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INFLAMMATION AND ACTIVITY AUGMENT BRAIN-DERIVED NEUROTROPHIC FACTOR PERIPHERAL RELEASE

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Abstract—Brain-derived neurotrophic factor (BDNF) release to nerve terminals in the central nervous system is crucial in synaptic transmission and neuronal plasticity. However, BDNF release peripherally from primary afferent neurons has not been investigated. In the present study, we show that BDNF is synthesized by primary afferent neurons located in the dorsal root ganglia (DRG) in rat, and releases to spinal nerve terminals in response to depolarization or visceral inflammation. In two-compartmented culture that separates DRG neuronal cell bodies and spinal nerve terminals, application of 50 mM K^+ to either the nerve terminal or the cell body evokes BDNF release to the terminal compartment. Inflammatory stimulation of the visceral organ (e.g. the urinary bladder) also facilitates an increase in spontaneous BDNF release from the primary afferent neurons to the axonal terminals. In the inflamed viscera, we show that BDNF immunoreactivity is increased in nerve fibers that are immuno-positive to the neuronal marker PGP9.5. Both BDNF and pro-BDNF levels are increased, however, pro-BDNF immunoreactivity is not expressed in PGP9.5-positive nerve-fiber-like structures. Determination of receptor profiles in the inflamed bladder demonstrates that BDNF high affinity receptor TrkB and general receptor p75 expression levels are elevated, with an increased level of TrkB tyrosine phosphorylation/activity. These results suggest a possibility of pro-proliferative effect in the inflamed bladder. Consistently we show that the proliferation marker Ki67 expression levels are enhanced in the inflamed organ. Our results imply that *in vivo* BDNF release to the peripheral organ is an important event in neurogenic inflammatory state.

Key words: BDNF, release, receptor, periphery, inflammation.

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Abbreviations: BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide; DAB, diaminobenzidine; DRG, dorsal root ganglion; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinases; IGF, insulin-like growth factor; NGF, nerve growth factor; NMDA, N-methyl-D-aspartate; SP, substance P; TNF-α, tumor necrosis factor-α.

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INTRODUCTION

Bidirectional communication between sensory neurons and peripheral organs is an important physiological process. Signals emanating from the target organ flow into sensory neurons located in the dorsal root ganglia (DRG) to ensure correct sensation of environmental cues. In response to peripheral stimulation, neurotransmitters are produced by sensory neurons, which carry the signals into the central nervous system, i.e. spinal cord, where signals are further organized. Neurotransmitters can also release along the distal branch of the sensory neuron axon into the peripheral organs where a paracrine action is undertaken to affect the cytological and physiological properties of the organ. Under pathological conditions such as peripheral inflammation or nerve injury, abnormal levels of neurotransmitters are generated by sensory neuronal cell bodies (Tonra et al., 1998; Obata and Noguchi, 2006; Qiao and Grider, 2007; Qiao et al., 2008; Lin et al., 2011). Neurotransmitters release to the spinal dorsal horn under these conditions often generate central sensitization contributing to painful sensation (Tonra et al., 1998; Lever et al., 2001; Kay et al., 2013). Excessive neurotransmitter release to the peripheral organs and their effects on the cellular changes in the organ have not been well characterized.

Brain-derived neurotrophic factor (BDNF) is one of the nerve growth factor (NGF) family members. As the same as all other neurotrophins, BDNF is initially generated as an unprocessed precursor, pro-BDNF (Mowla et al., 2001). In the cytoplasm, pro-BDNF can be cleaved within the endoplasmic reticulum or regulated secretory vesicles to generate mature BDNF (also called BDNF) (Greenberg et al., 2009). Pro-BDNF can also secret extracellularly where in the matrix pro-BDNF is either cleaved by plasmin to become BDNF, or functions as a ligand to facilitate cellular signals (Greenberg et al., 2009). Three cell surface receptors are responsible for BDNF and pro-BDNF action. The high affinity receptor TrkB is predominantly binding to BDNF. The low affinity receptor p75 can bind both pro-BDNF and BDNF. Sortilin binds a number of unrelated ligands including the pro-domain of the pro-neurotrophins (Nykjaer and Willnow, 2012). In neurons, BDNF binding to TrkB supports neuronal growth, survival and differentiation. Coupling of TrkB and p75 enhances BDNF action in growth and survive (Ho et al., 2011). On the other hand, pro-BDNF binding to p75 and Sortilin leads to apoptosis (Teng et al., 2005).

Different from NGF which is target-derived and is generated by the peripheral organs (Zhang and Qiao,

2012), BDNF is enriched in the sensory neuronal cell body in the DRG and is implicated to participate in sensory neuronal activation in a variety of states (Mannion et al., 1999; Obata and Noguchi, 2006; Cao et al., 2012; Xia et al., 2012; Yu et al., 2012; Qiao et al., 2013). In the DRG, BDNF is synthesized by a subpopulation of unmyelinated primary afferents and is packaged in large dense-core vesicles for anterograde transport to the axon terminals (Conner et al., 1997; Michael et al., 1997). It is extensively reported that anterograde transport of BDNF to the central terminals in the spinal cord dorsal horn (Conner et al., 1997; Michael et al., 1997) can interact with receptors and facilitate excitatory (glutamatergic) neurotransmission (Kerr et al., 1999), or modulate inhibitory (GABAergic/glycinergic) signaling in spinal neurons (Carrasco et al., 2007). BDNF release is often accompanied by other neurotransmitters including glutamate, substance P (SP), somatostatin, calcitonin gene-related peptide (CGRP), etc. in facilitation of spinal cord plasticity (Tonra et al., 1998; Lever et al., 2001; Luo et al., 2001; Ng et al., 2007; Ha et al., 2008). Peripheral release of CGRP and SP has been reported (White and Helme, 1985; Kilo et al., 1997). However, little information is known about peripheral release of BDNF.

The present study combined *in vitro* and *in vivo* approaches and investigated the possibility of BDNF release to the peripheral terminals of the DRG axons. We used a DRG-spinal nerve preparation along with an *in vivo* rat model of visceral inflammation to better understand the nature of BDNF peripheral release and its possible effect on peripheral organs. This is clinically significant because neurotransmitter release from primary afferent neurons at the peripheral terminals in response to the increase in the axonal terminal excitability may participate in the generation and maintenance of neurogenic inflammation.

EXPERIMENTAL PROCEDURES

Experimental animals

Adult male rats (200–250 g) from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) were used. All experimental protocols involving animal use were approved by the Institutional Animal Care and Use Committee at the Virginia Commonwealth University. Animal care was in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health guidelines. All efforts were made to minimize the potential for animal pain, stress or distress as well as to reduce the number of animals used.

Immunoprecipitation and western blot

After determination of protein concentration, protein extracts from tissues were separated on a 10% SDS–PAGE gel and transferred to a nitrocellulose membrane directly for western blot. Antibodies used for western blot include rabbit anti-TrkB (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-BDNF/pro-BDNF (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibody against p75 (1 μg/mL, Chemicon-Millipore, Darmstadt, Germany), and mouse antibody against β-actin antibody (1:3000, Sigma–Aldrich, St. Louis, MO, USA). To examine phospho-TrkB, protein extracts were pre-incubated with rabbit anti-TrkB primary antibody (1:200) overnight at 4 °C and then immunoprecipitated by Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA). The precipitates were boiled in laemmili loading buffer and loaded for western blot against phospho-tyrosine with a mouse monoclonal antibody (1:1000, Cell Signaling Technology, Inc., Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody/enhanced chemiluminescence (ECL) or IRDye/ODYSSEY infrared imaging was used to detect immunoreactive bands. Internal loading control used for normalization was obtained by re-probing the same membrane with anti-β-actin antibody after thorough stripping procedure. In immunoprecipitation, the supernatant after precipitation of TrkB was used to examine β-actin as loading control. Densitometric analysis was achieved by scanning and quantifying the density of the immunoreactive bands with software FluorChem 8800 (Alpha Innotech, San Leabdro, CA, USA).

BDNF release assay

BDNF release was tested in *ex vivo* preparation of DRG-spinal nerve complex as described previously by us (Yu et al., 2012). Briefly, the Campenot chamber was used to separate the ganglion and the nerve terminals. After initial equilibration in Dulbecco's Modified Eagle Medium (DMEM) (~2 h), the chambers were filled with fresh medium. The amount of DMEM used in both chambers kept consistently among experimental groups. In some of these preparations, a final concentration of 50 mM K^+ was added to either the terminal-containing chamber or the cell body-containing chamber. The relative amount of BDNF release into the nerve terminal-containing chamber was determined by slot blot (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All of medium from the terminal-containing chamber was collected and added to the designated slot. After all medium passed through, the membrane was subjected to primary antibody incubation followed by secondary antibody similar to the western blot procedure.

Quantitative real-time PCR

Total RNA was extracted using a RNA extraction kit RNAqueous (Ambion, TX, USA). RNA concentration was determined spectrophotometrically. cDNA was obtained by reverse transcription. Quantitative real-time PCR was performed on a 7300 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific Inc., Grand Island, NY, USA). Taqman probes (Applied Biosystems) were used for quantification of BDNF. β-actin expression in the same sample with the same amount of cDNA was used as internal control for normalization.

Enzyme-linked immunosorbent assay (ELISA)

BDNF ELISA kit (Promega Corporation, Madison, WI, USA) was used for examination of BDNF content.