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BMP5 EXPRESSION IN THE ADULT RAT BRAIN

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Abstract—Bone morphogenetic protein-5 (BMP5), a member of the transforming growth factor-β (TGF-β) superfamily, has many effects in several biological events. Although BMP5 expression has been well reported in the early development of the central nervous system (CNS), there is little information about its expression in the adult CNS. Thus, we analyzed BMP5 expression in the adult rat CNS by immunohistochemistry. Abundant BMP5 expression was observed in most neurons, and their dendrites and axons. Furthermore, strong BMP5 expression was also detected in the neuropil of the gray matters with high plasticity, such as the molecular layer of the cerebellum, locus coeruleus, and nucleus of the solitary tract. In addition, we showed BMP5 expression also in astrocytes, ependymal cells and meninges. Our data suggest that BMP5 is widely expressed throughout the adult CNS, and this abundant expression in the adult brain strongly supports the idea that BMP5 plays important roles not only in the developing brain but also in the adult brain.

Key words: axon, neuropil, immunohistochemistry.

INTRODUCTION

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β (TGF-β) superfamily (Bragdon et al., 2011). BMPs were at the beginning detected by their ability to promote ectopic bone formation, and are now reported to have various effects in several biological events (Bragdon et al., 2011). BMPs act on a cell via the induction of a heterodimeric complex of type I and type II BMP serine/threonine kinase receptors including bone morphogenetic protein receptor type I (BMPRIA, BMPRIB) and type II (BMPRII) (Bragdon et al., 2011). By binding to the type I BMPRs, BMPs activate the receptor-activated Smads (R-Smads; Smad1/5/8) which oligomerize with common-mediator Smad (Co-Smad; Smad4) in the cytoplasm. Then, the Smad complex enters the nucleus and initiates transcription (Moustakas and Heldin, 2009). Although most of the biological effects of BMPs have been related to the Smad-dependent signaling pathways, Smad-independent signaling pathways have been also shown (Massague, 2003). Functions of BMPs are also controlled extracellularly by secreted antagonistic regulators such as noggin, chordin, follistatin, and neurogenesin-1, which are reported to bind BMPs and inhibit their interaction with their receptors (Cho and Blitz, 1998; Ueki et al., 2003).

Bone morphogenetic protein-5 (BMP5) is also a member of the TGF-β superfamily. BMP5 is reported to be expressed in the thymus, bone marrow, spleen, skeletal muscle, heart, kidney, lung, pancreas, and prostate (Bragdon et al., 2011), and function in bone and cartilage morphogenesis, limb development and connective soft tissue morphogenesis (Bragdon et al., 2011). It is also known that BMP5 mutations cause shortened, slightly ruffled external ears due to a defective cartilage framework (King et al., 1994). BMP5 has also been reported to be involved in many critical developmental phenomena in the central nervous system (CNS). For example, BMP5 is known to be abundantly expressed in the dorsal neuroepithelium and be involved in the genesis of noradrenergic locus coeruleus neurons (Vogel-Hopker and Rohrer, 2002; Tilleman et al., 2010). In addition, Brederlau et al. (2002) have shown that BMP5 also acts on precursors of the dopaminergic and astroglial lineage and induces their differentiation. Furthermore, in the developing peripheral nervous system, BMP5 has been reported to enhance dendritic growth in cultured sympathetic neurons (Beck et al., 2001).

BMP5 expression has been investigated in the early development of the CNS (Furuta et al., 1997; Vogel-Hopker and Rohrer, 2002). However, there is little information about BMP5 expression in the adult CNS. Furthermore, BMP receptors (Miyagi et al., 2011, 2012), and BMP antagonists (Mikawa and Sato, 2011, 2014), have been also known to be abundantly expressed in the adult rat CNS. Thus, It is needed to investigate BMP5 expression more widely and more in detail in the adult rat brain. In the present study, we show that BMP5 protein is widely

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Abbreviations: BMP, bone morphogenetic protein; BMP5, bone morphogenetic protein-5; BMPR, bone morphogenetic protein receptor; BSA, bovine serum albumin; CNS, central nervous system; ELISA, enzyme-linked immunosorbent assay; GFAP, glial fibrillary acidic protein; IgG, immunoglobulin G; IR, like immunoreactivity; LTD, long-term depression; LTP, long term potentiation; NeuN, neuronal nuclei; PB, phosphate buffer; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SVZ, subventricular zone; TGF-β, transforming growth factor β; TTBS, Tris-buffered saline.

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expressed throughout the adult CNS, and that BMP5 protein is expressed in neurons, astrocytes, ependymal cells and meninges.

EXPERIMENTAL PROCEDURES

Animals and section preparation

Male Wister rats (n = 15, 7 weeks old; Japan SLC Inc., Shizuoka, Japan) were deeply anesthetized with diethyl ether, and then perfused with saline followed by 0.1 M phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde and 0.2% picric acid. The brains were quickly removed, and postfixed in the same fixative for 2 h at 4 °C. All brains were immersed in 10%, 20%, 25% buffered sucrose each, overnight at 4 °C, respectively. Frozen slices (20 μm for immunoperoxidase staining or 10 μm for immunofluorescence in thickness) were serially sectioned on a cryostat. All experiments conformed to the Guidelines for Animal Experimentation at Hamamatsu University School of Medicine on the ethical use of animals.

Immunohistochemistry

For immunoperoxidase staining, the sections were treated with 10% normal rabbit serum, 2% bovine serum albumin (BSA) and 0.2% Triton X-100 in 0.1 M PB for 2 h at room temperature (RT), and incubated further in goat anti-BMP5 (diluted 1:100, the final concentration, 1 μg/ml; R&D Systems, Inc., Minneapolis, MN, USA) overnight at 4 °C. After being washed with 0.1 M PB, the sections were incubated in rabbit anti-goat immunoglobulin G (IgG) with peroxidase complex (no dilution, ready-to-use; N-Histofine® Simple Stain™ Mouse MAX PO (G); Nichirei, Tokyo, Japan) for 2 h at RT. After being washed with 0.1 M PB, immunoreaction was visualized with 3,3′-diaminobenzidine (Wako, Osaka, Japan).

For double immunofluorescence with goat anti-BMP5 antibody (diluted 1:50; the final concentration, 2 μg/ml; R&D Systems, Inc.) and mouse anti-Tbx21 antibody (diluted 1:30; the final concentration, 17 μg/ml; Novus Biologicals, LLC, Littleton, CO, USA), or mouse anti-neuronal nuclei (NeuN) antibody (diluted 1:100; the final concentration, 10 μg/ml; Millipore, Temecula, CA, USA), or mouse anti-glial fibrillary acidic protein (GFAP) antibody (diluted 1:1000; the initial concentration is not available; Millipore), 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 RT, and incubated further in goat anti-BMP5 antibody, mouse anti-NeuN antibody and mouse anti-GFAP antibody overnight at 4 °C and in mouse anti-Tbx21 antibody for three nights at 4 °C. After being washed with 0.1 M PB, sections were incubated in both Alexa Fluor 594 donkey anti-goat IgG (diluted 1:250; the final concentration, 8 μg/ml; Molecular Probes, Inc., Oregon, USA) and Alexa Fluor 488 donkey anti-mouse IgG (diluted 1:500; the final concentration, 4 μg/ml; Molecular Probes, Inc.) for 1.5 h at RT. Furthermore, for double immunofluorescence with goat anti-BMP5 antibody (diluted 1:50) and mouse Fluoro anti-pan neuronal marker antibody cocktail (diluted 1:100; the initial concentration is not available; Millipore), 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 RT, and incubated further in goat anti-BMP5 antibody overnight at 4 °C. After being washed with 0.1 M PB, the sections were incubated in both Alexa Fluor 594 donkey anti-goat IgG (diluted 1:250) and mouse Fluoro anti-pan neuronal marker antibody cocktail (diluted 1:100) for 1.5 h at RT.

Brightfield and fluorescence images were recorded with an Eclipse 80i equipped with a DS-Ri CCD camera (Nikon, Tokyo, Japan) and were further processed by Adobe Photoshop (Tokyo, Japan).

Western blotting

A rat whole brain, or dissected rat cerebral cortex, hippocampus, thalamus, brain stem and cerebellum were homogenized in a 50 mM Tris–HCl (pH 7.4) buffer containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) to avoid degradation of proteins, and solubilized by adding 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. The sample was separated by SDS–PAGE (14% acrylamide; PAGEL, ATTO Corporation, Tokyo, Japan) and then transferred to PVDF membrane (Immobilon-P; Merck Millipore, Tokyo, Japan) by electroblotting (40 V overnight at 4 °C) using a transfer buffer (25 mM Tris, 192 mM glycine) containing 20% methanol. The membranes were blocked with 3% BSA in 50 mM Tris-buffered saline containing 0.05% Tween-20 (TTBS) for 2 h at RT and then incubated with goat anti-BMP5 antibody (diluted 1:200; the final concentration, 0.5 μg/ml; R&D Systems, Inc.) in TTBS containing 1% BSA for 40 min at RT. After being washed with TTBS, the blots were incubated with horseradish peroxidase-linked rabbit anti-goat Ig (diluted 1:4000; the final concentration, 0.125 μg/ml; KPL, Inc., Gaithersburg, MD, USA) in TTBS containing 1% BSA for 40 min at RT. After being washed with TTBS several times, signals were detected by SuperSignal West Pico chemiluminescent Substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The membranes were incubated with 15% H_2O_2 in phosphate-buffered saline (PBS) for 30 min to inactivate horseradish peroxidase. They were blocked with TTBS containing 3% BSA overnight at 4 °C and then incubated with rabbit anti-tubulin-β antibody (diluted 1:1000; the final concentration, 0.2 μg/ml; Thermo Fisher Scientific Inc.) in TTBS containing 1% BSA for 40 min at RT. After being washed with TTBS, the blots were incubated with horseradish peroxidase-linked goat anti-rabbit IgG (diluted 1:5000; the final concentration, 0.08 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in TTBS containing 1% BSA for 40 min at RT. After being washed with TTBS several times, signals were detected by SuperSignal West Pico chemiluminescent Substrate (Thermo Fisher Scientific, Inc.).