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Research report

# Brain-derived neurotrophic factor promotes vesicular glutamate transporter 3 expression and neurite outgrowth of dorsal root ganglion neurons through the activation of the transcription factors Etv4 and Etv5

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) is critical for sensory neuron survival and is necessary for vesicular glutamate transporter 3 (VGLUT3) expression. Whether the transcription factors Etv4 and Etv5 are involved in these BDNF-induced effects remains unclear. In the present study, primary cultured dorsal root ganglion (DRG) neurons were used to test the link between BDNF and transcription factors Etv4 and Etv5 on VGLUT3 expression and neurite outgrowth. BDNF promoted the mRNA and protein expression of Etv4 and Etv5 in DRG neurons. These effects were blocked by extracellular signal-regulated protein kinase 1/2 (ERK1/2) inhibitor PD98059 but not phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 or phospholipase C-γ (PLC-γ) inhibitor U73122. Etv4 siRNA and Etv5 siRNA effectively blocked the VGLUT3 expression and neurite elongation induced by BNDF. The overexpression of Etv4 or Etv5 potentiated the effects of BNDF-induced neurite elongation and growth-associated protein 43 (GAP-43), medium neurofilament (NF-M), and light neurofilament (NF-L) expression while these effects could be inhibited by Etv4 and Etv5 siRNA. These data imply that Etv4 and Etv5 are essential transcription factors in modulating BDNF/TrkB signaling-mediated VGLUT3 expression and neurite outgrowth. BDNF, through the ERK1/2 signaling pathway, activates Etv4 and Etv5 to initiate GAP-43 expression, promote neurofilament (NF) protein expression, induce neurite outgrowth, and mediate VGLUT3 expression for neuronal function improvement. The biological effects initiated by BDNF/TrkB signaling linked to E26 transformation-specific (ETS) transcription factors are important to elucidate neuronal differentiation, axonal regeneration, and repair in various pathological states.



## 1. Introduction

The vesicular glutamate transporters (VGLUTs) play an essential role in synaptic transmission by filling vesicles with glutamate (Glu) (Herman et al., 2014). The VGLUTs are considered to represent the most specific marker for neurons using Glu as a transmitter and are expressed in distinct subsets of neuronal populations at the spinal level (Oliveira et al., 2003). VGLUT3, a VGLUT subtype, is expressed in dorsal root ganglion (DRG) neurons as well as the brain (Atoji and Karim, 2014; Draxler et al., 2014). DRG neurons, as primary sensory neurons, play an important role in transmitting peripheral signals to the central nervous system (CNS). Vesicle transporters, which pack neurotransmitters into synaptic vesicles, are also involved in this process. VGLUT3-positive neurons in the DRG were shown to be important for mechanical hypersensitivity during inflammation and following nerve injury (Seal et al., 2009; Draxler et al., 2014). The expression pattern of VGLUT3 in the primary sensory neurons may represent a specific functional status of these group neurons. The expression of VGLUT3 in DRG neurons in the presence of brain-derived neurotrophic factor (BDNF) with the association of Etv4 and Etv5 expression or activation was explored in the present study.

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Etv4 and Etv5 are two members of the polyomavirus enhancer activator 3 (PEA3)/Etv4-subfamily of the ETS-domain transcription factors, which are transcriptional activators of downstream mitogen-activated protein kinase (MAPK) signaling (Willardsen et al., 2014). Etv4 and Etv5 are essential molecules of the transcriptional program linking neurotrophin signaling to sensory neuronal differentiation and neurite outgrowth (Fontanet et al., 2013). In particular, the expression of ETS transcription factors of the Pea3 subfamily induced in subpopulations of DRG sensory neurons controls several late aspects of neuronal differentiation, such as target terminal differentiation and neurite branching (Hippenmeyer et al., 2005). Etv4 interacts with various upstream neurotrophin signals to direct neurite extension and axonal outgrowth in model systems, including those for DRG cells (Kandemir et al., 2014). How neurotrophin interacts with Etv4 and Etv5 and how exactly Etv4 and Etv5 are involved in neuronal outgrowth remain unclear. BDNF is critical in sensory neuron survival and the establishment of neuronal projections to a peripheral target (Wan et al., 2014). BDNF and its cognate receptor, tyrosine kinase B (TrkB), are normally expressed in neurons and implicated in multiple pathological conditions (Tender et al., 2010). BDNF activates the extracellular signal-regulated protein kinase 1/2 (ERK1/2), phosphatidylinositol 3-kinase (PI3K), and phospholipase C-γ (PLC-γ) signaling pathways by binding to its receptor TrkB (Jeanblanc et al., 2013; Melo et al., 2013). Whether Etv4 and Etv5 could be induced by the BDNF/TrkB pathway and which downstream signaling pathway is involved in this process require more research. In the present study, primary cultured DRG neurons were used to investigate the Etv4 and Etv5 expression in the presence of BNDF with three pathway inhibitors (ERK1/2, PI3K, and PLC-γ).

As a unique neurotrophin, BDNF is synthesized by neurons and is transsynaptically transferred from neurons to other neurons (Song et al., 2008). BDNF is released in an activity-dependent manner to shape the structure and function of neuronal populations (Mariga et al., 2015). Our previous research revealed that BDNF exposure promoted neurite outgrowth, induced growth-associated protein 43 (GAP-43) expression and VGLUT3 upregulation in DRG neurons. In the presence of BDNF, blocking the PI3K/Akt or PLC-γ signaling pathways could inhibit vesicular monoamine transporter 2 (VMAT2) expression in DRG neurons (Liu et al., 2014). Whether Etv4 and Etv5 are involved in the regulation of BDNF in neurite outgrowth, GAP-43, VGLUT3, and VMAT2 expression in DRG neurons is unclear. In the present study, the knockdown of Etv4 or Etv5 by small interfering RNA (siRNA) was used to determine the roles those Etv4 and Etv5 play in the regulation of BDNF on neurite outgrowth as well as VGLUT3 and VMAT2 expression in DRG neurons. DRG neurons were also transfected with Flag-Etv4 or Flag-Etv5 constructs to determine the effects of Etv4 and Etv5 overexpression on neurite outgrowth and GAP-43 expression. The expression of medium neurofilament (NF-M) and light neurofilament (NF-L) was further detected to identify the specific mechanism of Etv4 and Etv5 on neurite outgrowth. And also, specific Etv4 and Etv5 siRNA knocking down experiments were also designed to further examine their roles in BDNF signaling pathway in regulating GAP-43, NF-M, and NF-L proteins. The data of the present study will provide a new understanding for the essential functions of Etv4 and Etv5 in the synapse transmission and neurite outgrowth of primary sensory neurons in response to neurotrophin signals.

## 2. Materials and methods

### 2.1. DRG cell culture preparations

All culture preparations utilized newborn rats (less than 24 h after birth) taken from the breeding colony of Wistar rats maintained in the Experimental Animal Center at the Shandong University of China. All procedures described herein were reviewed by and had prior approval from the Ethical Committee for Animal Experimentation of Shandong University. All surgery was performed under anesthesia, and all efforts were made to minimize the suffering of these animals. Under aseptic conditions, the bilateral dorsal root ganglia (DRGs) were removed from each animal, placed in culture medium, and digested with 0.25% trypsin (Sigma, St. Louis, MO) in D-Hanks solution at 37 °C for 20 min. The suspensions of DRG cells were centrifuged at $1 \times 10^3$ rpm for 5 min. The supernatants were removed, and the pellets were resuspended in Dulbecco's Modified Eagle's Medium with F-12 supplement (DMEM/F-12) medium (Gibco, Grand Island, NY) and triturated using a sterile modified Pasteur's glass pipette. The cells were then filtered using a 130-μm filter followed by counting. Dissociated DRG cells were cultured in 24-well clusters (Costar, Corning, NY) at 37 °C with 5% $CO_2$ for 24 h and then maintained in culture medium containing cytosine arabinoside (5 μg/ml) for another 24 h to inhibit the growth of non-neuronal cells. The cells were then cultured in different experimental conditions for an additional 24 h before observation. The DRG cells for fluorescent labeling were plated at $1 \times 10^5$ cells/well; the coverslips were precoated with poly-L-lysine (0.1 mg/ml, Sigma, St. Louis, MO) in each well. DRG cells for real-time PCR and Western blot assay were plated at a density of $5 \times 10^5$ cells/ml. The composition of the culture medium was D-MEM/F-12 (1:1) supplemented with 5% fetal bovine serum, 2% B-27 supplement (Gibco, Grand Island, NY), and L-glutamine (0.1 mg/ml, Sigma, St. Louis, MO).

### 2.2. Treatment with different agents for DRG neurons

To determine the effects of BDNF on Etv4 and Etv5 expression and the signaling pathways involved in these processes, DRG neurons at 48 h post-culture were treated with the following agents: (1) BDNF (10 ng/ml, Invitrogen Corporation, Camarillo, CA); (2) ERK1/2 inhibitor PD98059 (10 μmol/L, Cell Signaling Technology, Danvers, MA) 30 min prior to treatment with BDNF (10 ng/ml); (3) PI3K inhibitor LY294002 (10 μmol/L, Invitrogen Corporation, Camarillo, CA) 30 min prior to treatment with BDNF (10 ng/ml); (4) PLC-γ inhibitor U73122 (10 μmol/L, Sigma, St. Louis, MO) 30 min prior to treatment with BDNF (10 ng/ml); (5) Control group: The DRG neurons were continuously exposed to culture medium as a control.

All aforementioned cultures were incubated at 37 °C in a humidified 5% $CO_2$ air atmosphere. The mRNA and protein levels of Etv4 or Etv5 were evaluated with real-time PCR and Western blot assay, respectively. The percentage of Etv4-imunoreactive (IR) and Etv5-IR neurons were monitored by double fluorescent labeling of microtubule-associated protein 2 (MAP2) and Etv4 or Etv5. The coexpression of TrkB and Etv4 or Etv5 was determined by double fluorescent labeling of TrkB and Etv4 or Etv5 to further identify the effects of BDNF on Etv4 and Etv5 expression. Furthermore, the percentage of Etv4-IR or Etv5-IR neurons in PD98059 group and control group was also examined to avoid the possible additional effects of PD98059 towards neurons.

### 2.3. Etv4 and Etv5 knockdown by siRNA or Etv4 and Etv5 overexpression using recombinant plasmid vector

To further determine the different effects after Etv4 and Etv5 knockdown or Etv4 and Etv5 overexpression on neurite outgrowth and GAP-43, NF-M, and NF-L expression, DRG neurons at 48 h post-culture were either treated with Etv4 siRNA or Etv5 siRNA and transfection reagents (1 μl/well) (RiboBio, Guangzhou, China) and maintained in culture in the presence of BDNF for additional 24 h so as to knockdown the Etv4 and Etv5 expression; or transfected by Flag-Etv4 or Flag-Etv5 recombinant plasmid vec-