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Calcitonin gene-related peptide immunoreactive sensory neurons in the vagal and glossopharyngeal ganglia innervating the larynx of the rat

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ABSTRACT

We have examined whether calcitonin gene-related peptide-immunoreactive (CGRP-ir) neurons in the vagal and glossopharyngeal ganglia innervate the larynx. Many CGRP-ir neurons were located mostly in the superior glossopharyngeal–jugular ganglion complex that was fused the superior glossopharyngeal ganglion and the jugular ganglion in the cranial cavity. When Fluorogold was applied to the cut end of the superior laryngeal nerve (SLN) or the recurrent laryngeal nerve (RLN), many Fluorogold-labeled neurons were found in the superior glossopharyngeal–jugular ganglion complex and the nodose ganglion. Double-labeling for CGRP and Fluorogold showed that about 80% of Fluorogold-labeled neurons in the superior glossopharyngeal–jugular ganglion complex expressed CGRP-like immunoreactivity in the case of application to the SLN, and about 50% of Fluorogold-labeled neurons expressed CGRP-like immunoreactivity in the case of the RLN. Only a few double-labeled neurons were found in the nodose ganglion. The number of the Fluorogold-labeled neurons and double-labeled neurons in the superior glossopharyngeal–jugular ganglion complex in the case of the SLN was larger than that in the case of the RLN. These results indicate that sensory information from the larynx might be conveyed by many CGRP-ir neurons located in the superior glossopharyngeal–jugular ganglion complex by way of the SLN and the RLN.

1. Introduction

The larynx is sensitive to foreign objects during respiration and phonation (Bradley, 2000). The superior laryngeal nerve and the recurrent laryngeal nerve innervate the laryngeal wall. The superior laryngeal nerve descending along the pharynx innervates mainly the superior glottis region and the recurrent laryngeal nerve ascending along the trachea innervates mainly the inferior glottis region, respectively (Yoshida et al., 1992, 2000; Furusawa et al., 1996). Retrograde tracing studies using wheat germ agglutinin (WGA) or cholera toxin subunit B (CTb)-conjugated horseradish peroxidase (HRP) have shown that many labeled neurons are found in the jugular and the nodose ganglia after application of tracers to the superior laryngeal nerve, the recurrent laryngeal nerve or the laryngeal wall (Altschuler et al., 1989; Patrickson et al., 1991; Kano et al., 2011). In contrast to the human, the ganglia of the glossopharyngeal nerve and the vagus nerve of the rat often fuse to form a ganglion complex (Altschuler et al., 1989) at the jugular foramen and in the cranial cavity (Figs. 1 and 2A). The distributions of neurochemically identified sensory neurons projecting to the larynx have not been clearly defined in the jugular, the nodose and the superior glossopharyngeal ganglia in the rat.

Immunohistochemical studies have revealed that many calcitonin gene-related peptide immunoreactive (CGRP-ir) fibers and free nerve endings are distributed throughout the laryngeal mucosal membrane (Terenghi et al., 1986; Tanaka et al., 1993b; Yoshida et al., 1993). Many CGRP-ir neurons are located mostly in

Abbreviations: CGRP, calcitonin gene-related peptide; CTb, cholera toxin subunit B; DAB, 3,3′-diaminobenzidine; FG, Fluorogold; HRP, horseradish peroxidase; IJV, internal jugular vein; ir, immunoreactive; JF, jugular foramen; ND, nodose ganglion; NTS, nucleus tractus solitarii; n10, vagus nerve; n9, glossopharyngeal nerve; PACAP, pituitary adenylate cyclase-activating polypeptide; PB, phosphate buffer (pH 7.4); RLN, recurrent laryngeal nerve; SGPJG, superior glossopharyngeal–jugular ganglion complex; SLN, superior laryngeal nerve; TRPV1, transient receptor potentiated vanilloid 1; VR1, vanilloid receptor 1; WGA, wheat germ agglutinin; 5SP, spinal trigeminal tract.

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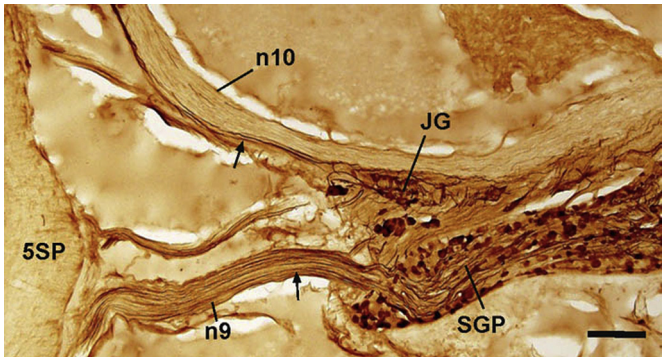

Fig. 1. Low-power photomicrograph of CGRP-ir neurons in a horizontal section of the superior glossopharyngeal ganglion (SGP) and the jugular ganglion (JG). Note that the SGP and the JG fuse to form the superior glossopharyngeal–jugular ganglion complex. Arrows indicate CGRP-ir fibers in the glossopharyngeal nerve and the vagus nerve. 5SP, spinal trigeminal tract; n9, radix of the glossopharyngeal nerve; n10, radix of the vagus nerve. Scale bar: 200 μm.

the superior glossopharyngeal and the jugular ganglia, and a few CGRP-ir neurons are located in the nodose ganglion (Helke and Hill, 1988; Hayakawa et al., 2010). Furthermore, CGRP-ir fibers and axon terminals are distributed characteristically in the lateral, the intermediate, the interstitial, the dorsal, and the commissural subnuclei of the nucleus tractus solitarii (NTS) (Sugimoto et al., 1997; Hayakawa et al., 2010). Transganglionic anterograde tracing studies using CTb or WGA-HRP have reported that the sensory neurons innervating the larynx project mainly to the interstitial subnucleus of the NTS (Hisa et al., 1985; Altschuler et al., 1989; Brining and Smith, 1996; Yoshida et al., 2000; Hayakawa et al., 2001). Thus, it is likely that the CGRP-ir neurons in the superior glossopharyngeal and the jugular ganglia innervate the laryngeal wall, and then send sensory information to the NTS. However, it is not clear which ganglion neurons send CGRP-ir fibers to the laryngeal region and send CGRP-ir axon terminals to the NTS.

Double labeling combining CGRP immunohistochemistry and Fluorogold retrograde tracing studies showed that about 40% of the neurons in the jugular ganglion that project to the cervical vagus nerve expressed CGRP-like immunoreactivity (Hayakawa et al., 2011). About 30% of the neurons in the superior glossopharyngeal ganglion innervating the oral and pharyngeal regions expressed CGRP-like immunoreactivity (Hayakawa et al., 2010). Thus, it is likely that the CGRP-ir neurons in the superior glossopharyngeal ganglion or the jugular ganglion project to the laryngeal wall by way of the laryngeal nerves.

In the present study, we attempted to clarify the distribution of the neurons innervating the larynx by way of the superior laryngeal nerve and the recurrent laryngeal nerve in the vagal ganglia by the application of Fluorogold to the cut end of the superior laryngeal nerve or the recurrent laryngeal nerve. We also clarified to what extent the CGRP-ir sensory neurons in the vagal ganglia send fibers to the larynx using double labeling combining immunohistochemistry for CGRP and retrograde tracing with Fluorogold.

2. Materials and methods

We used 15 male Sprague-Dawley rats weighing 250–300 g. All surgical procedures were carried out with the animals under sodium pentobarbital anesthesia (50 mg/kg, i.p.). The Animal Care and Use Committee of Hyogo College of Medicine approved the procedures.

2.1. Immunohistochemistry for CGRP

To investigate the distribution of CGRP-ir neurons in the glossopharyngeal and the vagal ganglia, three rats were perfused with saline followed by 500 ml of 4% paraformaldehyde–15% picric acid in 0.1 M phosphate buffer at pH 7.4 (PB). The occipital and the parietal bones were broken, and then the jugular foramen was opened to expose the glossopharyngeal and the vagus nerves entering the medulla oblongata. Then we removed the vagus nerve together with the glossopharyngeal nerve, including the jugular, the nodose, and the superior glossopharyngeal ganglia. The samples were immersed in the same fixative for 1 h, and then embedded with 10% gelatin. Horizontal frozen sections of the ganglia were cut at 40 μm. The frozen sections were incubated with rabbit anti-CGRP serum (Yanaihara, Fujinomiya, Japan; Y340, 1:3000) for 1 day. The primary antibody was localized with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; BA-1000, 1:200) for 5 h, and incubated with a Vectastain® ABC kit (Vector Laboratories) for 1 day, and reacted with a solution of 0.1% 3,3′-diaminobenzidine tetrahydrochloride (DAB) 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. Some sections for control experiments were incubated without the primary antibody, but with biotinylated goat anti-rabbit IgG, ABC kit and reacted with DAB.

2.2. Immunohistochemistry combined with retrograde tracing

We used ten adult male Sprague-Dawley rats weighing 250–450 g. To investigate the distribution of CGRP-ir neurons in the vagal ganglia innervating the larynx, Fluorogold (Flurochrome LLC, Denver, CO) was applied to the cut end of the left superior laryngeal or the left recurrent laryngeal nerve. The superior laryngeal or the recurrent laryngeal nerve at the level of the thyroid gland was cut, and then the cut end was inserted into an open tube. The open tube, including the cut end of the nerve, was inserted into another tube filled with 2% Fluorogold. Then the two tubes were fixed together with cyanoacrylate glue. For control experiment, we poured 10 μl of 2% Fluorogold on the surface of the larynx, or set a sealed tube filled with Fluorogold at the laryngeal area without contacting the cut nerve.

Three days after the application of tracers, the animals were perfused with saline followed by 500 ml of 4% paraformaldehyde–15% picric acid in 0.1 M PB. We checked leakage of Fluorogold from the sealed tube to the laryngeal area by observation using the surgical microscope. The left side of the vagus nerve, including the jugular, the nodose, and the superior glossopharyngeal ganglia were removed with the medulla oblongata as described above, and embedded with gelatin. Serial frozen sections were then made at a thickness of 40 μm. The sections were incubated with rabbit anti-CGRP serum (Yanaihara; 1:3000), and localized with Cy3-conjugated goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA; #111-165-003, 1:500). Fluorogold was viewed white with a U excitation filter (Fig. 2B), and Cy3 was viewed red with a G excitation filter (Fig. 2C). Fluorescence photomicrographs of Fluorogold-labeled and Cy3-labeled immunoreactive neurons were made using an Olympus BX51 microscope, and the merged photographs were made with Photoshop® CS5 (Fig. 2D). Comparing these three photographs, we observed Fluorogold-labeled, immunoreactive Cy3-labeled, and double-labeled neurons. We then counted Fluorogold-labeled neurons in every section of the vagal ganglia. To avoid double counting the labeled neurons, we checked consecutive photos of serial sections, identified neurons cut through the nucleus at corresponding positions within the sections, then counted only Fluorogold-labeled neurons containing a nucleus. We also counted neurons that were double labeled with Fluorogold and Cy3, and calculated percentages of double-labeled neurons per Fluorogold-labeled neuron.

3. Results

The vagus nerve emerged from the medulla oblongata at the level of the obex, while the glossopharyngeal nerve emerged from the dorsolateral medulla oblongata at the level of the rostral pole of the NTS. The two nerves then fused in the cranial cavity (Figs. 1 and 2A) and continued down to the jugular foramen. In the cranial cavity, the superior glossopharyngeal ganglion and the jugular ganglion fused to form the superior glossopharyngeal–jugular ganglion complex. At the jugular foramen, the trunk of the vagus nerve swells and contains the nodose ganglion (Fig. 2A). The vagus nerve together with the glossopharyngeal nerve exit the jugular foramen, separate and then innervate the pharynx, the larynx or the gastrointestinal tract. Immunohistochemistry for CGRP revealed that many CGRP-ir neurons were located in the superior glossopharyngeal–jugular ganglion complex (Fig. 1). The CGRP-ir nerve fibers proximal to the superior glossopharyngeal–jugular ganglion complex entered the medulla oblongata through the glossopharyngeal nerve or the vagus nerve (Fig. 1). There were a few CGRP-ir neurons in the nodose ganglion. There were no CGRP-ir neurons in the sections of the glossopharyngeal or vagal ganglia processed for immunohistochemistry without the primary antibody.