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Effects of insulin-like growth factor-1 on neurochemical phenotypes of cultured dorsal root ganglion neurons with excitotoxicity induced by glutamate

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Insulin-like growth factor-1 (IGF-1) is a neurotrophic factor and plays an important role in promoting axonal growth from dorsal root ganglion (DRG) neurons. The neuropeptide- and neurofilament (NF)-immunoreactive (IR) neurons are two major phenotypical classes in DRG. Whether IGF-1 affects neurochemical phenotypes of DRG neurons remains unknown. In the present study, primary cultured DRG neurons were used to determine the effects of IGF-1 on neurochemical phenotypes of the neurons with excitotoxicity induced by glutamate (Glu). DRG neurons were dissociated and cultured for 48 hours and then exposed to IGF-1 (20 nmol/L), Glu (0.2 mmol/L), Glu (0.2 mmol/L) plus IGF-1 (20 nmol/L) for additional 24 hours. The DRG neurons were continuously exposed to culture media as control. After that, all above cultured DRG neurons were processed for detecting mRNA levels of calcitonin gene-related peptide (CGRP) and neurofilament-200 (NF-200) by real time-PCR analysis. CGRP and NF-200 expression *in situ* was determined by fluorescent labeling technique. The results showed that CGRP mRNA, but not NF-200 mRNA, increased after IGF-1 administration in the absence or presence of Glu. IGF-1 could increase the percentage of CGRP-expressing neurons, but not NF-200-expressing neurons, in the absence or presence of Glu. The ability of IGF-1 on CGRP expression may play a role in neurogenic inflammation or nociception.

1. Introduction

Insulin-like growth factor-1 (IGF-1) is a polypeptide growth factor with a variety of functions in both neuronal and non-neuronal cells (Zheng and Quirion 2006; Wood et al. 2007). IGF-1 acts within the brain to enhance neuronal survival and plasticity (McCusker et al. 2006). Exogenous administration of IGF-1 after injury is neuroprotective and improves long-term neurological function (Guan 2008). Neurodegenerative disorders have been associated with decreased serum IGF-1 concentration (Svensson et al. 2006). IGF-1 promoted neuronal survival by activating its tyrosine kinase receptor IGF-1R (Zhong et al. 2004; McCusker et al. 2006). IGF-1 and its receptor (IGF-1R) are expressed in small dorsal root ganglion (DRG) neurons (Miura et al. 2011; Chirivella et al. 2012). IGF-1 plays an important role in promoting axonal growth from DRG neurons (Bomze et al. 2001; Jones et al. 2003; Seki et al. 2010) and in regulating sensory neuropeptide expression (Liu et al. 2010).

Glutamate (Glu) is an excitatory amino acid (Yamada et al. 2005; Edling et al. 2007) and induces neuronal excitotoxicity by activating N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) receptors to stimulate Ca^{2+} influx (Sanelli et al. 2007). This excitotoxicity is a calcium-dependent process and activation of the NMDA receptor subtype contributes mainly to neuronal damage, due to its high permeability to Ca^{2+} (Del Río et al. 2008).

The neuropeptide-immunoreactive (IR) and neurofilament (NF)-IR neurons are two major phenotypical classes in DRG. Neuropeptide-IR neurons are considered to be with unmyelinated or thinly myelinated nociceptive afferents which are considered to innervate skin and viscera. Calcitonin gene-related peptide (CGRP)-IR neurons represent neuropeptide-IR neurons in DRG (Hall et al. 1997; Liu et al. 2012). CGRP is a sensory neuron-associated neuropeptide (Gajda et al. 2005) and is expressed in primary sensory neurons and controls sensory transmission (Schaeffer et al. 2010). CGRP is released upon appropriate stimulation (Winston et al. 2001; Xing et al. 2006). NF-IR neurons typically have myelinated axons which are considered to innervate muscle spindle (Hall et al. 1997). NF-200-IR neurons represent NF-IR neurons in DRG (Wang et al. 2009, 2010). However, whether IGF-1 affects neurochemical phenotypes of DRG neurons remains unknown. In the present study, primary cultured DRG neurons were used to determine the effects of IGF-1 on neuronal phenotypes of the neurons with excitotoxicity induced by Glu.

2. Investigations and results

2.1. Effects of IGF-1 on CGRP mRNA levels

To test the effects of IGF-1 on CGRP mRNA expression in DRG neurons, cultures of E15 DRG at different experimental

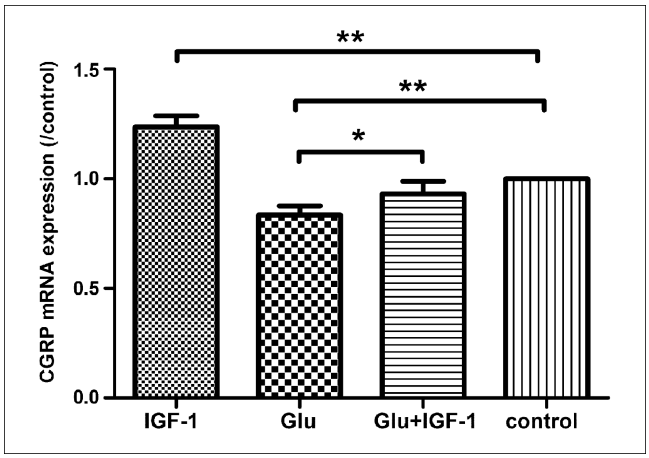

Fig. 1: Effects of IGF-1 on CGRP mRNA expression in DRG neurons at different experimental conditions were investigated by real time-PCR analysis. Normalized levels of CGRP mRNA in different groups are 1.24 ± 0.05 folds (IGF-1) *vs*. control, 0.84 ± 0.04 fold (Glu) *vs*. control, and 0.93 ± 0.06 fold (IGF-1 + Glu) *vs*. control, respectively. Bar graphs with error bars represent mean ± SD (n = 5). $^{*}P < 0.01$, $^{**}P < 0.001$

conditions were processed for real time-PCR analysis. The CGRP mRNA levels in IGF-1 (20 nmol/L), Glu (0.2 mmol/L), and Glu (0.2 mmol/L) plus IGF-1 (20 nmol/L) are 1.24 ± 0.05, 0.84 ± 0.04, and 0.93 ± 0.06 folds of control, respectively. Glu treatment decreased CGRP mRNA expression. IGF-1 could increase CGRP mRNA expression in the presence or absence of Glu in DRG cultures (Fig. 1).

2.2. Effects of IGF-1 on NF-200 mRNA levels

To test the effects of IGF-1 on NF-200 mRNA expression in DRG neurons, cultures of embryonic day 15 (E15) rat DRG at different experimental conditions were processed for real time-PCR analysis. The NF-200 mRNA levels in IGF-1 (20 nmol/L), Glu (0.2 mmol/L), and Glu (0.2 mmol/L) plus IGF-1 (20 nmol/L) are 1.04 ± 0.08, 0.83 ± 0.06, and 0.88 ± 0.05 folds of control, respectively. Glu treatment decreased NF-200 mRNA expression. IGF-1 did not affect NF-200 mRNA expression in DRG cultures in the presence or absence of Glu (Fig. 2).

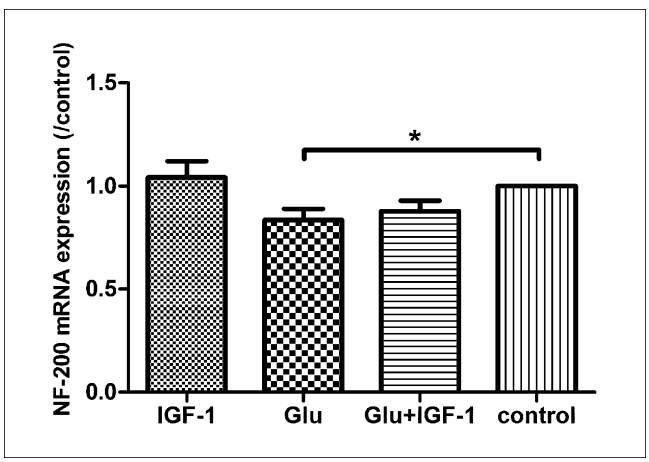

Fig. 2: Effects of IGF-1 on NF-200 mRNA expression in DRG neurons at different experimental conditions were investigated by real time-PCR analysis. Normalized levels of NF-200 mRNA in different groups are 1.04 ± 0.08 folds (IGF-1) *vs*. control, 0.83 ± 0.06 fold (Glu) *vs*. control, and 0.88 ± 0.05 fold (IGF-1 + Glu) *vs*. control, respectively. Bar graphs with error bars represent mean ± SD (n = 5). $^{*}P < 0.001$

2.3. Effects of IGF-1 on CGRP-expressing neurons

To test the effects of IGF-1 on CGRP expression in DRG neurons, cultures of E15 DRG at different experimental conditions were processed for double fluorescent labeling of MAP2 and CGRP and then DRG neurons containing CGRP were quantified. The percentage of CGRP-IR neurons in IGF-1 (20 nmol/L), Glu (0.2 mmol/L), Glu (0.2 mmol/L) plus IGF-1 (20 nmol/L) and control group is $33.08 \pm 1.10\%$, $24.04 \pm 1.27\%$, $28.86 \pm 1.01\%$, and $30.67 \pm 1.33\%$, respectively. IGF-1 could promote CGRP expression in DRG cultures (Fig. 3, 4,).

2.4. Effects of IGF-1 on NF-200-expressing neurons

To test the effects of IGF-1 on NF-200 expression in DRG neurons, cultures of E15 DRG at different experimental conditions were processed for double fluorescent labeling of MAP2 and NF-200 and then DRG neurons containing NF-200 were quantified. The percentage of NF-200-IR neurons in IGF-1 (20 nmol/L), Glu (0.2 mmol/L), Glu (0.2 mmol/L) plus IGF-1 (20 nmol/L) and control group is $27.58\% \pm 1.59\%$, $21.90\% \pm 2.16\%$, $22.52\% \pm 2.18\%$, and $26.04\% \pm 1.69\%$, respectively. IGF-1 did not have significant effect on the NF-200 expression in DRG cultures (Fig. 5, 6).

3. Discussion

IGF-1 has pleiotropic effects in the nervous system and can act both as a survival and a differentiation factor. IGF-1 supports to a population of predominantly nociceptive neurons in DRG which may contribute to neuropathic pain (Craner et al. 2002; Miura et al. 2011). IGF-1 has been shown to act as a neuroprotectant both in *in vitro* studies and in *in vivo* animal models (Miltiadous et al. 2010). The aim of the present study was to approach the question of neuronal phenotye expressing dependence on the presence of IGF-1 during development *in vitro*. In the present study, we found that IGF-1 promoted CGRP and its mRNA, but not NF-200 and its mRNA, expression in DRG cultures in the absence or presence of Glu.

It has been demonstrated that DRG neurons showed different phenotypes during the development. It has been shown that the developmental regulation of sensory neurons containing CGRP that predominantly contact visceral and cutaneous peripheral target end organs *in vivo* (Hall et al. 1997). The peptidergic neurons synthesize CGRP and extend nerve fibers peripherally to all tissues innervated by the sensory nervous system and centrally to the spinal dorsal horn (SDH). The release of CGRP from afferent nerve terminals in peripheral tissues plays a key role in neurogenic inflammation, while release from terminals in the SDH modulates pain transmission (Supowit et al. 2011). DRG primary sensory neurons that respond to nociceptive stimuli are characterized for expression of sensory neuropeptide. Release of sensory neuropeptide from peripheral endings causes a series of local inflammatory responses referred to as neurogenic inflammation (Trevisani et al. 2007). CGRP release may associate with the activation of transient receptor potential vanilloid type 1 (TRPV1) in viscera, because most of the visceral TRPV1-positive DRG neurons expressed CGRP (Avelino et al. 2002; Hwang et al. 2005). It has been demonstrated that IGF-1R expression colocalized with TRPV1 in DRG neurons (Miura et al. 2011) and IGF-1 enhanced TRPV1-mediated membrane currents in cultured DRG neurons (Van Buren et al. 2005). TRPV1 is an ion channel that mediates inflammatory thermal nociception and is present on sensory neurons. Sensory neuropeptide expression and TRPV1 expression may reflect nociceptive properties of DRG neurons (Abdulla et al. 2001; Tominaga et al. 2005; Tang et al. 2006). Sensory neuropeptide is

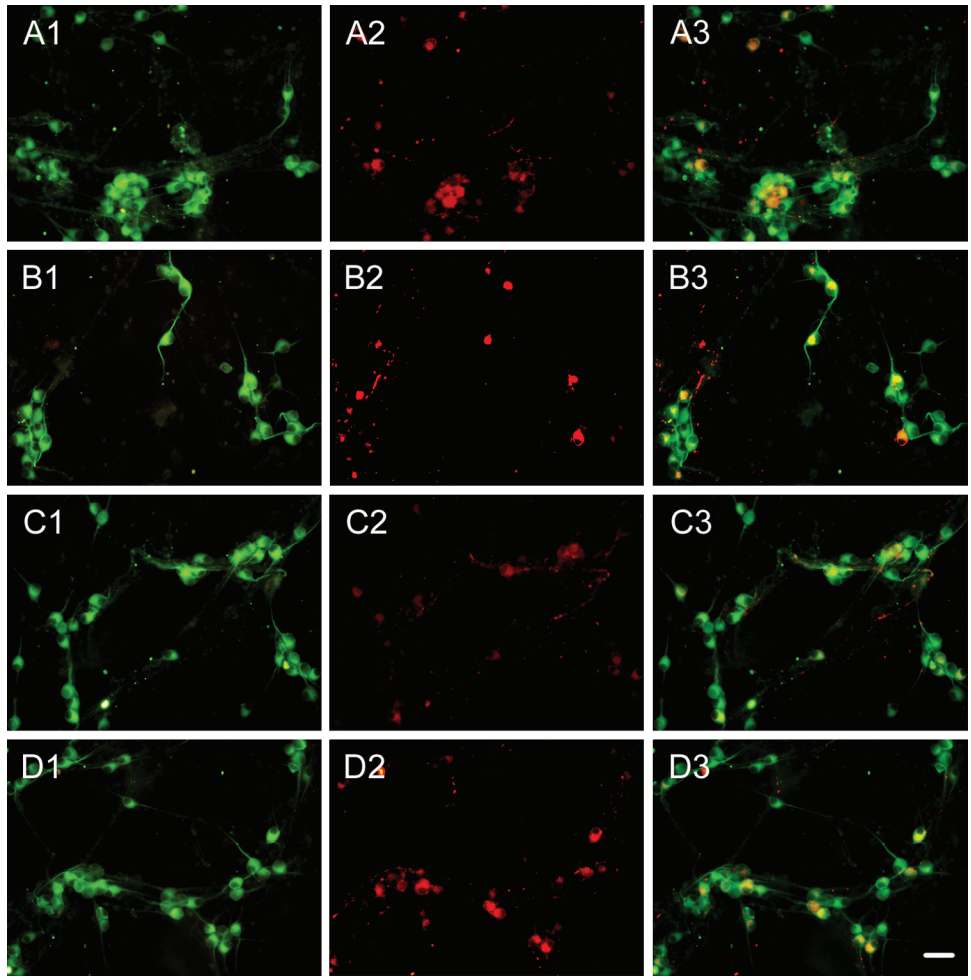

Fig. 3: Double fluorescent labeling of MAP2 and CGRP in DRG neurons. Panel A is DRG cultures with IGF-1 treatment (A1: MAP2; A2: CGRP; A3: overlay of A1 and A2). Panel B is DRG cultures with Glu treatment (B1: MAP2; B2: CGRP; B3: overlay of B1 and B2). Panel C is DRG cultures with Glu plus IGF-1 treatment (C1: MAP2; C2: CGRP; C3: overlay of C1 and C2). Panel D is control (D1: MAP2; D2: CGRP; D3: overlay of D1 and D2). Scale bar = 50 μm

often found in small DRG neurons (Hall et al. 1997). IGF-1 and its receptor are also expressed in small DRG neurons (Craner et al. 2002). CGRP-expressing neurons and IGF-1 supported neurons might be the same subpopulation of DRG neurons, or at least, they are largely overlap. In the present study, IGF-1 could increase the number of CGRP-expressing neurons in the absence or presence of Glu in DRG cultures. These results implicated that IGF-1 benefits the survival of CGRP-expressing neurons and rescues the damaged CGRP-expressing neurons by the presence of excitatory amino acid Glu *in vitro*. IGF-1 may play a role in neurogenic inflammation or nociception accoring to its targeting on specific CGRP-IR subpopulations of DRG neurons. Interestingly, CGRP release from DRG sensory neurons rapidly increases the production of the neuroprotective substance IGF-1 via increasing in its transcription (Harada et al. 2007; Okajima and Harada 2008; Harada et al. 2010; Umemura et al. 2010), and sensory neuron stimulation contributes to a reduction of spinal cord injury by inhibiting inflammatory responses in rats (Umemura et al. 2010). These findings and the results of our present study suggested that IGF-1 and CGRP may have mutual interactions on maintaining specific neuronal phenotype and inflammatory responses after nerve injury.

It has been shown that neurofilament-IR neurons are considered to innervate muscle spindle (Hall et al. 1997). Neurofilaments (NFs) are neuron-specific intermediate filaments which maintain and regulate neuronal cytoskeletal plasticity through the regulation of neurite outgrowth, axonal caliber and axonal transport (Kesavapany et al. 2003). They are classed into three groups according to their molecular masses: NF heavy, middle and light chains (NF-H, NF-M and NF-L). NF-IR neurons are described as large neurons (Averill et al. 1995). Abundant NF expression is characteristic of large neurons that innervate muscle spindles (Perry et al. 1991). NF-H plays an important role in healthy neurons (Gotow 2000). The appearance of NF-H represents a critical event in the stabilization of axons that accompanies their maturation (Yabe et al. 2001). Interestingly, it has been shown that IGF-1R expression did not colocalize with NF-200 in DRG neurons by double immunohistochemical labeling (Miura et al.

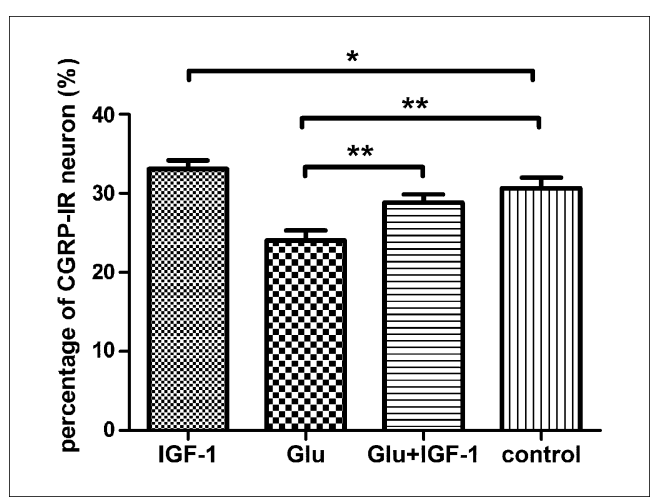

Fig. 4: The percentage of CGRP-IR neurons in DRG cultures at different experimental conditions (n = 5). *$P < 0.01$, **$P < 0.001$

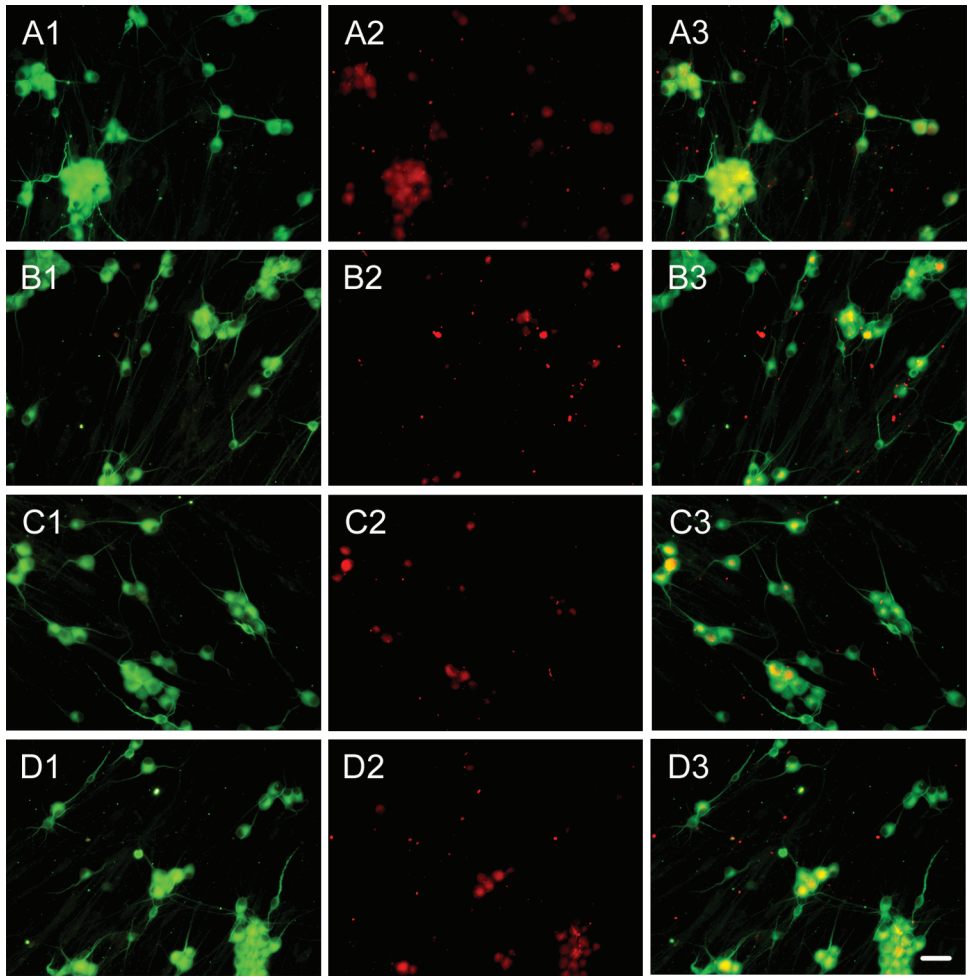

Fig. 5: Double fluorescent labeling of MAP2 and NF-200 in DRG neurons. Panel A is DRG cultures with IGF-1 treatment (A1: MAP2; A2: NF-200; A3: overlay of A1 and A2). Panel B is DRG cultures with Glu treatment (B1: MAP2; B2: NF-200; B3: overlay of B1 and B2). Panel C is DRG cultures with Glu plus IGF-1 treatment (C1: MAP2; C2: NF-200; C3: overlay of C1 and C2). Panel D is control (D1: MAP2; D2: NF-200; D3: overlay of D1 and D2). Scale bar = 50 μm

2011). In the present study, the percentage of NF-200-IR neurons did not change significantly after administration of IGF-1 in the presence or absence of Glu in DRG cultures. These results implicated that the alterations of NF-200-IR neurons were not sensitive to IGF-1, because NF-200-IR neurons did not express IGF-1R.

In conclusion, IGF-1 benefits the survival of CGRP-IR, but not NF-200-IR, neurons in the presence or absence of Glu in DRG cultures. IGF-1 predominantly rescues the damaged CGRP-expressing neurons by the presence of excitatory amino acid Glu *in vitro*. The ability of IGF-1 on CGRP expression may play a role in neurogenic inflammation or nociception. IGF-1 and CGRP may have mutual interactions on maintaining specific neuropeptidic neuronal phenotype after neuronal injury.

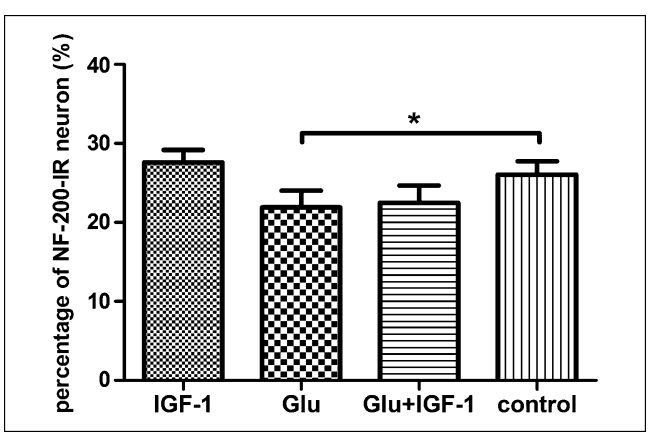

Fig. 6: The percentage of NF-200-IR neurons of cultured DRG neurons at different experimental conditions (n = 5). $*P < 0.05$

4. Experimental

4.1. DRG Cell culture

All culture preparations utilized rats taken from the breeding colony of Wistar rats maintained in the Experimental Animal Center at Shandong University of China. All procedures described herein were reviewed by and had prior approval by the Ethical Committee for Animal Experimentation of the Shandong University. All surgery was performed under anesthesia, and all efforts were made to minimize suffering. Embryonic rats at E15 were used for DRG culture preparations. Under aseptic conditions, the bilateral dorsal root ganglia (DRGs) were removed from each embryo, placed in culture medium, and digested with 0.25% trypsin (Sigma) in D-Hanks solution at 37 °C for 10 min. The suspension of DRG cells were centrifuged at 1×10^3 rpm for 5 min. The supernatants were removed and the pellets were resuspended in Dulbecco's Modified Eagle Medium with F-12 supplement (DMEM/F-12) media (Gibco) and triturated using a sterile modified Pasteur's glass pipette. Cells were then filtered using a 130 μm filter followed by counting. Dissociated DRG cells were cultured in 24-well clusters (Costar, Corning, NY, USA) at 37 °C with 5% CO_2 for 24 h and then maintained in culture media containing cytarabine (ara-C) (5 μg/ml) for another 24 h to inhibit growth of non-neuronal cells, and then cultured in culture media for additional 24 h at different experimental conditions before observation. DRG cells for double fluorescent labeling were plated at 1×10^5 cells/well which would contain a coverslip precoated with poly-L-lysine in each well. DRG cells for real time-PCR analysis were plated at a density of 5×10^5 cells/ml. The composition of the culture media is D-MEM/F-12 (1:1) supplemented with 5% fetal bovine serum, 2% B-27 supplement (Gibco), L-glutamine (0.1 mg/ml, Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml).

4.2. Exposure of Glu and IGF-1 on DRG neurons

At 48 hours of culture age, DRG neurons were exposed to IGF-1 (20 nmol/L, Peprotech), Glu (0.2 mmol/L, Sigma), Glu (0.2 mmol/L) plus IGF-1 (20 nmol/L) for additional 24 h. The DRG neurons were continuously exposed to culture media as control. All above cultures were incubated at 37 °C in a humidified 5% CO_2-air atmosphere.

4.3. Real time-PCR analysis for CGRP mRNA and NF-200 mRNA

After treatment with different agents, the mRNA levels of CGRP and NF-200 in DRG cultures at different experimental conditions were analyzed by real time-PCR. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also determined as an internal control. Total DRG cell RNA of each well of the clusters was isolated by TRIzol (TakaRa). cDNA was synthesized using cDNA synthesis kit (Fermentas) according to the manufacturer's instructions.

The synthetic oligonucleotide primer sequences for CGRP, NF-200 and GAPDH were as follows: 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). 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 by using SYBR Green dye (Fermantas) according to the manufacture's instructions. PCR were performed at 50 °C for 2 min, 94 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s.

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. The final results of real time-PCR were expressed as the ratio of mRNA of control.

4.4. Double fluorescent labeling of MAP2 and CGRP or NF-200

After treatment with different agents, DRG cultures were processed for double immunofluorescent labeling of microtubule associated protein 2 (MAP2) and CGRP or NF-200. The cells on coverslips were rinsed quickly one time in 0.1 mol/L phosphate buffer saline (PBS) to remove media. Then cells were fixed in 4% paraformaldehyde, pH 7.4, for 40 min at 4 °C. After washing in 0.1 mol/L PBS for 3 times, the cells were blocked by 10% normal goat serum in 0.6% Triton X-100 PBS to block non-specific sites and permeabilize cells. The samples were incubated with rabbit polyclonal anti-CGRP (1:400, Abcam) or anti-NF-200 (1:1000, Abcam) overnight at 4 °C, respectively. After washing in 0.1 mol/L PBS for 3 times, the samples were incubated by goat anti-rabbit conjugated to Cy3 (1:500, Abcam) for 60 min in the dark. After washing 3 times in 0.1 mol/L PBS, the cells were incubated with mouse monoclonal anti-MAP2 (1:400, Abcam) for 60 min in the dark. After washing 3 times in 0.1 mol/L PBS, the cells were incubated with goat anti-mouse conjugated to Cy2 (1:100, Abcam) for 60 min in dark. After washing in 0.1 mol/L PBS, the cells were coverslipped immediately with anti-fade mounting media (Santa Cruz Biotechnology) and stored at 4 °C until observation by fluorescent microscope.

4.5. Quantitative analysis of the percentage of CGRP-IR, and NF-200-IR neurons

CGRP-IR or NF-200-IR neurons were observed under a fluorescent microscope (Olympus) with a 20 × objective lens. CGRP-IR or NF-200-IR neurons in five visual fields in the central part of each coverslip were counted as the positive neurons in each sample. The numbers of total neurons (MAP2-IR) were also counted in the same five visual fields. Then the percentage of CGRP-IR or NF-200-IR neurons would be obtained.

4.6. Statistical analysis

Data are presented as mean ± SD. Statistical analysis was calculated with SPSS software by one-way ANOVA, followed by the Student-Newman-Keuls test for significance to compare the differences among various groups or two independent sample *t*-test for significance to compare the difference between two groups or nonparametric methods (Mann-Whitney test). Values of $P < 0.05$ were considered to be significant.

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