TRPM8 Function and Expression in Vagal Sensory Neurons and Afferent Nerves
Innervating Guinea Pig Esophagus

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Running Head: TRPM8 in esophageal vagal afferents

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ABSTRACT
Sensory transduction in esophageal afferent requires specific ion channels and receptors. TRPM8 is a new member of TRP channel family and participates in cold and menthol-induced sensory transduction, but its role in visceral sensory transduction is still less clear. This study aims to determine TRPM8 function and expression in esophageal vagal afferent subtypes. TRPM8 agonist WS-12-induced responses were first determined in nodose and jugular neurons by calcium imaging and then investigated by whole-cell patch clamp recordings in Dil-labeled esophageal nodose and jugular neurons. Extracellular single-unit recordings were performed in nodose and jugular C fiber neurons using ex vivo esophageal-vagal preparations with intact nerve endings in the esophagus. TRPM8 mRNA expression was determined by single neuron RT-PCR in Dil-labeled esophageal nodose and jugular neurons. TRPM8 agonist WS-12 elicited calcium influx in a sub-population of jugular but not nodose neurons. WS-12 activated outwardly rectifying currents in esophageal Dil-labeled jugular but not nodose neurons in a dose-dependent manner, which could be inhibited by TRPM8 inhibitor AMTB. WS-12 selectively evoked action potential discharges in esophageal jugular but not nodose C fibers. Consistently, TRPM8 transcripts were highly expressed in esophageal Dil-labeled TRPV1-positive jugular neurons. In summary, the present study demonstrated a preferential expression and function of TRPM8 in esophageal vagal jugular but not nodose neurons and C fiber subtypes. This provides a distinctive role of TRPM8 in esophageal sensory transduction and may lead to a better understanding of the mechanisms of esophageal sensation and nociception.
INTRODUCTION

Esophageal sensory transduction is initiated by the stimuli in the primary sensory afferent nerve endings in the wall of the esophagus and transmitted through both spinal and vagal pathways to the central nervous system. Those subtypes of sensory nerves, which are able to discriminate noxious and innoxious stimuli, are defined as nociceptors. In the esophagus, noxious mechanical (distension), chemical (acid reflux), and thermal (hot or cold) stimuli all are able to activate esophageal nociceptors and induce painful sensation such as heartburn or esophageal-related chest pain (17). At present, the cellular and molecular mechanisms underlying esophageal sensation and nociception are still less clear.

Vagal afferents in the esophageal not only participate in maintaining esophageal physiological functions but also play the important role in sensing potential tissue damage from noxious stimuli (4, 5). They are derived from two distinct embryonic tissues: cranial placodes and neural crest. Placode-derived sensory nerves innervating the esophagus have their neuronal cell bodies in nodose ganglion while neurocrest-derived sensory neurons are located in jugular (supranodose) ganglion. These two vagal ganglia have different neurotrophin regulation during development and play distinctive roles in esophageal sensory transduction (1, 7, 21). Our previous studies have characterized three subtypes of esophageal vagal afferent nerve fibers in guinea pig and demonstrated that in addition to the classical low threshold mechanosensitive vagal nodose Aδ-fibers (“tension receptors”), there are at least two high threshold nociceptive vagal afferent subtypes in the esophagus, namely vagal nodose C fibers and jugular C fibers (25).

These two vagal C fiber subtypes are able to discriminate noxious esophageal distension and respond strongly to nociceptor-selective stimuli such as agonists of TRPV1 and TRPA1 (3, 9, 23, 25). Like TRPV1 and TRPA1, transient receptor potential melastatin 8 (TRPM8) is a nonselective cationic channel of transient receptor potential (TRP) superfamily (10). TRPM8 is expressed selectively in a subpopulation of primary sensory
neurons and can be activated by cold temperature (6, 15). In addition, TRPM8 is
activated by chemicals that provide a sensation of cooling such as menthol and icilin (2, 18, 22). Clinical observations indicate that both cold and menthol can induce abnormal esophageal sensations. Drinking cold water, a stimulus for TRPM8 (and maybe TRPA1) can trigger esophageal-related chest pain, which is unlikely involving esophageal smooth muscle spasm (16). Menthol (and peppermint oil) usually worsens heartburn symptom but it also has been added in many antacid formulations to soothe heartburn. These suggest that cold and menthol might regulate esophageal afferent function via TRPM8 and possibly affect esophageal sensory transduction and nociception. TRPM8 expression and function have been well defined in dorsal root ganglion (DRG) and trigeminal ganglion (TG)(8,13, 27), but relatively little is known about the involvement of TRPM8 in the function of vagal sensory neurons. Moreover, virtually nothing is known about the role of TRPM8 in esophageal sensory transduction.

TRPM8 expression in vagal sensory neuron was first determined by an elegant study using single cell RT-PCR in Dil retrograde labeled rat vagal nodose neurons from the upper gut (26). Using the same approach, another group also detected TRPM8 transcript in rat nodose neurons and its expression was largely affected by tissue disruption (28). In mice and rats the nodose and jugular neurons often fuse into a single ganglion making it difficult to differential between the placodal vs neural crest derived nociceptors. In guinea pigs the vagal nodose and jugular ganglia are easily identified as separate ganglia. In the present study, we took advantage of such distinctive anatomic and physiological features in guinea pig vagal nodose and jugular ganglia to evaluate and characterize the relative contribution of TRPM8 in regulating the activity of nodose vs jugular C fibers neurons. Our results lead to the conclusion that in healthy animals, TRPM8 plays little role in nodose C fibers innervating the esophagus, but may play a major role in sensory transduction in jugular C fibers.
METHODS

Male Hartley guinea pigs (150-200g) were purchased from Hilltop Laboratory Animals (Scottsdale, PA). All experiments were approved by the Johns Hopkins University Animal Care and Use Committee.

Calcium imaging in dissociated nodose and jugular neurons

Nodose and jugular neurons were prepared as described previously (9). Briefly, nodose and jugular ganglia from guinea pigs were first dissected and collected in an ice-cold Kreb’s bicarbonate solution (118 mM NaCl, 1.0 mM NaH$_2$PO$_4$, 25.0 mM NaHCO$_3$, 5.4 mM KCl, 1.9 mM CaCl$_2$, 1.2 mM MgSO$_4$ and 11.1 mM Dextrose, pH 7.4, and gassed with 95% O$_2$ and 5% CO$_2$) and then treated with enzyme (2 mg/mL collagenase and 2 mg/mL dispase in Ca$^{2+}$/Mg$^{2+}$ free HBSS buffer) at 37°C for 2 hours. During the incubation, neurons were dissociated by mild trituration and then harvested by centrifugation. After re-suspending in fresh L15 media, these neurons were transferred onto coverslips (Warner Instrument) pre-coated with poly-D-lysine (0.1 mg/mL) and laminin (5 µg/mL), allowed adhere for 2 hours in an incubator at 37°C. Then, the cells were washed with fresh media and cultured in fresh L15 media overnight in the incubator at 37°C and used within 24 hours.

Calcium imaging studies were performed as described previously (9). Briefly, cultured vagal sensory neurons were loaded with 2 mM Fura-2-AM and 0.05% Pluronic F-127 dissolved in normal extracellular solution (ECS in mM: 140 NaCl, 5 KCl, 2 MgCl$_2$, 2 CaCl$_2$, 10 HEPES and 10 Glucose, adjusted to pH 7.4 with NaOH) in dark environment at 37°C for 45 min. After washing three times with ECS, these neurons were allowed to deesterify for at least 30 min before use. Fluorescence changes were measured with a Zeiss Upright Scope equipped with PTI-RatioMaster. Chemicals were applied with a custom-built perfuse system. At the end of each experiment, a 50 mM KCl buffer (95 mM NaCl, 50 mM KCl, 2 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM HEPES and 10 mM Glucose,
adjusted to pH 7.4 with NaOH) was applied to distinguish excitable cells. Only KCl-responsive cells were considered to be excitable cells and used for analysis.

**Patch clamp recording in Dil-labeled esophageal nodose and jugular neurons**

Retrograde labeling of nodose and jugular neurons from the esophagus with DiI (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) (Molecular Probe, Eugene, OR) were performed in 4 guinea pigs according to our previously described method (9). Briefly, under ketamine (50mg/kg) and xylazine (5mg/kg) anesthesia, the cervical esophagus was surgically exposed, DiI solution (1-2 μL, 1% in dimethyl sulfoxide and normal saline mixture) was injected in the wall of the esophagus at 50-60 mm above the gastric-esophageal junction (the injection site was confirmed by the time to dissect the ganglia). Each esophagus was received two to three injections. Postoperatively, animals were carefully monitored, and if necessary treated for pain, until totally recovery. After two-week, both nodose and jugular ganglia (two of each per animal) were collected and disassociated (see above) for whole cell patch clamp recordings or single neuron RT-PCR (see behind).

Whole cell patch clamp recordings in Dil-labeled esophageal nodose and jugular neurons were performed according to our previous described methods (9). Briefly, Borosilicate glass (WPI, Sarasota, FL) electrodes were 2–3 MΩ when filled with the pipette solution (mM): 140 CsCl, 1 MgCl₂, 5 MgATP, 2 EGTA, 10 HEPES (pH 7.2 with CsOH). Whole-cell patch clamp were performed using an Axopatch 200B patch-clamp amplifier and Axograph software (Axon Instruments, Foster City, CA). Currents were typically digitized at 10 kHz and filtered at 2 kHz. The whole cell currents were recorded using voltage ramp from -100 mV to 100 mV in 100 ms duration while cells were patched with a holding potential of 0 mV.

**Extracellular single-unit recording ex vivo in esophageal-vagal preparation**

Extra-cellular single unit recordings from nodose or jugular neurons were performed in ex vivo esophageal-vagal preparations with intact nerve endings in the esophagus.
according to our previous studies (23, 25). Briefly, Guinea pigs were killed by CO₂ inhalation and exsanguination, and the esophagus and trachea with intact bilateral extrinsic vagal innervation (including jugular and nodose ganglia) were dissected. The tissue was pinned in a small Sylgard-lined Perspex chamber filled with Krebs bicarbonate buffer. The two compartments were separately superfused with KBS (pH 7.4, 35°C, 4–6 mL/min). Polyethylene tubing was inserted 3–5 mm into the cranial and caudal esophagus and secured for esophageal distension. Isobaric (constant pressure) distension of the esophagus was achieved by increasing intraluminal esophageal pressure to 10, 30 and 60 mmHg. The pressure was generated by a calibrated device utilizing fluid (KBS) columns.

Extracellular recordings were performed using an aluminosilicate glass microelectrode (pulled with a Flaming-Brown micropipette puller, Sutter Instrument Company, Novato, CA, USA) and filled with 3 M sodium chloride (electrode resistance 2 MΩ). The electrode was placed into an electrode holder connected directly to the headstage (A-M Systems, Everett, WA, USA). A return electrode of silver–silver chloride wire and earthed silver–silver chloride pellet were placed in the perfusion fluid of the recording compartment. The recorded signal was amplified (Microelectrode AC amplifier 1800, A-M Systems) and filtered (low cut-off, 0.3 kHz; high cut-off, 1 kHz) and the resultant activity was displayed on an oscilloscope (TDS 340, Tektronix, Beaverton, OR, USA) and a model TA240 chart recorder (Gould, Cleveland, Ohio). The data were stored and analyzed on a Macintosh computer using the software TheNerveOfIt (sampling frequency 33 kHz; PHOCIS, Baltimore, MD, USA) and further processed using spreadsheet software (Microsoft Excel 2007).

The recording electrode was micromanipulated into the nodose or jugular ganglion (left or right). A distension-sensitive unit was identified when esophageal distension (with a rapid increase in intraluminal pressure to 60 mmHg for 5 s) evoked action potential discharge. The serosal surface of the esophagus was then searched with a stimulate electrode (pulse duration 1.5 ms, frequency 1 Hz) applied to the tissue. A mechanosensitive receptive field was located when the electronic stimulus evoked discharge of action potentials with waveforms identical to the action potentials evoked by
distension. Conduction time was measured as the time between the stimulation pulse and the action potential (visualized by oscilloscope). Conduction velocity was calculated by dividing the length of the approximated nerve pathway by conduction time. Isobaric esophageal distension for 20 s with an intraluminal pressure of 10, 30 and 60 mmHg separated by at least 60 s was used to determine the distension pressure–nerve activity relationship of an esophageal afferent fiber. To assess the reproducibility of distension-evoked activation, this distension protocol was repeated after at least 5 min. The distension-evoked response was quantified as the peak frequency of action potentials discharged during the 20 s of distension from which the spontaneous activity (if present) was subtracted. The peak frequency (Hz) was defined as the maximal frequency of action potential discharge.

After recording the baseline spontaneous activity and mechanical excitability (esophageal distension under the pressure of 10, 30 and 60 mmHg) of esophageal vagal C fiber, TRPM8 agonist WS-12 (1µM) was perfused to the serosal surface of the esophagus for 30 min. The action potential discharges of esophageal nodose or jugular C fibers induced by WS-12 were monitored continuously for 30 min and analyzed in 1 s bins (yielding the number of action potentials in each second, Hz). The esophageal distension evoked responses of these fibers were also detected at the end of agonist perfusion. Then WS-12 was washed out with fresh KBS (pH=7.4) for 30 min, AITC and capsaicin evoked action potential discharges were recorded (30-min washing with fresh KBS between each chemicals).

**Single cell RT-PCR in Dil-labeled nodose and jugular neurons**

*Single cell RT-PCR* studies were performed on individual neurons as described previously (21). The sensory ganglia were dissected, incubated in the enzyme buffer (2 mg ml\(^{-1}\) collagenase type 1A and 2mg ml\(^{-1}\) dispase II in Ca\(^{2+}\)-, Mg\(^{2+}\)-free Hanks’ balanced salt solution) for 3 x 15 min at 37\(^\circ\)C. Neurons were dissociated by trituration with three glass Pasteur pipettes of decreasing tip pore size between and after incubations, washed by centrifugation (three times at 1000 g for 2 min) and suspended in L-15 medium containing 10% fetal bovine serum (L-15/FBS). The cell suspension was
transferred onto Poly-D-Lysine/Laminin-coated coverslips. After the suspended neurons had adhered to the coverslips for 2 h, the neuron-attached coverslips were flooded with L-15/FBS and used within 8 hours. Coverslips with dissociated neurons were perfused with PBS, and the DiI-labeled neurons identified under fluorescent microscope (rhodamine filter) were individually harvested into a glass-pipette (tip 50-150µm) pulled with a micropipette puller (P-87, Sutter) by applying negative pressure. The pipette tip containing the cell was broken into a PCR tube containing RNAse Inhibitor (1µl, RNAseOUT, 2U/µl, Invitrogen), immediately frozen and stored at -80°C. Only the neurons free of debris or attached cells were collected. From one coverslip, one to five cells were collected. A sample of the bath solution was collected from some coverslips for no-template experiments (bath control). 

**RT-PCR:** First strand cDNA was synthesized from single neurons by using the Super-Script(tm) III CellsDirect cDNA Synthesis System (Life Technologies) according to the manufacturer’s recommendations. Samples were defrosted, lysed (10min, 75°C) and treated with DNase I. Then, poly(dT) and random hexamer primers (Roche Applied Bioscience) were added. The samples were reverse transcribed by adding SuperscriptIII RT for cDNA synthesis. 2µl of each sample (cDNA, RNA control or bath control, respectively) were used for PCR amplification by the HotStar Taq Polymerase Kit (Qiagen) according to the manufacturer’s recommendations in a final volume of 20µl. After an initial activation step 95°C for 15min, cDNAs were amplified with custom-synthesized primers (Life Technologies) by 50 cycles of denaturation 94°C for 30s, annealing 60°C for 30s and extension 72°C for 1 min followed by a final extension 72°C for 10min. Products were visualized in ethidium-bromide stained 1.5% agarose gels with 50bp or 100bp DNA ladder. The figures were constructed by using Microsoft PowerPoint and Apple Preview.

The primers were designed by using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). TRPV1 (sequence NM_001172652.1), forward primer (start 992), CCAACAAGAAGGGTTCACA, reverse primer (start 1159), ACAGGTCATAGAGCGAGGAG, predicted product 168 bp, predicted genomic product >1,000 bp. TRPM8 (sequence NM_001173090.1), forward primer (start 3031), ATCCCACTTCCCCCTTTGTG, reverse primer (start 3156),
GGTCTCGTTGTCCTCATTTTTG, predicted product 126 bp, predicted genomic product >1,000 bp. The primers are intron-spanning and no genomic product can be amplified because its predicted size >1,000 bp is not achievable with the extension time of 30s used for PCR thus preventing false positive results from.

Chemicals

All chemicals used in the experiments were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Collagenase/Dispase was purchased from Roche Applied Science (Indianapolis, IN). Fetal Bovine Serum, HBSS and Pluronic(R) F-127 were purchased from Life Technologies (Grand Island, NY). The stock solution of capsaicin (10 mM) was prepared in ethanol; those of Collagenase/Dispase(2mg/ml), laminin (5μg/mL) were in sterile Ca\(^{2+}/\)Mg\(^{2+}\) free Hank’s Balanced Salt Solution (HBSS), and Fura-2-AM (2M) was prepared in acetone and Poly-L-lysine (1mg/mL) was diluted in sterile water. WS-12 and AMTB (both from TOCRIS Bioscience, Bristol, UK) were diluted in dimethyl sulfoxide (DMSO). All the stock solution were separated into small aliquots and stored in -20°C and working solution were prepared 1~2 days before use.

Data analysis

In extra-cellular study, we only analyzed the results from capsaicin- or AITC-responsive C fibers, which were confirmed by the end of each recording to indicate that the nerve terminals were exposed to chemical perfusion. The agonist-evoked nerve response was quantified as the peak frequency of the action potential discharge within a 5-min period, and averaged from 6 recording periods for total 30-min. The peak frequency (Hz) of the action potential discharges were presented as means ± SEM and compared by paired \(t\)-test or one-way ANOVA. For all experiments, significance was defined as \(P < 0.05\).

In calcium imaging studies, neurons were defined as “responders” to a given compound if the mean response was greater than the mean baseline plus 2x the standard deviation using unpaired t-test. Patch clamp data were analyzed with Sigmaplot 11.0 (SPSS Inc. USA). Dose response curves for the agonist were fitted with a modified Hill equation. All
data are presented as means ± SEM. Statistical comparisons were made with unpaired Student’s $t$ test and Wilcoxon rank-sum test, and differences were considered significant at $P < 0.05$. 
RESULTS

TRPM8 selectively expressed in Dil-labeled TRPV1-positive jugular but not nodose neurons

We first evaluated the TRPM8 mRNA expression in the nodose and jugular neurons retrogradely labeled from the esophagus by using single cell RT-PCR. The neurons projecting the capsaicin-sensitive fibers (putative C fiber neurons) were identified by the expression of the capsaicin receptor TRPV1. The expression of TRPM8 was evaluated in these TRPV1-positive esophageal neurons. We found that TRPM8 was expressed in the vast majority (18/22) of jugular esophageal TRPV1-positive neurons. In contrast, only a minority (4/12) of the nodose TRPV1-positive esophageal C fiber neurons expressed TRPM8 (p<0.05, Fisher exact test compared to jugular)(Fig-1).

TRPM8 agonist WS-12 evoked calcium influx in jugular but not nodose neurons

WS-12 is a chemical analog of menthol that stimulates TRPM8 more selectively and more potently than menthol (12, 20). We performed calcium imaging assay in the nodose and jugular neurons from naïve guinea pigs (N=7) to determine their responses to WS-12. As shown in Fig-2, the population of WS-12 sensitive vagal neurons was limited only to those isolated from the jugular ganglia. We found that about 40% (61/163) jugular neurons were stimulated by WS-12. Nodose neurons were categorically unresponsive to WS-12 (0 of 135 nodose neurons responding)(Fig-2). By contrast the percentage of neurons responding to capsaicin or AITC was similar between those isolated from nodose and jugular ganglia. We found that 44.8 ± 5.7% (65 of 145) of nodose neurons were excited by 100 μM AITC comparing to that of 43.6 ± 4.3% (71 of 163) in jugular neurons (Fig-2A-D). Perfusion with capsaicin (100 nM) activated 48.3 ± 4.1% (70 of 145) of nodose neurons comparing to that of 46.6 ± 4.9% (76 of 163) in jugular neurons (Fig-2A-D). The Venn diagram in Fig-2E and F showed the overlaps among WS-12, AITC, and capsaicin responsive neurons in jugular and nodose ganglia.
TRPM8 agonist WS-12 induced inward current in Dil-labeled esophageal jugular but not nodose neurons

To further determine TRPM8 function in vagal sensory neurons specifically innervated the esophagus, we dissociated DiI retrogradely labeled esophageal nodose and jugular neurons for whole cell patch clamp recordings. WS-12 activated outwardly rectifying currents, with a reverse potential around 0 mV in about 40% of jugular neurons (7 of 17 DiI-labeled jugular neurons from 3 naive guinea pigs) (Fig-3C). A concentration-response analysis revealed that WS-12 stimulated the current with an EC$_{50}$ of 67.5 ± 10.6 nM (Fig-3D and E), which is consistent with the potency of WS-12 in activating TRPM8 in transfected HEK293 cells (20). The WS-12-evoked current was inhibited by AMTB, a selective inhibitor of TRPM8 (Fig-3F). In contrast, WS-12 did not evoked a current in nodose neurons (0 of 13 DiI-labeled nodose neurons from 3 guinea pigs responded) (Fig-3B).

TRPM8 agonist WS-12 selectively evoked action potential discharges in jugular but not nodose C fiber nerve endings in the esophagus,

Extra-cellular single-unit recording of action potential discharges in response to WS-12 were performed in nodose or jugular neurons with intact nerve endings in the esophagus using our established ex vivo esophageal-vagal preparations. We evaluated the activity one afferent C fiber (either nodose or jugular) per animal, so the total number of recorded C fibers (n) is equal to the number of animals (N) used in the experiments. In the present study, the average conduction velocity of nodose C fibers innervating the esophagus was 0.6±0.04 m/s (n=10), and that of jugular C fibers was 0.8±0.2 m/s (n=11).

Perfusion with WS-12 (1µM) for 30 min activated nearly every esophageal jugular C fiber studied (n=10/11) (Fig-4A). The peak discharge rate of action potentials averaged 5.80±0.96 Hz after perfusion with WS-12 (compared to a baseline of 1.30±0.26 Hz, P<0.001, n=10) (Fig-4B). Following WS-12 perfusion for 30 min, the peak discharge rates evoked by esophageal distension were compared. They were 4.80±2.20 Hz, 6.80±2.13 Hz and 8.71±2.21 Hz at distension pressures of 10, 30, and 60 mmHg respectively, which were not significantly changed comparing to those before WS-12
After washing with fresh KBS for 60-min, we tested repeat perfusion of WS-12-induced responses in 5 out of 10 WS-12 responsive jugular C fibers. The peak discharge rate of action potentials averaged 6.6±1.86 Hz after the first WS-12 perfusion (vs baseline at 1.4±0.4 Hz, P<0.05, n=5). The second WS-12 only activated 3 of those 5 jugular C fibers. The peak discharge rate of action potentials averaged 4.2±1.5 Hz after the second WS-12 perfusion (vs baseline at 2.0±0.71 Hz, P=0.063, n=5). After washing out WS-12 for 60 min, TRPA1 agonist-induced responses were determined. AITC (300-400µM) activated most of those WS-12 responsive C fibers (n=9/10). The peak discharge rate of action potentials averaged 12.70±3.02 Hz after AITC perfusion (compared with that before AITC at 2.20±0.42 Hz, P=0.006, n=10)(Fig-4C). At the end of each recording, after washing out of AITC for 30 min, TRPV1 agonist capsaicin (1 µ M) was perfused. Capsaicin also activated most of WS-12 responsive C fibers (n=9/10). The peak discharge rates averaged 16.70±3.31 Hz after capsaicin perfusion (compared with that before capsaicin at 1.80±0.44 Hz, P=0.001, n=10)(Fig-4D).

In contrast, perfusion of WS-12 (1µM) for 30 min did not evoke action potential discharges in most of esophageal nodose C fibers (n=9/10)(Fig-5A). The peak discharge rate of action potentials averaged 2.2±0.61 Hz after WS-12 perfusion (compared with that before WS-12 at 1.4±0.4 Hz, P>0.05, n=10)(Fig-5B). Following WS-12 perfusion for 30 min, the peak discharge rates evoked by esophageal distension were determined in these nodose C fibers. The peak discharge rates were 4.40±1.73 Hz, 6.50±2.58 Hz, and 7.80±2.72Hz at distension pressures of 10, 30, and 60mmHg, which were not significant different than those before WS-12 perfusion (4.00±1.58 Hz, 7.00±2.34 Hz, and 8.30±2.50Hz respectively, P>0.05, n=10). After washing out WS-12 for 60 min, TRPA1 agonist-induced response was determined. AITC (300-400µM) activated all these WS-12 non-responsive esophageal nodose C fibers (n=10). The peak discharge rate of action potentials averaged 19.7±4.04 Hz by AITC perfusion (compared with that before AITC at 1.30±0.37 Hz, P=0.001, n=10)(Fig-5C). At the end of each recording, after washing out AITC for 30 min, TRPV1 agonist capsaicin (1 µ M) was perfused. All these nodose C fibers could be activated by capsaicin. The peak discharge rates averaged 12.40±1.98 Hz
after capsaicin perfusion (compared with that before capsaicin at 1.80±0.47 Hz, P<0.001, n=10)(Fig-5D).
DISCUSSION

Vagal afferents innervated in the esophagus have two subtypes of C fibers. One type has its cell body situated in the nodose ganglion the other in the jugular ganglion. Both C fiber subtypes fit the definition of nociceptors in that they can distinguish noxious from non-noxious distension, and are stimulated by various inflammatory mediators commonly associated with nociceptor activation (23, 24, 25). However, these two nerve subtypes have different embryological origins, are under different neurotrophic control, innervate distinct aspects of the esophagus, and importantly, synapse with secondary neurons in distinct brain regions (5). For example, in viral tracing studies, jugular neurons have been suggested to have a more somatic central circuitry compared to the more visceral circuitry of the nodose fibers (14). This supports the idea that the jugular C fibers may transduce sensations and reflexes distinct from nodose C fibers. For these reasons it is critical to understand the activation profile of jugular vs nodose C fiber nociceptors.

Many chemical stimuli activate both nodose and jugular C fibers in the esophagus; e.g. tissue distention and all substances that can gate either TRPV1 or TRPA1 (although nodose C fiber are more strongly activated by TRPA1 agonists than jugular C fibers)(3, 23). Chemical stimuli have been identified that selectively activate nodose but not jugular C fibers including ATP via P2X2/3 receptors (11, 25), 5-HT via 5-HT3 receptor (24), and adenosine via A2a receptor agonists (18). Until the present study, no stimuli has been identified that would stimulate jugular but not nodose C fibers. The evidence provided here indicates that stimuli that gate TRPM8 may selectively activate jugular and not nodose C fibers in the esophagus.

The conclusion that TRPM8 activators will stimulate jugular but not nodose esophageal C fibers is based on four lines of evidence; First, single neuron RT-PCR revealed that TRPM8 mRNA were expressed in a large majority of esophageal-specific jugular C fiber neurons but only a minority of nodose neurons; Second, calcium image analysis revealed that a very selective TRPM8 agonist stimulated calcium influx in a majority of jugular
neurons but in virtually no nodose neurons; Third, patch clamp recording revealed that
jugular neurons responded to the TRPM8 agonist with a current properties consistent
with TRPM8, and sensitive to a TRPM8 inhibitor, whereas no such current was observed
in nodose neurons; And last, the nerve endings in the esophageal wall of jugular C fibers
but not nodose C fibers consistently responded with action potential discharge to TRPM8
activation.

The functional studies at the cell bodies and nerve endings showed a more categorical
distinction in TRPM8 responses between jugular and nodose neurons compared to the
gene expression data. Whereas very few if any nodose neurons responded to the TRPM8
agonist, about 25% of the esophageal-specific nodose neurons expressed some TRPM8
mRNA. This is not a quantitative analysis, so it may be that some neurons express small
amount of mRNA that is insufficient to produce a meaningful density of functional
channels.

These findings have potential clinical and experimental implications. Clinically it sets up
the conditions where by TRPM8 stimuli such a cold temperature and ingestion of
menthol related substances will lead to sensations and reflexes distinct from those stimuli
that activate nodose C fibers. Experimentally the present result provides a tool to
selectively activate jugular C fibers and not nodose C fibers. It will be of considerable
interest to characterize the physiological consequences of this activation compared to
selectively activating nodose but not jugular C fibers with stimuli such as the P2X3
agonist α, β methylene ATP. The selective expression and function of TRPM8 in vagal
afferent C fiber subtypes might also be helpful to further study the interaction of TRPV1
and TRPM8, relative contributions of TRPM8 and TRPA1 to cold, and integrated roles of
these TRP channels in esophageal sensation and nociception.

In summary, the present study adds new knowledge on TRPM8 function and expression
in vagal sensory neurons and afferents. Our data demonstrated that TRPM8 agonist WS-
12 selectively activated esophageal vagal jugular but not nodose nociceptive C fiber
neurons. TRPM8 highly expressed in esophageal jugular TRPV1-positive neurons. Such
preferential function and expression of TRPM8 in esophageal vagal jugular neurons and
C fibers provide a useful tool to further elucidate the cellular and molecular mechanisms
of menthol and cold-induced esophageal sensation and nociception.

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Figure Legend

Figure-1. TRPM8 mRNA expression in Dil-labeled esophageal nodose and jugular neurons using single neuron RT-PCR method. TRPM8 was expressed in the majority (18/22) of jugular esophageal TRPV1-positive neurons. In contrast, only a minority (4/12) of the nodose TRPV1-positive esophageal C fiber neurons expressed TRPM8 (P<0.05, Fisher exact test compared to jugular).

Figure-2. TRPM8 agonist WS-12 evoked calcium influx in vagal jugular but not nodose neurons. (A) Representative traces of calcium influxes in nodose neurons (left) and jugular neurons (right) evoked by TRP channel agonists (WS-12: 1 μM; AITC: 100 μM; Capsaicin: 100 nM) and KCl (50 mM). (B) Summary data of the responsive rates of nodose and jugular neurons to TRP channel agonists. (C) Venn diagram showing responsive overlaps of TRPM8 with TRPA1 and TRPV1 in jugular (right), but not nodose (left) neurons.

Figure-3. TRPM8 agonist WS-12 activated Dil-labeled esophageal jugular but not nodose neurons. (A) The protocol for whole-cell patch clamp experiments of TRPM8 agonist (WS-12)-induced responses in esophageal nodose and jugular neurons. (B, C) Representative current traces showing that WS-12 (1μM) activated esophageal jugular, but not nodose, neurons. (D) Representative current traces showing that WS-12 elicited currents in esophageal jugular neurons in a dose-dependent manner. (E) The dose-dependent activation curve of WS-12 in esophageal jugular neurons (n=7, N=3). (F) Representative current traces showing that TRPM8 inhibitor AMTB prevented WS-12-induced activation effect in esophageal jugular neurons.
Figure-4. TRPM8 agonist WS-12 activated esophageal jugular C fibers. (A) Summary of TRP channel agonists-induced responses in esophageal jugular C fibers; (B) TRPM8 agonist WS-12 (1 μM, 30-min) evoked action potential discharges in esophageal jugular C fibers (*P<0.05, vs baseline, n=10/11). (C) TRPA1 agonist AITC evoked action potential discharges in those WS-12 responsive jugular C fibers (*P<0.01, vs control, n=10). (D) TRPV1 agonist capsaicin evoked action potential discharges in those WS-12 responsive jugular C fibers (*P<0.01, vs control, n=10).

Figure-5. TRPM8 agonist WS-12 did not activate esophageal nodose C fibers. (A) Summary of TRP channel agonists-induced responses in esophageal nodose C fibers; (B) TRPM8 agonist WS-12 (1 μM, 30-min) did not evoke action potential discharges in esophageal nodose C fibers (*P>0.05, vs baseline, n=9/10). (C) TRPA1 agonist AITC evoked action potential discharges in those WS-12 responsive nodose C fibers (*P<0.01, vs baseline control, n=10). (D) TRPV1 agonist capsaicin evoked action potential discharges in those WS-12 responsive jugular C fibers (*P<0.01, vs baseline control, n=10).


Figure-2

(A) Nodose

(B) Jugular

(C) % Total Nodose Neurons

(D) % Total Jugular Neurons

(E) Venn Diagram: TRPA1, TRPV1, KCl

(F) Venn Diagram: KCl, TRPA1, TRPM8, TRPV1
Figure-3

A  Protocol  

B  Nodose

C  Jugular

D  Jugular

E  Normalized Current

F  Jugular

[Graphs and data showing protocols and responses for different conditions including concentrations of WS-12 and EC50 values]