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Nerve Growth Factor Regulates Synaptophysin Expression In Developing Trigeminal Ganglion Neurons *In Vitro*

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Abstract

The role of neuronal growth factors in synaptic maturation of sensory neurons, including trigeminal ganglion (TG) neurons, remains poorly understood. Here, we show that nerve growth factor (NGF) regulates the intracellular distribution of the synaptic vesicle protein synaptophysin (Syp) in newborn rat TG neurons *in vitro*. While reducing the number of Syp-positive cell bodies, NGF dramatically increases Syp immunoreactivity in both proximal and distal segments of the neurite. Intriguingly, the increase in Syp immunoreactivity occurs only in neuron-enriched cultures, in which the number of non-neuronal cells is significantly reduced. Together, our data indicate that NGF is a candidate molecule involved in early postnatal maturation of TG neurons, including control of presynaptic assembly, and thereby formation of synaptic connections.

Keywords

Development; *In Vitro*; Nerve Growth Factor; Sensory Neuron; Synaptophysin; Trigeminal Ganglion

INTRODUCTION

Primary sensory neurons with cell bodies in the trigeminal ganglion (TG) carry both nociceptive and non-nociceptive information from the craniofacial region, including several clinically significant structures, such as the meninges, temporomandibular joint, and teeth. The TG system remains plastic after birth, with as many as 90% of neurons innervating a specific target destined to be eliminated during the first 90 days of postnatal maturation (O'Connor and Van der Kooy, 1986). However, the cellular mechanisms of postnatal maturation of TG neurons remain largely unknown.

Only a few previous studies addressed the role of neurotrophic factors in postnatal maturation of TG neurons. These studies indicated that one member of the neurotrophin family of growth factors, nerve growth factor (NGF), is a major player in the establishment of trigeminal target innervation (Naftel et al., 1994; Qian and Naftel, 1996; Nosrat et al., 1997, 2001; Yang et al., 2006). NGF is expressed by peripheral targets of TG neurons (Nosrat et al., 1997, 2001), and increases the cell body diameter and neurite outgrowth of newborn TG neurons *in vitro* (Lillesaar et al., 2003). Deprivation of NGF during early postnatal development results in a

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decrease in the number of sensory neurons innervating the tooth pulp in adult rats, with small-diameter cells and unmyelinated axons being most severely affected (Qian and Naftel, 1996).

Previous studies have demonstrated that synaptophysin (synaptophysin I; Syp), an integral membrane protein of synaptic vesicles, is involved in several crucial aspects of synaptic vesicle trafficking, including the initiation of neurotransmitter release (Valtorta et al., 2004). Moreover, Syp plays an important regulatory role in activity-dependent competitive synapse formation in cultures of hippocampal neurons (Tarsa and Goda, 2002). The goal of the present study was to examine whether NGF could regulate synaptophysin in developing TG neurons, and thereby promote activity-dependent maturation of synaptic connections in trigeminal pathways.

METHODS

Animals

Postnatal day (P) 1 Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used for this study. All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon Health and Science University, and conformed to the Policies on the Use of Animals and Humans in Neuroscience Research approved by the Society for Neuroscience.

Preparation of TG cultures

Rat pups were deeply anesthetized by intraperitoneal injection of Euthasol (0.1 mg/kg) and decapitated. TGs were dissected and enzymatically dissociated as previously described by our laboratory (Buldyrev et al., 2006). TG cells were plated on poly-D-lysine- and laminin-coated glass coverslips (12 mm diameter) in 24-well tissue culture-treated plates (Falcon®, BD Biosciences, San Jose, CA), at the density of one ganglion per well. Cultures were grown for 3 days in Neurobasal-A medium supplemented with B-27 serum-free supplement (Invitrogen, Carlsbad, CA), 0.5 mM L-glutamine (Invitrogen), and 1% Penicillin-Streptomycin-Neomycin antibiotic mixture (Invitrogen). In order to rule out potential effects of active substances released from cells and cell debris during dissociation of the ganglia, the medium was changed 3-4 hours after cell plating and the data compared with those derived from cultures without the medium change.

In experiments designed to examine a potential contribution of non-neuronal cells, one half of dissociated TG cells (in 0.5 ml of plating medium) was layered on top of a two-layer gradient of Percoll (Sigma; 35%-60%; 1 ml each) and centrifuged for 10 minutes at $800 \times g$ at 4 °C. A vast majority of non-neuronal cells and cell debris formed a visible thin layer at the 'plating medium-35%' interface and, therefore, could be easily separated from neurons accumulated at the '35%-60%' interface. The neuron-enriched interface was collected and rinsed twice with culture medium before plating. The Percoll separation procedure inadvertently leads to a loss of a proportion of cells (e.g. during rinses) resulting in a lower count of plated neurons (Figure 2A, Control, Mixed vs. Neuron-enriched).

NGF treatment

NGF (Invitrogen) was dissolved in phosphate-buffered saline (PBS), applied within 30 minutes following cell plating (5 µl per well), and left for the duration of culture (3 days). Control wells were treated with 5 µl of PBS.

Immunostaining

Cultures were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 30 min at room temperature. Protein Gene Product 9.5 (PGP 9.5) and synaptophysin (Syp)

staining was performed at room temperature as previously described (Buldyrev et al., 2006), using rabbit anti-PGP 9.5 (1:1000; Accurate Chemical) and mouse monoclonal anti-Syp (1:500; Synaptic Systems, Germany), followed by goat anti-rabbit Alexa 488 (1:200; Invitrogen) and goat anti-mouse IgG-Cy3 (1:200; Jackson Immunoresearch). Control cultures, in which primary antibodies were omitted, were completely devoid of staining.

Digital image analysis

All specimens were analyzed with Olympus IX-71 inverted microscope (Olympus America Inc., Center Valley, PA) by an investigator blinded to treatment groups, and images captured with Hamamatsu ORCA-ER CCD camera and Wasabi software (Hamamatsu Corp., Bridgewater, NJ).

All PGP9.5- and Syp-immunoreactive neurons located within two middle strips (area of 26.4 mm²) of each coverslip were counted, and data presented per the entire coverslip (area of 113.04 mm²). Double PGP9.5/Syp immunoreactivity was determined by overlaying single-labeled images using Olympus Microsuite (vs. 5.0, Olympus America, Inc.).

The intensity of Syp immunoreactivity was examined in the proximal (adjacent to the cell body) and distal (farthest from the cell body) segments of the neurite, each 2 times the cell diameter in length. The cell diameter, 'Mean Gray Value' and 'Area' were determined using ImageJ software (National Institutes of Health, Bethesda, MD). 'Integrated Density' of Syp immunoreactivity was calculated as the product of 'Mean Gray Value' and 'Area' (ImageJ). The average cell diameter was $22.04 \pm 0.99 \mu\text{m}$ (n=39), and there were no significant differences in the cell diameter among cells, including various culture conditions and treatments, i.e. control vs. NGF.

Calculations and statistical analysis

Data are expressed as mean $\pm$ standard error. Samples were compared using ANOVA followed by Duncan's multiple comparison procedure, and $P < 0.05$ was considered significant.

RESULTS

We have examined the effects of nerve growth factor (NGF) on neuronal expression of synaptophysin (Syp), as an index of synapse formation during early postnatal maturation of TG neurons. P1 TG cultures were treated with either PBS vehicle or 100 ng/ml NGF as previously described (Edsjö et al., 2001), applied for 3 days from the day of plating, in the presence or virtual absence of non-neuronal cells. In control experiments, the culture medium was changed 3-4 hours after cell plating in order to remove potentially active substances released from cells and cell debris during preparation of mixed cultures. At the end of the 3-day culture period, all cultures were fixed and double-stained with antibodies against Syp and Protein Gene Product 9.5 (PGP 9.5), a pan-neuronal marker (Fig. 1).

Consistent with the previous study of growth factor dependence of P1 TG neurons demonstrating the predominant role of NGF in supporting survival of newborn TG neurons (Lillesaar et al., 2003), NGF significantly increased the number of PGP 9.5-immunoreactive (IR) P1 TG cells grown in neuron-enriched cultures (Fig. 2A).

A large subpopulation of PGP 9.5-IR cells also showed Syp immunoreactivity (Fig. 1, 2B). Among the Syp-IR cells, a considerable proportion was characterized by Syp immunoreactivity not only within cell bodies, but also neurites (Fig. 1). NGF treatment resulted in a significant decrease in the absolute number of neurons with Syp-IR cell bodies in mixed cultures (Fig. 2B), and the effect was not influenced by the culture medium change shortly after plating (Control: 851.7 ± 97.8 ; NGF: 519.3 ± 31.7 ; n=6 cultures; $p < 0.01$). In neuron-enriched cultures,

the effect of NGF on the number of Syp-IR cell bodies did not reach statistical significance, but the trend was very strong (Fig. 2B). The apparently weaker effect of NGF in neuron-enriched cultures could likely be explained by the fact that, under those conditions, NGF significantly increased the overall number of surviving neurons (Fig. 2A) and, consequently, the number of Syp-positive cells. The latter would counteract a direct effect of NGF on Syp expression. Indeed, when expressed as the percentage of PGP 9.5-IR cells, NGF decreased the number of Syp-positive cells by 21.9 % in neuron-enriched cultures (Control, 34.7 ± 0.07 %, $n=8$; NGF, 12.8 ± 0.03 %, $n=8$) compared to a significantly smaller, 9.6 % decrease in mixed cultures (Control, 15.5 ± 0.03 %, $n=9$; NGF, 5.9 ± 0.01 %, $n=7$). Together, these results indicate that NGF decreases Syp immunoreactivity in cell bodies of P1 TG neurons, suggesting a role for NGF in regulation of Syp expression, release and/or intra-axonal trafficking.

Very little is known about the effects of NGF on Syp expression in neurons. A previous study conducted in cultured dorsal root ganglion neurons indicates that NGF increases Syp levels in both the cell soma and neurites (Takazaki et al., 2002). Syp is an integral membrane protein of synaptic vesicles, which is synthesized in neuronal cell bodies and transported to the synaptic sites in neurites. Therefore, we hypothesized that the decrease in the number of Syp-IR cell bodies following NGF treatment could be due, at least in part, to NGF-mediated facilitation of Syp trafficking from the cell body to neurites and terminals. To test this hypothesis, we measured the density of Syp immunoreactivity in proximal and distal neurite segments of PGP 9.5-IR P1 TG cells, and compared between control and NGF-treated, grown in mixed and neuron-enriched cultures. For this analysis, we used neurons from the same population that was used for the analysis of Syp-IR in the cell soma (Fig. 2B). Fascinatingly, treatment with NGF resulted in a robust increase in the density of Syp-IR in both proximal (Fig. 3A) and distal (Fig. 3B) neurite segments. Moreover, the density of Syp-IR in distal neurites was over two-fold higher (213.3 ± 23.28 %, $n=24$) compared to Syp-IR in proximal neurites. The NGF-mediated increase in density of Syp-IR was observed exclusively in neuron-enriched, and not mixed, cultures (Fig. 3), and this differential effect was not dependent on the medium change, i.e. rinsing the mixed cultures with fresh medium, shortly after plating. Together, these data indicate that NGF induces recruitment of Syp-immunoreactivity to neurites of developing TG neurons, and the effect is prevented by the presence of non-neuronal cells.

DISCUSSION

The present study provides the first evidence that NGF regulates synaptophysin (Syp), an integral protein of synaptic vesicles, in newborn TG neurons. In a virtual absence of non-neuronal cells, NGF increases the intensity of Syp immunoreactivity in neurites while decreasing the number of Syp-IR neuronal cell bodies identified by immunoreactivity for the pan-neuronal marker PGP 9.5.

Very little is known about effects of NGF on Syp expression. In pheochromocytoma cell line PC12, exogenous NGF reduces the intracellular level and secretion of Syp (Vetter and Betz, 1989; Lah and Burry, 1993; Tao-Cheng et al., 1995; Lahiri et al., 2000), whereas in neuroblastoma-derived cell lines it has the opposite effect (Edsjö et al., 2001). On the other hand, NGF does not affect Syp expression in cerebellar granule cells *in vitro* (Coffey et al., 1997), and neither blockade nor activation of NGF function change the number of Syp-positive sites at cortical synapses *in vivo* (Debeir et al., 1999; Bruno et al., 2004). However, there is evidence that NGF treatment *in vivo* increases the level of Syp immunoreactivity in adult visual cortex (Liu et al., 1996). Previous studies have also shown that NGF treatment of dorsal root ganglion (DRG) cultures results in increases in Syp immunoreactivity in both perikarya and neurites (Takazaki et al., 2002).

Earlier studies suggested that regulation of Syp expression is uncoupled from mechanisms that govern neuronal differentiation, including process formation induced by NGF (Vetter and Betz, 1989). More recent studies indicate that, by stabilizing the microtubule system, NGF prevents accumulation of Syp immunoreactivity in the cell soma of cultured midbrain neurons (Jiang et al., 2006). These data are consistent with our observations that NGF treatment leads to a decrease in the number of Syp-immunoreactive cell bodies combined with an increase in Syp immunoreactivity in neurites of TG neurons. Considering the fact that Syp is a major component of the membrane proteins of small synaptic vesicles (Valtorta et al., 2004), our data suggest that NGF promotes synaptic vesicle trafficking, and thereby formation of synaptic connections in TG neurons. In support of this possibility, Syp has previously been shown to play an important role in activity-dependent synapse formation in hippocampal neuron cultures (Tarsa and Goda, 2002).

A vast majority of previous studies addressing changes in endogenous Syp expression following various treatments and conditions used quantitative immunoblotting of either whole-cell lysates (Vetter and Betz, 1989; Lah and Burry, 1993; Tao-Cheng et al., 1995; Coffey et al., 1997; Lahiri et al., 2000) or specific cell fractions, such as growth cones (Edsjö et al., 2001). Another commonly used approach is counting Syp-immunoreactive puncta along axons (Liu et al., 1996; Debeir et al., 1999). However, due to the high level of Syp expression and, consequently, clustering of single Syp-immunoreactive puncta in our culture system, the method of choice was the measurement of the fluorescence intensity of Syp staining. Given that the current data showing NGF-mediated redistribution of Syp immunoreactivity from the cell body to axons are based on the comparative analysis of the intensity of Syp immunoreactivity within the same neuron, factors such as differences in threshold levels, exposure time, or the affinity of the anti-Syp antibody, are unlikely to affect the outcome of these experiments.

Our data indicate that the role of NGF in both supporting survival of TG neurons (Fig. 2A) and increasing Syp expression in TG neurites (Fig. 3) is obscured in the presence of non-neuronal cells. This is consistent with earlier studies demonstrating that the NO-cGMP-mediated neurotrophin synthesis by glial cells supports survival of sensory neurons *in vitro* (Thippeswamy et al., 2005). Similarly, the numbers of Syp-IR cell bodies of TG neurons, normalized for the NGF effects on neuronal survival, are less severely decreased in mixed cultures, compared with neuron-enriched conditions (see text of the Results section above). To our knowledge, this is the first example of a potential role for non-neuronal cells in regulating distribution of Syp. One possibility is that the non-neuronal cells, acting in a paracrine fashion, provide a trophic support which prevails and makes the NGF treatment less effective, if not redundant, under these culture conditions. In fact, studies from other laboratories show that non-neuronal cells can influence neuronal development, including expression of transmitter phenotype (Sun et al., 1994; MacPhedran and Hall, 2001) and neurite outgrowth (Bandtlow et al., 1990; De Koninck et al., 1993). Therefore, it is plausible that the interaction between factors released from non-neuronal cells and NGF may occur *in vivo*, and that non-neuronal cells in the TG contribute to the regulation of Syp trafficking along neurites of TG neurons. Another possibility is that non-neuronal cells act as NGF scavengers, preventing the action of exogenously-derived NGF on TG neurons grown in mixed cultures. This scenario is supported by previous observations that both p75 and TrkA receptors for NGF are present on the satellite cells of dorsal root ganglia and, therefore, can act as NGF reservoirs (Pannese and Procacci, 2002).

In conclusion, the present study provides support for a novel role for NGF in regulating synaptophysin in TG neurons, and identifies NGF as a likely key player in mechanisms of postnatal functional maturation of TG neurons, including formation of synaptic connections.

Our data suggest a role for NGF in facilitating synaptic vesicle trafficking from the cell body to presynaptic terminals of developing TG neurons *in vivo*.

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Figure 1.

Synaptophysin (Syp) is expressed by a large subset of cultured P1 TG neurons identified by the pan-neuronal marker Protein Gene Product 9.5 (PGP 9.5). Micrographs of representative neuron-enriched cultures of dissociated P1 TG, treated for 3 days with either PBS vehicle (Control) or 100 ng/ml NGF (NGF), followed by double-immunostaining for PGP 9.5 (left panel; green) and synaptophysin (middle panel; red). A high density of synaptophysin staining can be seen in distal neurites of NGF-treated cultures, but not under control conditions. The right panel shows overlays of PGP 9.5 and Syp immunostaining, to demonstrate colocalization (yellow) of both markers.

Figure 2.

NGF decreases the number of synaptophysin-immunoreactive cell bodies of P1 TG neurons grown for 3 days in dissociate culture. Total number of TG neurons immunoreactive for (A) the pan-neuronal marker Protein Gene-Product 9.5 (PGP 9.5-IR) and (B) PGP9.5/Synaptophysin (Synaptophysin-IR), grown in mixed (left) or neuron-enriched (right) cultures, in the absence (Control) or presence (NGF) of 100 ng/ml NGF; Mixed/Control: n=15 (A) and n=9 (B), Mixed/NGF: n=13 (A) and n=7 (B), Neuron-enriched/Control n=14 (A) and n=8 (B), Neuron-enriched/NGF n=15 (A) and n=8 (B); n.s., not significant, * $p < 0.05$, *** $p < 0.001$.

Figure 3.

NGF increases synaptophysin immunoreactivity (IR) in neurites of P1 TG neurons grown in neuron-enriched cultures. Integrated density of synaptophysin immunoreactivity in (A) proximal and (B) distal segments of TG neurites, grown for 3 days in mixed (left) or neuron-enriched (right) cultures in the absence (Control) or presence (NGF) of 100 ng/ml NGF, and identified by the panneuronal marker PGP 9.5. The 'proximal' and 'distal' segments were defined as 'adjacent to the cell body' and 'farthest from the cell body', respectively, and the length of each corresponded to 2 times the cell diameter. There were no significant differences among the cell body diameter among groups. The 'Integrated Density' was calculated as the product of 'Mean Gray Value' and 'Area' (see Methods). (A), Proximal: n=11 (Mixed,

Control), n=14 (Mixed, NGF), n=14 (Neuron-enriched, Control), n=16 (Neuron-enriched, NGF); (B), Distal: n=16 (Mixed, Control), n=16 (Mixed, NGF), n=17 (Neuron-enriched, Control), n=20 (Neuron-enriched, NGF); n.s., not significant, ** $p < 0.01$.