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ORIGINAL STUDY

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Expression of mRNAs for PPT, CGRP, NF-200, and MAP-2 in cocultures of dissociated DRG neurons and skeletal muscle cells in administration of NGF or NT-3

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Abstract: Both neurotrophins (NTs) and target skeletal muscle (SKM) cells are essential for the maintenance of the function of neurons and nerve-muscle communication. However, much less is known about the association of target SKM cells with distinct NTs on the expression of mRNAs for preprotachykinin (PPT), calcitonin-gene related peptide (CGRP), neurofilament 200 (NF-200), and microtubule associated protein 2 (MAP-2) in dorsal root ganglion (DRG) sensory neurons. In the present study, a neuromuscular coculture model of dissociated dorsal root ganglion (DRG) neurons and SKM cells was established. The morphology of DRG neurons and SKM cells in coculture was observed with an inverted phase contrast microscope. The effects of nerve growth factor (NGF) or neurotrophin-3 (NT-3) on the expression of mRNAs for PPT, CGRP, NF-200, and MAP-2 was analyzed by real time-PCR assay. The morphology of DRG neuronal cell bodies and SKM cells in neuromuscular coculture at different conditions was similar. The neurons presented evidence of dense neurite outgrowth in the presence of distinct NTs in neuromuscular cocultures. NGF and NT-3 increased mRNA levels of PPT, CGRP, and NF-200, but not MAP-2, in neuromuscular cocultures. These results offer new clues towards a better understanding of the association of target SKM cells with distinct NTs on the expression of mRNAs for PPT, CGRP, NF-200 and MAP-2, and implicate the association of target SKM cells and NTs with DRG sensory neuronal phenotypes. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 2, 312–318)*

Key words: neurotrophins, substance P, calcitonin-gene related peptide, neurofilament 200, microtubule associated protein 2, dorsal root ganglion, skeletal muscle cell

Introduction

Targets of neuronal innervation play a vital role in regulating the survival and differentiation of innervating neurotrophin (NT)-responsive neurons [1]. Sensory nerve cross-anastomosis (sensory protection) provides a modified trophic environment by modulating neurotrophic factor synthesis in muscles [2]. Target tissues contribute to the phenotype and function of sensory neurons [3–5]. The neuropeptide--immunoreactive (IR) and neurofilament-IR neurons are two major phenotypical classes in DRG. Neuropeptide-IR neurons are considered to be unmyelinated or

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thinly myelinated nociceptive afferent neurons which are thought to innervate skin and viscera. Neurofilament (NF)-IR neurons typically have myelinated axons, many of which innervate muscle spindles [6].

Substance P (SP) is a nociceptive peptide [7] and a member of the tachykinin family of peptide neurotransmitters that are derived from the preprotachykinin (PPT) gene by alternative splicing [8]. Calcitonin gene-related peptide (CGRP) is a potent neuropeptide vasodilator [9] and controls sensory transmission [10]. Both SP and CGRP are expressed in primary sensory neurons [10, 11].

Neurofilaments (NFs) are neuron-specific intermediate filaments. Neurofilament heavy chain (NF-H) plays an important role in healthy neurons [12]. The appearance of NF-H represents a critical event in the stabilization of axons that accompanies their maturation [13]. Microtubule associated protein 2 (MAP-2) has been tentatively implicated in neuronal outgrowth and polarity of neuronal cells [14]. Within the neuronal cell, MAP-2 proteins are known to interact with microtubules, NFs and actin, and contribute to the maintenance of neuronal cytoarchitecture [15, 16].

Neurotrophins (NTs) are a family of growth factors that regulate the peripheral and central nervous system [17]. Nerve growth factor (NGF) and neurotrophin-3 (NT-3) are two members of the neurotrophin (NT) family, and all play important roles in the development of the peripheral sensory nervous system. Additionally, these growth factors are proposed to modulate the properties of the sensory system in the adult under pathological conditions [18, 19]. NGF utilizes two receptors, the NGF-specific tyrosine kinase receptor, TrkA, and also the non-specific neurotrophin receptor, p75(NTR) (p75). The specific contribution of each receptor to SP signaling remains to be determined [7]. Some neuronal phenotypes of neuropeptide are retained and can remain sensitive to NGF regulation in aged DRG neuronal cultures [20]. It has been demonstrated that NGF upregulates CGRP [21, 22]. NT-3, an important component of the Schwann cell autocrine survival loop, plays an important role in survival and differentiation of sensory neurons sprouting of neurites, synaptic reorganization, and axonal growth [19, 23].

Whether these factors regulate the expression of mRNAs for PPT, CGRP, NF-200, and MAP-2 in DRG neurons in the presence of target skeletal muscle (SKM) cells *in vitro* remains to be seen. In the present study, neuromuscular cocultures of dissociated DRG neurons and SKM cells were established. Using this unique culture system, the levels of mRNAs for PPT, CGRP, NF-200, and MAP-2 were determined after the administration of different NTs.

Material and methods

Neuromuscular coculture preparations and NT treatment. Animals used in this experiment were obtained from the Experimental Animal Center at Shandong University in China. All experiments involving animals were approved by the Ethical Committee of Shandong University. DRG cell cultures were prepared using Wistar rat pups at embryonic day 15 (E15). Under aseptic conditions, dorsal root ganglia (DRGs) were removed bilaterally using a sharp pair of forceps from each embryo and placed in Dulbecco's Modified Eagle Medium with F-12 supplement (DMEM/F-12) media (Gibco) in a half petri dish. After that, DRG explants were digested with 0.25% trypsin (Sigma) in D-Hanks solution at 37°C for 10 min and centrifuged for 5 min at 1×10^3 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. DRG cells were then filtered using a Millipore filtering apparatus with a 74 μm mesh, followed by counting. Dissociated DRG cells were plated at a density of 2×10^5 cells/ml in six-well clusters (Costar, Corning, NY, USA) which would contain a single layer of SKM cells prepared as follows.

SKM cell culture preparations utilized newborn Wistar rats. SKM cell cultures were prepared three days before DRG preparation. Under aseptic conditions and using the newborn rat, SKM was removed from the hind limb of each animal, minced with fine dissecting scissors into fragments approximately 0.5 mm in diameter, digested with 0.25% trypsin (Sigma) in D-Hanks solution at 37°C for 20 min, centrifuged, and triturated in growth media supplemented with 5% fetal bovine serum (Gibco). Isolated SKM cells were plated at a density of 2×10^5 cells/ml in six-well clusters (Costar, Corning, NY, USA) which would contain 24 mm diameter coverslips precoated with poly-L-lysine (0.1 mg/ml) and then incubated at 37°C in a 5% CO_2 incubator.

The neuromuscular cocultures were prepared as follows. The newly prepared DRG cell suspension was plated at three-day old SKM culture and coculture with SKM cell for an additional six days, with a medium change every two days.

All the cocultures were randomly divided into three groups. (1) NGF group: the neuromuscular cocultures were treated with NGF (10 ng/ml) during the six days of coculture. (2) NT-3 group: the neuromuscular cocultures were treated with NT-3 (10 ng/ml) during the six days of coculture. (3) control group: the neuromuscular cocultures were continuously exposed to growth media as control.
The composition of the culture medium was DMEM/F-12 (1:1) supplemented with 10% fetal bovine serum, 2% B-27 supplement (Gibco), L-glutamine (0.1 mg/ml, Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Living cell observation by inverted phase contrast microscopy. All cultures were observed using inverted phase con-

trast microscopy at different culture ages. The observers were blinded to the treatment protocol for the evaluation. The pictures were taken for monitoring morphological characteristics of neuromuscular cocultures of DRG and SKM cells with different NTs.

Real time-PCR analysis of mRNAs for PPT, CGRP, NF-200, and MAP-2. The mRNA levels of PPT, CGRP, NF-200, or MAP-2 in neuromuscular cocultures at six days of culture age with different NTs were analyzed by real time-PCR. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also determined as an internal control. The GAPDH gene is highly conserved and expressed in a wide variety of tissue types and so is useful as a reference 'house-keeping' gene. It is a stable reference gene for application in cell culture research using the rat. It allows determination of relative expression levels of other genes of interest. Total DRG cell RNA of each well of the clusters was isolated by TRIzol (TakaRa). cDNA was synthesized using a cDNA synthesis kit (Fermentas) according to the manufacturer's instructions.

The synthetic oligonucleotide primer sequences for PPT, CGRP, NF-200, MAP-2 and GAPDH were as follows: PPT 5'- CGA CAG TGA CCA AAT CAA GG -3' (coding sense) and 5'- CAA AGA ACT GCT GAG GCT TG -3' (coding antisense). CGRP 5'- CCT TTC CTG GTT GTC AGC ATC TT -3' (coding sense) and 5'- CAG TAG GCG AGC TTC TTC TTC AC -3' (coding antisense). NF-200 5'- AAA GTG AAC ACG GAT GCT ATG C -3' (coding sense) and 5'- GTG CTT TTC AGT GCC TCC AAC -3' (coding antisense). MAP-2 5'- GGC ACT CCT CCA AGC TAC TCT -3' (coding sense) and 5'- CTT GAC GTT CTT CAG GTC TGG -3' (coding antisense). GAPDH 5'- GGC ACA GTC AAG GCT GAG AAT G -3' (coding sense) and 5'- ATG GTG GTG AAG ACG CCA GTA -3' (coding antisense).

Real time-PCR was performed using SYBR green dye (Fermantas) according to the manufacturer's instructions. PCR was performed at 50°C for two minutes, 94°C for 15 minutes, followed by 40 cycles at 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds.

A comparative cycle of threshold fluorescence (Ct) method was used and the relative transcript amount of the target gene was normalized to that of GAPDH using the $2^{-\Delta\Delta Ct}$ method based on a previous study [24]. The final results of real time-PCR were expressed as the ratio of mRNA of control.

Statistical analysis. Data is expressed as mean ± SD. All the data was processed for verifying normality test for variables. If the normality test failed, the data was analyzed with SPSS software by non-parametric methods (Mann–Whitney test). If the normality test passed, statistical analysis was evaluated with SPSS software by one-way ANOVA followed by the Student–Newman–Keuls test (homogeneity of variance) or Dunnett's T3 test (heterogeneity of variance) for significance to compare the differences among various groups. Significance was determined as $p < 0.05$.

Results

Morphology of DRG neurons and SKM cells in coculture under inverted phase contrast microscope

The DRG neuronal cell bodies send axons passing over, or terminating on, the surfaces of SKM cells. The fusing SKM cells were fusiform, rod, bifurcate or 'starfish' in shape. The contraction of the fused SKM cells could be seen under an inverted phase contrast microscope because of the characteristic of spontaneous contraction. Axons from several neurons would cross each other, forming a fine network in areas of high cell density. The crossing axons adhered to each other; hence, displacement of a terminal axon on a contracting muscle cell would also oscillate the proximally crossing axonal network. The morphology of DRG neuronal cell bodies and SKM cells in coculture at different conditions was similar. The neurons exhibited evidence of dense neurite outgrowth in the presence of distinct NTs (Figure 1).

PPT mRNA expression at different culture conditions

To determine the mRNA levels of PPT, the neuromuscular cocultures at six days of culture age with different NT treatment were analyzed by real-time PCR. PPT mRNA levels increased in NGF (2.60 fold, $p < 0.01$) or NT-3 (2.02 fold, $p < 0.01$) treated cocultures compared to those in the control group (Figure 2).

CGRP mRNA expression at different culture conditions

To determine the mRNA levels of CGRP, the neuromuscular cocultures at six days of culture age with different NT treatment were analyzed by real-time PCR. CGRP mRNA levels increased in NGF (2.33 fold, $p < 0.05$) or NT-3 (1.98 fold, $p < 0.01$) treated cocultures compared to those in the control group (Figure 3).

NF-200 mRNA expression at different culture conditions

To determine the mRNA levels of NF-200, the neuromuscular cocultures at six days of culture age with different NT treatment were analyzed by real-time PCR. NF-200 mRNA levels increased in NGF (1.36

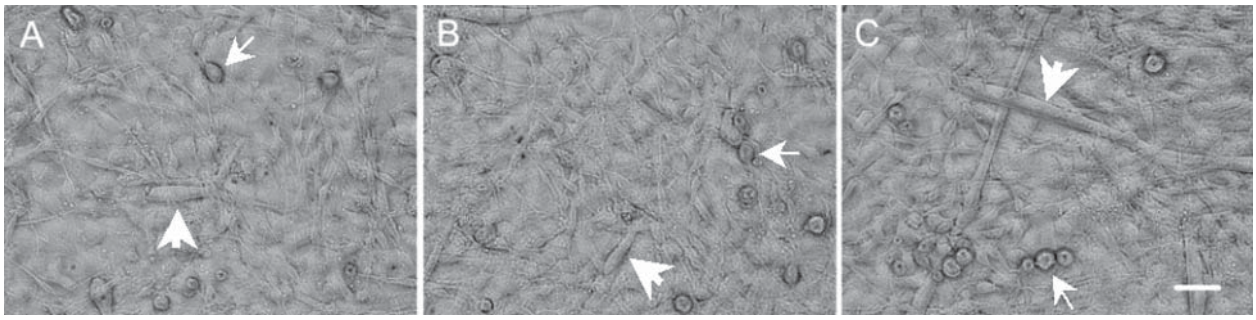

Figure 1. Inverted phase contrast microscopic photomicrographs of the living cells in neuromuscular coculture at six days of culture age. DRG neuronal cell body (thin arrows) sends axons across or terminating on SKM cells (thick arrows). Panel A: neuromuscular coculture with NGF treatment; Panel B: neuromuscular coculture with NT-3 treatment; Panel C: neuromuscular coculture without NT treatment. Scale bar = 50 μm

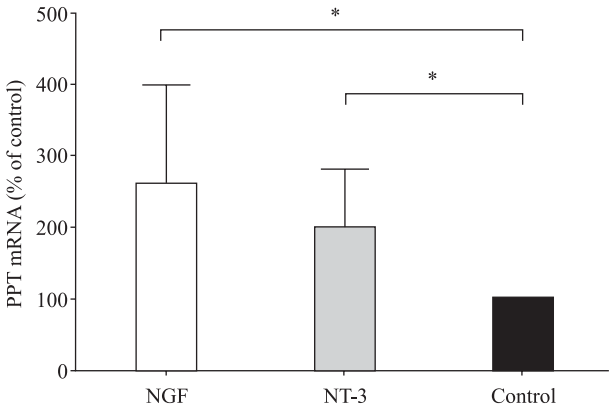

Figure 2. PPT mRNA expression at different culture conditions. Normalized levels of PPT mRNA were higher in NGF treated cocultures than control. NT-3 treatment increased PPT mRNA levels. Bar graphs with error bars represent mean ± SD (n = 12). *p < 0.01

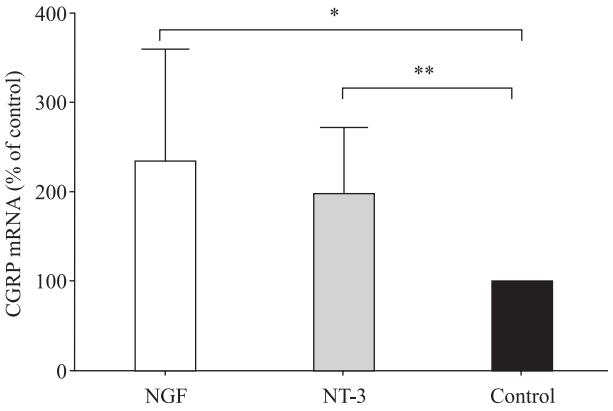

Figure 3. CGRP mRNA expression at different culture conditions. Normalized levels of CGRP mRNA were higher in NGF treated cocultures than control. NT-3 treatment increased CGRP mRNA levels. Bar graphs with error bars represent mean ± SD (n = 12). *p < 0.05, **p < 0.01

fold, p < 0.05) or NT-3 (1.55 fold, p < 0.001) treated cocultures compared to those in the control group (Figure 4).

MAP-2 mRNA expression at different culture conditions

To determine the mRNA levels of MAP-2, the neuromuscular cocultures at six days of culture age with different NT treatment were analyzed by real-time PCR. MAP-2 mRNA levels did not change significantly in the presence of NGF or NT-3 compared to those in the control group (Figure 5).

Discussion

During development, neurons extend axons to their targets, then become dependent for their survival on trophic substances secreted by their target cells [25]. The exogenous administration of NTs has protective properties for injured neurons, and stimulates axonal regeneration [26]. The expression of mRNAs for PPT, CGRP, NF-200, and MAP-2 may reflect the responsiveness of DRG sensory neurons to these factors.

In the present study, NGF and NT-3 increased mRNA levels of PPT, CGRP, and NF-200, but not MAP-2, in neuromuscular cocultures.

Many neurotrophic factors have been shown to promote neurite outgrowth by improving the microenvironment that is required for nerve regeneration [27]. It has been suggested that the concentrations of NGF were from 1 to 300 ng/ml for DRG neurons *in vitro* [28]. Serum concentrations of NGF were several pg/ml [29] which is much lower than that used in *in vitro*. The levels of growth factors in fetal bovine serum were much lower than the levels of exogenous growth fac-

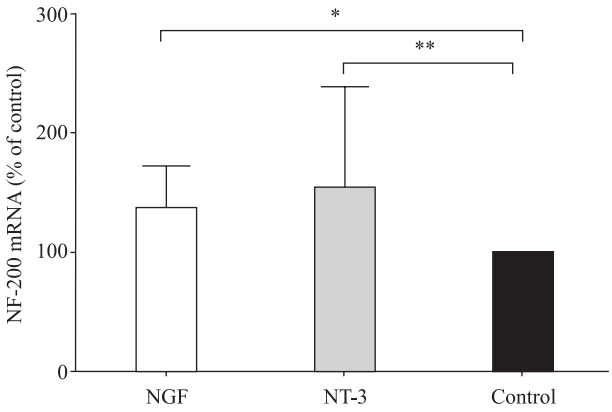

Figure 4. NF-200 mRNA expression at different culture conditions. Normalized levels of NF-200 mRNA were higher in NGF treated cocultures than control. NT-3 treatment increased NF-200 mRNA levels. Bar graphs with error bars represent mean ± SD (n = 12). *p < 0.05, **p < 0.001

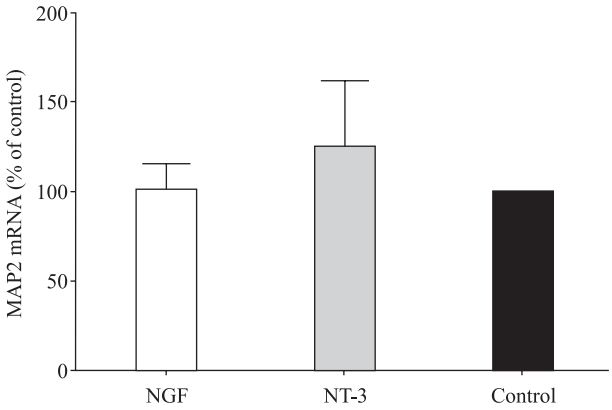

Figure 5. MAP-2 mRNA expression at different culture conditions. Normalized levels of MAP-2 mRNA were not changed significantly in NGF or NT-3 treated cocultures than control. Bar graphs with error bars represent mean ± SD (n = 6)

tors used in this experiment. In the present study, i.e. the exogenous administration of NGF or NT-3, respectively, to the neuromuscular cocultures of DRG neurons and SKM cells, the DRG neurons presented evidence of dense neurite outgrowth in the presence of distinct NT, suggesting that the microenvironment improved by distinct NT is suitable for neurite outgrowth of DRG neurons in the neuromuscular cocultures.

In DRG, NGF signaling is required not only for survival, but also for the differentiation of nociceptors which express SP [30]. NGF is a potent stimulator of SP which is produced in DRG sensory neurons [28, 31]. It has been demonstrated that expression of SP is regulated by NGF and exogenous exposure to high levels of NGF increases its cellular content and release. NGF up-regulation of SP expression requires the involvement of both TrkA and p75, although the specific contribution of each receptor to SP signaling remains to be determined [7, 32]. NGF has a key role in the survival and establishment of the phenotype of responsive primary afferent neurons during development [33]. In the present study, the exposure of exogenous NGF to neuromuscular cocultures of E15 DRG neurons and SKM cells could increase the levels of PPT mRNA encoding for SP, a finding consistent with previous studies. NGF also increased the levels of CGRP mRNA and NF-200 mRNA in neuromuscular cocultures of DRG neurons and SKM cells.

NT-3 signaling is required for the generation and survival of various DRG neuron classes, in particular proprioceptors [30]. NT-3 prevented abnormalities of NF-H in sensory neurons of diabetic rats [34]. The latest research demonstrated that NT-3 may have antinociceptive effects which may be mediated, at least in part, by its cognate receptor tyrosine-kinase C (TrkC) in the medium or large size DRG neurons [35].

In the present study, exposure of exogenous NT-3 to neuromuscular cocultures of E15 DRG neurons and SKM cells could increase the levels of mRNAs for PPT, CGRP, and NF-200. These results suggest that the effects of NT-3 on promoting neuronal survival might be through regulating expression of sensory neuropeptide and protein expressed in DRG neurons.

It has been suggested that at the initiation of radial growth, neurofilaments are likely to consist primarily of neurofilament light and medium, as neurofilament heavy expression is developmentally delayed [36]. In the present study, either NGF or NT-3 has a promotive effect on NF-200 mRNA expression, suggesting that both NGF and NT-3 were involved in regulation of the later stage of DRG development.

MAP-2 is a cytoskeletal protein and plays a regulatory role in neuronal plasticity and in maintaining the morphology of differentiated neurons [37]. MAP-2 has been tentatively implicated in neuronal outgrowth and polarity of neuronal cells [14]. MAP-2 is generally used as a neuronal marker [38]. In the present study, MAP-2 mRNA expression did not depend on the presence of distinct NTs in neuromuscular cocultures of DRG neurons and SKM cells suggested that target SKM cells is sufficiently on maitaining MAP-2 mRNA expression.

In conclusion, the neurons presented evidence of dense neurite outgrowth in the presence of distinct NTs in neuromuscular cocultures of DRG neurons

and SKM cells. NGF and NT-3 increased mRNA levels of PPT, CGRP, and NF-200, but not MAP-2, in neuromuscular cocultures. These results offer new clues towards a better understanding of the association of target SKM cells with distinct NTs on the expression of mRNAs for PPT, CGRP, NF-200, and MAP-2 and implicate the association of target SKM cells and NTs with DRG sensory neuronal phenotypes.

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