. 1B) exhibited floating dead cells and the attached neurons showed dramatic shrinkage and degeneration with disruption of neurites. In contrast, NGF deprived cultures that were treated with 10 μ M nicotine for 48 hours (experimental group I) had live neurons exhibiting phase-bright soma that retained most of the neurite network among them (Fig 1C).

3.2 Exposure to Nicotine on apoptosis in SCG neurons deprived of NGF

Since SCG neurons undergo apoptosis in the absence of NGF, we determined apoptosis in SCG neuron cultures in the five groups, control I, II and experimental I, II and III. The fluorescent microscopic assessment of TUNEL staining revealed that there were only a very few neurons that exhibited apoptotic cell death in control cultures in the presence of NGF (Fig. 2A) while SCG cultures deprived of NGF showed increased number of TUNEL positive cells (cells fluorescing green) indicating apoptotic death of neurons maintained in medium without NGF for 48 hours (Fig. 2B). However, NGF deprived SCG neurons treated with 10 μ M nicotine for 48 hours showed only a few TUNEL positive cells (Fig. 2C) and was comparable to control cultures with NGF.

When quantified, it was evident that apoptotic death was significantly reduced in the nicotine treated cultures although they did not have NGF (Fig. 3). When the $\alpha 7$ nAChRs were blocked by α -BTX, addition of nicotine still protected SCG neurons from apoptotic cell death. However when the non-specific nAChR antagonist that blocks almost all nAChRs in the SCG neurons was used, the neuroprotective effect of nicotine disappeared (Fig.3). ANOVA showed that there was a significant difference among the groups compared [$F = (DF 4, 40) 8.29, (**P < 0.000057)$]. Post-hoc analysis showed which of the individual groups differed significantly from each other. As shown in Fig. 3, it was evident that NGF withdrawal led to apoptosis among SCG neurons, presence of nicotine (10 μ M) did protect SCG neurons from apoptosis, and this neuroprotective effect of nicotine appeared to be negated by mecamylamine and not by α -BTX suggesting that nicotine rescued SCG neurons from NGF withdrawal- induced apoptosis mostly via the $\alpha 3$ nAChRs (Fig 3).

3.3 Nicotine exposure on viability of SCG neurons during NGF withdrawal

MTS assay which reflects metabolic function and thus the viability of cell was performed on SCG cultures that were established in 96-well plates. After four days, the cultures in the wells were treated as described in methods. Results of the MTS assay revealed that NGF withdrawal for 48 hours significantly reduced cell viability to only 25% when compared to the control SCG neurons maintained in NGF. In contrast, 10 μ M nicotine treatment had reduced the effect of NGF withdrawal and increased cell viability when compared to the control cultures maintained without NGF. There was a 20% increase in cell viability in cultures treated with 10 μ M nicotine and this increase in cell viability was statistically significant ($P < 0.01$, Fig.4). The different treatment groups showed significant differences in cell viability [$F = (DF 3, 12) 18.8, (**P < 0.00008)$]. Post-hoc analysis indicated that pretreatment of cultures with α -bungarotoxin prior to nicotine treatment neither increased nor reduced cell viability significantly (viability was almost same as control cultures maintained without NGF). However, cultures pretreated with mecamylamine and then with nicotine showed significantly less cell viability when compared to cultures treated with nicotine alone ($p < 0.01$, Fig 4) indicating that the cell viability afforded by nicotine is through nicotine acting via nAChR subtype primarily bearing $\alpha 3$ subunit.

3.4 Nicotine exposure on ERK1/2 expression in SCG neurons during NGF withdrawal

Western blot analysis of proteins present in the cell lysates of SCG neurons from the different groups were examined and showed a decreased expression of ERK1/2 (4.05 folds) in experimental cultures that did not have NGF when compared to control that had NGF (Fig. 5 B, C). Whereas, in nicotine treated experimental cultures devoid of NGF, expression of ERK1/2 was increased by 2.48 folds which was statistically significant ($P < 0.0002$) when compared to those that were deprived of both nicotine and NGF. While the presence of α -bungarotoxin which blocks $\alpha 7$ nAChRs reduced the nicotine-facilitated increase in ERK1/2 slightly (37% reduction, $p < 0.03$), the presence of mecamylamine that is an antagonist for most of the nAChRs including those with the $\alpha 3$ and $\alpha 4$ subunits significantly reduced the nicotine-facilitated increase in ERK1/2 (52% reduction, $p < 0.008$). These results (Fig.5C) show that the nicotine mediated increase in ERK1/2 expression in NGF deprived SCG neurons occurred by nAChR-mediated mechanisms.

4. Discussion

While there are a number of studies on nicotine's possible neuroprotective effects, such studies on autonomic neurons are lacking although autonomic neurons, especially the sympathetic neurons express nAChRs. Hence the present *in vitro* study was performed to evaluate the neuroprotective effect of nicotine on SCG neurons when challenged with NGF withdrawal. Although the satellite glial cells in SCG do play a role in synaptic regeneration (Hanani, 2010) they express predominantly muscarinic cholinergic receptors (Feldman-Goriachnik et al., 2018) and hence in our current study on determining the effect of nicotine we did not include the glial cells. Here, our results demonstrate that treatment with nicotine at 10 μ M concentration was able to attenuate the apoptotic cell death in SCG neurons upon NGF withdrawal. Further, increased expression of ERK1/2 in a group treated with nicotine alone indicated that the neuroprotection of nicotine is mediated through ERK1/2 which is known to be involved in signaling cell survival pathway.

In support of nicotine functioning as a neuroprotectant, several studies have identified different biochemical responses to nicotine that could contribute to its neuroprotective effects in CNS, including increased expression of growth/trophic factors such as NGF and FGF (Belluardo et al., 2000; Garrido et al., 2003; Harrod et al., 2011; Takarada et al., 2012), decreased nitric oxide generation (Shimohama et al., 1996; Toborek et al., 2000), decreased arachidonic acid release (Marin et al., 1997; Toborek et al., 2000) and decreased caspase signalling (Lu et al., 2017). Further increased expression/activation of ERK1/2 has been shown in PC12 cells (Nakayama et al., 2001) and spinal cord neurons (Toborek et al., 2007), which is attributed to nicotine-mediated neuroprotection. Similarly, nicotine-induced signaling of cell survival pathways have been identified in *Xenopus* oocytes (Hsu et al., 1997), hippocampal neurons and SH-SY5Y cell cultures (Dajas-Bailador et al., 2002), mouse primary cortical neurons (Steiner et al., 2007) and in mouse brains *in vivo* (Brunzell et al., 2003). Further, treatment with nicotine was shown to protect against apoptosis induced by NGF deprivation in PC12 cells (Yamashita and Nakamura, 1996; Xia et al., 1995), Fas-induced apoptosis in Jurkat cells (Holmstrom et al., 1998) and arachidonic acid induced apoptosis in spinal cord neurons culture (Toborek et al., 2007). It is interesting to note that ERK1/2 activation blocked the cell death induced by trophic deprivation of retinal ganglion cells and cerebellar granule neurons (Bonni et al., 1999; Villalba et al., 1997) similar to our findings here in sympathetic neurons.

In accordance with the above mentioned studies, results of the present study demonstrate that nicotine protected NGF deprived SCG neurons from undergoing apoptotic cell death. The nicotine-mediated neuroprotection observed in our study can be attributed to the increased expression of the ERK1/2 in NGF deprived SCG neurons treated with nicotine alone. From our results, the neuroprotective action of nicotine against NGF withdrawal in SCG neurons is shown to be mediated through nAChRs with both $\alpha 3$ and $\alpha 7$ subtypes while the contribution of $\alpha 3$ subtype being significant.

Similar to findings by others (Levi-Montalcini and Angeletti, 1968; Edwards and Tolkovsky, 1994) our results also showed that withdrawal of NGF over 48 hours resulted in apoptotic death in SCG neurons. Addition of nicotine was able to protect the SCG neurons from

apoptosis resulting from NGF withdrawal as the number of TUNEL positive cells upon nicotine treatment was very similar to that of the control cultures with NGF (Fig.3) in the present study. Our cell viability assay results strongly mirror these results (Fig.4) supporting our finding that nicotine does protect SCG neurons from cell death arising from NGF withdrawal. Addition of the non-specific nAChR antagonist mecamylamine that blocks most of the nAChRs was able to nullify this neuroprotective effect of nicotine while the addition of $\alpha 7$ nAChR –specific antagonist α -bungarotoxin reduced the neuroprotective effect of nicotine to some extent demonstrating that this anti-apoptotic action of nicotine on SCG neurons was dependent upon multiple types of nAChRs. Nicotine is known to exert its neuroprotective effects primarily through receptor-mediated mechanism(s) (Berg and Conroy, 2002; Bride and Leslie, 1999). Specifically, nicotine binding to nAChRs induces conformational changes of the receptor, opens the channel gate leading to a direct influx of calcium or an indirect increase in cytoplasmic calcium following neuronal excitation (Dajas-Bailador and Wonnacott, 2004; Xiao et al., 1998). Increased intracellular calcium can subsequently activate a variety of interrelated signal transduction mechanisms involving activation of calcium-dependent kinases. Our earlier study had shown that neonatal SCG neurons expressed nAChRs of both $\alpha 7$ and $\alpha 3$ subtypes and nicotine treatment during NGF withdrawal in these neurons increased expression of both $\alpha 7$ and $\alpha 3$ nAChRs (Srivatsan et al., 2006). The $\alpha 7$ nAChR are homomeric ligand-gated ion channels that allow Ca^{2+} influx, and is implicated in neuroprotection (Kihara et al., 1997) by transducing signals to PI3-kinase activating the anti-apoptotic kinase AKT (Kihara et al., 2001). On the other hand, nicotine can protect cells against apoptosis through the upregulation of XIAP and survivin in a $\alpha 3$ -nAChR-dependent manner (Dasgupta et al., 2006). Overall it appears that the cell proliferation and differentiation during development are mostly mediated by $\alpha 7$ nAChR and pro-survival effects are mediated by $\alpha 3$ or $\alpha 4$ nAChR (Dasgupta et al., 2006; Resende et al., 2008; 2008 a; Wang et al., 2017; West et al., 2003). These findings support our results indicating nicotine provides neuroprotection through both $\alpha 3$ and $\alpha 7$ nAChRs with a greater role for $\alpha 3$ nAChRs in preventing apoptosis upon NGF withdrawal in SCG neurons.

There are accumulating evidence to show that ERK1/2 which are serine/threonine kinases that participate in the Ras-Raf-MEK-ERK signal transduction cascade may mediate neuroprotection against damaging insults. For instance, ERK1/2 activation by BDNF was shown to protect cultured rat cortical neurons against apoptosis induced by DNA damage (Hetman et al., 1999; Gozdz et al., 2003). Similarly, Anderson and Tolkovsky (1999) showed that in cultured sympathetic neurons exposed to another DNA-damaging agent, cytosine arabinoside, MEK inhibitor PD98059 inhibited NGF-mediated protection, implicating ERK1/2 involvement. In the present study we observed that upon NGF withdrawal there was an increase in the number of TUNEL positive neurons indicating the DNA fragmentation in later stages of apoptotic cell death. However, nicotine at a concentration of 10 μM attenuated the effect of NGF withdrawal as indicated by decreased TUNEL positive neurons. Moreover the nicotine treatment resulted in the increased expression of ERK1/2 in NGF withdrawn SCG neurons implicating ERK1/2 involvement in neuroprotection. Since increased intracellular calcium can activate ERK1/2 (Veeranna et al., 2004) nAChR-mediated increase in intracellular calcium in SCG neurons could be responsible for the increased expression of ERK1/2 in the present study in response to

nicotine treatment when NGF was withdrawn (Fig. 5B and C). This increase in ERK1/2 was inhibited by the presence of nAChR antagonists confirming that the nAChR receptor-mediated mechanism enabled neuroprotection by nicotine in the absence of NGF in SCG neurons. Our results were consistent with the studies in which nicotinic receptor antagonists α -bungarotoxin (Toborek et al., 2007; Garrido et al., 2001; Serova and Sabban, 2002) and mecamylamine (Toborek et al., 2007) attenuated the protective effect of nicotine. These results indicate that nicotinic receptors play an important role in nicotine mediated-neuroprotection. Therefore, our results strengthen the notion that manipulations of the nAChR signaling pathway may be useful for neuroprotective interventions in diseases.

NGF is a target-derived growth factor for SCG neurons thus making sure only those neurons that make appropriate synaptic connections survive (Glebova and Ginty, 2004; Sharma et al., 2010). Our results show that nicotine exposure can prevent death of neurons even when NGF is not available. This suggests the possibility that pre or perinatal exposure to nicotine through cigarette smoke could interfere with this developmental regulation of survival of only those neurons that obtain NGF from targets. It would be interesting to confirm this possibility in future *in vivo* studies.

5. Conclusions

In summary, we have demonstrated that exposure to nicotine protected SCG neurons from apoptosis and increased ERK1/2 expression in cultured SCG neurons when challenged with NGF deprivation through nicotinic receptor-dependent mechanisms. Thus our data presented here show that nicotine can exert neuro protection in sympathetic neuron as it does in CNS neurons. Therefore, manipulations of the nAChR signaling pathway may be useful for interventions in pathologies related to sympathetic neurons. The results also suggest the possibility of pre and/or perinatal exposure to nicotine interfering with the developmentally programmed cell death.

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Abbreviations:

ERK1/2	extracellular signal-regulated kinases
MTS	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
SCG	superior cervical ganglion
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labeling

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Highlights

- Apoptotic cell death occurred among SCG neurons in culture when nerve growth factor (NGF) was withdrawn over a period of 48 hours as revealed by morphological changes, significantly increased TUNEL positive cells and much reduced population of metabolically active surviving cells.
- Treatment with 10 μ M nicotine showed significant reduction in cell death resulting from NGF withdrawal as shown by decreased TUNEL positive cells and increased cell survival.
- There was an increased expression of ERK1/2 in the nicotine treated cultures indicating that nicotine-mediated neuroprotection against apoptosis resulting from NGF deprivation may be by the activation of survival signaling kinases including ERK1/2.
- Neuroprotective effect of nicotine was seen even in the presence of nicotinic receptor blockers, α -bungarotoxin to a lesser extent and mecamylamine to a larger extent suggesting that nicotine mediated neuroprotection through both α 7 and α 3 nAChRs, however mostly via α 3 nAChR.
- These results are important because they show that pre and perinatal exposure to nicotine can interfere with developmentally regulated programmed cell death in sympathetic neurons. The results also underscore the therapeutic significance of nicotine for neuroprotection.

Figure 1: NGF withdrawal and nicotine exposure on morphology of SCG neurons

(A) shows SCG neurons in control medium with NGF exhibiting phase bright cell bodies, with long neurites profusely branching indicating healthy neurons. Occasionally a dead cell can be seen (arrow). In contrast, (B) shows many dying neurons (arrows) exhibiting shrunken cytoplasm along with disrupted neurites when exposed to medium without NGF for 48 hours. When the dead cells float, the disintegrating neurites can be seen without cell bodies. However addition of nicotine (10μM) over the 48 hours while NGF was withdrawn resulted in preventing cell death as seen by healthy neurons with profuse neurites seen in C.

Figure 2: NGF withdrawal and nicotine exposure on cell death in SCG neurons

Micrographs A, B and C are merged images of DAPI nuclear staining of all cells (blue fluorescence) and TUNEL staining of apoptotic nuclei (green fluorescence) while micrographs Aa, Bb and Cc show only the TUNEL staining of apoptotic cells. Control (A, Aa) culture of SCG neurons with NGF shows only a few apoptotic cells as seen by nuclear fragmentation and TUNEL positive green fluorescence. SCG neurons undergo apoptosis extensively when NGF is withdrawn from the culture medium for 48 hours (B, Bb) as shown by many nuclei being TUNEL positive while presence of 10μM nicotine in cultures devoid of NGF (C, Cc) reduced apoptosis as shown by fewer TUNEL positive nuclei.

Figure 3: Quantitative determination of apoptosis in SCG neurons during NGF withdrawal and exposure to nicotine

TUNEL positive cells were counted in control with NGF (Control), control without NGF (CON- NGF), 10 μ M nicotine treated without NGF (-NGF+10 uM NIC), 10 μ M nicotine treated with nAChR antagonist α - Bungaratoxin (-NGF+NIC+0.1uM BTX) and 10 μ M nicotine treated with nAChR antagonist mecamylamine (-NGF+NIC+10 uM MECA) as described in methods. ANOVA showed that there is a significant difference among the groups compared [$F = (DF 4, 40) 8.29, (**P < 0.00006)$]. Post-hoc analysis showed which of the individual groups differed significantly from each other, shown here by connecting lines and the levels of significance shown by * when $p < 0.05$ and ** when $p < 0.01$. Data shown are Mean $\pm$ SEM.

Figure 4: NGF withdrawal and exposure to nicotine on cell viability (as indicated by increased metabolic activity) of SCG neurons

Treatment of SCG cultures devoid of NGF with 10 μ M Nicotine significantly increased cell viability when compared to that of cultures without NGF. Treatment with nAChR antagonists α -bungarotoxin and mecamylamine prior to nicotine treatment showed a decreased cell viability attenuating the effect of nicotine action of neuroprotection. [n=3–5 independent experiments, [F= (DF 3, 12) 18.8, (**P<0.00008)]. Post-hoc analysis showed which of the individual groups differed significantly from each other, shown here by connecting lines and the levels of significance shown by * when p<0.05 and ** when p<0.01. Data shown are Mean $\pm$ SEM.

Figure 5: NGF withdrawal and exposure to nicotine on the expression of ERK1/2 in SCG neurons

(A) Changes in ERK1/2 expression in SCG neurons after different treatments were observed by quantitative western blot analysis. After 48 hours of different treatments, the proteins (10 μ g/lane) in cell lysates of SCG neurons along with protein standards were separated by SDS-PAGE. (B) Separated proteins were transferred to membrane and probed with mouse anti

ERK1/2 monoclonal primary antibody to identify the expressed ERK1/2 as shown here by a representative immunoblot. (C) The bar graph is the combined densitometry data obtained using Kodak image station software version 3.0 from four western blots. When NGF was withdrawn, treatment of SCG neurons with 10 μ M Nicotine with and without nAChR antagonists resulted in significant differences in ERK1 and ERK2 expressions [F (3, 12) = 9.6227 (*p<0.002)] and [F (3, 12) = 12.3118 (*p<0.0007)] respectively. Post-hoc analysis showed which of the individual groups differed significantly from each other, shown here by connecting lines and the levels of significance shown by * when p<0.05 and ** when p<0.01. Data shown are Mean $\pm$ SEM.