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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.

Keywords

TRPV1; P2X3; isolectin B4; cholera toxin B subunit; vagus nerve; confocal microscopy

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;

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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). Thus, 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 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 hour 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 minutes 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 μ l of a 4% solution in deionized water, Sigma-Aldrich) and CTb (1 to 2 μ l 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 μ m tip size, attached to a picospritzer (General Valve Inc.). Following injections, the parafilm was removed,

surgical wounds were closed with 4-0 monocril 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 μ m) 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 hours 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 hours 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 (VEKQSTDGAYSIGH) 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 hours 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 hours. Tissue sections were rinsed and then incubated in IB4 conjugated to biotin (5 ug/mL, Sigma-Aldrich) for 2 hours per the standard histochemical approach. Sections were rinsed again followed by incubation in streptavidin conjugated to Alexa 488 (1:800; Life Technologies) for 4 hours 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 subnucleus. 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 identical populations of fibers (Fig. 2). The antibody method showed higher intensity staining on the side ipsilateral to the injection site, suggesting that the concentration of IB4 is greater in fibers that had exogenous application. The abundance of dual-labeled fibers suggests that we successfully detected a large portion of the vagal fibers with our methods. By using IB4 as a tract tracer, we are able to ensure that the IB4 fibers studied are of vagal origin, rather than from other C-fiber afferents, and this approach allows us to do valid comparisons with CTb fibers from the vagus nerve. Only samples with verified successful tract tracer labeling within NTS were further studied to examine colocalization of TRPV1 or P2X3 proteins in vagal afferent central varicosities.

3.2 TRPV1 and P2X3 in NTS

The patterns of TRPV1 and P2X3 channel immunoreactivity (ir) in the brainstem were somewhat distinct (Fig 3). Specifically, within NTS, TRPV1-ir was most densely located in varicosities in the area immediately adjacent to the area postrema (Fig. 3A), in a pattern very similar to that seen with IB4-ir vagal afferents (Fig 1A). In contrast, P2X3 was also observed within the medial and dorsomedial subnuclei of NTS, with additional immunoreactivity observed within the solitary tract (Fig 3B). In order to examine the distribution of these molecules in specific populations of vagal afferents we selected regions of NTS that contained labeling of the fiber type of interest but also ensured that there was abundant detection of the channel of interest in the same area to avoid false negative

conclusions. Therefore, dual-labeling analyses were conducted in subpostremal dorsomedial NTS (see boxes Fig.1)

3.3 TRPV1 commonly in IB4, not CTb, varicosities

Confocal images show that IB4 fibers were fine caliber and have small varicosities while CTb-ir vagal afferents are larger in diameter and contain larger varicosities (Figs. 4 and 5). IB4- or CTb-ir varicosities within the dorsomedial subnucleus of NTS were selected for analysis as described above (See 2.6 *Quantification and Analysis*) and observers noted whether they contained TRPV1 or P2X3 channel immunoreactivity. The proportion of varicosities that contained the channel of interest were scored independently for each tracer. Thus, percentages are expressed as a percent of each fiber type identified by the tract tracer utilized, IB4 (n=5 animals; 125 varicosities) or CTb (n=5 animals; 125 varicosities). We found that TRPV1 was detected in 43% of the IB4-ir varicosities (Fig. 4). In contrast, CTb-ir vagal afferent fibers rarely (2%) contained TRPV1. These results suggest that TRPV1 is selectively localized to unmyelinated, IB4-ir, vagal afferent terminals in NTS.

3.4 P2X3 found in both IB4 and CTb varicosities

P2X3 staining was found in some CTb-ir varicosities (23%; Fig. 5), as well as in 18% of IB4-ir varicosities (Fig. 5). Previously we found that CTb was mostly associated with myelinated afferents, but the tracer was also detected in some unmyelinated afferents that were distinct from IB4 stained afferents (Hermes *et al.*, 2014). The present findings suggest that the P2X3 distribution within vagal afferents is not strictly related to fiber type.

3.5 Co-localization of TRPV1 and P2X3 in vagal afferents

We also tested for co-localization of TRPV1 and P2X3 in IB4- or CTb-ir vagal afferents, respectively. This summary is limited to varicosities that contained at least one of the channels (TRPV1 or P2X3) and we assessed whether these varicosities also contained the second channel (Fig. 6). As mentioned above, 23% of CTb-ir vagal afferents contained P2X3 and only 2% of CTb fibers contain TRPV1 (Fig. 6). When we tested for co-localization, only 1 of 125 CTb varicosities (1%) contained both TRPV1 and P2X3 (Fig. 6). For IB4-ir vagal afferents, the localization was primarily TRPV1 as mentioned above, with 43% of IB4 fibers containing TRPV1 and 18% containing P2X3. When we examined for co-localization we found that 10% of the IB4 varicosities (12 of 125) contained both P2X3 and TRPV1. These findings suggest that the TRPV1 channel is strongly associated with IB4-ir, presumably unmyelinated fibers, while the P2X3 channel was found in both types of fibers (Fig. 6). It is noteworthy that about half of the IB4 varicosities contained either TRPV1, P2X3, or both (Fig. 6; 65 of 125) while the majority of the CTb varicosities did not contain either TRPV1 or P2X3 (94 of 125 of CTb varicosities did not contain either channel). These results confirm the pharmacological finding that most myelinated vagal afferents lack P2X3 receptors (Jin *et al.*, 2004b).

4. DISCUSSION

Our study used two anterogradely transported tract tracers injected into the vagus nerve to preferentially label primary afferent sensory neurons with myelinated or unmyelinated

axons. The central terminal varicosities in NTS were then examined to detect two key presynaptic ion channels, TRPV1 and P2X3. We found that TRPV1 was primarily associated with IB4-labeled vagal varicosities – the unmyelinated marker, while P2X3 was primarily associated with CTb-ir vagal afferents. Thus, the main findings are consistent with pharmacological synaptic function studies using ligands for TRPV1 and P2X3 (Jin *et al.*, 2004b). However, some results were not consistent with prior pharmacological studies, including localization of TRPV1 in CTb afferents, P2X3 detected in IB4 afferents and co-localization of TRPV1 and P2X3 in the same varicosities. Each of these observations will be discussed.

TRPV1 in IB4-ir vagal afferents

Our finding that TRPV1 is primarily localized to IB4-ir vagal afferents is consistent with prior electrophysiological studies showing that TRPV1 modulates C-fiber activation (Jin *et al.*, 2004b). We previously showed by electron microscopy that IB4 is only detected in unmyelinated axons following vagal nerve applications (Hermes *et al.*, 2014), thus this tract tracer is effective at detecting C-fiber afferents. TRPV1 in peripheral nociceptors requires high (42°C) temperatures to reach threshold for activation and elicit perceptions of pain (Caterina *et al.*, 1999). TRPV1 is also differentially expressed in cutaneous versus visceral nociceptors (Hwang *et al.*, 2005) that project to the spinal cord. Although TRPV1 has been detected in the nodose ganglion cells and their afferent terminal fields in NTS, vagal afferent activation is not perceived as pain and indeed often produces anti-nociception (Randich and Aicher, 1988). In the relatively protected locales of these interoceptors, the role of TRPV1 in these central terminals is unlikely to be nociception. Our studies show that normal physiological temperatures (near 37°C) drive TRPV1 activation to cause spontaneous release of glutamate from cranial afferent terminals (Andresen *et al.*, 2012; Largent-Milnes *et al.*, 2014; Shoudai *et al.*, 2010). This action-potential autonomous TRPV1 activity and glutamate release may help to maintain synapse integrity in normal conditions of silent vagal afferents (Andresen *et al.*, 2012). Thus, although some authors refer to vagal afferents as nociceptors because they respond to stimuli considered noxious (Yu *et al.*, 2005), the overwhelming consensus in the literature holds that nociceptors are activated by tissue-damaging stimuli and evoke pain as well as aversive responses. Visceral nociception is mediated via spinal, not vagal afferents (Cervero, 1994; Cervero and Laird, 1999). In fact, vagal afferent stimulation produces analgesia, not pain (Aicher *et al.*, 1991; Bossut and Maixner, 1996). Thus, the role of TRPV1 in vagal afferents is likely to be homeostatic (Craig, 2002).

P2X3 in CTb-ir vagal afferents

Our observation that P2X3 is primarily found in CTb-ir vagal afferents is also consistent with prior electrophysiological studies. We have shown that CTb-ir is found in myelinated axons in the solitary tract following vagal nerve application (Hermes *et al.*, 2014) which would correspond to A-fibers in electrophysiological studies. Purinergic receptors are activated by ATP (Jahr and Jessell, 1983) and the P2X3 subtype is found most often in sensory neurons (Chen *et al.*, 1995). Purinergic receptors are strongly implicated in various enteric and autonomic reflexes (Burnstock, 2015). P2X3 receptors have been localized in

myelinated axons and axon terminals in the NTS and (Llewellyn-Smith and Burnstock, 1998) – findings consistent with our localization of P2X3 to CTb-ir vagal afferents.

TRPV1 in IB4-ir and P2X3 in CTb-ir vagal afferents

While our findings were generally consistent with pharmacological and electrophysiological studies suggesting segregation of TRPV1 to C-fibers (unmyelinated) and P2X3 to A-fibers (myelinated) (Jin *et al.*, 2004b), some of the current findings are not consistent with the strict segregation of TRPV1 and P2X3 into different afferent populations. Prior studies restricted to spontaneous transmission to neurons in the centralis subregion of NTS (Browning *et al.*, 2011) found more mixed results with pharmacological tests of spontaneous EPSC rates. These studies found that sensitivity to capsaicin was often accompanied by responsiveness to purinergic agonists – indicating that mixed populations of afferents activated sEPSCs in cNTS neurons, but these studies did not address whether TRPV1 and P2X3 are found together on single vagal afferents since the experiments were not conducted in the presence of TTX. Our evidence of some vagal terminals with both TRPV1 and P2X3 may be influenced by the possible lack of selectivity of the anterograde tracers. While CTb is primarily detected in myelinated axons, we and others have also detected CTb in some unmyelinated axons (Corbett *et al.*, 2005; Hermes *et al.*, 2014) and this may help account for some of the mixed results in the present studies. If a subset of our CTb-ir afferents are indeed C-fibers, this could explain why we found TRPV1 in a subset of these axons.

In the current studies, TRPV1 co-localized with P2X3 in a small subset of varicosities. The functional assays of synaptic glutamate transmission (Excitatory Postsynaptic Currents, EPSCs) and conduction velocity clearly separated TRPV1 effects on unmyelinated afferents from P2X3 effects on rapidly conducting myelinated afferents (Jin *et al.*, 2004b; Li and Schild, 2007). A number of factors and interpretations need to be considered. TRPV1 and P2X3 clearly have additional actions in afferent neurons beyond synaptic transmission for example (Benson *et al.*, 1999; McIlwrath *et al.*, 2009). Spatially, synaptic actions likely depend on the close proximity of the receptor to the release site within terminals to alter synaptic transmission (Fawley *et al.*, 2014) and thus some immunohistochemically detected sites may not functionally engage with the synaptic release of glutamate in vagal endings. This interpretation would suggest that co-localization of TRPV1 and P2X3 in the same axon terminal may not have detectable physiological consequences on assays of EPSCs, but could have other undetermined functions.

Another potential explanation of the disparities between our current anatomical studies and prior electrophysiological studies may be related to the strengths and limitations of each method. The functional assays of synaptic glutamate transmission (Excitatory Postsynaptic Currents, EPSCs) and conduction velocity clearly separated TRPV1 effects to unmyelinated neurons from P2X3 effects on rapidly conducting myelinated afferents (Jin *et al.*, 2004b; Li and Schild, 2007). Pharmacological evidence suggests that P2X3 activation triggers EPSCs from a subset of neurons that lack TRPV1 responses and corresponded to rapidly conducting vagal A-fibers (Jin *et al.*, 2004b). In contrast, no TRPV1-sensitive vagal afferent responded to P2X3 selective agents (Li and Schild, 2007). The less restricted association of P2X3 with both CTb-ir as well as IB4-ir varicosities in the present study warrants consideration of the

nature of the two approaches – one focused on synaptic transmission and the other on localization of markers in anatomical structures. The differing assays of the two studies – measures of glutamate release as EPSCs versus anatomical localization – may supply quite different, but complementary information. The pharmacological assays focused on release from sites which functionally expressed TRPV1 or P2X3, discriminated through antagonist compounds. This focus on synaptic function misses other potential functions that could be detected by other means. Likewise, our anatomical labeling studies focused on varicosities which are suspected as vagal afferent synaptic release sites (Andresen and Peters, 2008; Jin *et al.*, 2010). The presence of substantial proportions of varicosities without staining for either TRPV1 or P2X3 may reflect incomplete detection, but raises the possibility of non-releasing varicosities or alternative functions that are unrelated to the myelinated/unmyelinated phenotypic differences. In addition, by limiting our studies to varicosities, sites within vagal axons were not measured and these might be functionally important as well. We found P2X3 in about 20% of CTb fibers and we would have predicted more prevalent co-localization based on the more ubiquitous electrophysiological findings of A-fiber modulation. This could be due to the selectivity of the antibody which detects only a subset of purinergic receptors, since these receptors form complex heteromeric channels, or it could be due to limited access of the antibody to receptor sites within varicosities.

Our labeling studies suggested that P2X3 is found in IB4-ir fibers – a finding not consistent with pharmacological assessments at C-fiber terminals and nodose neurons (Jin *et al.*, 2004b). There are several possible explanations for these disparities. First, we only found P2X3 in a small population (18%) of IB4 fibers, thus it is possible that the afferent fibers containing P2X3 in our anatomical studies were not represented in the C-fiber populations studied in the prior electrophysiological studies. Previous studies have reported a similar proportion of nodose ganglion cells contain P2X3 in the rat (Wang *et al.*, 2014), although much higher proportions have been reported in humans (Sato *et al.*, 2014). The nodose ganglion is variably successful as a proxy for central terminals, since ion channels or receptors can be differentially trafficked to central and peripheral regions of the sensory neuron after synthesis in the ganglion. For example, calcium channels show currents in nodose cell bodies that are consistent with their roles in glutamate transmission (Mendelowitz *et al.*, 1995), but very different pharmacological profiles at their sensory endings (Kunze *et al.*, 1986). A second possible explanation may be that the immunocytochemistry identifies additional P2X3 receptors within IB4 fibers that are not coupled to synaptic release (e.g. different subcellular micro-domains within presynaptic terminals). In our confocal studies, we are only able to demonstrate that P2X3 is within the IB4 or CTb varicosity. Based on prior studies, we find that axonal varicosities correspond to synapses by electron microscopy (Bailey *et al.*, 2006), yet additional domains of localization outside the axon terminals may modify axonal conduction but not synaptic release at terminals (Hofmann *et al.*, 2014). Presynaptic receptors may be involved in distinct mechanisms that could be functionally segregated within the synaptic terminal. For example, our recent studies indicate that CB1 ligands modulate synaptically evoked glutamate release without altering spontaneous glutamate release or TRPV1 modulated glutamate release. These findings suggest that glutamate release sites on presynaptic terminals converging onto the same postsynaptic cell may involve distinct pools of synaptic vesicles (Fawley *et al.*,

2014). Thus, it is possible that P2X3 receptors may be anatomically localized to IB4 axons yet have no functional consequences for TRPV1-mediated glutamate release at these synapses. The demonstration of differentially regulated synaptic pools within vagal afferent terminals awaits further studies.

CONCLUSIONS

Our tract tracing combined with immunocytochemistry approach to the study of central vagal afferent varicosities has verified the relationship between IB4-ir fibers and TRPV1 channels, as well as the association of P2X3 with CTb-ir fibers. The additional presence of P2X3 receptors in IB4 fibers suggests presynaptic channels that may alter non-synaptic functions which have not been characterized. We found that TRPV1 was primarily detected in unmyelinated vagal afferents identified by IB4 tract tracing, supporting the hypothesis that this channel modifies synaptic transmission presynaptically for this subset of inputs to NTS. In contrast, P2X3 was found in myelinated and unmyelinated axons and thus it did not have good predictive value based on fiber type for the localization of this receptor within vagal afferent terminals.

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REFERENCES

- Aicher SA, Hermes SM, Hegarty DM. Corneal afferents differentially target thalamic- and parabrachial-projecting neurons in spinal trigeminal nucleus caudalis. *Neuroscience*. 2013; 232:182–193. [PubMed: 23201828]
- Aicher SA, Lewis SJ, Randich A. Antinociception produced by electrical stimulation of vagal afferents: Independence of cervical and subdiaphragmatic branches. *Brain Res*. 1991; 542:63–70. [PubMed: 2054659]
- Aicher SA, Reis DJ. Gigantocellular vasodepressor area is tonically active and distinct from caudal ventrolateral vasodepressor area. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 1997; 272:R731–R742.
- Amadesi S, Grant AD, Cottrell GS, Vaksman N, Poole DP, Rozengurt E, Bunnett NW. Protein kinase D isoforms are expressed in rat and mouse primary sensory neurons and are activated by agonists of protease-activated receptor 2. *J Comp Neurol*. 2009; 516:141–156. [PubMed: 19575452]
- Andresen MC, Hofmann ME, Fawley JA. The unsilent majority-TRPV1 drives “spontaneous” transmission of unmyelinated primary afferents within cardiorespiratory NTS. *American journal of physiology. Regulatory, integrative and comparative physiology*. 2012; 303:R1207–1216.
- Andresen MC, Peters JH. Comparison of baroreceptive to other afferent synaptic transmission to the medial solitary tract nucleus. *American journal of physiology. Heart and circulatory physiology*. 2008; 295:H2032–2042. [PubMed: 18790834]
- Andresen MC, Yang MY. Non-NMDA receptors mediate sensory afferent synaptic transmission in medial nucleus tractus solitarius. *American Journal of Physiology: Heart and Circulatory Physiology*. 1990; 90:H1307–H1311.
- Bailey TW, Hermes SM, Andresen MC, Aicher SA. Cranial visceral afferent pathways through the nucleus of the solitary tract to caudal ventrolateral medulla or paraventricular hypothalamus: target-specific synaptic reliability and convergence patterns. *Journal of Neuroscience*. 2006; 26:11893–11902. [PubMed: 17108163]
- Benson CJ, Eckert SP, McCleskey EW. Acid-evoked currents in cardiac sensory neurons: A possible mediator of myocardial ischemic sensation. *Circ Res*. 1999; 84:921–928. [PubMed: 10222339]

- Borges LF, Sidman RL. Axonal transport of lectins in the peripheral nervous system. *Journal of Neuroscience*. 1982; 2:647–653. [PubMed: 6176698]
- Bossut DF, Maixner W. Effects of cardiac vagal afferent electrostimulation on the responses of trigeminal and trigeminothalamic neurons to noxious orofacial stimulation. *Pain*. 1996; 65:101–109. [PubMed: 8826496]
- Browning KN, Wan S, Baptista V, Travagli RA. Vanilloid, purinergic, and CCK receptors activate glutamate release on single neurons of the nucleus tractus solitarius centralis. *American journal of physiology. Regulatory, integrative and comparative physiology*. 2011; 301:R394–401.
- Burnstock G. Purinergic signalling and the autonomic nervous system in health and disease. *Autonomic neuroscience : basic & clinical*. 2015
- Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D. A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature*. 1999; 398:436–441. [PubMed: 10201375]
- Cervero F. Sensory innervation of the viscera: Peripheral basis of visceral pain. *Physiological Reviews*. 1994; 74:95–138. [PubMed: 8295936]
- Cervero F, Laird JM. Understanding the signaling and transmission of visceral nociceptive events. *J Neurobiol*. 2004; 61:45–54. [PubMed: 15362152]
- Cervero F, Laird JMA. Visceral pain. *Lancet*. 1999; 353:2145–2148. [PubMed: 10382712]
- Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN. A P2X purinoceptor expressed by a subset of sensory neurons. *Nature*. 1995; 377:428–431. [PubMed: 7566119]
- Corbett EK, Sinfield JK, McWilliam PN, Deuchars J, Batten TF. Differential expression of vesicular glutamate transporters by vagal afferent terminals in rat nucleus of the solitary tract: projections from the heart preferentially express vesicular glutamate transporter 1. *Neuroscience*. 2005; 135:133–145. [PubMed: 16084661]
- Craig AD. How do you feel? Interoception: the sense of the physiological condition of the body. *Nat.Rev.Neurosci*. 2002; 3:655–666. [PubMed: 12154366]
- Fawley JA, Hofmann ME, Andresen MC. Cannabinoid 1 and transient receptor potential vanilloid 1 receptors discretely modulate evoked glutamate separately from spontaneous glutamate transmission. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2014; 34:8324–8332. [PubMed: 24920635]
- Fawley JA, Peters JH, Andresen MC. GABA(B)-mediated inhibition of multiple modes of glutamate release in the nucleus of the solitary tract. *Journal of Neurophysiology*. 2011; 106:1833–1840. [PubMed: 21734101]
- Guo A, Vulchanova L, Wang J, Li X, Elde R. Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X 3 purinoceptor and IB4 binding sites. *European Journal of Neuroscience*. 1999; 11:946–958. [PubMed: 10103088]
- Hegarty DM, Hermes SM, Largent-Milnes TM, Aicher SA. Capsaicin-responsive corneal afferents do not contain TRPV1 at their central terminals in trigeminal nucleus caudalis in rats. *J Chem Neuroanat*. 2014; 61-62C:1–12. [PubMed: 24996127]
- Hermes SM, Colbert JF, Aicher SA. Differential content of vesicular glutamate transporters in subsets of vagal afferents projecting to the nucleus tractus solitarii in the rat. *J Comp Neurol*. 2014; 522:642–653. [PubMed: 23897509]
- Hofmann ME, Largent-Milnes TM, Fawley JA, Andresen MC. External QX-314 inhibits evoked cranial primary afferent synaptic transmission independent of TRPV1. *J Neurophysiol*. 2014
- Hwang SJ, Oh JM, Valtchanoff JG. Expression of the vanilloid receptor TRPV1 in rat dorsal root ganglion neurons supports different roles of the receptor in visceral and cutaneous afferents. *Brain Res*. 2005; 1047:261–266. [PubMed: 15896726]
- Jahr CE, Jessell TM. ATP excites a subpopulation of rat dorsal horn neurones. *Nature*. 1983; 304:730–733. [PubMed: 6888539]
- Jin YH, Bailey TW, Li BY, Schild JH, Andresen MC. Purinergic and vanilloid receptor activation releases glutamate from separate cranial afferent terminals in nucleus tractus solitarius. *Journal of Neuroscience*. 2004a; 24:4709–4717. [PubMed: 15152030]
- Jin YH, Bailey TW, Li BY, Schild JH, Andresen MC. Purinergic and vanilloid receptor activation releases glutamate from separate cranial afferent terminals in nucleus tractus solitarius. *The*

Journal of neuroscience : the official journal of the Society for Neuroscience. 2004b; 24:4709–4717. [PubMed: 15152030]

- Jin YH, Cahill EA, Fernandes LG, Wang X, Chen W, Smith SM, Andresen MC. Optical tracking of phenotypically diverse individual synapses on solitary tract nucleus neurons. *Brain Res.* 2010; 1312:54–66. [PubMed: 19944080]
- Kalia M, Sullivan JM. Brainstem projections of sensory and motor components of the vagus nerve in the rat. *Journal of Comparative Neurology.* 1982; 211:248–264. [PubMed: 7174893]
- Kunze DL, Andresen MC, Torres LA. Do calcium antagonists act directly on calcium channels to alter baroreceptor function? *J Pharmacol Exp Ther.* 1986; 239:303–310. [PubMed: 2430091]
- Largent-Milnes TM, Hegarty DM, Aicher SA, Andresen MC. Physiological temperatures drive glutamate release onto trigeminal superficial dorsal horn neurons. *J Neurophysiol.* 2014; 111:2222–2231. [PubMed: 24598529]
- Li BY, Schild JH. Electrophysiological and pharmacological validation of vagal afferent fiber type of neurons enzymatically isolated from rat nodose ganglia. *J Neurosci Methods.* 2007; 164:75–85. [PubMed: 17512602]
- Llewellyn-Smith IJ, Burnstock G. Ultrastructural localization of P2X 3 receptors in rat sensory neurons. *NeuroReport.* 1998; 9:2545–2550. [PubMed: 9721930]
- Llewellyn-Smith IJ, Minson JB, Wright AP, Hodgson AJ. Cholera toxin B - gold, a retrograde tracer that can be used in light and electron microscopic immunocytochemical studies. *Journal of Comparative Neurology.* 1990; 294:179–191. [PubMed: 1692043]
- Loewy AD, Burton H. Nuclei of the solitary tract: Efferent projections to the lower brainstem and spinal cord of the cat. *Journal of Comparative Neurology.* 1978; 181:421–450. [PubMed: 690272]
- McIlwrath SL, Davis BM, Bielefeldt K. Deletion of P2X3 receptors blunts gastro-oesophageal sensation in mice. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society.* 2009; 21:890–e866. [PubMed: 19368663]
- Mendelowitz D, Reynolds PJ, Andresen MC. Heterogeneous functional expression of calcium channels at sensory and synaptic regions in nodose neurons. *Journal of Neurophysiology.* 1995; 73:872–875. [PubMed: 7760142]
- Paintal AS. Vagal sensory receptors and their reflex effects. *Physiological Reviews.* 1973; 53:159–227. [PubMed: 4568412]
- Peters JH, McDougall SJ, Fawley JA, Smith SM, Andresen MC. Primary afferent activation of thermosensitive TRPV1 triggers asynchronous glutamate release at central neurons. *Neuron.* 2010; 65:657–669. [PubMed: 20223201]
- Randich A, Aicher SA. Medullary substrates mediating antinociception produced by electrical stimulation of the vagus. *Brain Res.* 1988; 445:68–76. [PubMed: 3365559]
- Saeed AW, Ribeiro-da-Silva A. Non-peptidergic primary afferents are presynaptic to neurokinin-1 receptor immunoreactive lamina I projection neurons in rat spinal cord. *Molecular pain.* 2012; 8:64. [PubMed: 22963197]
- Sato D, Sato T, Urata Y, Okajima T, Kawamura S, Kurita M, Takahashi K, Nanno M, Watahiki A, Kokubun S, Shimizu Y, Kasahara E, Shoji N, Sasano T, Ichikawa H. Distribution of TRPVs, P2X3, and parvalbumin in the human nodose ganglion. *Cellular and molecular neurobiology.* 2014; 34:851–858. [PubMed: 24764033]
- Schild JH, Clark JW, Hay M, Mendelowitz D, Andresen MC, Kunze DL. A- and C-type rat nodose sensory neurons: model interpretations of dynamic discharge characteristics. *Journal of Neurophysiology.* 1994; 71:2338–2358. [PubMed: 7523613]
- Shoudai K, Peters JH, McDougall SJ, Fawley JA, Andresen MC. Thermally active TRPV1 tonically drives central spontaneous glutamate release. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2010; 30:14470–14475. [PubMed: 20980604]
- Stucky CL, Lewin GR. Isolectin B 4 -positive and -negative nociceptors are functionally distinct. *Journal of Neuroscience.* 1999; 19:6497–6505. [PubMed: 10414978]
- Talman WT, Perrone MH, Reis DJ. Evidence for L-glutamate as the neurotransmitter of primary baroreceptor afferent nerve fibers. *Science.* 1980; 209:813–815. [PubMed: 6105709]
- Wang L, Feng D, Yan H, Wang Z, Pei L. Comparative analysis of P2X1, P2X2, P2X3, and P2X4 receptor subunits in rat nodose ganglion neurons. *PloS one.* 2014; 9:e96699. [PubMed: 24798490]

Yu S, Undem BJ, Kollarik M. Vagal afferent nerves with nociceptive properties in guinea-pig oesophagus. *J Physiol.* 2005; 563:831–842. [PubMed: 15649987]

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Highlights

- Cholera toxin B (CTb) and Isolectin B4 (IB4) identify distinct groups of vagal afferents
- TRPV1 is localized in IB4 vagal afferents
- P2X3 is localized in both CTb and IB4 vagal afferents

HIGHLIGHTS

- IB4-ir, unmyelinated vagal afferents contain TRPV1.
- CTb-ir vagal afferents often contain P2X3.
- TRPV1 and P2X3 are rarely co-localized in vagal afferent varicosities.

Figure 1.

Injections of the tract tracers IB4 (**Panel A**) or CTb (**Panel B**) into the cervical vagus nerve resulted in abundant ipsilateral afferent labeling within NTS. **A.** IB4-ir vagal afferents were most densely observed in the subpostremal region, dorsal to Area Postrema (AP) in the ipsilateral medial (SolM) and dorsomedial subnuclei (SolDM) of the NTS as well as within the solitary tract (st). **B.** CTb-ir vagal afferents were also observed in the medial and dorsomedial subnuclei of NTS as well as the ventrolateral subnucleus (SolVL). Retrograde CTb immunoreactivity was also observed in the dorsal motor nucleus of the vagus (DMV). cc = central canal, GR = Gracile nucleus. Scale bar for both panels = 200 μ m. Dashed boxes in both panels indicate area sampled with confocal microscopy.

Figure 2.

Most Isolectin B4 positive afferents within NTS originate from the vagus nerve. Epifluorescent micrographs of NTS demonstrate that IB4 identified using histochemical methods (**Panel A**) is largely coincident with IB4 injected into the vagus nerve and identified with an antibody (**Panel B**). Higher magnification confocal micrographs of the histochemical method (**Panel C**) and antibody detection method (**Panel D**) illustrate a very large degree of overlap, seen as white (arrowheads) in the overlay (**Panel E**). The few fibers not detected with the antibody method indicate either potentially incomplete tract tracer labeling of all afferent fibers, or the possibility that some IB4 fibers in NTS arise from either the other vagus nerve or other afferent inputs. AP = area postrema, st = solitary tract, cc = central canal. Scale for panels A and B = 200 μ m. Panels C-E are a single optical section, 0.5 μ m thick; scale bar = 20 μ m.

Figure 3.

TRPV1 (**Panel A**) and P2X3 (**Panel B**) both display immunoreactivity within NTS. Preadsorption with the blocking peptide for P2X3 abolishes immunoreactivity for P2X3 (**Panel C**). TRPV1 immunoreactivity was most densely located in the medial (SolM) and dorsomedial subnuclei (SolDM) of NTS, adjacent to area postrema (AP), in a pattern that was very similar to that seen for IB4-ir vagal afferents (Fig. 1A). P2X3 immunoreactivity was also observed within SolM and SolDM as well as within the solitary tract (st). cc = central canal, Gr = Gracile nucleus. Scale bar for all panels = 200 μ m.

Figure 4.

TRPV1 immunoreactivity was often observed in IB4-ir vagal afferents but not in CTb-ir vagal afferents. Within subpostremal NTS IB4-ir vagal afferent varicosities (Magenta, **Panel A**) often contained TRPV1 immunoreactivity (Cyan, **Panel B**), indicated in **Panel C** by the white arrowheads. In contrast, CTb-ir (Magenta, **Panel D**) vagal afferent varicosities (open arrowheads) almost never contained TRPV1 immunoreactivity (Cyan, **Panel E**), indicated by the lack of colocalization seen in the overlay (**Panel F**). Images are confocal projections 1.0 μm thick comprised of 3 optical sections each. Scale bars for A-C and D-F = 10 μm .

Figure 5.

P2X3 immunoreactivity was found in both IB4-ir and CTb-ir vagal afferents. Approximately 18% of IB4-ir vagal afferent varicosities (Magenta, **Panel A**) contained P2X3 immunoreactivity (Green, **Panel B**), indicated in **Panel C** by white arrowheads. Similarly, 23% of CTb-ir vagal afferent varicosities (Magenta, **Panel D**) displayed colocalization with P2X3 immunoreactivity (Green, **Panel E**) also indicated by white arrowheads in **Panel F**. Black arrowheads indicate tracer-identified varicosities that did not contain P2X3. Images are confocal projections 1.0 μm thick comprised of 3 optical sections each. Scale bars for A-C and D-F = 10 μm .

Figure 6.

TRPV1 immunoreactivity was observed in close to half of IB4-labeled varicosities (43%), but only very rarely in CTb-labeled varicosities (2%). P2X3 immunoreactivity was detected in similar proportions of IB4- (18%) and CTb- (23%) labeled varicosities, but was not found in the majority of either type of labeled vagal afferent.