Different role of Tetrodotoxin-sensitive Voltage-gated Sodium Channel (NaV1) Subtypes in Action Potential Initiation and Conduction in Vagal Airway Nociceptors

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Running title: NaV1s in Vagal Airway Afferent Nerves

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Key points summary

The action potential initiation in the nerve terminals and its conduction along the axons of afferent nerves are not necessarily dependent on the same NaV1 subunits.

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The action potential initiation in jugular C-fibers within airway tissues is not blocked by TTX; nonetheless conduction of action potentials along the vagal axons of these nerves is often dependent on TTX-sensitive channels.

This is not the case for nodose airway Aδ-fibers and C-fibers where both action potential initiation and conduction is abolished by TTX, or selective Na\textsubscript{v}1.7 blockers.

The difference between initiation of action potentials within the airways vs. conduction along the axons should be considered when developing Na\textsubscript{v}1 blocking drugs that will be applied topically to the respiratory tract.

**ABSTRACT**

The action potential (AP) initiation in the nerve terminals and AP conduction along the axons do not necessarily depend on the same subtypes of Na\textsubscript{v}1s. We evaluated the role of tetrodotoxin (TTX)-sensitive and TTX-resistant Na\textsubscript{v}1s in vagal afferent nociceptor nerves derived from jugular and nodose ganglia innervating the respiratory system. Single cell RT-PCR was performed on vagal afferent neurons retrogradely labeled from the guinea pig trachea. Virtually all the jugular neurons expressed the TTX-sensitive channel Na\textsubscript{v}1.7 along with TTX-resistant Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9. Tracheal nodose neurons also expressed Na\textsubscript{v}1.7, but less frequently Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9. Na\textsubscript{v}1.6 was expressed in approximately 40% of the jugular and 25% of nodose tracheal neurons. Other Na\textsubscript{v}1 \(\alpha\) subunits were only rarely expressed. Single fiber recordings were made from the vagal nodose and jugular nerve fibers innervating the trachea or lung in the isolated perfused vagally-innervated preparations that allowed for selective drug delivery to the nerve terminal compartment (AP
initiation) or to the desheathed vagus nerve (AP conduction). AP initiation in jugular C-fibers was unaffected by TTX, but was inhibited by Na\textsubscript{\text{1.8}} blocker (PF-01247324) and abolished by combination of TTX and PF-01247324. However, AP conduction in the majority of jugular C-fibers was abolished by TTX. In contrast, both AP initiation and conduction in nodose nociceptors was abolished by TTX or selective Na\textsubscript{\text{1.7}} blockers. Distinctions between the effect of a drug at inhibiting AP in the nerve terminals within the airways vs. at conduction sites along the vagus nerve is relevant to therapeutic strategies involving inhaled Na\textsubscript{\text{1}} blocking drugs.

INTRODUCTION

Activation of sensory nerves involves the transduction of a stimulus (physical or chemical) to a membrane depolarization referred to as the “generator potential” or “receptor potential”. If the generator potential is of sufficient rate and magnitude it activates voltage-gated sodium channels (Na\textsubscript{1}s). This step is absolutely required for the genesis of action potentials and their conduction along the axon to the central terminals in CNS where it evokes the release of neurotransmitters at the synapse on secondary neurons (Hodgkin & Huxley, 1952). Among the nine subtypes of the pore-forming α subunit of Na\textsubscript{1} channels, Na\textsubscript{\text{1.1}}, 1.2, 1.3, 1.6, 1.7, 1.8, and 1.9 are commonly expressed in neurons, with differing nerve phenotypes expressing distinct subsets of Na\textsubscript{1} channels (Catterall, 2012; Schild & Kunze, 2012). Tetrodotoxin (TTX) potently blocks all of these channels except Na\textsubscript{\text{1.8}} and Na\textsubscript{\text{1.9}}. Although it is known that Na\textsubscript{1} are essential for the genesis and conduction of action potentials, it is not necessarily the case that these two processes depend equally on the same subtypes of Na\textsubscript{1}s. With respect to nociceptive C-fibers in the cornea, for example, it has been shown that the genesis of action potentials at the nerve terminals within the tissue
can occur independently of TTX-sensitive Na\textsubscript{\textit{1}}s, whereas the conduction of the action potentials in the trigeminal nerve is blocked with TTX (Brock \textit{et al.}, 1998).

The sensory nerves innervating the respiratory tract are primarily derived from neurons in vagal sensory ganglia (Mazzone & Undem, 2016). The majority of respiratory vagal afferent nerves are C-fibers that have nociceptive characteristics. Activation of these nerves can evoke parasympathetic reflex bronchospasm and secretions, as well as sensations of urge-to-cough and dyspnea. Two phenotypically distinct types of vagal C-fiber nociceptors innervate the guinea pig respiratory tract in approximately equal numbers, namely the epibranchial placode-derived nodose C-fibers and neural crest-derived jugular C-fibers. Another type of nociceptor that innervates the extrapulmonary airways of guinea pigs is the nodose A\textdelta-fiber (Undem \textit{et al.}, 2004). Stimulation of nodose A\textdelta-fibers evokes the cough reflex even in anesthetized animals, and they have been therefore referred to as cough receptors (Canning \textit{et al.}, 2004). Stimulation of vagal C-fibers in the guinea pig airways can also lead to cough, but only in conscious animals.

In acute and chronic inflammatory airways diseases it is thought that vagal nociceptors can become overstimulated leading to a hyperreflexic state that can result in bronchospasms, hypersecretions, and coughing that exceed their otherwise utilitarian function (Lee & Yu, 2014). If this is the case, quelling the activity of airway nociceptors would be a rational therapeutic strategy.

We have previously determined that vagal sensory neurons express primarily Na\textsubscript{\textit{1}}1.7, 1.8 and 1.9 mRNA (Kwong \textit{et al.}, 2008). We also determined that when Na\textsubscript{\textit{1}}1.7 was selectively
silenced using AAV-shRNA technology, the conduction of action potentials along the vagal fibers was largely inhibited (Muroi et al., 2011). Animals treated with NaV1.7shRNA lose both the C-fiber derived conscious cough as well as the nodose Aδ-fibers driven reflex unconscious cough (Muroi et al., 2011; Muroi et al., 2013).

The shRNA strategy to decrease Na\textsubscript{v}1.7 expression is predicted to diminish the density of Na\textsubscript{v}1.7 channels in the membranes of the afferent neuron cell body, along the conducting axons, and at the peripheral and central nerve terminals. The question remains how effective is blocking of Na\textsubscript{v}1.7 selectively at the fiber/terminals within the airway tissue in inhibiting action potential genesis. This question is critical inasmuch as a topical administration of therapeutic drugs (such as Na\textsubscript{v}1.7 blockers) to the airways by inhalation is a feasible and preferred route minimizing the systemic side effects. In the present study we addresses this key issue by taking advantage of recently discovered selective Na\textsubscript{v}1 inhibitors, combined with isolated innervated airways preparations that allow for delivery of the inhibitors selectively to the nerve ending-components within the tissue vs. to the conducting fibers in the vagus nerves.

METHODS

*Ethical Approval.* The experiments were approved by the Johns Hopkins School of Medicine Animal Care and Use Committee. The investigators understand the ethical principles under which the Journal of Physiology operates and their work complies with the animal ethics checklist as described by Grundy (Grundy, 2015). Male Hartley guinea pigs (Hilltop Laboratory Animals Inc., Scottsdale, PA, USA) weighing 100-200g. The animals were housed in an approved animal facility with controlled temperature and humidity, 24h day-
night cycle in groups in cages providing unrestricted access to food and water and species appropriate environmental enrichment. The animals were intraperitoneally injected with anticoagulant heparin (2000 IU kg⁻¹; diluted in saline 1000 IU ml⁻¹). Heparin prevents blood clot formation and was used to improve blood removal from the pulmonary circulation. After 20 min the animals were killed by exposure to CO₂ in rising concentration (CO₂ was sourced form compressed CO₂ gas cylinder and administered at a flow rate measured by flow meter displacing approximately 20%-30% of euthanasia chamber volume per minute conforming to (Grundy, 2015)) and exsanguination. The isolated preparations were studied at 37°C in Krebs bicarbonate solution (KBS); composed of (mM): 118 NaCl, 5.4 KCl, 1.0 NaH₂PO₄, 1.2 MgSO₄, 1.9 CaCl₂, 25.0 NaHCO₃, 11.1 dextrose, gassed with 95%O₂-5%CO₂ (pH 7.4).

**Tracheal tube preparation.**

A modification of the isolated vagally innervated tracheal preparation described previously (Riccio et al., 1996b) was used for extracellular recording of action potential from nodose and jugular vagal afferent nerve fibers that have defined receptive fields in the tracheal wall (Fig. 3A). The trachea with intact right-side extrinsic vagal innervation (including vagus nerve, superior laryngeal nerve, recurrent laryngeal nerve and nodose and jugular ganglia) was removed and placed in a dissecting dish containing KBS. Connective tissue was trimmed away, leaving the trachea, larynx with intact nerves. The rostral and caudal end of the tracheal tube was cannulated and the trachea was perfused (inside) with KBS (37°C) at a rate of 6-8 ml/min. Simultaneously, the tissue was also separately superfused (from outside) with KBS (6-8 ml/min, 37°C). The drugs were applied to the receptive field via the perfusion buffer in a rostral-caudal direction before stimulation the preparation. With this design any drug that could leak through the trachea would be immediately diluted by the superfusion KBS (see figure 1 for illustration of the preparation). This allows for the drugs to
be selectively applied to the tissue containing receptive fields inside the trachea, but not reaching vagus nerve and laryngeal nerves containing conducting axons. The right nodose and jugular ganglia, along with the rostral most vagus and superior laryngeal nerves, were gently pulled through a small hole into an adjacent compartment and separately superfused with KBS (6-8 ml/min, 37°C) for recording of single fiber activity as described previously.

Trachea-lung preparation.

The perfused vagal innervated tracheal-lung preparation was set up as previously described (Fig. 4A) (Undem et al., 2004). After isolating the lungs perfusion with KBS was used to wash the blood from the pulmonary circulation. The trachea and right lungs with intact right-side extrinsic vagal innervation including right jugular and nodose ganglia were dissected and placed in a two-compartment tissue bath. The right nodose and jugular ganglia along with vagus nerve were placed in one compartment, lung and trachea in the second compartment. The two compartments were separately superfused with KBS (6 ml min⁻¹, 37°C) containing indomethacin (3 µM). Indomethacin reduces the indirect influence of tissue prostanoids on C-fiber activation. The pulmonary artery and trachea were cannulated with PE tubing and continuously perfused with KBS (4 ml min⁻¹ and 2 ml min⁻¹, respectively). Drugs were added to the receptive field of the nerve via both the artery and trachea. The drugs in the perfusing buffer solution therefore exits the lungs via both these puncture ports as well as via the pulmonary veins and was immediately diluted into the superfusion buffer.

The single nerve activity was recorded with glass microelectrodes pulled with micropipette puller model P-87 (Sutter Instrument Company, Novato, CA, USA) and filled with 3 M sodium chloride (resistance ~ 2 MΩ). The signal was amplified (Microelectrode AC amplifier...
1800, A-M systems, Everett, WA, USA), filtered (low cut off, 0.3 kHz; high cut off, 1kHz), displayed on an oscilloscope (TDS 340, Tektronix, Beaverton, OR, USA) and a chart recorder (TA240), and recorded (sampling frequency 33 kHz) onto a Macintosh computer for offline analysis (TheNerveOft; PHOCIS, Baltimore, MD, USA).

The recording electrode was manipulated into either nodose or jugular ganglion.

A mechanosensitive receptive field was identified when a mechanical stimulus (Von Frey hair, 1800-3000 mN) bluntly applied to the lung surface evoked a burst of action potentials. Once a mechanosensitive receptive field was identified, a brief (<1 ms) electrical stimulus was delivered by a small concentric electrode positioned over this discrete mechanosensitive region to determine the conduction velocity of the fiber. The receptive field was stimulated electrically with a square pulse (0.5 ms) of increasing voltage (starting at 5 V) until an action potential was evoked. Conduction velocity was calculated by dividing the distance along the nerve pathway by the time between the shock artifact and the action potential evoked by electrical stimulation of the mechanosensitive receptive field. On those rare occasions where two units were recorded simultaneously, wave analysis software (TheNerveOft; PHOCIS, Baltimore, MD, USA) was used to distinguish between the two peaks. This method has been described previously (Undem et al., 2004).

**Action potential conduction in vagal axons**

A modification of the isolated vagally-innervated tracheal preparation described previously (Riccio et al., 1996b) was used. As shown in Figure 5A, the vagus nerve was routed through an additional chamber that was isolated by vaseline from both the recording chamber (containing vagal ganglia with the cell bodies of sensory neurons) and the tissue chamber.
(containing the trachea with receptive fields). Therefore, the access of drugs applied in the superfusion in this chamber was limited to the vagus nerve. The segment of the vagus nerve in the chamber was carefully desheathed. The receptive filed in trachea was stimulated by application of citric acid (Kollarik & Undem, 2002).

**Single Cell RT-PCR of airway specific vagal neurons.**

The neurons innervating the trachea were retrogradely labeled using retrograde tracer Dil (Invitrogen, Carlsbad, CA) in solution (1% in DMSO diluted 1:10 in PBS). Guinea pigs were anesthetized by injection of ketamine (50 mg/ml) and xylazine (2.5 mg/ml). Using sterile surgical approach cervical trachea was exposed through the midline 5mm cervical skin incision, the needle connected to Hamilton syringe was inserted into the tracheal lumen by penetrating the anterior tracheal wall and the dye was injected into 2 sites (5-10µl per site) in the dorsal tracheal wall. Subcutaneous layers and the skin were sutured. Following the surgery animals recovered on a heated pad under close observation for 2–3 hours. The animals were monitored for wound healing and the presence of distress and pain daily. No animal in this study met conditions under which early euthanasia would be considered as defined in our animal protocol. 14-21 days after the injection, the animals were killed by CO₂ asphyxiation and exsanguination. Jugular and nodose ganglia were harvested, enzymatically dissociated (collagenase 2 mg/ml and dispase II 2 mg/ml dissolved in Ca-, Mg-free Hanks’ balanced salt solution) while repeatedly triturated with a glass fire-polished Pasteur pipettes, washed by centrifugation and suspended in L-15 medium containing 10% fetal bovine serum (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 (37°C), the neuron-attached coverslips were flooded with the L-15 medium (10% FBS). Coverslips with dissociated neurons were constantly perfused by PBS. The Dil-labeled cells were identified.
using fluorescence microscopy, and labeled single neurons were collected into a glass pipette (tip diameter 50–150 µm, pulled with a micropipette puller P-87 Sutter Instruments, Novato, CA) by applying negative pressure within. The pipette tip was then broken in a PCR tube containing 1 µl of resuspension buffer (from SuperScript III CellsDirect described below) and RNAse inhibitor (RNaseOUT; 2 U/µl). The tubes were immediately snap frozen and stored on dry ice. Only one to four cells were collected from each coverslip.

The major concern in this type of experiments is whether the sample that also contains some superfusing fluid is contaminated by RNA/DNA from adjacent and/or damaged/lysed cells. To control for this problem, we collected only the neurons that were clean, and not attached to other neurons, other cells or debris. In addition, our approach using constant flow perfusion of the coverslips during the cell collection repeatedly proved effective to essentially eliminate any contamination. In each experiment, the samples of superfusing fluid from the vicinity of the collected neurons was obtained. These negative controls were found nearly uniformly negative (in 2 instances when positive, all cells from the experiment were discarded).

Single-Cell RT-PCR. First strand cDNA was synthesized from single neurons by using the Super-Script(tm) III CellsDirect cDNA Synthesis System (Thermofisher - 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, water control or bath control) 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 prepared by using Microsoft PowerPoint and Apple Preview.

Intron-spanning primers specific for each guinea pig Na\(_\alpha\) subunit were designed based on indicated Pubmed sequences with aid of UCSC (University of California, Santa Cruz) Genome Browser using Primer3 (v.0.4.0) program(Rozen & Skaletsky, 2000) (Table 1). These primers were reported previously (Kocmalova et al., 2017). For most primers 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. The selectivity of each primer was evaluated by aligning all Na\(_\alpha\) subunits with each primer using ClustalW. RNA for positive control was selected based on established Na\(_\alpha\) subunit expression pattern: whole brain RNA for Na\(_\alpha\)1.1, 1.2, 1.3, and 1.6; skeletal muscle RNA for Na\(_\alpha\)1.4; heart muscle RNA for Na\(_\alpha\)1.5; and dorsal root ganglia (DRG) or vagal sensory ganglia RNA for Na\(_\alpha\)1.7, 1.8, and 1.9. The guinea pig RNA was isolated by using RNAeasy mini kit (Qiagen).

**Patch clamp electrophysiology**

The jugular and nodose ganglia were dissected, cleared of adhering connective tissues, cut into a few pieces, respectively, and digested enzymatically using type 1A collagenase (2mg/ml) and dispase II (2mg/ml) as previously described (Kwong et al. 2010).
nodose and jugular neurons were kept separately at 37°C in L-15 medium containing 10% of fetal bovine serum for use within 24 hours.

Conventional whole-cell patch clamp technique was employed to record the sodium current ($I_{\text{Na}}$) using an Axopatch 200B amplifier interfaced with Axon Digidata 1550A and driven by pCLAMP 10 software (Molecular Devices, Sunnyvale, CA, USA). Bath solution contained (mM): choline-Cl 126, NaCl 10, CsCl 3, TEA-Cl 5, CdCl$_2$ 0.1, MgCl$_2$ 1, CaCl$_2$ 1, HEPES 10 and glucose 10 with pH adjusted to 7.35 with CsOH. Pipette solution contained (mM): CsF 140, NaCl 10, MgCl$_2$ 1, CaCl$_2$ 0.1, EGTA 1.1 and HEPES 10 with pH adjusted to 7.2 with CsOH. Pipettes with a tip resistance about 1 MΩ were used so that a lower access resistance (usually between 1.5 and 4 MΩ) could be obtained. Cell capacitance and series resistance were compensated electronically by ~80% to ensure an adequate voltage control.

To record $I_{\text{Na}}$, cells were held at -110 mV and depolarized every 5 seconds for 50 ms to voltages ranging from -90 to +20 mV at an increment of 5 mV. The current was sampled at 50 kHz and filtered at 10 kHz. Since pilot experiments showed a run-up of $I_{\text{Na}}$ and a negative shift of its voltage dependence following membrane rupture, the families of $I_{\text{Na}}$ in all cells were recorded 8 to 10 minutes after the whole-cell formation when the current become relatively stable. The steady-state inactivation of TTX-resistant $I_{\text{Na}}$ was studied using a conventional double-pulse protocol composed of conditioning pulses of 1 sec from a holding potential of -110 mV to voltages from -90 to +10 mV followed by a test pulse to -20 mV.

Amphotericin B-perforated whole-cell patch clamp technique was used to record the membrane potentials under current clamp mode. Action potentials (AP) were evoked by current pulses of 100ms at an increment of 5pA. Bath solution contained (mM): NaCl 136,
KCl 5.4, MgCl$_2$ 1, CaCl$_2$ 1.5, HEPES 10 and glucose 10 with pH adjusted to 7.35 with NaOH.

Pipette solution contained (mM): KCl 20, K-gluconate 125 and HEPES 10 with pH adjusted to 7.2 with KOH. Freshly prepared amphotericin B was added to the pipette solution (300 μg ml$^{-1}$) before experiments. The junction potential (-14 mV estimated using Clampex calculator) was corrected offline for the readings of membrane potential. All patch clamp experiments were performed at room temperature.

Patch clamp data were analyzed using Clampfit 10 and SigmaPlot softwares. The amplitude of $I_{Na}$ was measured as the difference between peak currents and the steady-state current at the end of 50 ms pulses. The chord conductance of Na$^+$ channels ($G_{Na}$) was calculated using equation $G_{Na}=I_{Na}/(V_m-V_{rev})$, where $I_{Na}$ is the current amplitude obtained at depolarizing voltages ($V_m$) and $V_{rev}$ is reversal potential determined as the voltage where the fitted linear portion of the current-voltage ($I$-$V$) curve established in each cell intercepts with the zero-current voltage axis. The activation curve was established by plotting the $G_{Na}$ against $V_m$ and fitting the data to the Boltzmann function: $G_{Na}/G_{max} = 1/(1+exp(V_{0.5}-V_m)/k)$, where $G_{max}$ is the maximal conductance, $V_{0.5}$ the voltage for half-maximal activation and $k$ the slope factor.

The steady-state inactivation curve was established by plotting the amplitude of $I_{Na}$ recorded at test pulse against the conditioning voltages ($V_m$), and fitting the data points to the Boltzmann function: $I/I_{max}=1/(1+exp(V_m-V_{0.5})/k)$, where $I_{max}$ is the maximal current amplitude recorded at the test pulse following the most hyperpolarizing conditioning voltage, $V_{0.5}$ the half inactivation voltage and $k$ the slope factor. Pooled data are expressed as mean ± SEM.

**Guinea pig isolated trachea (tissue based Na$_V$1.7 assay)**

We have previously reported that the parasympathetic nerve controlling cholinergic contractions of the guinea pig isolated trachea express Na$_V$1.7 as the only TTX-sensitive Na$_V$1 a subunit(Kocmalova et al., 2017). Therefore we can use this assay as to assess the potency of selective Na$_V$1.7 drugs in an isolated tissue environment. The trachea is isolated
and set up in a standard tissue bath apparatus, the nerves are activated by electrical field stimulation and the cholinergic contractions are recorded exactly as we have previously described (Kocmalova et al., 2017). The concentration response curve for PF-05089771 inhibiting the Na\textsubscript{v}1.7-mediated responses was obtained in a cumulative fashion.

**Statistical analysis**

The single fiber recording data are presented as a total number of action potentials evoked by indicated stimuli and expressed as mean±SEM. The statistical tests used are indicated in the text.

**Drugs and solutions**

The compounds used were prepared as socks solutions stored at -20°C and were diluted in Krebs solution to their final concentrations on the day of use. The stock solutions of tetrodotoxin 1mM (Alomone Laboratories Jerusalem, Israel), bradykinin 10mM (Sigma-Aldrich), and citric acid (Sigma-Aldrich) were dissolved in distilled water. Lidocaine (Sigma-Aldrich) 1M was dissolved in ethanol. ICA121431, PF05089771 and PF01247324 (all Tocris Minneapolis, MN) and compound 13 (first described in WO2012004706, Almirall Laboratories, Barcelona, Spain) were dissolved to 10mM in DMSO.

**RESULTS**

A. **TTX blocks initiation of action potentials in nodose but not in jugular tracheal vagal nociceptors**

The afferent innervation of the guinea pig trachea comprises nociceptive fibers derived from the jugular and nodose ganglia. The jugular ganglion-derived fibers are TRPV1 expressing...
capsaicin sensitive C- and Aδ-fibers. The nodose ganglion-derived fibers are mainly capsaicin-insensitive Aδ-fibers that are low threshold punctate mechanosensors and acid-sensors. The tracheal nodose Aδ-fibers are consistent with bona fide cough receptors (Canning et al., 2004). Activation of both types of nociceptors can drive parasympathetic reflexes. An image of a C-fiber and an Aδ-fiber in a whole mount of the trachea is shown in Figure 1A-B (Mazzone & Undem, 2016). In previous studies evaluating the tracheal cross sections we noted that both the Aδ-fibers and the C-fibers terminate close to tracheal lumen (Fig. 1C) (Mazzone et al., 2009), with the Aδ-fibers terminating beneath the epithelial basal membrane, whereas the C-fibers are found more superficial branching also above the basal membrane between the epithelial cells (Watanabe et al., 2005; Watanabe et al., 2006; Mazzone et al., 2009). Figure 1 illustrates the diffusion path of drugs in order to access nodose and jugular nerve fiber terminals in the tracheal lumen.

Na\textsubscript{V}1 \textalpha subunits mRNA expression

Single neuron RT-PCR of TRPV1-negative nodose neurons (presumed nodose Aδ-fiber neurons) and TRPV1-positive jugular neurons (presumed jugular C-fiber neurons) retrogradely labeled from the guinea pig trachea revealed that the Na\textsubscript{V}1.7, Na\textsubscript{V}1.8, and Na\textsubscript{V}1.9 mRNA was the most frequently expressed (Fig. 2). While the expression of TTX-sensitive NaV1.7 was similar between tracheal specific nodose TRPV1-negative and jugular TRPV1-positive neurons, the TTX-resistant Na\textsubscript{V}1.8 and Na\textsubscript{V}1.9 were more frequently expressed in jugular TRPV1-positive neurons (9/17 vs. 26/31 P<0.05, Fisher exact test, and 8/17 vs. 26/31, P<0.05, Fisher exact test). Approximately 20% and 40% of tracheal specific nodose TRPV1-negative and jugular TRPV1-positive neurons, respectively, also expressed Na\textsubscript{V}1.6. The other Na\textsubscript{V}1 subunits were rarely expressed.
**Action potential discharge**

Tracheal jugular C-fibers and nodose Aδ-fibers in the trachea are strongly and reproducibly activated by acid, although different ion channels are likely involved in the generator potentials (Kollarik & Undem, 2002). When TTX (1 µM) was perfused through the lumen of the trachea for 30 minutes before citric acid application, the citric acid-induced burst of action potentials was abolished in 6 of 6 experiments with nodose Aδ fibers (Fig. 3B). In two additional experiments the number of citric-acid-induced action potentials was 81 and 47 before and 0 and 0, after treatment with 10 µM of the NaV1.7 selective blocker Compound 13 (Kocmalova et al., 2017). We have previously noted that Compound 13 is selective for NaV1.7 and blocks guinea pig NaV1.7-mediated parasympathetic responses in the guinea pig trachea with an IC50 of ~0.5 µM and a maximal inhibitory effect at 10 µM (Kocmalova et al., 2017).

In striking contrast to the nodose Aδ-fibers in the trachea, TTX had only a very modest effect on citric acid-induced action potential discharge in the tracheal jugular C-fibers (Fig. 3C) even in the large number of experiments (N=31). In eight of these experiments lidocaine was applied following TTX and this essentially abolished the response in all 8 jugular C-fibers tested (Fig. 3C) demonstrating effective access of drugs applied into the lumen to nerve terminals. We also examined the effect of TTX on the electrical threshold of action potentials evoked by electrical stimulation of receptive fields of the jugular C-fibers in the trachea. TTX modestly, but significantly increased the voltage required to evoke action potentials. The voltage threshold before and after TTX (1µM) averaged 29 ± 2V and 48 ± 8V, respectively (n=6, P <0.05, paired T-test). Adding lidocaine abolished the electrical
response to 150V (i.e. no action potentials was evoked by a maximum voltage output from stimulator) in 5 of these 6 C-fibers, in the remaining 1 C-fiber lidocaine increased the threshold voltage to 90V.

B. TTX blocks initiation of action potentials in nodose but not in jugular intrapulmonary vagal nociceptors

As discussed above, the nodose and jugular ganglia project two distinct subtypes of nociceptors into the trachea - nodose Aδ-fibers and jugular C-fibers. In contrast, both nodose and jugular nociceptors in the lungs are C-fibers(Undem et al., 2004). Although these nerves have unique activation profiles, and likely terminate in different areas in the CNS, both nodose and jugular C-fibers in the lungs are strongly and consistently activated by bradykinin and capsaicin(Undem et al., 2004).

As we found in the nodose Aδ-fibers in the trachea, TTX (delivered selectively to the intrapulmonary compartment) abolished action potential discharge in nodose C-fibers evoked by bradykinin (Fig. 4B). Consistent with its marginal effect on the tracheal jugular C-fibers, TTX had no appreciable effect on the bradykinin-induced action potential discharge in the jugular C-fibers terminating within the lungs (Fig. 4C).

The blockade of the bradykinin-induced responses in nodose C-fibers by TTX was mimicked by two selective Na\textsubscript{V}1.7 blockers, Compound 13 and PF-05089771 (Fig. 4B). The concentration of PF-05089771 was chosen based on the concentration needed to abolish
Na\textsubscript{v}1.7-mediated parasympathetic responses in the guinea pig isolated trachea performed as described previously (Kocmalova et al., 2017). We found that PF-05089771 blocked the Na\textsubscript{v}1.7 responses in the guinea pig trachea with IC\textsubscript{50} of 0.5 ± 0.2 µM and complete blockade at 3 µM (n=4, data not shown). The concentrations of Na\textsubscript{v}1.7 blockers Compound 13 and PF-05089771 required to inhibit the Na\textsubscript{v}1.7-mediated response in native environment of nerve terminals with the airway was about >10 fold greater than that required to inhibit Na\textsubscript{v}1.7 channels (in the inactivated state) in heterologous systems (Kocmalova et al., 2017).

We addressed the hypothesis that Na\textsubscript{v}1.8 supports the induction of TTX-resistant action potential discharge in the jugular C-fibers. In patch clamp recordings at the cell soma we found that the Na\textsubscript{v}1.8 blocker PF-01247324 (Payne et al., 2015) effectively, but not potently, inhibited the fast TTX-resistant current in guinea pig vagal sensory neurons (Fig. 4D). At 10 µM PF-01247324 the TTX-resistant current was inhibited by ~75% (Fig. 4D). In nerve-lung preparation experiments the bradykinin-induced action potential discharge in 11 jugular C-fibers was reduced by ~50% in the presence of the Na\textsubscript{v}1.8 blocker PF-01247324 10 µM (Fig. 4C). When TTX 1µM was combined with PF-01247324 10µM the response was nearly abolished (Fig. 4C).

C. TTX blocks conduction of action potentials in both nodose and jugular the vagal nociceptors.

Although TTX had no appreciable effect on action potential initiation in jugular C-fibers within the trachea or lungs, it effectively blocked the conduction of action potential in the majority of jugular C-fibers in the vagus nerve. In these experiments a segment of vagus nerve was
desheathed and TTX was applied to the vagus nerve outside the trachea either in superfusion or in a separate chamber and action potentials were evoked by citric acid applied to the tracheal lumen (Fig. 5A-B). TTX 1µM abolished the action potential discharge in 6 of 8 jugular C-fibers tested. The number of citric acid-induced action potentials was 57 ± 14 and 0 ± 0 before and after TTX. In 1 of 2 remaining jugular C-fibers that were not abolished by TTX 1µM applied to vagus nerve, TTX reduced the response by ~50% (from 91 to 47 action potentials) and adding PF-01247324 10µM to TTX completely abolished the response (0 action potentials). In the other jugular C-fiber TTX alone or together with PF-01247324 failed to inhibit action potential conduction, however, it was completely abolished by lidocaine 1mM.

In another set of experiments action potentials in jugular C-fibers were evoked by electrical stimulation (1Hz) of recurrent laryngeal nerve which caries jugular C-fibers that mostly terminate in the trachea or esophagus. TTX 1µM applied to the desheathed vagus nerve abolished electrically-induced action potentials in 6 of 9 jugular C-fibers. The action potential discharge frequency was reduced from 1±0 Hz to 0±0 Hz. Therefore, combined data from these two sets of experiments show that TTX 1µM applied to desheathed vagus nerve completely blocked action potential conduction in 70% (12/17) vagal jugular C-fibers (Fig. 5C).

We have previously found that TTX or a selective knockdown of Na\(_v\)1.7 gene expression in the nodose ganglion blocks action potential conduction in vagal nodose fibers (Muroi et al., 2011). Here we recorded action potentials in nodose Aδ-fibers innervating trachea evoked by electrical stimulation of their receptive field. We found that in all tested fibers, the action...
potential conduction was abolished by the NaV1.7 selective inhibitor Compound 13 1-10µM (Kocmalova et al., 2017) when applied to the desheathed vagus nerve (n=3).

D. Patch Clamp Electrophysiology

Figure 6A shows representative recordings of $I_{Na}$ from a nodose and a jugular neuron in the absence and presence of 1 µm TTX. Digital subtraction of currents recorded in the presence of TTX (TTX-resistant, TTX-R) from those in the absence of TTX gave the TTX-sensitive $I_{Na}$ (TTX-S). In both nodose and jugular neurons, TTX eliminated a fast activated and deactivated current component and unveiled the slower activated and deactivated TTX-R. Mean I-V curves of TTX-S and TTX-R $I_{Na}$ for nodose and jugular neurons are plotted in Figure 6B. The voltage-dependent properties of the $I_{Na}$ are similar between jugular and nodose neurons. In both cell types the activation for TTX-S $I_{Na}$ begins at about -65 mV and peaks around -30 mV; the main TTX-R component activates and peaks at voltages 10-15 mV more positive than those of TTX-S, consistent with previously described properties of NaV1.7-mediated TTX-S $I_{Na}$ and NaV1.8-mediated TTX-R $I_{Na}$ (Vijayaragavan et al., 2001). A much smaller (approximately 2-times smaller, Fig. 6B) component of TTX-R $I_{Na}$ observed at more negative voltage range under the present experimental conditions is also visible in nodose cells and may be mediated by NaV1.9 as previously reported (Coste et al., 2004).

The current density of NaV1.8-mediated TTX-R was 2-times greater in jugular neurons. (Fig. 6B). Indeed, the $G_{max}$ of TTX-R channels is significantly higher in jugular neurons than in nodose cells while the $G_{max}$ of TTX-S channels is not different in two types of cells (Fig. 6C). These results indicate that, in the presence of TTX, a larger amount of $I_{Na}$ is available to be activated at depolarized potentials in jugular neurons.
To evaluate whether the different TTX-R current density observed in nodose vs. jugular neurons is due to differences in channel regulation, we have examined the voltage dependence of steady-state activation and inactivation of TTX-R $I_{Na}$ in both cell types (n=6 each group). As shown in Table 2, the half-activation and half-inactivation voltages of TTX-R $I_{Na}$ in nodose and jugular neurons were the same, while the $G_{max}$ in jugular neurons was again higher than in nodose neurons (Tab. 2). These results suggest that the higher TTX-R current density observed in jugular neurons may be due to a higher number of functional NaV$_{1.8}$ channels.

To evaluate whether the increased TTX-R current density observed in jugular neurons plays a role in maintaining the cellular excitability after the TTX-sensitive $I_{Na}$ is eliminated, we have measured the minimal amount of current required to evoke an action potential in nodose and jugular neurons in the absence and presence of 1 µM TTX (Tab.3). TTX decreased the excitability of both nodose and jugular neurons leading to an increase in the amount of current required to elicit an action potential (Tab.3); the inhibitory effect of TTX effect, however, was substantively greater in the nodose vs. jugular neurons (10-fold vs. 4-fold increase in current, $P<0.01$, unpaired T-test, Tab.3).

Discussion

The data support two major conclusions regarding voltage-gated sodium channels and vagal nociceptors in the respiratory tract. First, action potential generation in nodose Aδ-fibers and nodose C-fibers terminating in the trachea and lungs, respectively, as well as the conduction of the action potentials along their fibers in the vagus nerve is strongly dependent on the
activity of Na\(_V\)1.7 channels. Second, the generation of action potentials in the jugular C-fiber nociceptors terminating in the trachea or lungs is not strictly dependent on Na\(_V\)1.7 or other TTX-sensitive channels, but the conduction of the action potential in the majority of their fibers in the vagus nerve is prevented by blocking TTX-sensitive channels (most likely Na\(_V\)1.7 and possibly Na\(_V\)1.6). The results from studies on jugular C-fibers presents a caveat when inferring data about Na\(_V\)1 subtype involvement at the nerve terminations within tissues from studies of action potentials conducted along the nerve trunk or in the neuronal cell bodies.

TTX applied only to the tracheal compartment, abolished citric acid-induced action potential discharge in capsaicin-insensitive nodose A\(\delta\)-fibers (cough receptors). This was most likely due to blockade of Na\(_V\) 1.7. This conclusion is based on the finding that in many cases Na\(_V\)1.7 was the only TTX-sensitive Na\(_V\)1 subunit expressed in the TRPV1-negative nodose neurons retrogradely labeled from the trachea (putative nodose A\(\delta\)-fiber neurons). In addition, the selective Na\(_V\)1.7 blocker, Compound 13, that we previously characterized in guinea pig airway parasympathetic nerves(Kocmalova et al., 2017), also abolished the response to citric acid in tracheal nodose A\(\delta\)-fibers.

When TTX was applied selectively to the tracheal compartment it had little effect on citric acid-induced action potential discharge in capsaicin-sensitive jugular C-fibers. Moreover, TTX only modestly increased the electrical stimulus voltage threshold for action potential genesis in the tissue. This lack of effect of TTX was unlikely due to insufficient TTX access to the jugular C-fibers in the tissue. The jugular C-fiber terminals are often found situated more superficially (closer to the lumen) than the nodose A\(\delta\)-fiber terminals (Fig. 1C)(Hunter
Undem, 1999) and are readily influenced by acid, bradykinin, capsaicin, lidocaine and other drugs applied to the lumen (Riccio et al., 1996a; Kajekar et al., 1999; Kollarik & Undem, 2002).

We addressed the question of whether the difference in TTX sensitivity was due to distinctions in ganglionic origin, i.e. the placodes-derived nodose neurons versus the neural crest-derived jugular neurons (Baker & Bronner-Fraser, 2001), or due to a difference in nerve phenotype; tracheal nodose fibers were \( \text{A}\delta\)-fibers, whereas the jugular fibers were unmyelinated C-fibers (Riccio et al., 1996a). We took advantage of the fact that both nodose and jugular neurons project C-fibers that terminated in the intrapulmonary compartment (Undem et al., 2004). Since the bradykinin-induced response in nodose C-fibers was completely abolished by selectively applying TTX to the intrapulmonary compartment (Fig. 4B), whereas the same treatment had no effect on the intrapulmonary jugular C-fibers, it would appear that the distinction is a consequence more of the ganglionic origin of the neuron than the afferent fiber phenotype (\( \text{A}\delta\)-fiber vs. C-fiber). As with the nodose \( \text{A}\delta\)-fiber in the trachea, the TTX-sensitivity of the nodose C-fibers in the lungs is likely due to blockade of Na\( _{\text{V}}\)1.7 channels. This is supported by two selective Na\( _{\text{V}}\)1.7 blockers, Compound 13 and PF-05089771 (Alexandrou et al., 2016; Kocmalova et al., 2017), mimicking TTX (Fig. 4B).

It was not surprising that blocking Na\( _{\text{V}}\)1.7 also almost completely (>95%) blocked the conduction of action potential in the nodose nerve fibers within the vagus. This is consistent with our previous finding where TTX or selectively blocking Na\( _{\text{V}}\)1.7 gene expression using AAV-shRNA technology, effectively blocked action potential conduction of nodose A- and C-
fibers (Muroi et al., 2011). It was more interesting to note that the conduction of action potentials along the jugular C-fiber in the vagus was also, in most cases (70%, 12/17 jugular C-fibers), abolished by TTX (Fig. 5). This reflected a stark distinction between the NaV1 subtypes required in jugular C-fibers for the genesis of action potentials within the tissue (TTX-insensitive) vs. their conduction in the vagus nerve (TTX-sensitive). This is the not the first time such an observation has been made. In elegant studies carried out on trigeminal C-fibers terminating in the cornea it was likewise found that the spike occurring at or near the nerve terminals was TTX-resistant (but blocked by lidocaine), whereas the conduction of the action potential to the CNS could be abolished by TTX (Brock et al., 1998).

It is likely that NaV1.8 channels play a dominant role in the induction of action potentials in jugular C-fibers. We found PF-01247324 (Payne et al., 2015) to be an effective, but not very potent, inhibitor of the putative NaV1.8 current in guinea pig vagal sensory neurons (Fig. 4D). At a concentration that inhibited this current by about 75%, the bradykinin-induced action potential discharge in jugular C-fibers was inhibited by an average of about 50% (range of 5%-95% inhibition) (Fig. 4C). In those neurons where the response was only partially inhibited by PF-01247324, it would appear it is due to a redundant effect of TTX-sensitive and NaV1.8 channels, as adding TTX to PF-01247324 led to the near abolition of the response (Fig. 4C).

The single neuron RT-PCR analysis indicates that nearly every airway specific nodose and jugular neurons expressed NaV1.7 and NaV1.8 (and some neurons expressed NaV1.6 as well). Moreover, the data obtained from whole cell patch clamp studies shows that either NaV1.7 or NaV1.8 is sufficient to support action potential genesis at the cell soma (Kwong et
The data also indicate that the density of Na$_{V}1.8$ in the jugular C-fibers within the tissue is sufficient to support acid- or bradykinin-induced action potential genesis, but this is not the case for nodose C-fibers and nodose A$\delta$-fiber. This may be due to a greater overall density of Na$_{V}1.8$ channels relative to other Na$_{V}1$ channels in the membranes of the neural crest-derived jugular neurons compared to the epibrachial placode-derived nodose neurons. Consistent with this is the observation that with respect to current densities at the cell soma, the Na$_{V}1.8$ to Na$_{V}1.7$ ratio is significantly larger in jugular neurons than nodose neurons (Fig. 6C). After TTX the current needed to evoke an action potential was similar between nodose and jugular neurons, but TTX had a much greater net effect (10-fold vs. 4-fold) on excitability due to the fact that the nodose neurons were more excitable at baseline than jugular neurons. This is also consistent with a recent RNAseq analysis of vagal C-fiber neurons in the mouse where the NaV1.8 expression is quantitatively greater in capsaicin-sensitive jugular neurons compared to capsaicin-sensitive nodose neurons (Wang et al., 2017).

The results have practical implications in the use of Na$_{V}1$ blockers as therapeutic drugs. We have previously shown that Na$_{V}1.7$ blocking drugs can effectively abolish bronchoconstriction due to activation of vagal post-ganglionic cholinergic nerves in human and guinea pig airways (but not mouse airways)(Kocmalova et al., 2017). In addition, we have found in previous studies and in the present study that blocking Na$_{V}1.7$ blocked action potential conduction in the vagus nerves and inhibited cough reflexes(Muroi et al., 2011; Muroi et al., 2013). One might surmise that drugs that block the neuronal communication between the respiratory tract and brain will not only inhibit cough, but may also vagal reflex bronchospasm and mucus secretion as well as dyspneic sensations. If the Na$_{V}1.7$ blocker drug were to be delivered via aerosol, however, the present findings indicate that it may effectively inhibit sensations and reflexes secondary to activation of respiratory nodose.
afferent nociceptors, but not jugular nociceptors. There are at least two caveats when
drawing therapeutic implications from this work: first, there may be distinctions between
human and guinea pig vagal sensory nerves with respect to NaV1 function; two, all studies
were carried in healthy animals, and Na\textsubscript{v1} subtype expression and function may change in
the face of tissue inflammation associated with airway pathology(Chahine & O'Leary, 2014).

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Author contributions.

M. Kol., H. S. and B. J. U. contributed to conception or design of the work, acquisition, analysis or interpretation of data for the work, and drafting the work or revising it critically for important intellectual content.

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F.R., S. N. M., M. Koc. and R. A. H contributed to acquisition, analysis or interpretation of data for the work, and drafting the work or revising it critically for important intellectual content.

All authors approved the final version of the manuscript, agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved, all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Competing Interests

The authors have no competing interests.

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Tables

Table 1. The guinea pig primer sequences for single cell RT-PCR

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<th>Target</th>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Product size (bp)</th>
<th>Genomic size (bp)</th>
<th>NCBI Reference Sequence:</th>
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<td>Forward</td>
<td>TCTCTCCAGCCCTCCTCC</td>
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Table 2. Steady-state activation and inactivation properties of TTX-R

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<th>Jugular neurons</th>
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<td>6</td>
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<tr>
<td>(G_{\text{max}}) (nS/pF)</td>
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<td>6.05 ± 0.64*</td>
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<tr>
<td>(V_{0.5}) (mV)</td>
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<td>-25.8 ± 3.5</td>
</tr>
<tr>
<td>k</td>
<td>5.2 ± 0.6</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>Inactivation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>(V_{0.5}) (mV)</td>
<td>-42.8 ± 1.6</td>
<td>-46.4 ± 2.0</td>
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<tr>
<td>k</td>
<td>-4.2 ± 0.3</td>
<td>-3.8 ± 0.2</td>
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</table>

* \(p<0.01\) vs. nodose neurons

Table 3. Effects of TTX on resting potential and excitability

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<table>
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<tr>
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<th>Nodose neurons</th>
<th>Jugular neurons</th>
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</thead>
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<td>RP (mV)</td>
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<tr>
<td>n</td>
<td>5</td>
<td>7</td>
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<tr>
<td>Before TTX</td>
<td>-69.8 ± 2.1</td>
<td>-66.9 ± 1.4</td>
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<tr>
<td>With TTX</td>
<td>-67.7 ± 1.7</td>
<td>-65.9 ± 1.3</td>
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<tr>
<td>Current to evoke AP</td>
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<td></td>
</tr>
<tr>
<td>(pA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Before TTX</td>
<td>25 ± 6</td>
<td>86 ± 27</td>
</tr>
<tr>
<td>With TTX</td>
<td>200 ± 45*</td>
<td>220 ± 78*</td>
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<tr>
<td>Fold increase</td>
<td>9.5 ± 2.7</td>
<td>4.0 ± 1.2&quot;</td>
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</tbody>
</table>

*P<0.05 vs. before TTX. **P<0.05 vs. nodose neurons.

Figures

Figure 1. Putative nerve terminals of nodose Aδ-fibers and jugular C-fibers in the guinea pig trachea. (A) Putative nodose Aδ-fiber and (B) jugular C-fiber visualized by the expression of green fluorescent protein (GFP) in wholemount of guinea pig trachea. Confocal images, adopted from (Mazzone & Undem, 2016). (C) Putative nerve terminals of nodose Aδ-fibers...
and jugular C-fibers are located close to tracheal lumen. Putative nodose Aδ-fiber is visualized by staining for α3 subunit NaK-ATPase (red, denoted by asterisk), and putative jugular C-fibers are visualized by staining for substance P (green, indicated by arrowheads). Confocal image of a thin cross section of the guinea pig trachea, adopted from (Mazzone et al., 2009). Note that the autoflorescence of epithelial cells (Epi) in green channel.

Figure 2. The expression of NaV1 α subunits mRNA in putative tracheal (A) nodose Aδ-fiber neurons and (B) jugular C-fiber neurons. Vagal nodose and jugular neurons were retrogradely labeled from the trachea. The putative nodose Aδ-fiber neurons were identified.
as labeled nodose neurons negative for TRPV1 mRNA (N=17), while putative jugular C-fiber neurons were identified as labeled jugular neurons positive for TRPV1 mRNA (N=31).

Figure 3. The effect of TTX on tracheal nociceptors. (A) A photograph and a schematic drawing of the experimental setup used for selective delivery of drugs to the tracheal lumen. (B) TTX 1µm applied to tracheal lumen (30 min) completely abolished citric acid (1mM)-induced action potential discharge in nodose Aδ-fibers (N=6, *P<0.05, paired T-test). (C) TTX 1µM only marginally inhibited citric acid (1mM)-induced action potential discharge in jugular C-fibers (N=31, *P<0.05, paired T-test). Adding lidocaine 1mM completely abolished this response in all jugular C-fiber tested (N=8, **P<0.01, paired T-test).
Figure 4. The effect of TTX and Na\textsubscript{v}1 blockers on intrapulmonary nociceptors. (A) A photograph and a schematic drawing of the experimental setup used for selective delivery of drugs to the lung. (B) TTX 1µm applied for 30 min into the lungs via airways and right pulmonary artery abolished bradykinin-induced action potential discharge in nodose C-fibers (N=6). The Na\textsubscript{v}1.7 selective blockers Compound 13 and PF-05089771 had similar effect (combined data are shown, Compound 13 10µM inhibited the response by 100±0%, N=3, and PF-05089771 3µM by 96±4%, N=4). (C) TTX 1µM alone had only an insignificant effect on bradykinin-induced action potential discharge in jugular C-fibers (N=9), however, the response to bradykinin was by 50% inhibited by Na\textsubscript{v}1.8 blocker PF-01247324 10 µM (N=11, *P<0.05, paired T-test) and >80% inhibited by combination of PF-01247324 10 µM and TTX.
1µM (N=7, *P<0.05, paired T-test). (D) Evaluation of the efficacy of PF-01247324 to inhibit TTX-resistant $I_{Na}$ in jugular neurons.

Figure. 5. TTX blocks conduction of action potentials in the majority of jugular C-fibers in the vagus nerve. (A) Schematic drawing of the isolated vagally-innervated tracheal preparation in an experimental chamber in which TTX and other drugs were applied selectively to the desheathed vagus nerve. (B) Representative trace of a jugular C-fiber response to citric acid
applied to the trachea before and 30 min after TTX 1µM was applied to the desheathed vagus nerve. The arrow denotes the artifact indicating the start of citric acid challenge. (C) The proportion of trachea jugular C-fibers in which the response to citric acid was completely abolished (i.e. no action potential was evoked by citric acid) by TTX 1µM applied to receptive field (0/31) vs. TTX 1µM applied to desheathed vagus nerve (12/17, **P<0.01, Fisher exact test). See text for details.

Figure 6. (A) Representative recordings of the total, TTX-resistant (TTX-R) and TTX-sensitive (TTX-S) I\textsubscript{Na} obtained from a nodose and a jugular neuron. TTX-R was recorded in

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the presence of 1µM TTX. TTX-S was obtained by digital subtraction of TTX-R from the total $I_{Na}$. (B) Mean I-V curves of TTX-S and TTX-R currents in nodose (n=13) and jugular (n=11) neurons. (C) Mean $G_{max}$ of TTX-S and TTX-R channels obtained in nodose (n=13) and jugular (n=12) neurons by Baltzmman fits to the individual conductance-voltage curves. * $p<0.001$. 

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