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Expression of NPR-B in neurons of the dorsal root ganglia of the rat

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ABSTRACT

C-type natriuretic peptide (CNP) is an abundant neuropeptide in the central nervous system, which exerts its physiological effects through natriuretic peptide receptor B (NPR-B). Recently, the CNP/NPR-B system has been recognized as an important regulator for the development of sensory axons. The dorsal root ganglion (DRG) contains neurons transmitting several kinds of spinal sensory stimuli to the central nervous system. In this study, we characterized NPR-B receptor expression in the rat DRG, using reverse transcription-polymerase chain reaction, Western blotting and immunohistochemistry. Immunostaining revealed that NPR-B was expressed in neuronal cell bodies and processes of the DRG, with NPR-B immunoreactivity mainly prominent in small and medium-sized DRG neurons. Double-immunolabeling showed that NPR-B was expressed in calcitonin gene-related peptide- and isolectin B4-positive neurons. Furthermore, NPR-B expression was co-localized with calcitonin gene-related peptide in the dorsal horn of the spinal cord. Together, our data suggest that the natriuretic peptides may perform several biological actions on sensory neurons via their binding to NPR-B in the DRG.

1. Introduction

Sensory information is transmitted from the periphery to the central nervous system through a heterogeneous population of primary sensory neurons which have their cell bodies in dorsal root ganglia (DRG). Then sensory information is processed within the dorsal horn before it is passed to the central nervous system [30]. It has been proposed that small- and medium-sized DRG cells consist of two neurochemically distinct subpopulations, defined according to their expression of neuropeptides and response to neurotrophic factors [21,26]. Peptidergic cells express neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P. The second cell population is called "non-peptidergic" or "IB4 cells" [8,9,29]. CGRP is important for neuronal modulation in the primary spinal sensory pathway, including the pain pathway [10,16]. It is expressed in small-diameter neurons in the DRG and produced from afferent fibers in the lamina I–II of the dorsal horn of the spinal cord [14,23]. Levels of CGRP mRNA and protein are increased in the DRG and dorsal horn in acute inflammatory pain models [27,32]. On the other hand, isolectin-B4 (IB4) binds selectively to relatively small DRG neurons which are associated with afferent fibers involved largely in conduction of thermal, mechanical, and chemical stimuli [12]. Also, it has been reported that IB4-positive DRG cells can express CGRP [29].

The natriuretic peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and the type-C natriuretic peptide (CNP) [20], are three structurally related peptides. The physiological effects of the natriuretic peptides are mediated by three receptors. Atrial natriuretic peptide and BNP show the highest binding affinity for natriuretic peptide receptor A (GC-A, NPR-A), whereas CNP binds to natriuretic peptide receptor B (GC-B, NPR-B) [20]. All three hormones bind natriuretic peptide receptor-C, which is mainly a clearance receptor [17]. Our previous studies showed that natriuretic peptides and their receptors are expressed in neuronal structures and may modulate several physiological neuronal functions [1–4,6,7]. BNP and NPR-A immunoreactivity has been reported in small DRG neurons and their expression levels are upregulated by inflammation [31]. Also, BNP immunoreactivity has been found in afferent fibers in the spinal lamina I–II of rats and pigs [15,22]. It has been reported that CNP is expressed in the dorsal horn of the embryonic spinal cord inducing bifurcation of growing sensory axons via its receptor NPR-B [24,33]. Furthermore, in cultures of embryonic DRG neurons, CNP enhances the survival effects for sensory neurons [11].

Previous in situ hybridization studies demonstrated that CNP and NPR-B transcripts are found during embryonic development of the DRG [11,25]. However, to our knowledge, the expression of NPR-B protein has not been investigated in detail in the DRG of any species. To further understand the actions of natriuretic peptides in

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the peripheral nervous system, we characterized NPR-B expression in the rat DRG.

2. Materials and methods

2.1. Animals

Twelve male Wistar rats (Clea Japan, Tokyo, Japan) weighing 200–250 g were used. All procedures were approved by the Institutional Animal Care and Use Committee of Shiga University of Medicine, and designed to minimize the number of animals and their suffering in accordance with the 1996 NIH Guide for Care and Use of Laboratory Animals.

2.2. Tissue preparation

Rats were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg), and transcardially perfused with 10 mM phosphate-buffered saline (PBS, pH 7.4) at 20 °C. For Western blot and reverse transcription-polymerase chain reaction (RT-PCR) analysis, L4 to L6 DRG were quickly extracted and stored at −80 °C. For immunohistochemical analysis, rats were similarly anesthetized, perfused with PBS and further perfused with an ice-cold fixative of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Fourth to sixth lumbar DRG and spinal cord segments were collected and post-fixed by immersing for 24 h in the same fixative at 4 °C. After cryoprotection for 2–3 days in phosphate buffer containing 15% sucrose at 4 °C, 20 μm sections were cut in a cryostat. The sections were collected and stored in 0.1 M PBS containing 0.3% Triton X-100 (PBST) at 4 °C.

2.3. Western blotting

Western blot analysis was performed according to the method previously reported [6]. Briefly, DRG samples were homogenized in 6 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 0.1% Triton X-100 and protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany). The homogenates were centrifuged at 15,000 rpm for 20 min at 4 °C, and then the supernatants were collected as a crude protein fraction. Protein concentrations were detected using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Protein samples (40 μg) were electrophoresed on SDS–PAGE (7% gels) and transferred onto polyvinylidene difluoride membranes. Non-specific protein binding sites on the membranes were blocked by a 2-h incubation at room temperature in 10% skim milk in 25 mM Tris-buffered saline (TBS, pH 7.4), then incubation for 4 h at 4 °C with a rabbit polyclonal antibody against NPR-B (Santa Cruz Biotechnology, Santa Cruz, CA), at a dilution of 1:1000 in 25 mM TBS containing 0.1% Tween-20 (TBST). The blots were washed 4 times for 10 min each with 25 mM TBST, and then incubated for 1 h at room temperature with peroxidase-conjugated anti-rabbit IgG (1:10,000; Jackson ImmunoResearch Lab, Inc). After extensive washing with 25 mM TBST, the blots were developed using SuperSignal West Pico Chemiluminescent substrate (Pierce), and visualized using an LAS-3000 FujiFilm Lumino-Image Analyzer (FujiFilm, Tokyo, Japan).

2.4. RT-PCR

Total RNA was extracted from DRG samples using a Fastpure RNA kit (Takara bio, Otsu, Japan) and treated with DNAse (Turbo DNAse, Ambion, Austin, TX). Five micrograms of total RNA was reverse-transcribed into cDNA using oligo dT primer, using SuperScript III (Invitrogen). PCR amplification of cDNA was carried out using the following NPR-B primers: 5′ TCATGACAGCCCATGGGAAA (sense), and 5′ GGTGACAATGCAGATGTTGG (antisense). The amplification profile consisted of an initial step of denaturation at 95 °C for 10 min, 36 cycles of 30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C, followed by a final extension at 72 °C for 8 min. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

2.5. Immunohistochemistry

Serial cryostat sections (20 μm) were floated in 0.1 M PBST (pH 7.4), for 4 days at 4 °C and treated in a free-floating state. Endogenous peroxidase activity was blocked by incubating the sections in 0.5% H_2O_2 in PBST for 30 min. After the sections underwent three 10-min rinses with PBST, they were incubated in 4% bovine serum albumin (BSA) for 1 h at room temperature, and then they were incubated for 72 h at 4 °C with a rabbit polyclonal antibody against human NPR-B (1:400 dilution; Santa Cruz Biotechnology). The primary antibody was diluted in 1% BSA in PBST. After sections had been rinsed three times with PBST (10 min each time), they were incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (1:3000 dilution; Vector Laboratories, Burlingame, CA) before being rinsed another three times with PBST. The sections were finally incubated for 1 h at room temperature with an avidin–biotin–peroxidase complex (1:4000 dilution; ABC Elite; Vector Laboratories) and the peroxidase-labeled sections were developed in 0.02% 3,3-diamine-benzidine tetrahydrochloride with 0.07% nickel ammonium sulfate in 50 mM Tris–HCl (pH 7.6), with 0.005% H_2O_2. Negative control sections were subjected to the same procedures without primary antibody.

2.6. Double-label immunofluorescence

The sections were blocked for 60 min with 4% BSA in PBST, then incubated for 2 days at 4 °C with a mixture of rabbit polyclonal NPR-B antibody (diluted 1:200; Santa Cruz Biotechnology), and either guinea-pig polyclonal CGRP antibody (1:200; T-5053, Peninsula Lab, San Carlos, CA.), mouse monoclonal neurofilament 200 (NF200) antibody (200 kDa; diluted 1:400; Amersham), or TRITC-GS1-IB4 antibody (1:200; EY Laboratories, Inc., San Mateo, CA). Negative controls for specificity were included which omitted the primary antibody in each case. After three rinses with PBST, sections were incubated for 2 h at room temperature with the appropriate secondary antibodies. The secondary antibodies were Alexa Fluor 555-conjugated anti-rabbit IgG, Alexa Fluor 488-conjugated anti-guinea pig IgG, Alexa Fluor 488-conjugated anti-rabbit IgG, or Alexa Fluor 488-conjugated anti-mouse IgG (all antibodies diluted 1:400, all from Molecular Probes). Negative control sections were subjected to the same procedures without primary antibody. After three washes with PBST, the sections were mounted on gelatin-coated glass slides and examined using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Heidelberg, Germany).

3. Results

3.1. Expression of NPR-B in the rat DRG

The specificity of the NPR-B antibody used in this study has been previously confirmed [6]. Furthermore, our Western blot analysis showed that the NPR-B antibody recognized a single band of the expected size (approximately120 kDa) in the rat DRG homogenates, corresponding to NPR-B protein. Taken together, these findings suggest that the protein recognized by the NPR-B antibody used in this work is indeed NPR-B.

Expression of NPR-B in the rat DRG was detected by RT-PCR, Western blotting and immunohistochemistry. RT-PCR analysis showed a positive NPR-B band of the expected size (209 bp) in the