Autonomic Neuroscience: Basic and Clinical 216 (2019) 25–32

Contents lists available at ScienceDirect

Autonomic Neuroscience: Basic and Clinical

journal homepage: www.elsevier.com/locate/autneu

Nicotine is neuroprotective to neonatal neurons of sympathetic ganglion in rat

Mahadevappa P. Badanavalu[1], Malathi Srivatsan*

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

ARTICLE INFO

Keywords:
Superior cervical ganglion
Nicotinic receptor
Nerve growth factor
Apoptosis
Cell survival
TRK receptor

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 NGF was withdrawn in rat neonatal SCG neuron cultures which were then treated with either 10 μM nicotine alone or with nAChR antagonists 0.1 μM α-bungarotoxin (antagonist for α7 subunit bearing nAChR) and 10 μM mecamylamine (non-specific antagonist for ganglionic nAChR) for 48 h. 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 μM 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 μM 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.

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 et al., 2004; Xue et al., 2000). Absence of Trk receptors in developing mice (Smeyne et al., 1994) or injection of antibody against Trk receptor to pregnant rat results in death of SCG neurons by apoptosis (Johnson Jr. et al., 1989). 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 Jr., 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 α1–α10, β1–β4, γ, δ, and ε of which the neurons express α and ß 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

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

* Corresponding author at: Department of Biological Sciences, 3712 Ridgeway Circle, Jonesboro, AR 72404, United States.
E-mail address: msrivatsan@astate.edu (M. Srivatsan).
[1] Present Address: Department of Life Science, Bangalore University, Bengaluru 560056, India.

https://doi.org/10.1016/j.autneu.2018.08.004
Received 11 May 2018; Received in revised form 13 July 2018; Accepted 31 August 2018

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 α7 and the heteromeric α4β2 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 kinase1 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; World Health Organization, 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 μM 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 deoxyriboneuclease (75 μg/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 μL sterile pipette tip until the cell clumps dissociated into single cell suspension. The cell suspension was then centrifuged for 3 min at 250 × *g* at 4 °C to gently pellet the cells. The cell pellet was re-suspended in fresh DMEM containing 1 mM glutamax (Gibco) and 1 mM glutamine (Invitrogen), antibiotic solution (100 U/ml Penicillin, 100 mg/ml Streptomycin, 250 ng/ml Amphotericin B, Sigma-Aldrich), uridine (70 μM/ml, Sigma-Aldrich), 5-fluoro-2-deoxyuridine (30 μM, Sigma-Aldrich), NGF (50 ng/ml), and N2 supplement and pre-plated onto uncoated 100 mm culture dishes for 45 min 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 (80 μg/ml, Sigma-Aldrich) and collagen (100 μg/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 × 10^4 cells were plated in 60 mm 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%CO_2. The medium was changed after 24 h of plating and every second day thereafter.

2.2. Nicotine treatment

10 mM stock solution of (−) Nicotine hydrogen tartrate (Sigma-Aldrich) was prepared in 0.01 M phosphate buffered saline (PBS) pH 7.4. 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 60 mm dishes. Initially various concentrations of nicotine hydrogen tartrate such as 0.1 μM, 1 μM, 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 (Kristufek 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 10 μM nicotine in the absence of NGF for a period of 48 h. 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 α3/ß2 or α3/ß4 and α7 subunits of nAChRs (Signore et al., 2004; Srivatsan et al., 2006) of which α7 as well as α3 receptors bind to mecamylamine (Meca, a nonspecific nAChR antagonist, well known as ganglionic blocker) at 10 μM concentration while α7 receptors bind to α-bungarotoxin (BTX, a α7 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 min prior to nicotine treatment with either 0.1 μM 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 h of treatment, cell lysates collected from 60 mm 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 min 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 min 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. 20 µL 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% CO_2 for 3 h. 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 10 µM nicotine alone or in the presence of 0.1 µM α-bungarotoxin and 10 µM mecamylamine (nicotinic receptor antagonists) for 48 h. The cells were then washed once in 2 mL of prewarmed 0.01 M PBS at pH 7.4 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 13,200 × *g* for 10 min 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-mercaptoethnol, 5% of 2% (w/v) bromophenol blue in 0.5 M Tris-HCl pH 6.8 and boiled for 3 min. 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 h at 0.2 A. 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 min 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 min 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.

2.6. 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 ± SEM from 3 to 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 µM) and 5-fluoro 2-deoxyuridine (30 µM) 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.

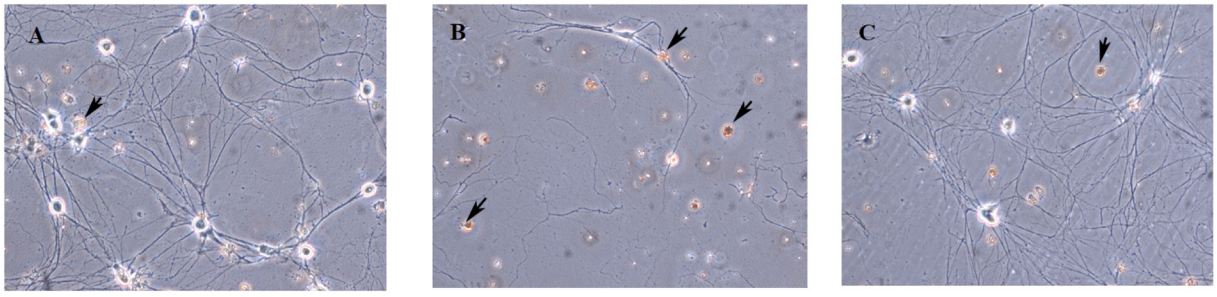

Fig. 1. NGF withdrawal and nicotine exposure on morphology of SCG neurons.
(A) shows SCG neurons in control medium with NGF exhibiting phase bright cell bodies, with long neurites profusely branching indicating healthy neurons. Occasionally a dead cell can be seen (arrow). In contrast, (B) shows many dying neurons (arrows) exhibiting shrunken cytoplasm along with disrupted neurites when exposed to medium without NGF for 48 h. When the dead cells float, the disintegrating neurites can be seen without cell bodies. However addition of nicotine (10 µM) over the 48 h while NGF was withdrawn resulted in preventing cell death as seen by healthy neurons with profuse neurites seen in C.

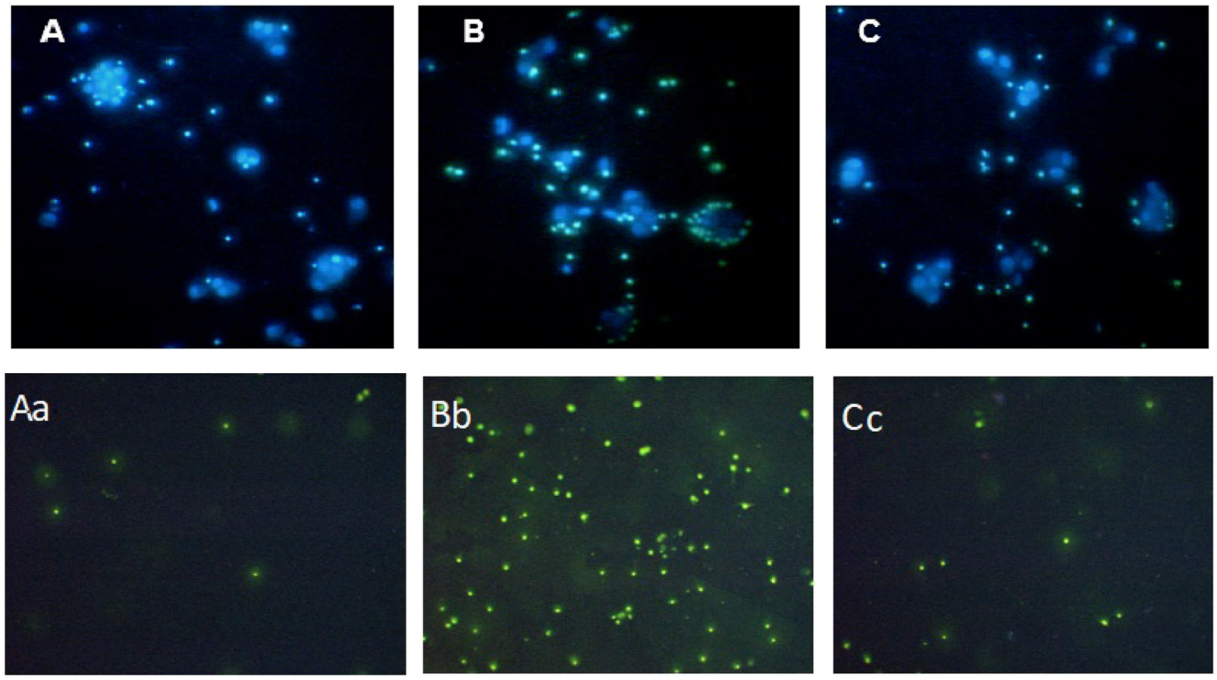

Fig. 2. NGF withdrawal and nicotine exposure on cell death in SCG neurons.
Micrographs A, B and C are merged images of DAPI nuclear staining of all cells (blue fluorescence) and TUNEL staining of apoptotic nuclei (green fluorescence) while micrographs Aa, Bb and Cc show only the TUNEL staining of apoptotic 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 h (B, Bb) as shown by many nuclei being TUNEL positive while presence of 10 μM nicotine in cultures devoid of NGF (C, Cc) reduced apoptosis as shown by fewer TUNEL positive nuclei. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Such healthy four day old SCG cultures were used for the experiments. Within 24 h of NGF deprivation, SCG neurons began showing signs of membrane disruption, cellular shrinkage and vacuolization. By 48 h, 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 μM nicotine for 48 h (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 1, 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 h (Fig. 2B). However, NGF deprived SCG neurons treated with 10 μM nicotine for 48 h 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 α7 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

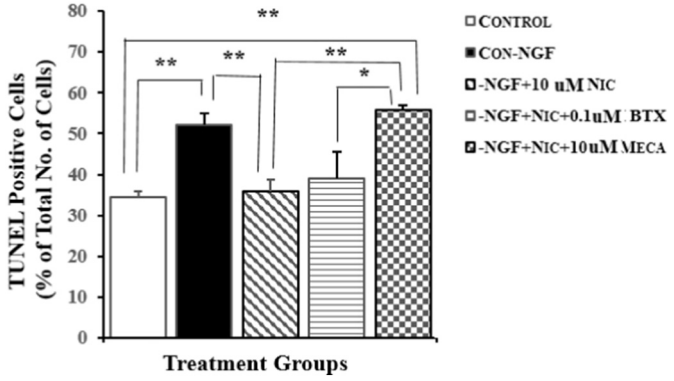

Fig. 3. Quantitative determination of apoptosis in SCG neurons during NGF withdrawal and exposure to nicotine.
TUNEL positive cells were counted in control with NGF (Control), control without NGF (CON-NGF), 10 μM nicotine treated without NGF (−NGF + 10 μM NIC), 10 μM nicotine treated with nAChR antagonist α-Bungaratoxin (−NGF + NIC + 0.1 μM BTX) and 10 μM nicotine treated with nAChR antagonist mecamylamine (−NGF + NIC + 10 μM 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 ± SEM.

evident that NGF withdrawal led to apoptosis among SCG neurons, presence of nicotine (10 μM) 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 α3 nAChRs (Fig. 3).

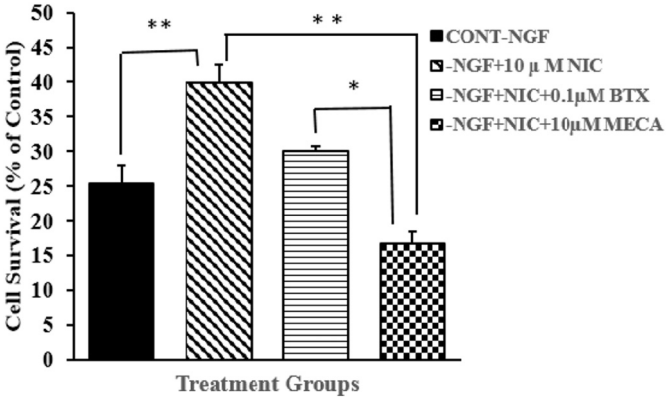

Fig. 4. NGF withdrawal and exposure to nicotine on cell viability (as indicated by increased metabolic activity) of SCG neurons.
Treatment of SCG cultures devoid of NGF with 10 μM Nicotine significantly increased cell viability when compared to that of cultures without NGF. Treatment with nAChR antagonists 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 ± SEM.

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 h significantly reduced cell viability to only 25% when compared to the control SCG neurons maintained in NGF. In contrast, 10 μM 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 μM 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 α3 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. 5B, 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 α7 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 α3 and α4 subunits significantly reduced the nicotine-facilitated increase

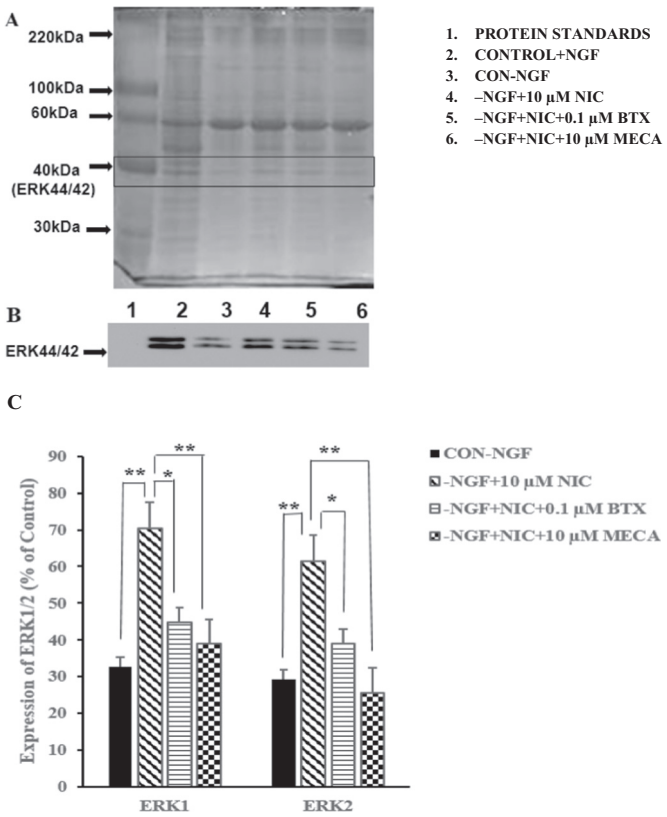

Fig. 5. NGF withdrawal and exposure to nicotine on the expression of ERK1/2 in SCG neurons.
(A) Changes in ERK1/2 expression in SCG neurons after different treatments were observed by quantitative western blot analysis. After 48 h of different treatments, the proteins (10 μg/lane) in cell lysates of 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 version3.0 from four western blots. When NGF was withdrawn, treatment of SCG neurons with 10 μM 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 ± SEM.

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 μM 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 signaling (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 culures (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., 1996), 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 neuroprtoection observed in our study can be attributed to the increased expression of the ERK1/2 in NGF derprived 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 α3 and α7 subtypes while the contribution of α3 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 h 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 α7 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 α7 and α3 subtypes and nicotine treatment during NGF withdrawal in these neurons increased expression of both α7 and α3 nAChRs (Srivatsan et al., 2006). The α7 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 α3-nAChR-dependent manner (Dasgupta et al., 2006). Overall it appears that the cell proliferation and differentiation during development are mostly mediated by α7 nAChR and pro-survival effects are mediated by α3 or α4 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 α3 and α7 nAChRs with a greater role for α3 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.

Declarations of interest

None.

Acknowledgements

This work was supported by National Institute of Drug Abuse (NIDA) Grant R15 DA019971 and Arkansas Biosciences Institute, United States access to equipment (to M. Srivatsan). The contents of this work are solely the responsibility of the authors and do not necessarily reflect the official views of the federal government.

References

Akaike, A., Takada-Takatori, Y., Kume, T., Izumi, Y., 2010. Mechanisms of neuroprotective effects of nicotine and acetylcholinesterase inhibitors: role of alpha4 and alpha7 receptors in neuroprotection. J. Mol. Neurosci. 40, 211–216.

Albuquerque, E.X., Alkondon, M., Pereira, E.F., Castro, N.G., Schrattenholz, A., Barbosa, C.T., Bonfante-Cabarcas, R., Aracava, Y., Eisenberg, H.M., Maelicke, A., 1997. Properties of neuronal nicotinic acetylcholine receptors: pharmacological characterization and modulation of synaptic function. J. Pharmacol. Exp. Ther. 280, 1117–1136.

Albuquerque, E.X., Pereira, E.F., Alkondan, M., Rogers, S.W., 2009. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol. Rev. 89, 73–120.

Anderson, C.N., Tolkovsky, A.M., 1999. A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside. J. Neurosci. 19, 664–673.

Bell, K.A., O'Riordan, K.J., Sweatt, D., Dineley, K.T., 2004. MAPK recruitment by ß-amyloid in organotypic hippocampal slice cultures depends on physical state and exposure time. J. Neurochem. 91, 349–361.

Belliveau, D.J., Krivko, I., Kohn, J., Lachance, C., Pozniak, C., Rusakov, D., Kaplan, D., Miller, F.D., 1997. NGF and neurotrophin-3 both activate TrkA on sympathetic neurons but differentially regulate survival and neuritogenesis. J. Cell Biol. 136, 375–388.

Belluardo, N., Mudo, G., Blum, M., Amato, G., Fuxe, K., 2000. Neurotrophic effects of central nicotinic receptor activation. J. Neural Transm. Suppl. 60, 227–245.

Berg, D.K., Conroy, W.G., 2002. Nicotinic alpha 7 receptors:synaptic options and downstream signaling in neurons. J. Neurobiology 53, 512–523.

Bonni, A., Brunet, A., West, A.E., Datta, S.R., Takasu, M.A., Greenberg, M.E., 1999. Cell survival promoted by the ras–MAPK signaling pathway promotes cell survival via a dual transcription-dependent and transcription-independent mechanism. Science 286, 1358–1362.

Bride, R.S., Leslie, F.M., 1999. The alpha 7 acetylcholine receptor in neuronal plasticity. Mol. Neurobiol. 20, 1–16.

Brunzell, D.H., Russell, D.S., Picciotto, M.R., 2003. *In vivo* nicotine treatment regulates mesocorticolimbic creb and erk signaling in c57bl/6j mice. J. Neurochem. 84, 1431–1441.

Cohen, G., Han, Z.Y., Grailhe, R., Gallego, J., Gaultier, C., Changeux, J.P., Lagercrantz, H., 2002. Beta 2 nicotinic acetylcholine receptor subunit modulates protective responses to stress: a receptor basis for sleep-disordered breathing after nicotine exposure. Proc. Natl. Acad. Sci. U. S. A. 99, 13272–13277.

Cohen, G., Roux, J.C., Grailhe, R., Malcolm, G., Changeux, J.P., Lagercrantz, H., 2005. Perinatal exposure to nicotine causes deficits associated with a loss of nicotinic receptor function. Proc. Natl. Acad. Sci. U. S. A. 102, 3817–3821.

Dajas-Bailador, F., Wonnacott, S., 2004. Nicotinic acetylcholine receptors and the regulation of neuronal signaling. Trends Pharmacol. Sci. 25, 317–324.

Dajas-Bailador, F.A., Lima, P.A., Wonnacott, S., 2000. The alpha7 nicotinic acetylcholine receptor subtype mediates nicotine protection against NMDA excitotoxicity in primary hippocampal cultures through a Ca (2+) dependent mechanism. Neuropharmacology 39, 2799–2807.

Dajas-Bailador, F.A., Soliakov, L., Wonnacott, S., 2002. Nicotine activates the extracellular signal-regulated kinase 1/2 via the alpha7 nicotinic acetylcholine receptor and protein kinase A, in SH-SY5Y cells and hippocampal neurones. J. Neurochem. 80, 520–530.

Dasgupta, P., Kinkade, R., Joshi, B., Decook, C., Haura, E., Chellappan, S., 2006. Nicotine inhibits apoptosis induced by chemotherapeutic drugs by up-regulating XIAP and survivin. Proc. Natl. Acad. Sci. U. S. A. 103, 6332–6337.

Deckwerth, T.L., Johnson Jr., E.M., 1993. Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. J. Cell Biol. 123, 1207–1222.

Dwoskin, L., Crooks, P., 2001. Competitive neuronal nicotinic receptor antagonists: a new direction for drug discovery. J. Pharmacol. Exp. Ther. 298, 395–402.

Edwards, S.N., Tolkovsky, A.M., 1994. Characterization of apoptosis in cultured rat sympathetic neurons after nerve growth factor withdrawal. J. Cell Biol. 124, 537–546.

Feldman-Goriachnik, R., Wu, B., Hanani, M., 2018. Cholinergic response of satellite glial cells in the superior cervical ganglia. Neurosci. Lett. 671, 19–24.

Ferchmin, P.A., Hao, J., Perez, D., Penzo, M., Malsonado, H.M., Gonzalez, M.T., Rodriquez, A.D., de Vellis, J., 2005. Tobacco cembranoids protect the function of acute hippocampal slices against NMDA by a mechanism mediated by alpha4beta2 nicotinic receptors. J. Neurosci. Res. 82, 631–641.

Ferrea, S., Winterer, G., 2009. Neuroprotective and neurotoxic effects of nicotine. Parmacopsychiatry 42, 255–265.

Garrido, R., Mattson, M.P., Hennig, B., Toborek, M., 2001. Nicotine protects against arachidonic-acid-induced caspase activation, cytochrome c release and apoptosis of cultured spinal cord neurons. J. Neurochem. 76, 1395–1403.

Garrido, R., King-Pospisil, K., Son, K.W., Hennig, B., Toborek, M., 2003. Nicotine upregulates nerve growth factor expression and prevents apoptosis of cultured spinal cord neurons. Neurosci. Res. 47, 349–355.

Glebova, N.O., Ginty, D.D., 2004. Heterogeneous requirement of NGF for sympathetic target innervation in vivo. J. Neurosci. 24, 743–751.

Gozdz, A., Habas, A., Jaworski, J., Zielinska, M., Albrecht, J., Chlystun, M., Jalili, A., Hetman, M., 2003. Role of *N*-methyl-D-aspartate receptors in the neuroprotective activation of extracellular signal-regulated kinase1/2 by cisplatin. J. Biol. Chem. 278, 43663–43671.

Grove, K.L., Sekhon, H.S., Brogan, R.S., Keller, J.A., Smith, M.S., Spindel, E.R., 2001. Chronic maternal nicotine exposure alters neuronal systems in the arcuate nucleus that regulate feeding behavior in the newborn rhesus macaque. Clin. Endocrinol. Metab. 86, 5420–5426.

Hanani, M., 2010. Satellite glial cells in the sympathetic and parasympathetic ganglia: in search of function. Brain Res. Rev. 64, 304–327.

Harrod, S.B., Lacy, R.T., Zhu, J., Hughes, B.A., Perna, M.K., Brown, R.W., 2011. Gestational IV nicotine produces elevated brain-derived neurotrophic factor in the mesocorticolimbic dopamine system of adolescent rat offspring. Synapse 65, 1382–1392.

Hetman, M., Kanning, K., Cavanaugh, J.E., Xia, Z., 1999. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. J. Biol. Chem. 274, 22569–22580.

Holmstrom, T.H., Chow, S.C., Elo, I., Coffey, E.T., Orrenius, S., Sistonen, L., Eriksson, J.E., 1998. Suppression of Fas/APO-1-mediated apoptosis by mitogen-activated kinase signaling. J. Immunol. 160, 2626–2636.

Hsu, N.-Y., Edwards, S., Wecker, L., 1997. Nicotine enhances the cyclic AMP-dependent protein kinase-mediated phosphorylation of a4 subunits of neuronal nicotinic receptors. J. Neurochem. 69, 2427–2431.

Johnson Jr., E.M., Osborne, P., Taniuchi, M., 1989. Destruction of sympathetic and sensory neurons in the developing rat by a monoclonal antibody against the nerve growth factor (NGF) receptor. Brain Res. 478, 166–170.

Kaneko, S., Maeda, T., Kume, T., Kochiyama, H., Akaike, A., Shimohama, S., Kimura, J., 1997. Nicotine protects cultured cortical neurons against glutamate-induced cytotoxicity via alpha7-neuronal receptors and neuronal CNS receptors. Brain Res. 765, 135–140.

Kihara, T., Shimohama, S., Sawada, H., Honda, K., Nakamizo, T., Shibasaki, H., Kume, T., Akaike, A., 2001. α7 nicotinic receptor transduces signals to phosphatidylinositol 3-kinase to block A beta-amyloid-induced neurotoxicity. J. Biol. Chem. 276, 13541–13546.

Kihara, T., Shimohama, S., Sawada, H., Kimura, J., Kume, T., Kochiyama, H., Maeda, T., Akaike, A., 1997. Nicotinic receptor stimulation protects neurons against beta-amyloid toxicity. Ann. Neurol. 42, 159–163.

Kristiansen, M., Ham, J., 2014. Programmed cell death during neuronal development: the sympathetic neuron model. Cell Death Differ. 21, 1025–1035.

Kristufek, D., Stocker, E., Boehm, S., Huck, S., 1999. Somatic and prejunctional nicotinic receptors in cultured rat sympathetic neurones show different agonist profiles. J. Physiol. 516, 739–756.

Kuruvilla, R., Zweifel, L.S., Glebova, N.O., Lonze, B.E., Valdez, G., Ye, H., Ginty, D.D., 2004. A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell 118, 243–255.

Levi-Montalcini, R., 1987. The nerve growth factor: thirty-five years later. EMBO J. 6, 1145–1154.

Levi-Montalcini, R., Angeletti, P.U., 1968. Nerve growth factor. Physiol. Rev. 48, 534–569.

Levi-Montalcini, R., Booker, B., 1960. Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve-growth protein. Proc. Natl. Acad. Sci. U. S. A. 46, 384–391.

Lindstrom, J., 1997. Nicotinic acetylcholine receptors in health and disease. Mol. Neurobiol. 15, 193–222.

Lu, J.Y.D., Su, P., Barber, J.E.M., Nash, J.E., Le, A.D., Lui, F., Wong, A.H.C., 2017. The neurotrophic effect of nicotine in Parkinson's disease models is associated with inhibiting PARP-1 and caspace-3 cleavage. Peer J. 5, E3933. https://doi.org/10.7717/peerj.3933.

Marin, P., Hamon, B., Glowinski, J., Premont, J., 1997. Nicotine-induced inhibition of neuronal phospholipase A2. J. Pharmacol. Exp. Ther. 280, 1277–1283.

Mudo, G., Belluardo, N., Fuxe, K., 2007. Nicotinic agonists as neuroprotective/neurotrophic drugs. Progress in molecular mechanisms. J. Neural Transm. (Vienna) 114, 135–147.

Nakayama, H., Numakawa, T., Ikeuchi, T., Hatanaka, H., 2001. Nicotine-induced phosphorylation of extracellular signal-regulated protein kinase and CREB in PC12h cells. J. Neurochem. 79, 489–498.

Papke, R., Sanberg, P.R., Shytle, R.D., 2001. Analysis of mecamylamine sterioisomers on human nicotinic receptor subtypes. J. Pharmacol. Exp. Ther. 297, 646–656.

Resende, R.R., Alves, A.S., Britto, L.R., Ulrich, H., 2008. Role of acetylcholine receptors in proliferation and differentiation of P19 embryonal carcinoma cells. Exp. Cell Res. 314, 1429–1443.

Roncarati, R., Scali, C., Comery, T.A., Grauer, S.M., Aschmi, S., Bothmann, H., Jow, B., Kowal, D., Gianfriddo, M., Kelley, C., Zanelli, U., Ghiron, C., Haydar, S., Dunlop, J., Terstappen, G.C., 2009. Procognitive and neuroprotective activity of a novel α7 nicotinic acetylcholine receptor agonist for treatment of neurodegenerative and

cognitive disorders. J. Pharmacol. Exp. Ther. 329, 459–468.
Sekhon, H.S., Keller, J.A., Benowitz, N.L., Spindel, E.R., 2001. Prenatal nicotine exposure alters pulmonary function in newborn rhesus monkeys. Am. J. Respir. Crit. Care Med. 164, 989–994.
Serova, L., Sabban, E.L., 2002. Involvement of alpha seven nicotinic acetylcholine receptors in gene expression of dopamine biosynthetic enzymes in rat brain. J. Pharmacol. Exp. Ther. 303, 896–903.
Sharma, G., Vijayaraghavan, S., 2002. Nicotinic receptor signaling in nonexcitable cells. J. Neurobiol. 53, 524–534.
Sharma, N., Deppmann, C.D., Harrington, A.W., St. Hillaire, C., Chen, Z., Lee, F., Ginty, D.D., 2010. Long distance control of synapse assembly by target-derived NGF. Neuron 67, 422–434.
Shimohama, S., Akaike, A., Kimura, J., 1996. Nicotine-induced protection against glutamate cytotoxicity. Nicotinic cholinergic receptor-mediated inhibition of nitric oxide formation. Ann. N. Y. Acad. Sci. 777, 356–361.
Signore, A.D., Gotti, C., Rizzo, A., Moretti, M., Paggi, P., 2004. Nicotinic acetylcholine receptor subtypes in the rat sympathetic ganglion: pharmacological characterization, subcellular distribution and effect of pre- and postganglionic nerve crush. J. Neuropathol. Exp. Neurol. 63, 138–150.
Slotkin, T.A., 1998. Fetal nicotine or cocaine exposure: which one is worse? J. Pharmacol. Exp. Ther. 285, 931–945.
Smeyne, R.J., Klein, R., Schnapp, A., Long, L.K., Bryant, S., Lewin, A., Lira, S.A., Barbacid, M., 1994. Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature 368, 246–249.
Srivatsan, M., Treece, J., Shotts, E.E., 2006. Nicotine alters nicotinic receptor subunit levels differently in developing mammalian sympathetic neurons. Ann. N. Y. Acad. Sci. 1074, 505–513.
Steiner, R.C., Heath, C.J., Picciotto, M.R., 2007. Nicotine-induced phosphorylation of ERK in mouse primary cortical neurons: evidence for involvement of glutamatergic signaling and CaMKII. J. Neurochem. 103, 666–678.
Takarada, T., Nakamichi, N., Kawagoe, H., Ogura, M., Fukumori, R., Nakazato, R., Fujikawa, K., Kou, M., Yoneda, Y., 2012. Possible neuroprotective property of nicotinic acetylcholine receptors in association with predominant upregulation of glial cell line-derived neurotrophic factor in astrocytes. J. Neurosci. Res. 90, 2074–2085.
Takeuchi, H., Yanagida, T., Inden, M., Takata, K., Kitamura, Y., Yamakawa, K., Sawada, H., Izumi, Y., Yamamoto, N., Kihara, T., Uemura, K., Inoue, H., Taniguchi, T., Akaike, A., Takahashi, R., Shimohama, S., 2009. Nicotinic receptor stimulation protects nigral dopaminergic neurons in rotenone-induced Parkinson's disease models. J. Neurosci. Res. 87, 576–585.
Toborek, M., Garrido, R., Malecki, A., Kaiser, S., Mattson, M.P., Hennig, B., Young, B., 2000. Nicotine attenuates arachidonic acid-induced overexpression of nitric oxide synthase in cultured spinal cord neurons. Exp. Neurol. 161, 609–620.
Toborek, M., Son, K.W., Pudelko, A., King-Pospisil, K., Wylegala, E., Malecki, A., 2007. ERK 1/2 signaling pathway is involved in nicotine-mediated neuroprotection in spinal cord neurons. J. Cell. Biochem. 100, 279–292.
Valjent, E., Pagès, C., Hervé, D., Girault, J.A., Caboche, J., 2004. Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain. Eur. J. Neurosci. 19, 1826–1836.
Veeranna, K.T., Boland, B., Odrljin, T., Mohan, P., Basavarajappa, B.S., Peterhoff, C., Cataldo, A., Rudnicki, A., Amin, N., Li, B.S., Pant, H.C., Hungund, B.L., Arancio, O., Nixon, R.A., 2004. Calpain mediates calcium-induced activation of the erk1, 2 MAPK pathway and cytoskeletal phosphorylation in neurons: relevance to Alzheimer's disease. Am. J. Pathol. 165, 795–805.
Villalba, M., Bockaert, J., Journot, L., 1997. Pituitary adenylate cyclase-activating polypeptide (PACAP-38) protects cerebellar granule neurons from apoptosis by activating the mitogen-activated protein kinase (MAP kinase) pathway. J. Neurosci. 17, 83–90.
Wang, J., Lu, Z., Fu, X., 2017. Alpha-7 nicotinic receptor signaling pathway participates in the neurogenesis induced by ChAT-positive neurons in the subventricular zone. Transl Stroke Res 8, 484–493.
West, K.A., Brognard, J., Clark, A.S., Linnoila, I.R., Yang, X., Swain, S.M., Harris, C., Belinsky, S., Dennis, P.A., 2003. Rapid Akt activation by nicotine and a tobacco carcinogen modulates the phenotype of normal human airway epithelial cells. J. Clin. Invest. 111, 81–90.
World Health Organization, 2011. World Health Organization Report on the Global Tobacco Epidemic, Warning About the Dangers of Tobacco (Last accessed on 05/10/2018) Available at. http://whqlibdoc.who.int/publications/2011/9789240687813_eng.pdf?ua=1.
Xia, Z., Dudek, H., Miranti, C.K., Greenberg, M.E., 1996. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J. Neurosci. 16, 5425–5436.
Xiao, Y., Meyer, E.L., Thompson, J.M., Surin, A., Wroblewski, J., Kellar, K.J., 1998. Rat α3/β4 subtype of neuronal nicotinic acetylcholine receptor stably expressed in a transfected cell line: pharmacology of ligand binding and function. Mol. Pharmacol. 54, 322–333.
Xue, L., Murray, J.H., Tolkovsky, A.M., 2000. The Ras/phosphatidylinositol 3-kinase and Ras/ERK pathways function as independent survival modules each of which inhibits a distinct apoptotic signaling pathway in sympathetic neurons. J. Biol. Chem. 275, 8817–8824.
Yamashita, H., Nakamura, S., 1996. Nicotine rescues PC12 cells from death induced by nerve growth factor deprivation. Neurosci. Lett. 213, 145–147.