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## Nerve growth factor regulates galanin and neuropeptide Y expression in primary cultured superior cervical ganglion neurons

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Both galanin and neuropeptide Y (NPY) are expressed in superior cervical ganglion (SCG) neurons. Following nerve transection or axotomy galanin is strongly upregulated and NPY is downregulated in SCG neurons because target-derived nerve growth factor (NGF) content decreased. It is not known whether or to what extent NGF affects both galanin and NPY expression in primary cultured SCG neurons. In the present study we examine whether exogenous NGF affects expression of neuropeptides for galanin and NPY in primary cultured SCG neurons. In addition, we explore whether mRNAs for galanin and NPY are affected by administration of exogenous NGF in SCG cultures. The significance of expression of galanin and NPY and their mRNAs was revealed by performing experiments without and with administration of exogenous NGF. Galanin and its mRNA expression was attenuated by administration of exogenous NGF in SCG cultures. The enhancement of NPY and its mRNA expression by administration of exogenous NGF in SCG cultures was dose-dependent. The physiological or pathophysiological mechanisms of the alterations of galanin and NPY expression affected by NGF in primary cultured SCG neurons are still unknown. The present data provide basic knowledge about the expression of galanin and NPY in primary cultured SCG neurons of rats, which may further improve our understanding of the functional significance of galanin and NPY expression affected by NGF.

### 1. Introduction

Galanin is a 29-amino-acid neuropeptide which is widely distributed throughout the nervous system including superior cervical ganglion (SCG) neurons (Norberg et al. 2004; Holmberg et al. 2005). Galanin has developmental and trophic effects during development and plays a trophic role after nerve injury in the adult animal (Brumovsky et al. 2006). Galanin-like immunoreactivity is normally seen in only a few neurons of SCG. Galanin overexpressed mice exhibited a strong galanin-like immunoreactivity in most SCG neuron profiles. The overexpression of the peptide in SCG neurons was paralleled by increased mRNA levels (Brumovsky et al. 2006). Galanin is induced in autonomic neurons after peripheral nerve lesion. Following transection of the two efferent carotid nerves galanin is strongly upregulated in the neuronal cell bodies of rat and mouse SCG (Holmberg et al. 2005).

Neuropeptide Y (NPY) is a 36-amino-acid neuropeptide which is expressed in SCG neurons. NPY expression was used for identifying sympathetic neuronal phenotypes in the rat SCG (Li and Horn 2006). Following axotomy NPY mRNA expression in SCG neurons decreased in wild-type mice and increased in nerve growth factor (NGF)-overexpressing mice (Holmberg et al. 2001). Sym-

pathectomy increases inflammation lesions in rat mandible which was associated with an almost complete loss of NPY-immunoreactive nerve fibers in the inflammatory jaws suggesting a close relationship between NPY expression and inflammation (Haug and Heyeraas 2003). The decrease in NGF availability after axotomy is mainly responsible for the decrease in NPY expression (Shadiack et al. 2001). NGF increases sympathetic NPY mRNA content in chick embryonic sympathetic cells in culture (Medina-Ortiz and Garcia-Ararras 2000).

NGF, a member of the neurotrophin family, initially interested neurobiologists because of its effects in the developing nervous system (Petruska and Mendell 2004). In the course of the last years, several lines of evidence converged to indicate that NGF participates in regulation of expression of several kinds of neuropeptides in SCG neurons as well as in dorsal root ganglion (DRG) sensory neurons (Shadiack et al. 2001; Winston et al. 2001; Skoff and Adler 2005; Thippeswamy et al. 2007; Yang et al. 2007). It has been known that NGF is one of the well characterized regulators of galanin and NPY expression (Shadiack et al. 2001; Thippeswamy et al. 2007). Axonal transection of adult sympathetic neurons leads to a decrease in their content of target-derived NGF. NGF regulates galanin and NPY expression occurring in SCG neu-

rons after axotomy (Shadiack et al. 2001). It is not known whether or to what extent NGF affects both galanin and NPY expression in primary cultured SCG neurons. Studies were performed using primary cultured newborn rat SCG neurons to determine exposure (4 days) of NGF at different concentrations (5 ng/ml, 10 ng/ml, 20 ng/ml, respectively) on the expression of galanin and NPY.

## 2. Investigations and results

### 2.1. Galanin mRNA and galanin peptide expression with NGF treatment

The effects of NGF treatment on galanin mRNA and galanin peptide expression in cultured SCG neurons were investigated by RT-PCR and Western blot, respectively. NGF inhibited galanin mRNA and galanin peptide expression as compared with control at the same time point (Figs. 1, 2).

### 2.2. NPY mRNA and NPY peptide expression with NGF treatment

The effects of NGF treatment on NPY mRNA and NPY peptide expression in cultured SCG neurons were investigated by RT-PCR and Western blot, respectively. NGF promoted NPY mRNA and NPY peptide expression in a dose-dependent manner as compared with control at the same time point (Figs. 3, 4).

## 3. Discussion

Different lines of evidence have shown that neuropeptides, including galanin and NPY, are expressed in SCG neurons (Norberg et al. 2004; Holmberg et al. 2005; Li et al.

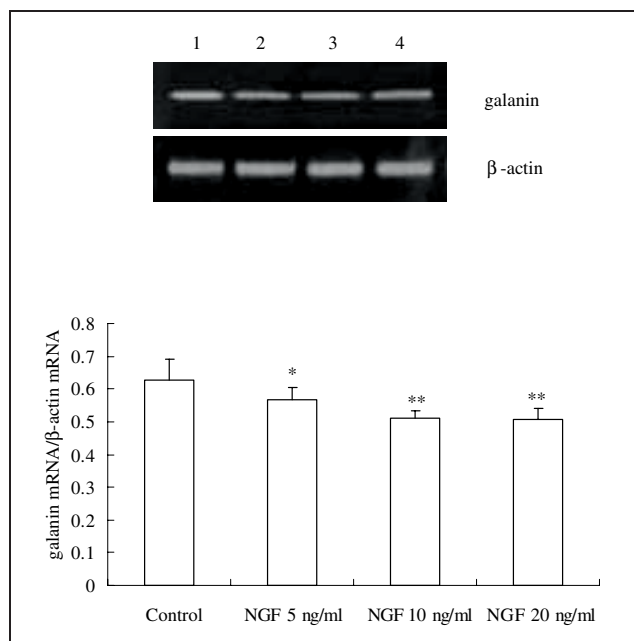


Fig. 1: Effects of NGF on galanin mRNA expression in primary cultured SCG neurons. Galanin and  $\beta$ -actin mRNA were analyzed by RT-PCR. Lane 1: Normal control (galanin mRNA/ $\beta$ -actin mRNA = 0.6275  $\pm$  0.0651). Lane 2: Exposure of NGF 5 ng/ml (galanin mRNA/ $\beta$ -actin mRNA = 0.5658  $\pm$  0.0398). Lane 3: Exposure of NGF 10 ng/ml (galanin mRNA/ $\beta$ -actin mRNA = 0.5095  $\pm$  0.0247). Lane 4: Exposure of NGF 20 ng/ml (galanin mRNA/ $\beta$ -actin mRNA = 0.5070  $\pm$  0.0340). Bar graphs with error bars represent mean  $\pm$  SD (n = 5). \*P < 0.05 vs. control, \*\*P < 0.01 vs. control.

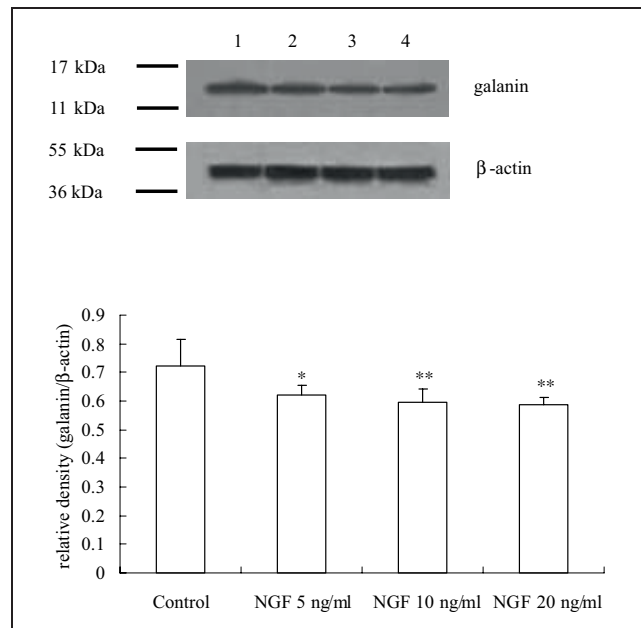


Fig. 2: Effects of NGF on galanin peptide expression in primary cultured SCG neurons. Galanin peptide expression was analyzed by Western blot. Lane 1: Normal control (galanin/ $\beta$ -actin = 0.7205  $\pm$  0.0933). Lane 2: Exposure of NGF 5 ng/ml (galanin/ $\beta$ -actin = 0.6209  $\pm$  0.0343). Lane 3: Exposure of NGF 10 ng/ml (galanin/ $\beta$ -actin = 0.5943  $\pm$  0.0464). Lane 4: Exposure of NGF 20 ng/ml (galanin/ $\beta$ -actin = 0.5877  $\pm$  0.0244). Bar graphs with error bars represent mean  $\pm$  SD (n = 5). \*P < 0.05 vs. control, \*\*P < 0.01 vs. control.

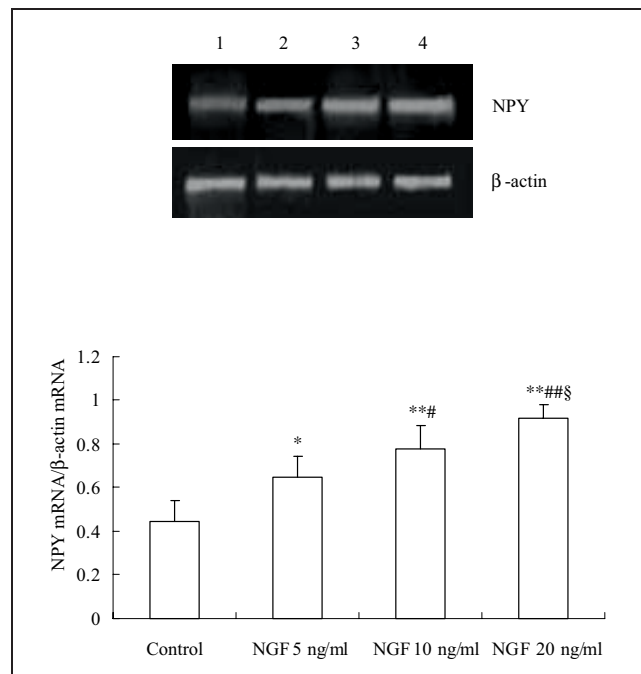


Fig. 3: Effects of NGF on NPY mRNA expression in primary cultured SCG neurons. Galanin and  $\beta$ -actin mRNA were analyzed by RT-PCR. Lane 1: Normal control (NPY mRNA/ $\beta$ -actin mRNA = 0.4468  $\pm$  0.0940). Lane 2: Exposure of NGF 5 ng/ml (NPY mRNA/ $\beta$ -actin mRNA = 0.6459  $\pm$  0.0994). Lane 3: Exposure of NGF 10 ng/ml (NPY mRNA/ $\beta$ -actin mRNA = 0.7753  $\pm$  0.1068). Lane 4: Exposure of NGF 20 ng/ml (NPY mRNA/ $\beta$ -actin mRNA = 0.9161  $\pm$  0.0632). Bar graphs with error bars represent mean  $\pm$  SD (n = 5). \*P < 0.01 vs. control, \*\*P < 0.001 vs. control, #P < 0.05 vs. NGF 5 ng/ml, ##P < 0.001 vs. NGF 5 ng/ml, \$\$\$P < 0.05 vs. NGF 10 ng/ml.

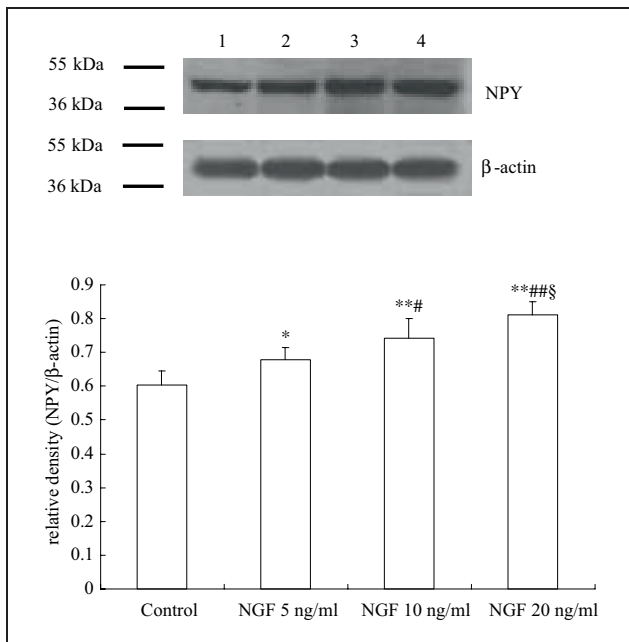


Fig. 4: Effects of NGF on NPY peptide expression in primary cultured SCG neurons. Galanin peptide expression was analyzed by Western blot. Lane 1: Normal control (NPY/β-actin =  $0.6050 \pm 0.0390$ ). Lane 2: Exposure of NGF 5 ng/ml (NPY/β-actin =  $0.6790 \pm 0.0341$ ). Lane 3: Exposure of NGF 10 ng/ml (NPY/β-actin =  $0.7428 \pm 0.0562$ ). Lane 4: Exposure of NGF 20 ng/ml (NPY/β-actin =  $0.8109 \pm 0.0404$ ). Bar graphs with error bars represent mean  $\pm$  SD (n = 5). \*P < 0.05 vs. control, \*\*P < 0.001 vs. control, #P < 0.05 vs. NGF 5 ng/ml, ##P < 0.001 vs. NGF 5 ng/ml, §P < 0.05 vs. NGF 10 ng/ml.

2007). And also, following nerve transection or axotomy galanin is strongly upregulated and NPY is downregulated in SCG neurons of rat or mouse (Holmberg et al. 2001; Holmberg et al. 2005). Because axonal transection of adult sympathetic neurons leads to a decrease in their content of target-derived NGF, the alterations of neuropeptide expression may reflect a trophic role or regenerative event.

In the present study, galanin and its mRNA were downregulated and NPY and its mRNA were upregulated in primary cultured rat SCG neurons after administration of exogenous NGF. These *in vitro* observations are consistent with the observed upregulation of galanin (Hyatt-Sachs et al. 1996) and downregulation of NPY (Shadiack et al. 2001) following axotomy because of deprivation of target-derived NGF. The different expression patterns of galanin and NPY after nerve injury may reflect the different roles of these neuropeptides in neurite outgrowth or nerve regeneration. However, the mechanisms of the alterations of galanin and NPY expression affected by NGF are still unclear. In contrary, galanin and NPY may share the same function on reducing cholinergic transmission. It has been shown that both galanin and NPY could attenuate vagal inhibitory activity through activating GalR1 and NPY Y2 receptors in nerve terminals of parasympathetic neurons to reduce acetylcholine release (Smith-White et al. 2003).

Interestingly, a high degree of coexistence for galanin and NPY in axotomized neurons in SCG suggested a possible role in trophic and regenerative events (Landry et al. 2000). Galanin, via activation of GalR2, plays a pro-nociceptive role in the periphery (Kerekes et al. 2003; Jimenez-Andrade et al. 2004, 2005, 2006) and enhances neurite outgrowth from adult neurons (Mahoney et al. 2003; Suarez et al. 2006). Galanin may play a role in the adaptive response of the peripheral nervous system (PNS) to

injury and modulates pain transmission (Holmes et al. 2005). Sympathectomy increases inflammation lesions in rat mandible associated with an almost complete loss of NPY-immunoreactive nerve fibers in the inflammatory jaws. And also, inflammation causes sprouting of NPY-immunoreactive nerve fibers in non-sympathectomy animals (Haug and Heyeraas 2003). These *in vivo* observations showed that the close relationship of galanin and NPY which are involved in trophic and regenerative events after peripheral inflammation or nerve injury.

A study of immunocytochemical developmental patterns of the thoracolumbar sympathetic chain in the chick showed that galanin was strongly expressed in sympathetic ganglion cells from day 4 onwards (Sánchez-Montesinos et al. 2005). A study of galanin expression properties of rat sympathetic ganglionic neurons during early postnatal development revealed that galanin-immunoreactive neurons were scarce up to 10 days of life, after which their number increased to reach a maximal value in 30-day-old animals and then declined again. This result showed a distinct chronological pattern of this neuropeptide (Masliukov and Timmermans 2004). Studies on neurochemical differentiation of functionally distinct populations of sympathetic neurons showed that the topography of NPY immunoreactive neurons is established during late embryonic and early fetal stages of development and reflects that found in the mature animal by the end of the early fetal period (Anderson et al. 2001). Interestingly, a developmental *in vivo* study of the guinea pigs showed that selective expression of NPY by subpopulations of sympathetic neurons occurs prior to innervation of their targets suggesting that target contact is not required to establish appropriate patterns of expression of peptide neurotransmitters by sympathetic neurons during development (Morris et al. 2001). Studies on changes in the expression of NPY of human sympathetic ganglionic neurons during maturation revealed that the appearance of NPY in the principal sympathetic ganglionic neurons defines not only a qualitatively new level in the functional regulation of target organs at birth, but serves as an index of neonatal maturity (Roudenok 2000). A recent postnatal ontogenesis study on mouse sympathetic ganglionic neurons revealed that there were increases in the proportion of cells contained NPY from birth to two months suggested that maturation of neurotransmitter NPY in sympathetic neurons was complete by the second month of life (Maslyukov et al. 2006). The results of these studies demonstrate that these neuropeptides are involved in developmental processes.

In the present study, we observed not only that the downregulated galanin peptide is paralleled with galanin mRNA but also that the upregulated NPY peptide is paralleled with NPY mRNA which may reflect that the biosynthesis of these neuropeptides is affected by exogenous NGF in primary cultured SCG neurons. A possible explanation for these phenomena may be that the cultured neurons seem to represent a suitable model as axotomized neurons, since the neurons are axotomized during culture preparation and their axons regenerate during the culture period (Kerekes et al. 1997; Shadiack et al. 2001). Yet, the physiological or pathophysiological mechanisms of the alterations of galanin and NPY expression affected by NGF are unknown. The present data provide basic knowledge about the expression of galanin and NPY in primary cultured SCG neurons of rat, which may further our understanding of the functional significance of galanin and NPY expression affected by NGF.

## 4. Experimental

### 4.1. SCG cell culture preparations

Bilateral SCG was dissected out from newborn Wistar rats. The animals were obtained from the Experimental Animal Center of Shandong University of China. Under aseptic conditions, bilateral SCG were taken out by using a sharp pair of forceps and the SCG explants were put in a drop of Dulbecco's Modified Eagle Medium with F-12 supplement (DMEM/F-12) media (Gibco) in the half of petri dishes. SCG prior to establishment in culture was digested with 0.25% trypsin (Sigma) in D-Hanks solution at 37 °C for 10 min and centrifuged for 5 min at  $1 \times 10^3$  rpm. The supernatants were removed and the pellets were resuspended in DMEM/F-12 media and triturated using a sterile modified Pasteur's glass pipette. SCG cells were then filtered through a 130  $\mu$ m filter followed by counting. Dissociated SCG cells were then cultured in flasks (Costar, Corning, NY, USA) for detecting expression of mRNAs for galanin and NPY by RT-PCR and peptides for galanin and NPY by Western blot. SCG cells were plated at a density of  $5 \times 10^5$  cells/ml in flasks which were pre-coated with poly-L-lysine prior to plating. Then SCG cells were cultured in culture media at 37 °C with 5% CO<sub>2</sub> for 24 h and then maintained in culture media containing cytarabine (ara-C) (5  $\mu$ g/ml) for another 24 h to inhibit growth of non-neuronal cells, and then cultured in culture media for another 4 days with media change every 2 days. The composition of the culture media is D-MEM/F-12 (1:1) supplemented with 5% fetal bovine serum, 2% B-27 supplement (Gibco), insulin (0.25  $\mu$ g/ml, Sigma), L-glutamine (0.1 mg/ml, Sigma), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml).

### 4.2. Exposure of NGF on SCG neurons

To determine whether NGF influences galanin and NPY expression in SCG cultures, NGF (5 ng/ml, 10 ng/ml, 20 ng/ml, respectively) was added to the culture media at 2 days of culture age. These cultures were then incubated for additional 4 days with media change every 2 days. The culture media would contain NGF (5 ng/ml, 10 ng/ml, 20 ng/ml, respectively) during the 4 days incubation. SCG neurons were cultured continuously in culture media for 6 days as control.

### 4.3. RNA extraction and RT-PCR for detecting mRNAs for galanin and NPY

The mRNA levels of galanin and NPY were analyzed by RT-PCR after NGF treatment on SCG neurons. The expression of  $\beta$ -actin was also determined as an internal control. Total SCG cell RNA of each flask was isolated by TRIzol. cDNA synthesis was performed with M-MLV reverse transcriptase. The gene-specific primers were synthesized by use of the published cDNA sequences for galanin, NPY, and  $\beta$ -actin. The synthetic oligonucleotide primer sequences for galanin, NPY, and  $\beta$ -actin were as follows:

galanin 5'-ATG CCA ACA AAG GAG AAG AG-3' (upper primer) and 5'-AGG TGC AAG AAA CTG AGA AA-3' (lower primer).

NPY 5'-GGG CTG TGT GGA CTG ACC-3' (upper primer) and 5'-GGA AGG GTC TTC AAG CCT-3' (lower primer).

$\beta$ -actin 5'-ATC ATG TTT GAG ACC TTC AAC-3' (upper primer) and 5'-CAT CTC TTG CTC GAA GTC CA-3' (lower primer).

The predicted sizes of the amplified galanin, NPY, and  $\beta$ -actin DNA products were 224 bp, 264 bp, and 317 bp, respectively.

PCR amplification was performed for 35 cycles. The cycle profile included denaturation for 45 s at 94 °C, annealing for 60 s at 53 °C, and extension for 45 s at 72 °C. PCR was performed within the range that demonstrates a linear correlation between the amount of cDNA and the yield of PCR products.

The amplified products were analyzed by standard agarose gel electrophoresis and stained with ethidium bromide, visualized by a UV transilluminator and photographed. The photographs were scanned and the electrophoresis gel images were analyzed quantitatively by using an Imaj 1.39u image analysis software. The levels of galanin mRNA and NPY mRNA were expressed as the ratio of the gene to  $\beta$ -actin.

### 4.4. Western blot analysis for galanin and NPY expression

Galanin and NPY protein expression was analyzed by Western blot. Fresh cultured SCG neurons after NGF treatment at different concentrations (5 ng/ml, 10 ng/ml or 20 ng/ml) were homogenized in 10 mmol/L Tris homogenization buffer (pH 7.4) with protease inhibitors (1 tablet in 50 ml; Sigma). The samples were centrifuged at 12,000 rpm for 20 min and the supernatants collected for Western blot. After determining the protein concentrations of the supernatants (BCA method, standard: BSA), 50  $\mu$ g protein of each sample was loaded onto the 8% SDS gel, separated by electrophoresis and transferred to PVDF membrane. The membranes were incubated with goat anti-galanin polyclonal IgG (1:1000, Santa Cruz Biotechnology), mouse anti-rat NPY monoclonal IgG (1:1000, Santa Cruz Biotechnology) or mouse anti- $\beta$ -actin monoclonal IgG (1:1000, Santa Cruz Biotechnology). After being washed three times for 10 min with

washing solution, the membranes were incubated with donkey anti-goat IgG-HRP (1:2000, Santa Cruz Biotechnology) or goat anti-mouse IgG-HRP (1:2000, Santa Cruz Biotechnology). The immunoreactive bands were visualized by an ECL Western blotting detection kit (Pierce) on light sensitive film.

### 4.5. Statistical analysis

Data are expressed as mean  $\pm$  SD. Statistical analysis was evaluated with SPSS software by one-way ANOVA followed by the Student-Newman-Keuls test for significance to compare the differences among various groups. Significance was accepted at  $P < 0.05$ .

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