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Structure of vagal afferent nerve terminal fibers in the mouse trachea

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Abstract

The structure of primary afferent nerve terminals profoundly influences their function. While the complex vagal airway nerve terminals (stretch receptors, cough receptors and neuroepithelial bodies) were thoroughly characterized, much less is known about the structure of airway nerves that do not form distinct complex terminals (often termed free nerve fibers). We selectively induced expression of GFP in vagal afferent nerves in the mouse by transfection with AAV-GFP virus vector and visualized nerve terminals in the trachea by whole organ confocal imaging. Based on structural characteristics we identified four types of vagal afferent nerve fiber terminals in the trachea. Importantly, we found that distinct compartments of tracheal tissue are innervated by distinct nerve fiber terminal types in a non-overlapping manner. Thus, separate terminal types innervate tracheal epithelium vs. anterolateral tracheal wall containing cartilaginous rings and ligaments vs. dorsal wall containing smooth muscle. Our results will aid the study of structure-function relationships in vagal airway afferent nerves and regulation of respiratory reflexes.

Graphical Abstract

Keywords

vagus nerve; airways; nerve terminals; afferent; C-fibers; nerve structure

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1. Introduction

The main function of vagal afferent nerves in the airways is to regulate reflexes that protect respiratory system and optimize its function (Lee and Yu, 2014; Mazzone and Undem, 2016). The understanding of the structure of afferent nerve terminals is essential because the structure critically influences nerve function. A major progress has been made in elucidating nerve terminal structures of complex afferent nerve terminals in the airways (Adriaensen et al., 2015; Lee and Yu, 2014; Mazzone and Canning, 2013; Mazzone and Undem, 2016). Three types of complex afferent nerve terminals in the airways and lungs have been thoroughly characterized. The first type are nerve terminals termed smooth muscle associated terminals (SMATs) in the intrapulmonary airways that are functionally considered to be pulmonary stretch receptors (slowly adapting receptors, SARs) (Adriaensen et al., 2015; Yu et al., 2003). The second type are cough receptors found in the larynx, trachea and main bronchi (Baluk and Gabella, 1991; Mazzone et al., 2009; Yamamoto et al., 1994) that initiate cough (Canning et al., 2004). Finally, the third type are neuroepithelial bodies (NEBs) that are densely innervated groups of pulmonary neuroendocrine cells in the epithelium (Adriaensen et al., 2006; Adriaensen et al., 2015). NEB were implicated in several sensory functions, most notably airway oxygen sensing. In each of these complex terminals - SMATs (stretch receptors), cough receptors and NEBs - the knowledge of their structure was instrumental for advancing the understanding of their function.

In contrast, much less is known about the structure of vagal afferent nerve fibers that do not form distinct complex terminal structures. These afferent nerve fibers in the airways are often referred to as “fine nerve fibers”, “free nerve fibers” or “non-specialized nerve fibers”. It has been well established that fine nerve fibers form an extensive network (plexus) in the epithelium, and are also found in subepithelial layers including lamina propria, smooth muscle and adventitia (Baluk et al., 1992; Lamb and Sparrow, 2002; McDonald et al., 1988; Terada et al., 1992; Watanabe et al., 2005; Yamamoto et al., 1998). Beyond these seminal papers little is known about the structural organization and topographic distribution of fine non-specialized nerve fibers in the airways. Specifically, it is unknown what structural types of these fibers are present in the airways and what areas and layers of the airways they innervate.

The study of these questions has been hampered by the high density of nerve fibers in the airways and methodological difficulties to unequivocally distinguish vagal afferent nerve fibers from parasympathetic and sympathetic nerve fibers. Here we used transgene expression of GFP to selectively label vagal afferent nerve fibers and whole-organ confocal imaging to capture whole trachea with high resolution. We found that there are four distinct types of terminal fibers based on structural characteristics and tracheal areas and layers innervated. Specifically, we found that subepithelial layers in dorsal vs. anterolateral wall of the trachea are supplied by two distinct fiber types in a non-overlapping manner, and that the epithelium is innervated by additional separate fiber types. Our results provide the basis for the study of structure-function relationships in the vagal afferent nerve fibers in the trachea for better understanding of afferent regulation of respiratory reflexes.

2. Materials and methods

2.1 Transfection and tissue harvest

The experiments were approved by the Johns Hopkins Animal Care and Use Committee in accordance with National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Male C57/BL6 mice (6–10 weeks) were used. Left or right rostral cervical vagus nerve including afferent ganglia was surgically exposed in a brief anesthesia (50 mg/kg ketamine and 10 mg/kg xylazine, i.p.). AAV 2/8 (adeno-associated virus 2-based vectors pseudotyped with AAV8 serotype capsid (Penn Vector Core, Gene Therapy Program, University of Pennsylvania) in concentration of 10^{12} PFU and volume of 0.1–0.3 μ l was slowly (over 1–2 min) injected through a pulled aluminum-silicate pipette (tip diameter 50–100 μ m) into the vagal jugular/nodose ganglion complex. After 4–5 weeks, the animals were killed by CO₂ asphyxiation and exsanguination. The trachea was harvested, cut longitudinally through the anterior or dorsal wall, pinned stretched mucosal side up in a sylgard-lined dish and fixed in 4% formaldehyde at 4°C overnight. The tracheal tissues were then rinsed 3–4 times a day for 3–4 days at 4°C in PBS. In all experiments vagal ganglia were harvested and in some experiments brainstem was also harvested.

2.2 The whole-mount immunostaining of trachea

The tracheal wholemount tissues were incubated with antibodies in 0.5 ml tubes in order to expose both mucosal and adventitial surface to antibodies. In such conditions it is possible that a portion of the tissue will adhere to the tube wall or another portion of the tissue. In order to minimize the possibility that this adhering will interfere with the staining the tissues were frequently (as indicated below) repositioned - i.e. manipulated with blunt forceps in order to expose all surfaces and minimize adhering to the wall. The tissues were washed in PBS in 50 ml tubes on the rotor as indicated. The tissues were permeabilized in 1% Tween 20 (Sigma-Aldrich, St. Louis, MO) in filtered phosphate buffered saline (PBS) at room temperature for 6 hours, washed in filtered PBS (3 $\times$ 20 min on rotator), incubated with rabbit anti-GFP primary antibody (cat. number A11122, Molecular Probes, Thermofisher Scientific, Grand Island, NY) diluted 1:500 in 1% bovine serum albumin in PBS (PBS/BSA) for 48 hours at 4°C (repositioned 5–6 times during incubation), washed in filtered PBS (10 $\times$ 30 min on rotator) at 4°C, incubated with goat biotin - XX conjugate anti-rabbit IgG (H+L) secondary antibody (cat. number B2770, Molecular Probes) diluted 1:100 in PBS/BSA overnight at room temperature (repositioned 1–2 times during incubation), washed in filtered PBS (10 $\times$ 20 min on rotator) at 4°C, incubated with streptavidin, Alexa Fluor® 488 conjugate (cat. number S11223, Molecular Probes) diluted 1:100 in filtered PBS for 5 hours at room temperature, washed in filtered PBS (3 $\times$ 20 min on rotator at 4°C), incubated in anti-fade (pH=8.6) glycerol (Sigma-Aldrich) for 24 hours at room temperature and stored in anti-fade (pH=8.6) glycerol at 4°C. The trachea was then positioned mucosal side up on a glass slide, covered with coverslip 24 $\times$ 55 mm and weighted with load approximately 900 g for at least 48–72 h optimized in preliminary experiments in order to flatten the tissue without damaging epithelium and other layers. In the first stained preparation we found numerous randomly distributed oval solid artifacts (these artifacts are denoted by asterisks in Fig. 1, 3A and 4B). Optimization of staining procedure revealed that these artifacts

attributable to the use of 1% goat blocking serum in the first experiment. In control experiments omitting goat serum eliminated these artifacts, while including goat serum reproduced them. Therefore in all subsequent experiments goat serum was omitted.

2.3 Whole trachea imaging and analysis

Zeiss Axio Examiner microscope with LSM 880 confocal system and argon laser 488 nm was used for imaging (Carl Zeiss, Jena, Germany). Zeiss Zen software and NIH ImageJ (version 2.0.0-rc-34/1.50a) were used for image acquisition and processing. In order to investigate the course of the nerve fibers and nerve terminal structures throughout the whole thickness and whole area of the tissue the complete image of the whole organ (trachea) was acquired. This was accomplished by using Tile and Z-stack modules of Zen software. As illustrated in Figure 1, the whole area of the organ was divided into individual tiles by using the Tile function (each tile was the size of a single visual field that was determined by the properties of the objective used, see below) and for each tile confocal optical sections spaced 0.76–1 µm were obtained throughout the whole thickness of the tissue (up to 200 optical sections in the tissue on concave areas at the edges). In most experiments 20x objective (Zeiss Plan-Apochromat 20x/0.8 M27) with a single visual field of 424.7 µm x 424.7 µm was used and the image was acquired with the resolution of 1024 pixels x 1024 pixels. In one experiment (preparation 1) 10x objective was used to acquire visual fields of 849.8 µm x 849.8 µm with the resolution of 2048 pixels x 2048 (i.e. the overall resolution was the same). Because of logistic reason (acquisition duration >12h) most tracheas were imaged in 2 sessions. All nerve terminal structures were reviewed by at least 2 investigators.

3. Results

3.1 Expression of GFP in vagal afferent neurons following AAV-GFP injection into vagal jugular/nodose ganglion complex (JNG)

AAV2/8 was injected into the right and left vagal ganglion complex in 4 and 2 mice, respectively. The transfection was successful in all instances (Fig. 2). The efficacy of transfection was similar between the animals. Based on semi-quantitative evaluation of all wholemount vagal ganglia and the evaluation of GFP-positive profiles in cryostat sections of two ganglia, we estimate that 20–30% of vagal sensory neurons expressed GFP. As described before, the vagal sensory neurons in the mouse often form a single structure jugular/nodose ganglion (JNG) complex. The mouse JNG contains the neurons developmentally derived from embryonic placodes (nodose neurons) in the distal portion and the neurons derived from neural crest (jugular neurons) in the more proximal portion (Nassenstein et al., 2010; Surdenikova et al., 2012). We found that the neurons in both nodose and jugular portion of JNG expressed GFP following AAV-GFP injection (Fig. 2B). This is consistent with our previous observation that AAV-GFP induced GFP expression in both nodose and jugular neurons in the guinea pig (Kollarik et al., 2010b). As expected no GFP positive neurons were observed in contralateral JNGs that were not injected. Consistent with our previous report no GFP-positive neurons were observed in the brainstem including areas containing motor nuclei of the vagus nerves (N=2).

As described in methods, the tracheas were cut longitudinally through the middle of the anterior or dorsal wall and mounted mucosal side up. Stretch and pressure that were optimized in preliminary experiments were applied during fixation and mounting in order to flatten the trachea without damage to the epithelium and other layers. The GFP signal in the tracheal tissue was amplified by staining with anti-GFP antibody (see Methods). A complete image of each wholemount tracheal preparation was acquired by using tile and Z-stack functions of Zen imaging software with the resolution of approximately 0.4µm in X- and Y-axes and 0.75–1µm in Z-axis. By using this approach the GFP-positive vagal afferent nerve fibers could be studied in great detail and followed through the different layers and different areas of the tissues.

In total we evaluated 6 wholemount tracheal preparations numbered 1 through 6. Preparations 1 to 4 were from animals with AAV-GFP injection into right vagal ganglia, and preparations 5 and 6 from the animals with AAV-GFP injection into left vagal ganglia. The trachea anatomically consists of the cartilaginous rings connected by ligaments that together comprise the anterolateral tracheal wall, and the dorsal membranous wall containing airway smooth muscle (Inset in Fig. 1). In order to preserve complete dorsal wall the tracheas were cut through the anterior wall (preparations 1 and 2). In order to preserve complete anterolateral wall, the tracheas were cut through the dorsal wall (preparations 3 and 4). It should be noted that the membranous part was stretched in preparations cut through the anterior wall (preparations 1 and 2) but contracted in the preparations cut through dorsal wall (preparations 3 and 4).

For the purpose of this discussion we chose descriptive terms to label different vagal afferent fiber terminal types in the trachea based on their location and gross microscopic structure. We identified four distinct groups of vagal afferent fiber terminal types: large branching mucosal terminals, small intraepithelial terminals, anterolateral segmental arrays and dorsal terminal structures.

3.2 Large branching mucosal terminals

Large branching mucosal terminals have a composite structure as branching nerve terminals innervating overlapping areas of epithelial cells and thus forming a complex network (Fig. 3). In the lamina propria beneath the epithelium there were several (up to 4) generations of branching of the parental axons (Fig. 3C). Terminal branches of large branching mucosal terminals in the epithelium run around lateral and basal domains of epithelial cells, often around the whole circumference of individual epithelial cells (Fig. 3E–G). Large branching mucosal terminals were localized exclusively in the laryngeal end of the mouse trachea. The largest and most complex large branching mucosal terminals were found near the midline of dorsal tracheal wall above cricotracheal ligament (Fig. 3I, Fig. 9B). The parental axons of large branching mucosal terminals were arranged in bundles. In some instances the large branching mucosal terminals could be traced back to a single axon.

3.3 Small intraepithelial terminals

In addition to large branching mucosal terminals we also observed small intraepithelial nerve terminals (Fig. 4). The size of these intraepithelial nerve terminals was 150–350µm

compared to large branching mucosal terminals that were typically $>1500\mu\text{m}$ (Fig. 3). The number of small intraepithelial nerve terminals was relatively small in most preparations, specifically 5, 0, 12 and 2 in preparations 1, 2, 3 and 4, respectively (Fig. 9B). Small intraepithelial terminals were located in the membranous part of the trachea and sometimes in the proximal laryngeal portion, but not in the anterolateral wall (e.g. not above cartilage rings or ligaments) (Fig. 4D, Fig. 9B). It is possible that there were more than one type of small intraepithelial terminals (examples in Fig. 4), however, the variability of their structure and low numbers precluded more thorough analysis.

3.4 Dorsal terminal structures

The term dorsal terminal structure was chosen because of localization of these structures in the dorsal membranous part of the trachea. Only rarely small branches of these structures extended into ligamentous or cartilage parts (Fig. 5E, G Fig. 9B). (Fig. 6A).

The bundles, branches and terminations of dorsal terminal structures were found in all subepithelial layers of tracheal dorsal wall, from mucosal lamina propria through tracheal smooth muscle to inner adventitia (Fig. 5A–C), but not in the epithelium. Most of dorsal terminal structures arose from a bundle of several axons in adventitia (example is shown in Fig. 6A). Occasionally, a single dorsal terminal structure could be reliably traced to a single axon that ramifies richly (example in Fig. 6C). Several generations of ramification (from two to five) typically sprouting into various directions can be observed (Fig. 6C). The branches often traveled through all layers (i.e. from lamina propria to adventitia), sometimes repeatedly, before further branching or terminating (Fig. 5C1–C3). The predominant orientation of the branches in lamina propria was parallel to the mucosal surface, while they often run through smooth muscle perpendicularly to the mucosal surface (Fig. 5C1–C3).

Individual dorsal terminal structures as well as different parts of same dorsal terminal structure often overlapped and crossed each other branches. Smaller individual dorsal terminal structures ranged $200\text{--}500\mu\text{m}$ in diameter (examples in Fig. 9B, preparation 2). Because of the overlaps it was not always possible to establish the size of larger dorsal terminal structures. The largest dorsal terminal structure traced to a single axon occupied an area approximately $1300\mu\text{m} \times 400\mu\text{m}$ (Fig. 6C) demonstrating that some of dorsal terminal structures are relatively large. It should be noted that the membranous part was retracted in tracheal preparations cut through the dorsal wall (preparations 3 and 4, Fig. 9B) therefore the circular dimension of dorsal terminal structures was reduced.

3.5 Anterolateral segmental arrays

Figures 7 and 8 show examples of a complex structure and a relatively simple structure of anterolateral segmental arrays, respectively, to illustrate morphological characteristics of this vagal afferent nerve fiber type. Anterolateral segmental array were localized in the mucosa almost exclusively above tracheal (angular) ligaments (i.e. in the ligamentous part). A typical example of this localization is shown in Fig. 7C and the localizations of all visualized anterolateral segmental arrays is shown in Fig. 9B. Most branches of segmental fibers run in lamina propria just underneath the epithelium but the branches did not extend between the epithelial cells.

Anterolateral segmental arrays run in parallel with cartilage rings and ramify richly (Fig. 7, 8). They often and repeatedly branch into the opposite direction (example in Fig. 8A) or form a U-shaped loops (Fig. 8D). The U-shaped loops are typically found at the border of ligamentous and membranous part, but could be anywhere in course of segmental terminal fibers. The majority of axons and branches comprising anterolateral segmental array were located above the ligaments, nonetheless, short portions of branches extended into the mucosa above the cartilage rings, and occasionally branches crossing the cartilage ring were observed (Fig. 8). With few exceptions all segmental fiber innervated mucosa only above a single ligament. In some segmental fibers a connection to a single fiber ascending through deeper layers from adventitia (possibly a parent axon) could be identified (Fig. 8).

3.6 Topographical distribution of vagal afferent nerve terminal types in the mouse trachea

For mapping of GFP-positive nerve terminals in the trachea we used two strategies that we termed here as detailed drawing and schematic drawing for the purpose of the discussion. Detailed drawing was performed by using graphic functions of Zen software directly over optical sections and projections and all detectable GFP-positive fibers were captured and drawn. Examples of detailed drawings are shown in Fig. 6A, 6C, 7D, 8D. This approach was also used to map the whole preparation 1 as shown in Fig. 9A. However, this approach was extremely effort intensive and therefore schematic drawing was explored as an alternative approach. Schematic drawing was performed by using graphic software paint.net based on optical section and projections, was less effort intensive and captured main structural characteristics of the fibers. Comparison of detailed and schematic drawing of the same preparation 1 (Fig. 9A vs. Fig. 9B, preparation 1) shows that the schematic drawing sufficiently captures the size and complexity of terminal fibers and structures. This observation is also corroborated by comparison of detailed drawings of segmental terminal fibers in preparations 3 (Fig. 8) and in preparation 4 (Fig. 7), vs. the same structures drawn schematically in preparations 3 and 4 (Fig. 9B) as well as the detailed drawing of deep terminal arrays in preparation 3 (Fig. 5D–E) vs. schematic drawing of the same structures in preparation 3 (Fig. 9B).

Thorough evaluation of the mapped preparations revealed distinct distribution of vagal afferent nerve terminals in the mouse trachea. The large branching mucosal terminals locate exclusively in the rostral trachea, dorsal terminal structures in the dorsal membranous part of the trachea, anterolateral segmental arrays in the ligamentous part between cartilage rings with characteristic orientation parallel to cartilage rings, and small intraepithelial terminals to the membranous part of the trachea. The comparison of preparations from animals with transfected right (preparations 1–4) and left (preparations 5 and 6) vagal ganglion complex revealed that both right and left vagal afferent neurons project the same nerve fiber types and innervate both ipsilateral and contralateral trachea (Fig. 9B).

It should be noted that intentionally only a proportion (estimated 20–30%) of vagal afferent neurons were transfected (Fig. 2). Therefore, we presume that only approximately 20–30% of vagal afferent nerve fibers in the trachea are visualized. Because of that and because only variable portions of main bronchi were present in each preparation, it was not surprising that we did not observe many stretch receptors that are typically found in smaller airways (Yu et

al., 2003). In the 6 preparations analyzed only 2 structures that resembled the stretch receptors were observed. Figure 10 shows one such structure.

4. Discussion

We identified at least four distinct types of nerve terminal structures of fine (“free”) vagal afferent nerve fibers in the mouse trachea. We descriptively termed these structures as large branching mucosal terminals, small intraepithelial terminals, anterolateral segmental arrays and dorsal terminal structures. Importantly, we found that distinct compartments of mouse tracheal tissue are innervated by distinct types of afferent nerve terminal structures in an exclusive almost non-overlapping manner. Thus, large branching mucosal terminals and small intraepithelial terminals innervate the epithelium, anterolateral segmental arrays innervate subepithelial layers of anterolateral tracheal wall, and dorsal terminal structures innervate subepithelial layers of dorsal membranous wall including the tracheal smooth muscle. While many aspects of afferent innervation of the airways have been elucidated before, this is to our knowledge the first comprehensive description of different types of nerve terminal structure of fine vagal afferent nerve fibers.

Our results pave the way for the study of structure-function relationships in vagal airway afferent nerves. Our results also enable the analysis of expression of receptors and other molecular markers in the terminals of different types of fine vagal afferent nerve fibers in the trachea by using transgenic mice and/or immunostaining techniques (i.e. characterization of neurochemical phenotype). In addition, our results laid the foundation evaluating neurophysiological properties of different fiber types as well as the reflex consequences of their activation.

Neurons in both jugular and nodose portion of jugular/nodose ganglion complex were effectively transfected (Fig. 2). Our previous studies showed that AAV-GFP vectors transfect both A- and C-fiber neurons (Kollarik et al., 2010a). It is therefore expected that all types of vagal afferent nerve terminals in the airways were visualized. The injection of AAV-GFP into vagal afferent ganglia (JNG) leads to very selective expression of GFP in vagal afferent neurons. Indeed, consistent with our previous studies (Kollarik et al., 2010a) no GFP-positive neurons were found in the brainstem including the areas containing vagal motor nuclei. Similarly, no GFP positive neurons were found in the airway parasympathetic ganglia. This supports the conclusion that all GFP-positive fibers in the trachea were vagal afferent nerve fibers. Such selectivity is a major advantage of AAV-GFP transfection approach over many tracing and staining techniques that do not discriminate between afferent nerve fibers and other types of nerve fibers. Another advantage is that by decreasing the dose of virus the number of transfected neurons could be intentionally decreased. Thus, only a proportion of nerve terminals is visualized and individual terminals can be analyzed more easily as in the condition when all terminals are transfected.

Large branching mucosal terminals were located exclusively in the rostral trachea most often near the midline of dorsal tracheal wall (Fig. 9). These fibers repeatedly branched in lamina propria and terminated between epithelial cells (Fig. 3). Nerve fibers structurally identical to the mouse large branching mucosal terminals have been previously described in all species

studied thus far. Specifically, by using PGP9.5 or TRPV1 immunostaining, these fibers (described as dense plexus or network of fine varicose fibers) were demonstrated in the tracheal epithelium of rats (Baluk et al., 1992; Jeffery and Reid, 1973; Kusindarta et al., 2004; Terada et al., 1992), guinea pigs (Watanabe et al., 2006; Watanabe et al., 2005), dogs (Yamamoto et al., 1998; Yamamoto et al., 2007), pigs (Lamb and Sparrow, 2002) and humans (Lamb and Sparrow, 2002; West et al., 2015). Similar to our findings, previous studies showed that these intraepithelial fibers are produced by several generations of branching of parental axons in lamina propria (Lamb and Sparrow, 2002).

Because these fibers were positive for substance P (evaluated in rats and pigs) (Baluk et al., 1992; Lamb and Sparrow, 2002) it is often concluded that they are C-fibers terminals. This is corroborated by their sensitivity to capsaicin treatment demonstrated in rats (Baluk et al., 1992; Bowden et al., 1996) and TRPV1 expression demonstrated in dogs and guinea pigs (Watanabe et al., 2006; Watanabe et al., 2005; Yamamoto et al., 2007). Because substance P is found in vagal jugular neurons, but only in a few nodose neurons, it is very probable that these fibers are C-fibers originating from the jugular ganglia. Indeed, in the guinea pig this fiber type is considered to be a jugular C-fiber (Mazzone and Undem, 2016) consistent with previous observations in the rat (McDonald et al., 1988). It is therefore probable that large branching mucosal terminals in the mouse trachea are terminals of jugular C-fibers. Their appearance is similar to that reported in epithelium from human biopsies (West et al., 2015).

The topographic distribution of branching mucosal terminals was only reported in the rat in which their density was the largest in the proximal trachea and decreased progressively to less than a half of that in the bronchus (Baluk et al., 1992). Although the studies from other species referenced above do not explicitly comment on the distribution of these terminals, they were found in various portions of the trachea. Such distribution contrasts with our observation that large branching mucosal terminals were located exclusively in the proximal trachea of the mouse (Fig. 9). This suggests interspecies differences in the innervation by large branching mucosal terminals between mouse and other species. Nonetheless, such restricted location in the mouse may be advantageous for the study of their properties and function.

The terminal fiber type analogous to small intraepithelial terminals described in this study has not been to our knowledge reported in the trachea. This is most probably due to their relative paucity (3 of 4 mouse tracheas had only 0 to 5 small intraepithelial terminals). Their identification in other species may be further impeded by a dense intraepithelial innervation by large branching mucosal terminals that are more widely distributed in other species (see discussion above). It cannot be excluded that some of the small intraepithelial terminals innervate neuroepithelial bodies (NEB) or are part of some other specialized sensory structures (Krasteva et al., 2011).

Anterolateral segmental arrays were located in the lamina propria of the anterolateral wall above the ligaments connecting cartilaginous rings and extended above the edges of the rings (Fig. 7, 8 and 9). Anterolateral segmental arrays had characteristic orientation parallel to cartilaginous rings (Baluk et al., 1992). In most instances (>80%) a single anterolateral segmental array innervated the area of a single ligament (i.e. single segment) (Fig. 9,

example in Fig. 7). In the remaining instances a single segmental array innervated two neighboring ligaments and adjacent cartilaginous rings (example in Fig. 8). Because of this segmental arrangement, anterolateral segmental arrays can in theory provide the information on the localization of the stimulus on the long tracheal axis, i.e. whether the stimulus is located more rostrally or caudally. Whether this organotopic arrangement has any functional consequences remains to be investigated.

Tracheal segmental arteries also run above the ligaments and have orientation parallel to cartilaginous rings. Because blood vessels were not visualized in our experiments, future studies are needed to elucidate the relationship of anterolateral segmental arrays to tracheal vasculature that is quite complex in anterolateral wall (McDonald, 1988). Previous studies demonstrated substance P-positive fibers running along the segmental arterioles in lamina propria above the ligaments in the rat (Baluk et al., 1992) and CGRP-positive fibers with similar orientation were found in lamina propria in the same area in the dog (Terada et al., 1992). The association of substance P-positive fibers with blood vessels was also noted in the guinea pig (Mazzone et al., 2010).

Nerve fibers in the tracheal muscle have been described in all species evaluated including mice (Baluk and Gabella, 1991; Baluk et al., 1992; Cadieux et al., 1986; Nassenstein et al., 2015; Uddman et al., 1984; Watanabe et al., 2006; Watanabe et al., 2005) and humans (West et al., 2015). The majority of nerve fibers in the smooth muscle are thought to be efferent parasympathetic cholinergic and in some species also sympathetic adrenergic nerve fibers. Nonetheless, markers often expressed in afferent fibers including substance P, CGRP and TRPV1 were noted in a small proportion of nerve fibers in airway smooth muscle (Baluk et al., 1992; Watanabe et al., 2006; Watanabe et al., 2005).

Our tracing technique allowed for a comprehensive description of nerve fiber terminal structures in the tracheal muscle. We found that the only vagal afferent fiber type innervating the muscle in the mouse trachea were dorsal terminal structures (termed so descriptively because of their exclusive location to dorsal tracheal wall) (Fig. 5A–C). Importantly, dorsal terminal structures innervated not only the tracheal smooth muscle, but also other subepithelial layers including lamina propria and adventitia, extensively and repeatedly ramified often traveling up and down through all layers before further branching or terminating (Fig. 5C1–C3), and were sometimes very large (Fig. 6A, C). Therefore dorsal terminal structures directly connect mucosa (lamina propria) with tracheal smooth muscle which may be an anatomical substrate for local axon reflexes. In this context it is potentially important that no terminal structures were found to innervate both smooth muscle and anterolateral wall, or both smooth muscle and epithelium. As discussed above large branching mucosal fibers and small intraepithelial terminals were restricted to the mucosa, and the anterolateral segmental arrays were restricted to anterolateral wall (Fig. 9). Intriguingly, functional staining of TRPV1 agonist responsive (TRPV1-positive) nerve fibers in the mouse trachea revealed subepithelial TRPV1-positive nerve fibers similar to dorsal terminal structures (Canning, 2011) suggesting possibility that dorsal terminal structures are a subset of C-fibers.

As expected cough receptors identified in the guinea pig and dog (Baluk and Gabella, 1991; Mazzone and McGovern, 2006, 2008; Mazzone et al., 2009; Yamamoto et al., 1995; Yamamoto et al., 1994) were not present in the mouse trachea. The cough receptors are also virtually absent in the rat trachea (Baluk and Gabella, 1991; Mazzone et al., 2009; Yamamoto et al., 1999). This is consistent with the notion that the mechanical stimulation of the trachea is ineffective to evoke cough in the mouse and rat (Mazzone et al., 2009) (although chemical stimuli were reported to evoke cough in the mice (Zhang et al., 2017)). It is improbable that the cough receptors were not labeled in our experiments, because AAV-GFP transfection is very effective to visualize cough receptors in the guinea pig (Kollarik et al., 2010b) and was also effective to label stretch receptor (SMATs that is structurally similar to cough receptor) in the mouse bronchus (Fig. 10).

Unilateral injection into the right vagal ganglia resulted in bilateral visualization of nerve fibers in the trachea (Fig. 9B). The comparison of the results obtained in two animals in which AAV-GFP was injected into left vagal ganglia also showed similar bilateral pattern (Fig. 9B). These observations indicate that vagal afferent nerves from both left and right vagal ganglia innervate the trachea bilaterally.

We cannot rule out the possibility that some nerve terminals are not entirely filled with GFP that is synthesized in the cell bodies and transported through the axons. Nonetheless, the known airway and other visceral nerve terminal structures were previously found to be effectively visualized by our technique. For example, we demonstrated AAV-GFP visualization of the guinea pig tracheal cough receptors and esophageal intraganglionic laminar endings (Kollarik et al., 2010a) which were identical to those reported previously by others (Mazzone et al., 2009; Zagorodnyuk et al., 2001; Zagorodnyuk et al., 2003). Furthermore, the large intraepithelial terminals described in this study (Fig. 3) are structurally similar to those reported by others using immunostaining approaches (for example (Lamb and Sparrow, 2002)). However, the possibility that some structures are less effectively visualized by GFP should be kept in mind.

Neurophysiological properties of vagal afferent nerve fibers innervating the mouse trachea have not been to our knowledge reported. It is possible that our results will stimulate the progress in the understanding of the function of different types vagal afferent nerve fibers in the trachea in a manner similar to how the understanding of the terminal structure of stretch receptors and NEB has advanced the understanding of their function (Adriaensen et al., 2006; Adriaensen et al., 2015; Yu et al., 2003). Such functional studies will be probably also aided by histological analysis of expression of receptors, ion channels and other relevant molecules (i.e. neurochemical phenotype) in different terminal fiber types enabled by our study. Our results also provide anatomical substrate for the speculation that stimulation of the dorsal tracheal may evoke different reflex responses than the stimulation of anterolateral wall as dorsal vs. anterolateral walls are innervated by distinct fiber types (dorsal terminal structures vs. anterolateral segmental arrays). To our knowledge such hypothesis has not been investigated yet.

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Highlights

- Little is known about terminal structures of free afferent nerve fibers in airways.
- Virus vector was used to visualize vagal afferent terminals with GFP in the mouse.
- Distinct compartments of trachea are innervated in non-overlapping manner.
- Distinct fiber types innervate epithelium vs. anterolateral vs. dorsal tracheal wall.

Figure 1. Imaging of the whole trachea

The image was obtained by joining 11×7 square tiles as indicated by the grid. Each tile is a single optical section $849.8 \mu\text{m} \times 849.8 \mu\text{m}$ imaged with resolution of 2048 pixels $\times$ 2048 pixels. Because the rostral portion of trachea is thinner than the caudal portion, this image shows the epithelium (E) in the rostral part and deeper structures including cartilage rings (C) and membranous part (M) in the more distal parts. *Inset*: Schematic drawing of this tracheal preparation, (L) denotes ligamentous parts between tracheal rings. *Asterisk indicates accumulation of the staining artifacts in this preparation (see methods for details).

Figure 2. Transfection of mouse jugular/nodose ganglion (JNG) complex
(A) Wholemount microphotograph of right JNG four weeks following the injection of AAV-GFP into the JNG. (B–C) Higher magnification showing transduced GFP-positive neurons in both the nodose and jugular portion of JNG.

Figure 3. Large branching mucosal terminals

(A) Average projection of optical sections from the epithelium showing the whole large branching mucosal terminal structure. (B) Scaled down (approximately 1:3) image from panel (A) indicating locations of panels (C) through (G). (C) Repeated branching (arrowheads) of one of the main axons. The topmost optical sections with some intraepithelial structures were removed in order to better visualize branching points. This axon supplied multiple overlapping intraepithelial terminal structures exemplified in (D–F). (D–F) Details of intraepithelial terminals. Note that segments of intraepithelial terminal fibers run between epithelial cells. (G) Detail in adventitia of two axons supplying these large branching mucosal terminals. The whole terminal structure could be with reasonable confidence traced back to these two parental axons. (H) Scaled down image in panel (A) showing how the areas with intraepithelial terminals were demarcated for terminal mapping in panel (I). (I) The location and size of these large branching mucosal terminals in the wholemount trachea preparation. Note the location of large branching mucosal terminals to the rostral part of the trachea (see Fig. 9 for the distribution of large branching mucosal

terminals in all preparations). Preparation number 1 was a trachea cut along the long axis through the anterior wall. *The asterisks in (A, B and H) denote staining artifacts (see methods for details).

Figure 4. Small intraepithelial terminals

(A –C) Examples of various structures of small intraepithelial terminals. Arrowheads indicate parent axons. The numbers 1–3 in (C) indicate terminal structures originating from the same parent axon, the structures 1 and 2 overlapped. (D) The location and size of these small intraepithelial terminals in the wholemount trachea preparations (see Fig. 9 for the distribution of all identified small intraepithelial terminals). Preparation number 1 was a trachea cut along the long axis through the anterior wall, and preparation 3 was cut through the dorsal wall. *The asterisks in (B) denote staining artifacts (see methods for details).

Figure 5. Dorsal terminal structures

(A) Schematic drawing of the layers in the dorsal membranous part of trachea (does not contain cartilage rings). The tissue layers from the lumen are: mucosa, composed of surface epithelium (Epi), subepithelial lamina propria and submucosa (LP), smooth muscle (SM), and adventitia (Ad). (B) Average projections of optical sections from the epithelium and (C) from subepithelial lamina propria through smooth muscle and an inner portion of adventitia. Note that there are no GFP-positive fibers or nerve terminals in the epithelium (B) despite dense innervation of subepithelial layers (C). (C1–C3) The layers combined in panel (C) are shown separately. (C1) Average projections of optical sections from lamina propria only, (C2) from smooth muscle only, and (C3) from the inner portion of adventitia only. Note that in the subepithelial layer (C1) the nerve fiber segments run in parallel with the luminal surface, but they mostly run perpendicularly to luminal surface in the smooth muscle (C2, some examples are indicated by arrowheads). The fibers were much less organized in adventitia (C3).

Figure 6. Dorsal terminal structures

(A) Detailed drawing of the dorsal terminal structures shown in Fig. 5B–C. These dorsal terminal structures originated from several separate axons indicated by arrowheads. The area shown in Fig. 5B–C is indicated. (B) The location and size of the dorsal terminal structures from (A) in the wholemount trachea preparation. Preparation 3 was a trachea cut along the long axis through the dorsal wall. (C) An example of a dorsal terminal structure from a different trachea (preparation 1) that was traced to a single parental axon (arrowhead). (D) The location of the dorsal terminal structure from (C) in the wholemount tracheal preparation. Preparation 1 was a trachea cut along the long axis through the anterior wall. Note that the dorsal terminal structures locate in the membranous part of the trachea (see Fig. 9 for the distribution of all identified dorsal terminal structures).

Figure 7. Anterolateral segmental array

(A) Average projection of optical sections from lamina propria. Because of a concave shape of the cartilaginous portion of the trachea epithelial cells are visible in the upper part of the image. (B) Detailed drawing of the whole anterolateral segmental array terminal structure. The area shown in (A) is highlighted. (C) The location and size of this anterolateral segmental array in the wholemount trachea preparation. Note the typical location and orientation of anterolateral segmental array above the tracheal (angular) ligament parallel to tracheal rings (see Fig. 9 for the distribution of all identified anterolateral segmental arrays). Preparation number 4 was a trachea cut along the long axis through the dorsal wall.

Figure 8. Anterolateral segmental array innervating two neighboring ligaments

An example of a relatively simple anterolateral segmental array that can be traced to a single parent axon and innervates two neighboring ligaments. (A–C) Details of branching and terminations. Average projections of optical sections that include the epithelium to illustrate the proximity of the anterolateral segmental array in the lamina propria to the epithelium. Long segments of terminal branches are located just underneath the epithelium but do not penetrate between the epithelial cells. Arrowhead indicates the parent axon. The asterisks indicate subepithelial glands. (D) Detailed drawing of this anterolateral segmental array. The areas shown in panels (A–D) are indicated. (E) The location and size of this anterolateral segmental array in the wholemount trachea preparation. Preparation number 3 was a trachea cut along the long axis through the dorsal wall.

Figure 9. Maps of vagal afferent nerve terminal types in the mouse trachea

(A) Detailed drawing of the terminal fibers and structures in the preparation 1. Detailed drawing was performed by using graphic functions of Zen software directly over optical sections/projections. By this approach all detected GFP-positive terminal fibers in the preparation 1 were captured. Vagal nerve branches and nerve boundless in adventitia are omitted for clarity. The areas in which the epithelium has been damaged are denoted in gray.

(B) Schematic drawing of the terminal fibers and structures in 6 evaluated preparations. Schematic drawing was performed by using graphic software paint.net based on optical sections and projections. The comparison of detailed drawing of preparation 1 in (A) and

schematic drawing of the same preparation 1 in (B) shows that the schematic drawing that was more effort effective similarly captures the size and complexity of terminal fibers and structures. See text for more details. Note characteristic location of large branching mucosal terminals in the rostral trachea, dorsal terminal structures in the dorsal membranous part of the trachea, anterolateral segmental arrays in the ligamentous part between cartilage rings with characteristic orientation parallel to cartilage rings, and small intraepithelial terminals to the membranous part of the trachea. Preparations 1 to 4 were from animals with AAV-GFP injection into right vagal ganglia, and preparations 5 and 6 from the animals with AAV-GFP injection into left vagal ganglia. Preparations 1, 2, 5 and 6 were tracheae cut along the long axis through the dorsal wall, and preparations 3 and 4 were cut through the anterior wall. Preparations 5 and 6 were tracheae cut along the dorsal wall. Because only 25–30% of vagal afferent neurons were intentionally transfected it is presumed that only approximately 25–30% of nerve fibers in each trachea are transfected.

Figure 10. Putative stretch receptor in the bronchial muscle

An example of a nerve terminal with the structure similar to that of stretch receptors described in other species (Yu et al., 2003). This structure was located in a main bronchus in the layers topographically consistent with the airways smooth muscle.