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BMP2, BMP4, NOGGIN, BMPRIA, BMPRIB, AND BMPRII ARE DIFFERENTIALLY EXPRESSED IN THE ADULT RAT SPINAL CORD

M. MIYAGI,[a,b] S. MIKAWA,[b] T. SATO,[b] T. HASEGAWA,[a] S. KOBAYASHI,[a] Y. MATSUYAMA[a] AND K. SATO[b]*

[a]*Department of Orthopaedic Surgery, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashiku, Hamamatsu, Shizuoka 431-3192, Japan*

[b]*Department of Anatomy and Neuroscience, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashiku, Hamamatsu, Shizuoka 431-3192, Japan*

Abstract—Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF-β) superfamily. BMPs, such as BMP2 and BMP4, exert its biological functions by interacting with membrane bound receptors belonging to the serine/threonine kinase family including bone morphogenetic protein receptor I (BMPRIA, BMPRIB) and type II (BMPRII). Functions of BMPs are also regulated in the extracellular space by secreted antagonistic regulators such as noggin. Although BMP2, BMP4, noggin, BMPRIA, BMPRIB, and BMPRII expressions have been well described in the central nervous system, little information is available for their expressions in the spinal cord. We, thus, investigated these protein expressions in the adult rat spinal cord using immunohistochemistry. Here, we show that BMP2, BMP4, noggin, BMPRIA, BMPRIB, and BMPRII are widely and differentially expressed in the spinal cord. Besides abundant BMP2, BMP4, noggin, BMPRIA, BMPRIB, and BMPRII protein expressions in neurons, we detected them also in astrocytes, oligodendrocytes, and ependymal cells. In addition, we found BMPRIA, BMPRIB, and BMPRII protein expressions in microglia. Interestingly, we also observed that these proteins are strongly expressed in many kinds of axons in both ascending and descending tracts. These data indicate that BMP2, BMP4, noggin, BMPRIA, BMPRIB, and BMPRII proteins are more widely expressed in the adult spinal cord than previously reported, and their continued abundant expressions in the adult spinal cord strongly support the idea that BMP signaling plays pivotal roles in the adult spinal cord.

Key words: BMP2, BMP4, noggin, BMPRIA, BMPRIB, BMPRII.

Bone morphogenetic proteins (BMPs) constitute the largest subgroup of the transforming growth factor β (TGF-β) superfamily (Bragdon et al., 2011). BMPs were initially detected by their ability to direct ectopic bone formation, are now shown to play an important role in multiple biological events (Bragdon et al., 2011). The activities of the BMPs are mediated by a heterodimeric complex of type I and type II BMP serine/threonine kinase receptors, composed of a short extracellular domain, a single transmembrane domain, and the intracellular serine/threonine kinase domain, including bone morphogenetic protein receptor type I (BMPRIA, BMPRIB) and type II (BMPRII) (Bragdon et al., 2011). On BMP binding, the type I BMPRs activate the receptor activated Smads (R-Smads; Smad1/5/8), which oligomerize with common-mediator Smad (Co-Smad; Smad4). The Smad complex then translocates to the nucleus and acts as a transcription regulator (Moustakas and Heldin, 2009). Although many of the biological effects of the BMPs have been related to the Smad-dependent pathways, Smad-independent pathways have been also reported (Massague, 2003). Functions of BMPs are also regulated in the extracellular space by secreted antagonistic regulators such as noggin, chordin, follistatin, neurogenesin-1, which are reported to bind BMPs and prevent their interaction with their receptors (Cho and Blitz, 1998; Ueki et al., 2003).

In the CNS, BMP signaling has been known to be involved in many pivotal events. For example, BMP2 promotes generation of astrocytes both *in vivo* and *in vitro* (Gross et al., 1996; Bonaguidi et al., 2005). In addition, BMP4 is implicated in repression of the oligodendroglial lineage and generation of the astroglial lineage during brain maturation (Gross et al., 1996; Mabie et al., 1999). In addition, in the adult CNS, BMP2 and BMP4 expressed in the type B/C cells in the adult subventricular zone potently inhibit neurogenesis, and its antagonist noggin secreted from the ependymal cells makes a niche for adult neurogenesis (Lim et al., 2000).

BMPs also play pivotal roles in the development of the spinal cord. BMPs are expressed in the dorsal neural tube and overlying dorsal ectoderm, and thought to contribute to the patterning of the dorsoventral axis of the neural tube, and the correct specification of cell types in dorsal regions (Ulloa and Briscoe, 2007). For example, the roof plate resident BMPs have sequential functions in the developing spinal cord, establishing cell fate and orienting axonal trajectories (Hazen et al., 2011). BMPs drastically inhibit oligodendrocyte precursor cell differentiation into mature oligodendrocytes, instead promoting astrogliogeneis (Feigenson et al., 2011). Smad1-dependent BMP signaling is developmentally regulated and governs axonal growth in dorsal root ganglion neurons (Parikh et al., 2011).

BMP signaling is also reported to be deeply involved in the process of spinal cord injury (SCI) (Matsuura et al., 2008; Sahni et al., 2010; Parikh et al., 2011). However, these results are controversial. For example, Matsuura et al. have shown that BMP inhibits axonal growth of pyrami-

*Corresponding author. Tel: +81-53-435-2582; fax: +81-53-435-2582.
E-mail address: ksato@hama-med.ac.jp (K. Sato).

Abbreviations: BMP, bone morphogenetic proteins; BMPRIA, BMPRIB, bone morphogenetic protein receptor I; BMPRII, bone morphogenetic protein receptor type II; BSA, bovine serum albumin; GFAP, glial fibrillary acidic protein; IRs, immunoreactivities; PB, phosphate buffer; SCI, spinal cord injury.

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dal tract in SCI (Matsuura et al., 2008). In contrast, Parikh et al. have shown that reactivation of Smad1-dependent BMP signaling results in sensory axon regeneration (Parikh et al., 2011). Previously we have reported that BMP ligands, BMP2 and BMP4, their antagonist, noggin, and their receptors, BMPRIA, BMPRIB, and BMPRII are abundantly and differentially expressed in the adult rat brain (Mikawa et al., 2006, 2011; Sato et al., 2010; Miyagi et al., 2011). In spite of the importance of BMP signaling in the spinal cord, detailed anatomical evaluations for these proteins have not been done yet in the spinal cord. In the present study, we, thus, investigated BMP2, BMP4, noggin, BMPRIA, BMPRIB, and BMPRII protein expressions in the adult rat spinal cord using immunohistochemistry.

EXPERIMENTAL PROCEDURES

Animals and section preparation

Under deep diethyl ether anesthesia, C3–5 spinal cord samples were isolated from male Wistar rats (n=10, 7 weeks old; Japan SLC Inc., Shizuoka, Japan). For immunohistochemistry, the rats were perfused transcardially with saline followed by 0.1 M phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde and 0.2% picric acid. The spinal cords were removed rapidly, and then postfixed in the same fixative for 2 h at 4 °C. The spinal cords were immersed in 10%, 20%, 30% buffered sucrose overnight at 4 °C. Frozen sections (20 μm in thickness) were cut on a cryostat. All experiments conformed to the Guidelines for Animal Experimentation at Hamamatsu University School of Medicine on the ethical use of animals. Especially, we tried to minimize the number of animals used and their suffering.

Immunohistochemistry

For immunoperoxidase staining, the sections were treated with 10% normal rabbit serum or 10% normal goat serum, 2% bovine serum albumin (BSA), and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature, and incubated further with primary antibodies overnight at 4 °C. After washing with 0.1 M PB, sections were incubated with a secondary antibody for 2 h at room temperature. After washing with 0.1 M PB, immunoreaction was visualized with 3,3′-diaminobenzidine (DAB) (Wako, Osaka, Japan). A goat anti-BMP2 (1:50 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), a mouse anti-BMP4 (1:100 dilution; Novocastra, Newcastle, UK), a rabbit anti-noggin (1:100 dilution; Santa Cruz Biotechnology, Inc.), a goat anti-BMPRIA (1:50 dilution; Santa Cruz Biotechnology, Inc.), a goat anti-BMPRIB (1:200 dilution; Santa Cruz Biotechnology, Inc.), or a goat anti-BMPRII (1:50 dilution; Santa Cruz Biotechnology, Inc.) was used as the primary antibody. And then, a rabbit anti-goat IgG with peroxidase complex (Histofine Simple Stain Rat MAX-PO(G); Nichirei, Tokyo, Japan), a goat anti-mouse IgG with peroxidase complex (Histofine Simple Stain Rat MAX-PO(M); Nichirei, Tokyo, Japan), or a goat anti-rabbit IgG with peroxidase complex (EnVision™ System, K4002; Dako, Tokyo, Japan) was used as the secondary antibody. The anti-BMP2 antibody is an affinity purified goat polyclonal antibody raised against a peptide of 14 amino acids (QAKHKQRKRLKSSC), corresponding to the amino acids 280–293 of the rat BMP2 protein. The anti-BMP4 antibody is a monoclonal antibody raised against the recombinant mouse BMP4 protein. The anti-noggin antibody is a rabbit polyclonal antibody raised against amino acids 1–232 representing full length noggin of human origin. The anti-BMPR1A antibody and the anti-BMPR1B antibody are affinity purified goat polyclonal antibodies raised against the N-terminal domains of each BMPR protein of human origin. The anti-BMPRII antibody is an affinity purified goat polyclonal antibody raised against the cytoplasmic domain of BMPRII protein of human origin.

For double immunofluorescence, in the cases of BMP2, BMPRIA, BMPRIB, or BMPRII and astrocytes staining, the sections were treated with 10% normal donkey serum, 2% BSA, and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature. And then, the sections were incubated further with the anti-BMP2 antibody (1:50 dilution), anti-BMPRIA antibody (1:50 dilution), anti-BMPRIB antibody (1:200 dilution), or anti-BMPRII antibody (1:50) and a mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:1000 dilution; Chemicon, Temecula, CA, USA). After washing with 0.1 M PB, sections were incubated with the Alexa Fluor 594 donkey anti-goat IgG (1:500 dilution; Molecular Probes, Inc., Eugene, OR, USA) and Alexa Fluor 488 donkey anti-mouse IgG (1:500 dilution; Molecular Probes, Inc.) for 1 h at room temperature. In the case of BMP4 and astrocytes staining, the sections were treated with 10% normal chicken serum, 2% BSA, and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature. And then, the sections were incubated further with the anti-BMP4 antibody (1:20 dilution) and a goat anti-GFAP antibody (1:50 dilution; Santa Cruz Biotechnology, Inc.). After washing with 0.1 M PB, the sections were incubated with the Alexa Fluor 594 chicken anti-mouse IgG (1:500 dilution; Molecular Probes, Inc.) and Alexa Fluor 488 chicken anti-goat IgG (1:500 dilution; Molecular Probes, Inc.) for 1 h at room temperature. In the case of noggin and astrocytes staining, the sections were treated with 10% normal goat serum, 2% BSA, and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature. And then, the sections were incubated further with the anti-noggin antibody (1:100 dilution) and a mouse anti-GFAP antibody (1:1000 dilution; Chemicon). After washing with 0.1 M PB, sections were incubated with the Alexa Fluor 594 goat anti-rabbit IgG (1:500 dilution; Molecular Probes, Inc.) and Alexa Fluor 488 goat anti-mouse IgG (1:500 dilution; Molecular Probes, Inc.) for 1 h at room temperature.

In the cases of BMP2, BMPRIA, BMPRIB, or BMPRII and oligodendrocytes staining, the sections were treated with 10% normal donkey serum, 2% BSA, and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature. And then, the sections were incubated further with the anti-BMP2 antibody (1:50 dilution), anti-BMPRIA antibody (1:50 dilution), anti-BMPRIB antibody (1:200 dilution), or anti-BMPRII antibody (1:50 dilution) and a mouse anti-oligodendrocytes antibody (1:40,000 dilution; Milipore, Tokyo, Japan), which recognizes both early and mature oligodendrocytes. After washing with 0.1 M PB, sections were incubated with the Alexa Fluor 568 donkey anti-goat IgG (1:500 dilution; Molecular Probes, Inc.) and Alexa Fluor 488 donkey anti-mouse IgG (1:500 dilution) for 1 h at room temperature.

In the case of BMP4 and oligodendrocytes staining, the sections were treated with 10% normal donkey serum, 2% BSA, and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature. And then, the sections were incubated further with the mouse anti-BMP4 antibody (1:20 dilution) and a rabbit anti-CNPase antibody (1:50 dilution; Bioworld Technology, Inc., St. Louis, MN, USA). After washing with 0.1 M PB, the sections were incubated with the Alexa Fluor 488 donkey anti-mouse IgG (1:500 dilution) and Alexa Fluor 594 donkey anti-rabbit IgG (1:500 dilution; Molecular Probes, Inc.) for 1 h at room temperature. In the case of noggin and oligodendrocytes staining, the sections were treated with 10% normal donkey serum, 2% BSA, and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature. And then, the sections were incubated further with the anti-noggin antibody (1:100 dilution) and the anti-oligodendrocytes antibody (1:40,000 dilution). After washing with 0.1 M PB, sections were incubated with the Alexa Fluor 594 donkey anti-rabbit IgG (1:500 dilution) and Alexa Fluor 488 donkey anti-mouse IgG (1:500 dilution) for 1 h at room temperature.

In the cases of BMPRIA, BMPRIB, or BMPRII and microglia staining, the sections were treated with 10% normal donkey serum, 2% BSA, and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature. And then, the sections were incubated further with