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Localization of TRPV1 and P2X3 in unmyelinated and myelinated vagal afferents in the rat

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ABSTRACT

The vagus nerve is dominated by afferent fibers that convey sensory information from the viscera to the brain. Most vagal afferents are unmyelinated, slow-conducting C-fibers, while a smaller portion are myelinated, fast-conducting A-fibers. Vagal afferents terminate in the nucleus tractus solitarius (NTS) in the dorsal brainstem and regulate autonomic and respiratory reflexes, as well as ascending pathways throughout the brain. Vagal afferents form glutamatergic excitatory synapses with postsynaptic NTS neurons that are modulated by a variety of channels. The organization of vagal afferents with regard to fiber type and channels is not well understood. In the present study, we used tract tracing methods to identify distinct populations of vagal afferents to determine if key channels are selectively localized to specific groups of afferent fibers. Vagal afferents were labeled with isolectin B4 (IB4) or cholera toxin B (CTb) to detect unmyelinated and myelinated afferents, respectively. We find that TRPV1 channels are preferentially found in unmyelinated vagal afferents identified with IB4, with almost half of all IB4 fibers showing co-localization with TRPV1. These results agree with prior electrophysiological findings. In contrast, we found that the ATP-sensitive channel P2X3 is found in a subset of both myelinated and unmyelinated vagal afferent fibers. Specifically, 18% of IB4 and 23% of CTb afferents contained P2X3. The majority of CTb-ir vagal afferents contained neither channel. Since neither channel was found in all vagal afferents, there are likely further degrees of heterogeneity in the modulation of vagal afferent sensory input to the NTS beyond fiber type.

1. Introduction

The vagus nerve contains afferent fibers arising from most visceral organs including the heart, lungs, and abdominal organs, which send excitatory glutamatergic projections to the nucleus of the tractus solitarius (NTS) (Andresen and Yang, 1990; Corbett et al., 2005; Kalia and Sullivan, 1982; Paintal, 1973; Talman et al., 1980). Past work focused on the arrangement of afferents from different visceral organs within the NTS (Loewy and Burton, 1978). Recent studies indicate that fiber type based on myelination is importantly influenced by very different ion channel expression in A-fiber compared to C-fiber afferents underlying important functions of vagal afferent systems (Schild et al., 1994). Most vagal afferents have the cellular phenotype of unmyelinated, C-fibers, whereas a smaller number are lightly or heavily myelinated A-fibers (Andresen et al., 2012). Electrophysiology studies suggest that synaptic transmission at A-fibers and C-fibers within NTS are differentially modulated by ion channel receptors such as TRPV1 and P2X3 (Jin et al., 2004a). These functional studies suggested that these molecules should be anatomically segregated to distinct fiber types.

Our previous studies indicate the tract tracer isolectin B4 (IB4) is only associated with unmyelinated vagal afferents (Hermes et al., 2014), allowing us to specifically mark distinct groups of fibers based upon their degree of myelination. Cholera Toxin B subunit (CTb) preferentially labels fibers with myelinated axons, but also detects a small population of unmyelinated afferents (Corbett et al., 2005). Using IB4 and CTb tracing in the present study, we ask whether TRPV1 and P2X3 receptors are located on presynaptic vagal afferents differing in myelination by examining labeled varicosities within NTS. TRPV1 channels are cation channels which regulate spontaneous release of glutamate from C-fiber axon terminals in NTS (Fawley et al., 2011; Peters et al., 2010). P2X3, an ATP-sensitive channel, modulates glutamate release from capsaicin-insensitive NTS afferents (Jin et al., 2004b). TRPV1 and P2X3 in the peripheral endings of sensory fibers may be involved in a variety of visceral reflexes (Cervero and Laird, 2004), but these channels have quite different mechanisms

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of activation and modulation at the central sensory terminals in the brain. The present studies will determine if there is an anatomical basis for the differential modulation of vagal afferents based on fiber type.

2. Materials and methods

2.1. Animals

All methods were approved by the Institutional Animal Care and Use Committee (IACUC) at OHSU. Male Sprague-Dawley rats ($N = 7$, 200–400 g; Charles River Laboratories) served as subjects in all experiments. Rats were housed in pairs on a 12 h light/dark cycle and had food and water available at all times except during surgery.

2.2. Anterograde labeling of vagal afferents

Each rat was given atropine (0.1 mg/ml, s.c.) 15 min prior to surgery to prevent airway secretions during surgery, anesthetized (4% isoflurane), laid supine and cervical portions of the left vagus nerve was isolated from surrounding tissues. A small piece of parafilm was placed under the left vagus nerve to minimize diffusion to surrounding tissues and the tract tracers IB4 (1 to 2 μl of a 4% solution in deionized water, Sigma-Aldrich) and CTb (1 to 2 μl of 1–2% solution in deionized water, List Biological Laboratories) were pressure injected slowly into the nerve with a single-barrel glass micropipette, 20 to 40 μm tip size, attached to a picospritzer (General Valve Inc.). Following injections, the parafilm was removed, surgical wounds were closed with 4–0 monocryl suture (Ethicon Inc.), and animals were kept warm on a heated pad to recover until they were returned to their home cage.

2.3. Perfusion and immunocytochemistry

Seven days after injections, rats were overdosed with sodium pentobarbital (150 mg/kg i.p.) and perfused transcardially with 10 ml heparinized saline followed by 600 ml of 4% paraformaldehyde (in 0.1 M phosphate buffer (PB)). The brain was removed and placed in fixative solution for 30 min on a rotator. The medulla was then sectioned (40 μm) on a vibrating microtome (Leica Biosystems) and collected into 0.1 M PB. Sections were incubated in a 1% sodium borohydride solution for 30 min to increase antigenicity, rinsed, and incubated in 0.5% Bovine Serum Albumin solution for 30 min to reduce non-specific binding of antibodies. Tissue sections were rinsed again and placed in a primary antibody cocktail for 40 h at 4 °C on a shaker table. Consecutive alternating sections through the medulla were processed for examination of colocalization of P2X3 and TRPV1 with either IB4- or CTb-labeled vagal afferents.

To examine channel content within IB4-labeled vagal afferents, tissue sections were incubated in an antibody cocktail consisting of a polyclonal goat antibody directed against Griffonia (Bandeiraea) simplicifolia lectin I (IB4) (1:1000; Vector Laboratories, Cat: AS-2104) along with a polyclonal rabbit antibody directed against P2X3 (1:7500; Neuromics, Cat: RA10109) and a polyclonal guinea pig antibody directed against the TRPV1 receptor (1:6000; Chemicon, Cat: AB5566). To examine channel content within CTb-labeled vagal afferents, alternating sections were incubated in the same cocktail with the exception of a polyclonal goat antibody directed against cholera toxin subunit B (1:25000; List Biological Laboratories, Cat: 703) instead of the IB4 antibody. The antibody directed against TRPV1 was preincubated in sections of naïve rat cerebellum for 2 h prior to utilization, to reduce nonspecific binding. Bound antibodies were visualized with donkey secondary antibodies conjugated to AlexaFluor 488, AlexaFluor 546, (1:800; Life Technologies) and Cy5 (1:800; Jackson ImmunoResearch). Unless otherwise noted, all incubations were carried out on a shaker table at room temperature. Sections were mounted onto gelatin-coated slides, coverslipped with Prolong™ Antifade Media (Life Technologies) and stored at −20 °C.

All antibodies used for this study were commercially available. The antibodies directed against the tract tracers IB4 (Vector, Cat: AS-2104) and CTb (List labs, Cat: 703), have been previously utilized by our lab, as well as others (Aicher et al., 2013; Aicher and Reis, 1997; Borges and Sidman, 1982; Hermes et al., 2014; Llewellyn-Smith et al., 1990; Stucky and Lewin, 1999). The antibodies directed against the channels P2X3 (Neuromics, Cat: RA10109) and TRPV1 (Chemicon AB5566) have also been utilized in a number of prior studies (Amadesi et al., 2009; Guo et al., 1999; Hegarty et al., 2014; Saeed and Ribeiro-da-Silva, 2012). Per the manufacturer's specifications, the rabbit polyclonal P2X3 antibody recognizes residues 383 to 397 (VEKQSTDSGAYSIGH) of the carboxy terminus of rat P2X3. Incubation of the P2X3 antibody with the corresponding blocking peptide (Neuromics, Cat: P10108) resulted in the abolishment of any observable immunoreactivity. The guinea pig polyclonal TRPV1 antibody recognizes a 22 amino acid peptide at the carboxy-terminus of the rat TRPV1 receptor protein.

2.4. Histochemical verification of immunocytochemical detection of IB4

In order to verify that our IB4 tract tracing method would detect similar populations to those found using histochemical methods we combined immunocytochemical detection of IB4 with histochemical detection. Briefly, the procedure for the immunocytochemical approach involved incubation of brainstem sections in the goat primary antibody directed against IB4 (1:1000, Vector Laboratories) for 40 h at 4 °C on a shaker table. After rinses, tissue sections were incubated with a donkey anti-goat secondary antibody conjugated to Alexa 546 (1:800; Life Technologies) for 2 h. Tissue sections were rinsed and then incubated in IB4 conjugated to biotin (5 μg/ml, Sigma-Aldrich) for 2 h per the standard histochemical approach. Sections were rinsed again followed by incubation in streptavidin conjugated to Alexa 488 (1:800; Life Technologies) for 4 h to allow for fluorescent visualization.

2.5. Confocal imaging

Only cases with tract tracer transport to NTS were included in analyses. Of the seven animals that received anterograde injections, three animals displayed successful vagal afferent labeling from both IB4 and CTb. Of the four remaining animals, half displayed successful transport of only IB4 ($n = 2$) and half displayed successful transport of only CTb ($n = 2$). No difference in the labeling pattern of either tracer of interest was apparent between rats that displayed successful transport of only a single tracer and those that displayed successful transport of both. The result was five animals with successful IB4 labeling and five animals with successful CTb labeling.

Z stacks bounded by the vertical extent (Z-axis) of the labeling within NTS were captured using the single pass, multi-tracking format on an LSM 510 confocal microscope (Zeiss, Thornwood, NY). A 488 nm laser (Argon/2), a 543 nm (HeNe1) laser, and a 633 nm laser (HeNe2) were used to excite AlexaFluor 488, AlexaFluor 546 (or NeuroTrace 530), and Cy5, respectively. Emitted wavelengths passed through an HFT UV/488/543/633 nm dichroic mirror and then the appropriate band pass filter sets; 500–550 nm for AlexaFluor 488, 565–615 nm for AlexaFluor 546, and 650–710 nm for Cy5 before collection. Confocal micrographs presented in figures were adjusted for optimal brightness and contrast utilizing the Zeiss ZEN software and Adobe Photoshop (CS5), and figures were created in Adobe Illustrator (CS5).

2.6. Quantification and analysis

To examine localization of TRPV1 or P2X3 in the putative synaptic terminals of vagal afferent fibers, we analyzed 25 distinct varicosities within the subpostremal NTS region in each animal. Varicosities were defined operationally as a discrete regions approximately twice the diameter of the fiber of origin containing the anterograde tracer and counted in each animal (see boxed area, Fig. 1). Varicosities were counted within a single vibratome section from each animal, thereby eliminating the possibility of duplicate counting of the same varicosities between sections. Each varicosity was located on a distinct fiber and was evaluated for colocalization by two independent observers. Five animals were included in the analyses concerning the localization of the P2X3 and TRPV1 channels in IB4- or CTb-ir vagal afferents, for a total of 125 varicosities for each tract tracer.

3. Results

3.1. IB4- and CTb-immunoreactive (ir) afferents in NTS

IB4 or CTb varicosities revealed distinct patterns of vagal afferent labeling within NTS (Fig. 1). IB4-ir fibers were most dense in the medial subnucleus located within subpostremal NTS and indicated that unmyelinated varicosities were quite concentrated in the area adjacent to the area postrema, as well as in the solitary tract ipsilateral to the injection (Fig. 1A). As we have shown previously (Hermes et al., 2014), CTb-ir vagal afferents were scattered throughout the subnuclei of subpostremal NTS, with the most dense localization in more lateral subregions (Fig. 1B). The overall distribution of CTb labeling was distinct from that of IB4 labeling within NTS with an overlapping distribution observed in the dorsomedial and medial subnuclei. In addition to the anterogradely labeled fibers in NTS, CTb was found prominently in cell bodies of vagal motor neurons within the dorsal motor nucleus of the vagus (DMV; Fig. 1B).

We used IB4 as a tract tracer by applying it directly to the left vagus nerve. This technique is distinct from many other studies that detect IB4 fibers using a histochemical method. In order to verify that our tract tracing method would detect similar populations to those found using histochemical methods; we combined immunocytochemical detection of IB4 with histochemical detection and found that both methods identified largely