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Brain-derived neurotrophic factor immunoreactive vagal sensory neurons innervating the gastrointestinal tract of the rat

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ABSTRACT

We have determined whether brain-derived neurotrophic factor immunoreactive (BDNF-ir) neurons in the vagal ganglia innervate the gastrointestinal tract. Many BDNF-ir neurons were medium in size and located throughout the jugular and nodose ganglia. When Fluorogold was injected into the wall of the cervical esophagus, many retrogradely Fluorogold-labeled neurons were found in both the jugular ganglion and the nodose ganglion. When Fluorogold was injected into the body of the stomach or applied to the cut end of the subdiaphragmatic vagus nerve, numerous Fluorogold-labeled neurons were found mostly in the nodose ganglion. Double-labeling combining immunohistochemistry for BDNF and retrograde tracing with Fluorogold showed that more than 90% of the neurons in the jugular ganglion and the nodose ganglion projecting to the cervical esophagus contained BDNF-like immunoreactivity. In the cases of both Fluorogold injection into the stomach and Fluorogold application to the subdiaphragmatic vagus nerve, almost all Fluorogold-labeled neurons in the nodose ganglion contained BDNF-like immunoreactivity. These results indicated that almost all vagal sensory neurons located in either the jugular ganglion or the nodose ganglion that innervate the gastrointestinal tract are BDNF-ir neurons.

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Introduction

Retrograde tracing studies have revealed that the larynx and the cervical esophagus receive projections from the sensory neurons in both the jugular ganglion and the nodose ganglion (Hayakawa et al., 2012, 2014; Nomura and Mizuno, 1983), while the subdiaphragmatic gastrointestinal tract receives projections from vagal sensory neurons located mostly in the nodose ganglion (Altschuler et al., 1989; Green and Dockray, 1987; Hayakawa et al., 2011; Neuhuber et al., 2006). Immunohistochemical studies have shown that there are many calcitonin gene-related peptide-immunoreactive (CGRP-ir) neurons in the jugular ganglion (Hayakawa et al., 2011; Helke and Hill, 1988), but only a few CGRP-ir neurons are located in the nodose ganglion. Double-labeling studies combining immunohistochemistry and retrograde tracing have reported that many CGRP-ir neurons in the jugular ganglion project to the larynx and the cervical esophagus, but not to the subdiaphragmatic esophagus, the stomach or the intestines (Hayakawa et al., 2011, 2012). Thus, the sensory neurons in the jugular ganglion are thought to send somatic sensory information from the larynx and the cervical esophagus, and the neurons in the nodose ganglion are thought to send visceral sensory information from the gastrointestinal tract.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotropic family, which is essential for neuronal proliferation and differentiation during development (Barde et al., 1982; Klein, 1994; Zhou and Rush, 1996). In the spinal dorsal root ganglia, BDNF-ir neurons are small- to medium-sized neurons that have unmyelinated or finely myelinated fibers (Luo et al., 2001). BDNF-ir axon terminals were found in the superficial laminae of the spinal dorsal horn (Zhou and Rush, 1996). Since BDNF-ir neurons often contain CGRP, vanilloid receptor-1 (VR-1) or P2X3 receptors, they are considered to play a role in nociceptive function in the spinal cord. Immunohistochemical studies have reported that there are many BDNF-ir neurons in the trigeminal, the geniculate, the vestibulocochlear, and the vagal ganglia in addition to the spinal dorsal root ganglia (Ichikawa et al., 2006; Zhou et al., 1998). In the vagal ganglia, about 50% of the neurons in the jugular ganglion express BDNF-like immunoreactivity, while more than 80% of the

Abbreviations: 5SP, spinal trigeminal tract; AmC, compact formation of the nucleus ambiguus; BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide; DMV, dorsal motor nucleus of the vagus nerve; IGLEs, intraganglionic laminar endings; ir, immunoreactive; n9, glossopharyngeal nerve; n10, vagus nerve; PB, 0.1 M phosphate buffer at pH 7.4; VGlut, vesicular glutamate transporters; VR-1, vanilloid receptor-1..

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neurons in the nodose ganglion express BDNF-like immunoreactivity (Ichikawa et al., 2007; Zhou et al., 1998). Thus, it is likely that the BDNF-ir neurons in the vagal ganglia project to the gastrointestinal tract. However, it is not clear whether the BDNF-ir sensory neurons in the vagal ganglia project to the cervical or the abdominal gastrointestinal tract.

In the trigeminal ganglion, many neurons innervating the cutaneous regions (47%) were BDNF-ir neurons, but a few neurons innervating the tooth pulp (13%) expressed BDNF-like immunoreactivity (Ichikawa et al., 2006). In the jugular ganglion, about 80% of the neurons projecting to the larynx were CGRP-ir neurons, while about 40% of the neurons projecting to the cervical esophagus expressed CGRP-like immunoreactivity (Hayakawa et al., 2014). These results suggested that a different number of BDNF-ir neurons in the jugular and the nodose ganglia project to the different parts of the gastrointestinal tract. However, it is not clear to what proportion of the BDNF-ir neurons in the jugular and the nodose ganglia project to the cervical esophagus, the stomach or the subdiaphragmatic gastrointestinal tract. Several chemical substances have been identified as markers for different types of vagal ganglion neurons (Green and Dockray, 1987; Helke and Hill, 1988; Helke and Niederer, 1990; Ichikawa and Sugimoto, 2003; Patterson et al., 2003; Raab and Neuhuber, 2003). However, there are few characteristic neurochemical substances contained in the neurons in the nodose ganglion projecting to the subdiaphragmatic gastrointestinal tract.

In the present study, we attempted to determine whether BDNF-ir neurons in the vagal ganglia project to the gastrointestinal tract by using double labeling with immunohistochemistry for BDNF and the retrograde tracer Fluorogold. Then, we determined the percentage of the neurons in the jugular ganglion and the nodose ganglion that project to the cervical esophagus or the subdiaphragmatic gastrointestinal tract that are immunoreactive for BDNF.

Materials and methods

Sixteen male Sprague-Dawley rats weighing 250–300 g were used. All surgical procedures were carried out with the animals under 7% of sodium pentobarbital anesthesia (50 mg/kg, i.p.), and approved by The Animal Care and Use Committee of Hyogo College of Medicine.

Immunohistochemistry for BDNF

To determine the distribution of BDNF-ir neurons in the vagal ganglia, three rats were perfused with 0.9% NaCl in 0.1 M phosphate buffer at pH 7.4 (PB) followed by 500 ml of 4% paraformaldehyde-15% picric acid in PB. The occipital and parietal bones were removed, and then the jugular foramen was opened to expose the vagus nerve entering the medulla oblongata. We then removed the left jugular and nodose ganglia together with the medulla oblongata. The samples were immersed in the same fixative for 1 h. Then they were embedded with a solution of 10% gelatin and immersed in 10% sucrose in PB for 2 days. We made serial frozen horizontal sections at a thickness of 30 μm. The frozen sections were incubated for 1 h with 1% bovine serum albumin, 0.9% NaCl in PB containing 0.3% Triton X-100 (PBSX). The sections were incubated for 1 day at 4 °C with a polyclonal rabbit anti-BDNF serum (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200 in PBSX, sc-20981). The treated sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:200 in PBSX) for 5 h at room temperature, then incubated with Vectastain® ABC reagents (Vector Laboratories) for one day and, finally, reacted with a solution of 0.1% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.1 M Tris–HCl buffer at pH 7.4 for 5 min to produce brown reaction products. Every fourth sections were processed for control experiments. They were incubated with 1% normal rabbit serum (Vector Laboratories, S5000) or without the primary antiserum, but with the biotinylated goat anti-rabbit IgG. Photomicrographs of BDNF-ir neurons were taken from several sections. For cell size analysis of BDNF-ir neurons, the cross-sectional area of BDNF-ir cell bodies that contained nucleus was measured in the photomicrographs of the jugular ganglion and the nodose ganglion by using Image J (NIH) software.

Immunohistochemistry combined with retrograde tracing

We used 13 adult male Sprague-Dawley rats weighing 250–300 g. To investigate the distribution of BDNF-ir neurons in the vagal ganglia that innervate the gastrointestinal tract, 2% Fluorogold (Fluorochrome LLC, Denver, CO) was injected into the cervical esophagus, the stomach, or applied to the cut end of the subdiaphragmatic vagus nerve. The cervical esophagus was exposed by removing the sternohyoid and the sternothyroid muscles. The stomach was exposed by opening the abdominal wall, and by pushing aside the liver. The left (ventral trunk) vagus nerve was separated from the esophageal wall at the level of the subdiaphragmatic esophagus. Then the vagus nerve was cut and inserted in an open tube. The open tube, including the cut end of the nerve, was inserted in another tube filled with 2% Fluorogold. Then the two tubes were fixed together with cyanoacrylate glue (Hayakawa et al., 2013). The left side of the cervical esophagus at the level of the thyroid gland or the ventral side of the body of the stomach was injected with 10 μl of 2% Fluorogold by pressure using a glass micropipette (tip diameter = 100 μm) affixed to a 10-μl Hamilton syringe. Cotton swabs were used during the injections to prevent the spread of the tracer to adjacent structures. For control experiments, we poured 10 μl of 2% Fluorogold on the surface of the cervical esophagus and the anterior vertebral muscles in one animal, and the abdominal cavity in one animal. Three days after the injection, the animals were perfused with 80 ml of 0.9% NaCl in PB followed by 500 ml of 4% paraformaldehyde-15% picric acid in PB. In the case of application of the vagus nerve, we confirmed that Fluorogold did not leak out from the sealed tube at the abdominal cavity using a dissection microscope. The left jugular and nodose ganglia were removed together with the medulla oblongata and the first segment of the spinal cord. Then they were embedded with a solution of 10% gelatin and immersed in 10% sucrose in PB for 2 days. Serial frozen horizontal sections were made at a thickness of 30 μm. The sections were incubated with the rabbit anti-BDNF serum (Santa Cruz Biotechnology; 1:200 in PBSX) for one day at 4 °C, and then with Cy3-conjugated goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA; 1:500 in PBSX) for 5 h at room temperature. Fluorogold was viewed using a U excitation filter (blue-white), and Cy3 was viewed using a G excitation filter (red). Fluorescence photomicrographs of Fluorogold-labeled and Cy3-labeled immunoreactive neurons were made with an Olympus BX51 microscope, and merged photographs were made with Adobe Photoshop® CS5 (Adobe) software. Comparing these three photographs, we observed Fluorogold-labeled, Cy3-labeled immunoreactive, and double-labeled neurons. We counted Fluorogold-labeled neurons and the neurons that were double labeled with Fluorogold and Cy3 in every section of the vagal ganglia, and calculated percentages of double-labeled neurons per Fluorogold-labeled neurons. To avoid double counting the labeled neurons, we checked photos of consecutive serial sections, identified neurons cut through the nucleus at the corresponding positions within the sections, and then counted only Fluorogold-labeled neurons containing a nucleus.

Results

The jugular ganglion of the rat is located in the cranial cavity, while the nodose ganglion is distal to the jugular ganglion along the internal jugular vein at the jugular foramen. The glossopharyngeal nerve often enters the jugular ganglion and then fuses with the superior glossopharyngeal ganglion to form the superior glossopharyngeal-jugular ganglion complex in the cranial cavity. Immunohistochemistry for BDNF showed that numerous BDNF-ir neurons are located throughout the jugular ganglion and the nodose ganglion (Fig. 1A). The BDNF-ir neurons are round or oval (Fig. 1B) and medium-sized in the jugular ganglion ($546.9 \pm 69.5\ \mu m^2$, mean ± S.E.M., $n = 417$), and in the nodose

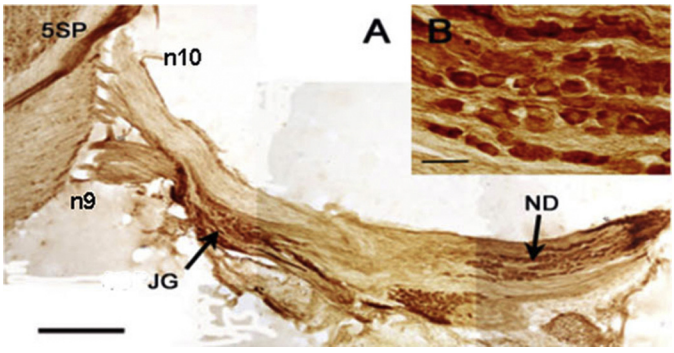

Fig. 1. Distribution and morphology of BDNF-ir neurons in the vagal ganglia. (A) Many BDNF-ir neurons are located in both the jugular ganglion (JG) and the nodose ganglion (ND) in the cranial cavity. 5SP, spinal trigeminal tract; n9, glossopharyngeal nerve; n10, vagus nerve. (B) High-power photomicrograph of BDNF-ir neurons in the nodose ganglion. Scale bars: 500 μm in (A), and 50 μm in (B).

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