

TRPV1 induction in airway vagal low-threshold mechanosensory neurons by allergen challenge and neurotrophic factors

Tina Marie Lieu, Allen C. Myers, Sonya Meeker and Bradley J. Undem

Am J Physiol Lung Cell Mol Physiol 302:L941-L948, 2012. First published 17 February 2012;
doi: 10.1152/ajplung.00366.2011

You might find this additional info useful...

This article cites 32 articles, 16 of which you can access for free at:

<http://ajplung.physiology.org/content/302/9/L941.full#ref-list-1>

Updated information and services including high resolution figures, can be found at:

<http://ajplung.physiology.org/content/302/9/L941.full>

Additional material and information about *American Journal of Physiology - Lung Cellular and Molecular Physiology* can be found at:

<http://www.the-aps.org/publications/ajplung>

This information is current as of May 15, 2013.

TRPV1 induction in airway vagal low-threshold mechanosensory neurons by allergen challenge and neurotrophic factors

Tina Marie Lieu, Allen C. Myers, Sonya Meeker, and Bradley J. Undem

The Johns Hopkins University Medical Institutions, Baltimore, Maryland

Submitted 22 November 2011; accepted in final form 16 February 2012

Lieu T, Myers AC, Meeker S, Undem BJ. TRPV1 induction in airway vagal low-threshold mechanosensory neurons by allergen challenge and neurotrophic factors. *Am J Physiol Lung Cell Mol Physiol* 302: L941–L948, 2012. First published March 2, 2012; doi:10.1152/ajplung.00366.2011.—We addressed the hypothesis that allergic inflammation in guinea pig airways leads to a phenotypic switch in vagal tracheal cough-causing, low-threshold mechanosensitive A δ neurons, such that they begin expressing functional transient receptor potential vanilloid (TRPV1) channels. Guinea pigs were actively sensitized to ovalbumin (OVA) and beginning 21 days later exposed via aerosol to OVA daily for 3 days. Tracheal-specific neurons were identified in the nodose ganglion using retrograde tracing techniques. Tracheal specific neurons were isolated, and mRNA expression was evaluated at the single-neuron level using RT-PCR analysis. Electrophysiological studies have revealed that the vast majority of vagal nodose afferent nerves innervating the trachea are capsaicin-insensitive A δ -fibers. Consistent with this, we found <20% of these neurons express TRPV1 mRNA or respond to capsaicin in a calcium assay. Allergen exposure induced de novo TRPV1 mRNA in a majority of the tracheal-specific nodose neurons ($P < 0.05$). The allergen-induced TRPV1 induction was mimicked by applying either brain-derived neurotrophic factor (BDNF) or glial-derived neurotrophic factor (GDNF) to the tracheal lumen. The BDNF-induced phenotypic change observed at the level of mRNA expression was mimicked using a calcium assay to assess functional TRPV1 ion channels. Finally, OVA exposure induced BDNF and GDNF production in the tracheal epithelium, the immediate vicinity of the nodose A δ -fibers terminations. The induction of TRPV1 in nodose tracheal A δ -fibers would substantively expand the nature of stimuli capable of activating these cough-causing nerves.

asthma; C-fiber; capsaicin; vagus

IN THE SOMATOSENSORY SYSTEM, inflammation can lead to a so-called phenotypic switch in which low-threshold touch-sensitive mechanosensitive A β -fibers take on a nociceptor phenotype by expressing de novo substance P, a neuropeptide typically limited to C-fiber neurons. When substance P is released from the central terminals of the A β -fibers upon light touch, the excitability of spinal cord neurons is increased, leading to the hypothesis that the phenotypic switch may contribute to the phenomenon of allodynia (21). We have noted that allergic inflammation of the respiratory tract leads to a similar phenotypic change in low-threshold vagal nodose mechanosensory neurons innervating the lung (8) and trachea (19), such that they express substance P and calcitonin gene-related peptide (CGRP), neuropeptides typically limited to C-fibers in these tissues (and a subset of jugular A δ -fibers in the trachea) (24). Zhang et al. (31) have extended the idea of a phenotypic switch by showing that transient receptor potential

vanilloid (TRPV1), a key ion channel limited to nociceptors in healthy airways, was expressed in intrapulmonary low-threshold mechanosensors (rapidly and slowly adapting receptors) following allergic inflammation of rat airways (31). These data indicate that allergic inflammation can qualitatively alter A-fiber phenotype in a manner that may contribute to inappropriate sensations and reflexes.

The guinea pig trachea is innervated by neurons situated in both the jugular and nodose ganglion. The jugular ganglion projects capsaicin-sensitive (TRPV1-expressing nociceptive) fibers to the trachea. The vast majority of nodose neurons (80–90%), by contrast, are of the A δ phenotype (24). These fibers are capsaicin insensitive (do not express TRPV1) but exquisitely sensitive to light touch and evoke cough upon activation (7, 24). Should these cough-evoking nerve fibers be induced to express TRPV1, it would lead to a qualitative change in the nature of stimuli capable of their activation and could in principle contribute to the excessive coughing often associated with inhaled allergens.

Neurotrophic factors are capable of interacting with sensory nerve terminals in a manner that leads to changes in gene expression and nerve phenotype (32). Inflammatory airway diseases (asthma, chronic obstructive pulmonary disease, bronchitis) are associated with an increased production of neurotrophic factors in the airways (20, 25). The responsiveness of a neuron to a given neurotrophic factor depends on its expression of selective high-affinity neurotrophic factor receptors. Neurotrophins are a family of neurotrophic factors comprising of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 3 and 4 (NT-3 and NT-4) (22). These bind with high affinity to certain Trk receptors. NGF binds to TrkA; BDNF and NT-4 bind to tropomyosin receptor kinase B (TrkB); and NT-3 binds to TrkC (2, 3). Another family of neurotrophic factors that can induce plasticity in sensory neurons is referred to as glial cell-derived neurotrophic factor (GDNF)-family ligands (15). This family comprises GDNF, neurturin, artemin, and persephin (1). These factors bind with high affinity to GDNF-family receptors (GFR- α receptors). GDNF binds to GFR- α 1, neurturin to GFR- α 2, artemin to GFR- α 3, and persephin to GFR- α 4 (1). Each of the GFR- α receptors use the tyrosine kinase RET as a cosignaling receptor molecule. We have recently characterized the pattern of neurotrophic factor receptor expression in distinct vagal sensory nerve afferent innervating the respiratory tract of the adult guinea pig. We found that the nodose neurons innervating the guinea pig trachea nearly uniformly express TrkB and GFR- α 1/RET (14).

In the present study, we address the hypothesis that allergic airway inflammation can induce the expression of TRPV1 in tracheal A-fiber neurons. In addition, we hypothesize that this is secondary to neurotrophic factor production. Specifically, we

Address for reprint requests and other correspondence: B. J. Undem, Johns Hopkins Asthma and Allergy Center (3A.44), 5501 Hopkins Bayview Circle, Baltimore, MD 21224 (e-mail: bundem@jhmi.edu).

evaluated the effect of allergen challenge on TRPV1 expression in nodose Aδ tracheal neurons, and, based on their expression of specific neurotrophic factor receptors, we addressed the hypothesis that the allergen effect could be mimicked by exogenous application of BDNF and/or GDNF.

MATERIALS AND METHODS

Animals. All experiments were performed with approval from the Johns Hopkins University Animal Use and Care Committee. Male Hartley guinea pigs (200–300 g) were obtained from Hilltop Laboratory Animals (Scottsdale, PA).

Retrograde tracing of neurons innervating trachea. Guinea pigs were anesthetized by injection of ketamine (50 mg/ml) and xylazine (2.5 mg/ml) dissolved in PBS. The trachea was retrogradely labeled using a fluorescent retrograde tracer DiI (Invitrogen, Carlsbad, CA) solution (2% dissolved in ethanol). For tracheal labeling, the ventral upper tracheal region was exposed and pierced with a microinjecting needle attached to a Hamilton syringe. The 1,1-dioctadecyl-3,3,3-tetramethylindocarbocyanine perchlorate (DiI) (5 μ l) was directly injected into several sites directly into the tracheal wall. Upon histological assessment, we found that the dye stays delimited to the trachea. The dye does not diffuse appreciably caudally; no dye was seen in the main bronchi, esophagus, or heart. The incisions were sutured, and animal was allowed to recover for ~2 wk for sufficient labeling of cell bodies in the vagal ganglia. All animals were closely monitored, and any animal that displayed behaviors indicating excessive pain or infection was euthanized immediately via CO₂ asphyxiation.

Immune sensitization and allergen challenge. Guinea pigs were actively sensitized by injecting intraperitoneally 10 mg/kg ovalbumin (OVA) three times at 48-h intervals. Experiments were conducted on animals 6–8 wk of age, beginning 3 wk following the active sensitization. Control guinea pigs were actively sensitized and exposed only to aerosolized PBS for 5 min. Additional control studies were carried out in naïve (neither sensitized nor challenged) animals.

One group of animals received what we refer to as “threshold” dosing. The actively sensitized guinea pigs were challenged by exposing animals to increasing concentrations of aerosolized OVA (0.01, 0.03, 0.10, and 0.30% OVA dissolved in PBS) during normal breathing in a Plexiglas chamber (8 l volume) for 5 min at each concentration. Animals were removed to ambient air at the first point they showed signs of an overt allergic response (gasping, rapid breathing), irrespective of the OVA dose. This was repeated on three consecutive days.

Another group of animals received what we referred to as “maximal” dosing. Guinea pigs are very sensitive to the cardiopulmonary effects of histamine. This limits the OVA concentration that can be safely given to guinea pigs (4). Therefore, this group of animals was injected with the H1 receptor antagonist pyrilamine (0.1 mg/kg) 20 min before they were exposed to a maximal (0.3%) OVA challenge for 5 min. This was repeated on three consecutive days. These animals typically did not show overt signs of discomfort.

One day following the final OVA challenge, the animals were killed, the nodose ganglia were isolated for gene expression analysis of tracheal specific neurons, or calcium influx studied. Additionally, the trachea were isolated and studied histologically.

BDNF in Matrigel. To provide a sustained presence of growth factor, we initially evaluated the effect of BDNF embedded in Matrigel. Matrigel is liquid at room temperature but solidifies at body temperature. Growth factors leach out of solidified Matrigel slowly over time; in vitro assays have shown that this can occur over days to a couple weeks (9), but the precise kinetics will depend on the experimental design and particular growth factor studied. Animals were anesthetized by injection of ketamine (50 mg/ml) and xylazine (2.5 mg/ml) dissolved in PBS. Tracheotomy was performed, and BDNF dissolved in Matrigel (growth factor reduced, phenol-free) (BD

Bioscience, Bedford, WA) was delivered to the trachea. Animals were treated with 50 μ l of Matrigel alone or Matrigel containing BDNF at 50 or 200 ng/ml. The Matrigel (30 μ l) was injected as a bead in the region between the esophagus and trachea (corresponding to the region of trachea previously injected with the DiI), and the remaining 20 μ l was positioned on the tracheal surface and closed off with surrounding tissue. The incisions were sutured, and the animal was allowed to recover. Approximately 2 wk later the animals were killed, and the nodose ganglia were isolated.

BDNF/GDNF/NGF instillation. Animals previously labeled with DiI were anesthetized by injection of ketamine/xylazine. One-centimeter midline incision of the skin was made over the cervical trachea. The cervical trachea was exposed, and 50 μ l (at designated concentrations) of human recombinant BDNF, human recombinant GDNF (BioSource, Invitrogen), human recombinant NGF (R&D Systems, Minneapolis, MN), or PBS (vehicle) was injected into the site along the upper tracheal as described above for DiI injections. At the stated time point, the ganglion were harvested and processed for single-cell RT-PCR.

Ganglion dissociation and cell picking. The blood from circulation was washed out by in situ perfusion with Krebs's bicarbonate solution, composed of (in 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). The nodose ganglia were harvested. Each ganglion was incubated in the enzyme buffer (2 mg/ml collagenase and 2 mg/ml dispase II dissolved in Ca²⁺-, Mg²⁺-free Hanks' balanced salt solution) for 30 min at 37°C. The cells were gently dissociated by trituration with glass fire-polished Pasteur pipette. Two additional enzymatic digestions at 37°C for 20 min were carried out. The cells were washed by centrifugation (three times at 1,000 g for 2 min) and suspended in L-15 medium containing 10% FBS. The cell suspension was transferred onto poly D-lysine (0.1 mg/ml)-laminin (0.004 mg/ml)-coated cover slips. After the suspended neurons had adhered to the cover slips for 2 h in an incubator (37°C), the neuron-attached cover slips were flooded with the L-15 medium (10% FBS). All chemicals were purchased from Fisher Scientific Products (Pittsburg, PA).

Neurons were picked, and gene expression was evaluated on the same day as the dissociation. Cover slips containing dissociated neurons were constantly perfused by Krebs's solution bubbled with 95% O₂-5% CO₂. The tracheal-specific neurons were identified by using fluorescence microscopy, and single neurons were drawn into a glass pipette (tip diameter 50–150 μ m) pulled with a micropipette puller (Model P-87; Sutter Instruments, Novato, CA) by applying negative pressure, within 8 h of the dissection (14). The pipette tip was then broken in a PCR tube containing 1 μ l of Invitrogen Resuspension Buffer and RNase Inhibitor (RNaseOUT, 2 U/ μ l). The tubes were immediately snap frozen and stored on dry ice.

Only neurons found to be unattached to other neurons and ostensibly free from other cells or debris were attempted to be collected for single-cell RT-PCR analysis. Every neuron collected for the study met the appropriate positive controls in our PCR analysis.

For our single-cell RT-PCR analysis, we used 50 PCR cycles. Therefore, the major concern in these experiments is whether the superfusing fluid is contaminated by RNA/DNA from any contaminating cells. To control for this, only clearly labeled neurons found accessible and not attached to other neurons, other cells, or even cellular debris were collected for single-cell RT-PCR analysis. This careful approach limits the number of neurons that can be successfully collected from any given ganglion. For this study we characterized the mRNA expression in 642 neurons isolated from 108 nodose ganglia (5–8 tracheal-specific neurons were obtained per ganglion, each of which was placed into individual PCR tubes). 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 RT-PCR from the superfusing

fluid from the vicinity of the collected cells (the negative control) was nearly uniformly negative.

Single-neuron RT-PCR. Single neurons were processed using Superscript III Cells Direct cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. Samples were lysed by the addition of 10 μ l of Resuspension Buffer and incubated at 75°C for 10 min. Each sample was next treated with 5 μ l of DNase I and 1.6 μ l with 10 $\times$ DNase I Buffer. After 5 min of incubation at room temperature of DNase treatment, 1.2 μ l of 25 mM EDTA was added to each sample and placed in a thermocycler for an incubation at 70°C for 5 min. Subsequently, 1 μ l of oligo (dT), random primers (Invitrogen), and 10 mM dNTP Mix were added to each sample and then incubated at 70°C for another 5 min. The final addition of 5 $\times$ RT Buffer (6 μ l), RNase OUT (1 μ l), and 0.1 M DTT (1 μ l) were added to each sample. Water was added to one-third of the sample for the negative RT control while the remaining two-thirds of the final volume were reverse transcribed by adding Superscript III Reverse Transcriptase for cDNA synthesis. Each tube was transferred to the thermal cycler preheated to 50°C for 50 min with an inactivated step set at 85°C for 5 min. The reaction was chilled at 4°C, and cDNA was stored at -20°C until PCR amplification.

The PCR reaction mixture contained 0.5 U of HotStar Taq Polymerase, 2.5 mM MgCl₂, and 10 $\times$ PCR buffer (Qiagen). Additionally 10 mM dNTP and custom-synthesized intron-spanning primers (Invitrogen) were added to the reaction mixture in a volume of 2 μ l as previously described (14). As a positive control, RNA was isolated from whole nodose ganglion and reverse transcribed using Omniscript RT Kit (Qiagen). The PCR reaction conditions were on a 50-cycle basis with initial activation at 95°C for 15 min, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. Control experiments testing the amplification of the "no RT" for each individual neuron and bath control by using either one or multiple primer pairs did not produce a specific product.

Immunofluorescence staining. For immunohistochemical experiments, four guinea pigs were killed by an overdose of pentobarbital (150 mg/kg, intraperitoneal), transcardially perfused with PBS (pH 7.4; 60 ml) containing 0.1% procaine and heparin (10 U/ml), followed by 4% formaldehyde in PBS (60 ml), and the trachea with the esophagus were removed and fixed for an additional 2 h (4°C). Two guinea pigs were challenged with OVA according to the threshold dosing plan described above, and two animals were naïve animals.

The tracheal tissues were harvested and placed in 10% formaldehyde fix solution. Cryostat sections of tracheas were blocked with a solution containing 10% donkey serum, 1% BSA, and 0.1% Tween 20 in PBS, applied for 1 h at room temperature. For double immunofluorescent staining, the sections were then incubated (4°C) overnight in primary antibodies diluted in PBS containing 1% BSA and 0.5% Triton X-100, rinsed, and then covered in species-specific secondary antibodies conjugated with Alexa dyes 350, 488, and 594 (Invitrogen-Molecular Probes) recognizing species-specific primary antibodies. We used rabbit anti-GDNF at 1:200 dilution (SC-328; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-BDNF at 1:1,000 dilution (AB15435P; Chemicon, Temecula, CA). For negative controls, separate sections were processed similarly, but either the primary antibody was replaced with off-target IgG to evaluate nonspecific staining or with primary antibodies preabsorbed with antigenic peptide with the ratio of 10:1 concentration (GDNF, BDNF). Serial sections of the trachea from control and allergen-challenged animals were analyzed. The slides were evaluated using micrographs taken by fluorescent microscope (Olympus BX-50) equipped with a camera (Q-Imaging Retiga Exi camera); a micrograph field of view of the entire stained section was taken. Imaging software (iVision, Exton, PA) was used to analyze areas of positive staining in each digitized micrograph, where simple bi-level thresholding was performed: the microscope was focused on an area of no staining, and from this the computer defined "background"; a target threshold value above this

background was set for positive staining and applied the same to all images. The total area of the region of interest (the epithelial layer) was outline by the observer and objectively measured by the software. The software measurement of the positively stained area within the region of interest was used to calculate the percent area of positive immunostaining (positive area divided by the entire area of region of interest).

Calcium imaging. Dissociated nodose neurons from BDNF-treated animal (200 ng/ml in Matrigel for 2 wk) ganglia and control animals were plated on coverslips were loaded with fura-2 AM (8 μ M) (Molecular Probes) in L-15 media containing 10% FBS and incubated (40 min, 37°C). For imaging, the coverslip was placed in a custom-built chamber (bath volume of 600 μ l) and superfused at 4 ml/min with Locke solution at 34°C, composed of (in mM): 136 NaCl, 5.6 KCl, 1.2 MgCl₂, 2.2 CaCl₂, 1.2 NaH₂PO₄, 14.3 NaHCO₃ (gassed with 95% O₂-5% CO₂, pH 7.3-7.4), for 15 min before and throughout each experiment by an infusion pump. Changes in intracellular free calcium concentration; intracellular [Ca²⁺]^{free} were measured by digital microscopy (Universal; Carl Zeiss, Thornwood, NY) equipped with in-house equipment for ratiometric recording of single cells.

DiI-labeled cells were monitored by sequential dual excitation, 352 and 380 nm, and the analysis of the image ratios used methods previously described to calculate changes in intracellular [Ca²⁺]^{free} (26). The ratio images were acquired every 6 or 12 s. Superfused buffer was stopped 30 s before each drug application, when 300 μ l buffer was removed from the bath and replaced by 300 μ l of capsaicin (1 μ M) added between image acquisitions. At the end of the dissociated vagal neuronal studies and all calcium imaging experiments, dissociated vagal neurons were exposed to 300 μ l of ionomycin (30 s, 1 μ M) to obtain a maximal response.

For the analysis of fura-2 AM-loaded cells, the measurement software converted ratiometric information to intracellular [Ca²⁺]^{free} using Tsien parameters: [Ca²⁺] = Kd [(R - R_{min})/(R_{max} - R)](b) (26). Preliminary calibration studies yielded an R_{min} (352/380-nm ratio under calcium-free conditions) of 0.3 for guinea pig sensory neurons and an R_{max} (352/380-nm ratio under calcium saturating conditions) of 15 for neurons. In the following experimental studies, we did not specifically calibrate the relationship between ratiometric data and absolute calcium concentration for each specific cell, choosing instead to use the parameters provided from the calibration studies and relate all measurements to the peak ionomycin response in each viable cell. This effectively provided the needed cell-to-cell calibration for enumerating individual cellular responses. Only cells that had a robust response to ionomycin were included in analyses. At each time point for each cell, data were presented as the percentage change in intracellular [Ca²⁺]^{free}, normalized to ionomycin: Response x = 100 $\times$ ([Ca²⁺] _{x} - [Ca²⁺]_{bl})/([Ca²⁺]_{max} - [Ca²⁺]_{bl}), where [Ca²⁺] _{x} was the apparent [Ca²⁺]^{free} of the cell at a given time point, [Ca²⁺]_{bl} was the mean baseline of the apparent [Ca²⁺]^{free} of the cell measured over 120 s, and [Ca²⁺]_{max} was the peak apparent [Ca²⁺]^{free} of the cell during ionomycin treatment. Neurons were defined as "responders" to a given compound if the mean response was greater than the mean baseline plus 2 $\times$ the standard deviation. Given that nodose ganglia are probably composed of heterogeneous neuronal populations, it is important to emphasize the point that results are presented in two distinct ways. First, the number of DiI-labeled neurons responding (based on the criteria described above) to a given stimulus compared with the total number of DiI-labeled neurons is reported. Second, the mean percentage change in intracellular [Ca²⁺]^{free} normalized to ionomycin of those neurons that (based on the above criteria) were defined as responders is reported.

Statistical analysis. The variable quantified in the gene expression analysis was the percentage of neurons expressing a given gene (5-8 tracheal-specific neurons are studied/nodose ganglion). In each gel there was or was not a visible band denoting mRNA expression. In nearly all instances the bands were unambiguous (see Fig. 2 for an example of 28 neurons). When two investigators viewed a gel, the

concordance in the determination of the number of neurons expressing a given gene was always 100%. To illustrate consistency among ganglia, the percentage of labeled neurons expressing a given gene in a given ganglion is presented as the mean $\pm$ SE (n = number of ganglia from which the neurons were obtained). For statistical analysis, the neurons from all ganglia within a treatment group were pooled, and the proportion of neurons expressing TRPV1 was subjected to a χ^2 analysis. The percentage of the epithelium stained with anti-BDNF and anti-GDNF (as determined by the computer program discussed above) was compared with control using Student's t -test.

RESULTS

We have previously reported with the use of a vagally innervated tracheal preparation that 80–90% of guinea pig nodose ganglion neurons innervating the trachea are A δ -fibers (conduction velocity $\sim$ 5 m/s) (13). These nerves are exquisitely sensitive to light touch of the mucosa and rapid decreases in pH but are not activated by the TRPV1 agonist capsaicin (12, 24). They evoke cough upon activation, even in lightly anesthetized animals and accordingly have been referred to as cough receptors (7). The remaining 10–20% of the nerve fibers projecting from the nodose to the trachea are capsaicin-sensitive C-fibers with conduction velocities of $\sim$ 1 m/s (13, 24). As predicted from this functional analysis and consistent with our previous reports (13, 14), we found that the vast majority ($\sim$ 80%) of nodose neurons retrogradely labeled from the trachea do not express TRPV1 mRNA (see controls in Fig. 1).

Allergen challenge. After 3 days of OVA challenge (see MATERIALS AND METHODS), we evaluated the dye-labeled (tracheal-specific) nodose neurons for TRPV1 mRNA expression. The results show that exposing actively sensitized animals to OVA aerosol increased substantially the percentage of tracheal nodose neurons expressing TRPV1 ($P < 0.05$). In unsensitized/nonchallenged (naïve animals) $20.3 \pm 4.2\%$ of the tracheal-specific nodose neurons expressed TRPV1 ($n = 16$ ganglia). Likewise, in another group of animals that were actively sensitized with OVA but not challenged, $19.4 \pm 4.2\%$ of the neurons expressed TRPV1 ($n = 6$ ganglia). These TRPV1-expressing neurons likely reflect the small percentage of no-

dose C-fibers innervating the trachea (13). In nodose ganglia isolated from actively sensitized guinea pigs that were previously exposed to a symptom threshold dose of OVA, $37.1 \pm 4.1\%$ of the neurons expressed TRPV1, $n = 8$ ganglia, $P < 0.05$ (Fig. 1). Under the antihistamine cover (as described in MATERIALS AND METHODS), guinea pigs inhaled 0.3% OVA (the maximum dose we tried) without overt cardio-pulmonary symptoms. After 3 consecutive days of this maximal dose of OVA, we observed that $51.1 \pm 13.6\%$ of the neurons express TRPV1 mRNA, with six ganglia analyzed $P < 0.05$ (Fig. 1). The results demonstrated that allergic inflammation can induce neuroplasticity in the nodose A δ tracheal neurons in the adult animal with respect to the expression of TRPV1 mRNA.

BDNF treatment. We have previously characterized the expression of neurotrophin receptors in nodose neurons specifically innervating the trachea and found that $>80\%$ of these neurons express TrkB, with a minority of neurons expressing TrkC, and only about 10–20% expressing TrkA (14). Given the importance of neurotrophic factors in regulating gene expression, we evaluated whether BDNF, an agonist for TrkB, could mimic the effect of allergen challenge.

In our initial experiments, the trachea of the animal was treated with 50 μ l of Matrigel alone or Matrigel containing 50 ng/ml or 200 ng/ml (10 ganglia analyzed) of BDNF. Matrigel allows for slow release of the growth factor over time (see MATERIALS AND METHODS). Two weeks following treatment with Matrigel alone, about 20% of the nodose tracheal neurons expressed TRPV1 ($n = 6$ ganglia analyzed, Fig. 2A). This proportion was similar to other control values as mentioned above and likely reflects a small proportion of nodose C-fibers innervating the trachea. Matrigel containing 50 ng/ml BDNF did not significantly increase the proportion of nodose trachea neurons expressing TRPV1 mRNA ($n = 6$ ganglia, Fig. 2A). However, after treatment with Matrigel containing 200 ng/ml BDNF, $>60\%$ of the tracheal-specific nodose neurons expressed TRPV1 ($n = 8$ ganglia analyzed $P < 0.01$ Fig. 2A). In fact, in some nodose ganglia from the BDNF-treated tracheas, nearly every neuron labeled from the trachea expressed TRPV1 (Fig. 2B).

We next evaluated the time course of the BDNF effect (Fig. 3). In these studies, BDNF was dissolved in PBS at 200 ng/ml and instilled into the rostral trachea of the animal. This provides an immediate but transient exposure of the trachea to the growth factor. Animals were killed 1 day, or 1, 2, or 3 wk following this acute BDNF treatment. Control animals received instillations of PBS alone, and, as expected, $<20\%$ of the neurons express TRPV1 mRNA ($n = 10$ ganglia). However, as early as 1 day following the acute BDNF treatment, we observed that $>50\%$ of the tracheal-specific TrkB+ neurons expressed TRPV1 ($P < 0.05$, $n = 10$ ganglia, Fig. 3). One week following acute BDNF treatment, there was still a significant increase in the percentage of nodose tracheal neurons expressing TRPV1. By the second and third weeks following acute treatment, the percentage of nodose tracheal neurons expressing TRPV1 was greater than control values, but the difference did not reach statistical significance (Fig. 3).

NGF treatment. As stated above, only about 20% of tracheal-specific nodose neurons express TrkA (with respect to tracheal vagal innervation, TrkA is selectively expressed in jugular neurons) (14). Because few of the tracheal-specific nodose neurons express TrkA, we predicted that NGF (an agonist for

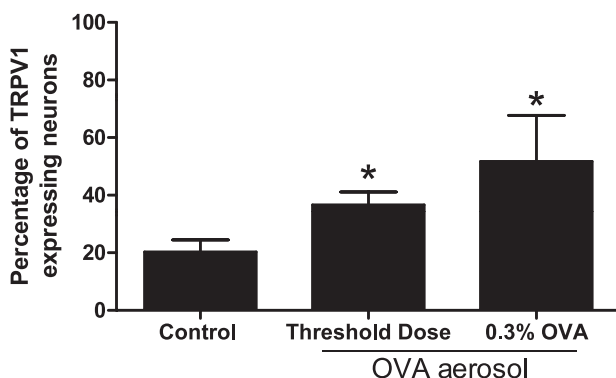


Fig. 1. Allergen-induced transient receptor potential vanilloid (TRPV1) mRNA expression in tracheal-specific nodose neurons. Percentage of tracheal-specific nodose neurons that express TRPV1 mRNA. Neurons were isolated from nodose ganglia of guinea pigs exposed to PBS only ($n = 6$ ganglia), guinea pigs exposed to threshold dose of ovalbumin (OVA) aerosol ($n = 8$ ganglia), and guinea pigs exposed to 0.3% OVA aerosol ($n = 6$ ganglia). Data are expressed as means $\pm$ SE, * $P < 0.05$ based on χ^2 analysis. The animals exposed to 0.3% OVA were pretreated with pyrilamine (see MATERIALS AND METHODS).

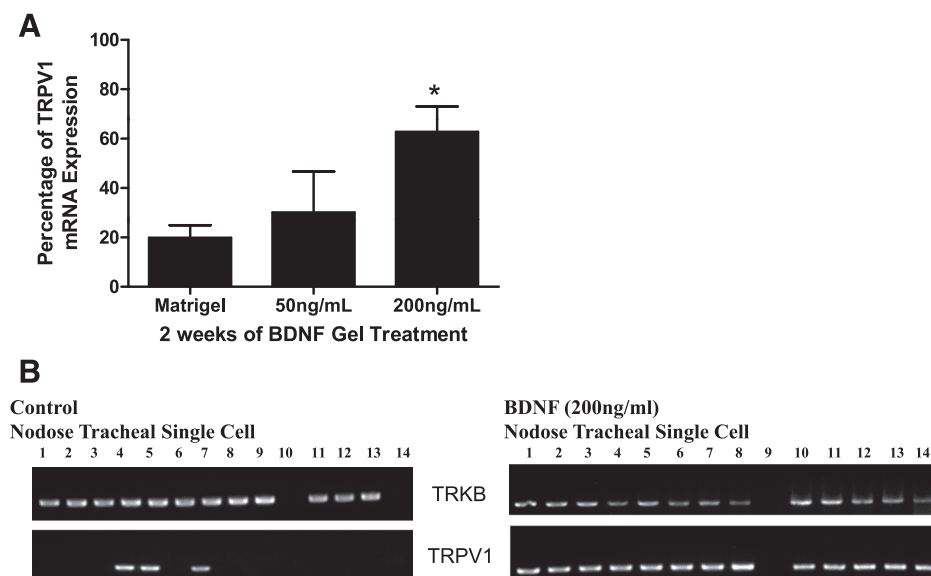


Fig. 2. Brain-derived neurotrophic factor (BDNF) (in Matrigel)-induced TRPV1 mRNA expression in tracheal-specific nodose neurons. **A**: percentage of tracheal-specific nodose neurons expressing TRPV1 mRNA 2 wk after BDNF or control treatment. Neurons were isolated from nodose ganglia of guinea pigs exposed to Matrigel alone (6 ganglia), exposed to Matrigel with 50 ng/ml BDNF (6 ganglia), or exposed to Matrigel with 200 ng/ml BDNF (10 ganglia). Data are expressed as means $\pm$ SE, * $P < 0.01$ based on χ^2 analysis. **B**: example of gel results of gene expression in single tracheal-specific nodose neurons. Expression of tropomyosin receptor kinase B (TrkB) and TRPV1 mRNA are shown in 14 tracheal-specific neurons (2 ganglia) isolated from a Matrigel-only-treated animal on the left, and 14 neurons in 2 nodose ganglia isolated from a guinea pig treated with 200 ng/ml BDNF in Matrigel.

TrkA) would have little effect on neuronal phenotype. We directly instilled NGF at 200 ng/ml into the rostral tracheal of the guinea pig. One day later, we found that the percentage of tracheal-specific neurons that expressed TRPV1 mRNA was not significantly different than control ($n = 6$ ganglia Fig. 4).

GDNF treatment. In addition to previously characterizing the neurotrophin receptors expressed by nodose A δ tracheal neurons, we also characterized the expression of GFR- α s. Similar to neurotrophins, GDNF family ligands can bind to their respective GFR receptor and signal via retrograde transport to induce gene expression within the cell body (27, 28). We found that the 80% of the tracheal-specific neurons primarily express GFR- α 1 and that over 60% expressed both GFR- α 1 and RET (14). Thus we hypothesize that GDNF, an agonist for GFR- α 1/RET could induce TRPV1 gene expression in nodose tracheal A δ neurons. One day after GDNF (200 ng/ml in PBS) was instilled into the trachea, we observed significant increase such that >50% of the nodose tracheal

neurons expressed TRPV1 mRNA ($P < 0.05$ compared with control, $n = 10$ ganglia) (Fig. 5).

Calcium recording. We wanted to insure that the neuroplasticity seen in the tracheal-specific nodose neurons was not limited to mRNA expression but also occurred at the level of functional TRPV1 channels. TRPV1 is a nonselective cation channel that when activated allows for entry of calcium that can be monitored at the level of the cell bodies using conventional fura-2 AM imaging techniques. We induced TRPV1 induction in nodose tracheal neurons using 200 ng/ml of BDNF in Matrigel for 2 wk. This provided a strong and persistent (>1 wk) induction in TRPV1 (see above), allowing ample time for functional channels to accumulate in the membrane. The calcium response to capsaicin was monitored specifically in dissociated nodose neurons that were labeled from the trachea (Fig. 6A). In control (Matrigel alone)-treated animals, <20% of the nodose tracheal neurons responded to capsaicin with an elevation of intracellular calcium (Fig. 6B). By contrast, after BDNF treatment, fully 50% of the nodose tracheal neurons responded to 1 μ M of capsaicin with a brisk rise in intracellular calcium ($P < 0.05$) (Fig. 6B). These results are consistent with

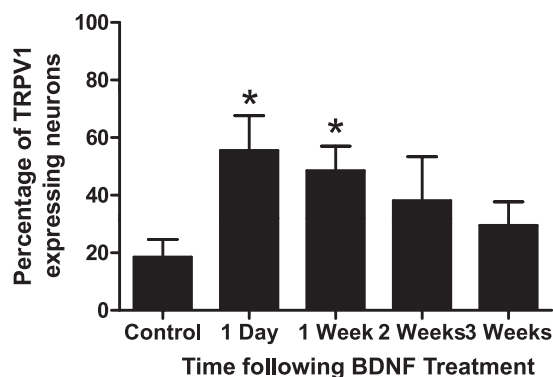


Fig. 3. Time course of acute BDNF-induced TRPV1 mRNA expression in tracheal-specific nodose neurons. Percentage of tracheal-specific nodose neurons expressing TRPV1 mRNA in ganglia isolated from PBS-treated animals (control) and in ganglia isolated from animals at various times following a single acute treatment to the rostral trachea of BDNF 200 ng/ml dissolved in PBS. Data are presented as means $\pm$ SE, * $P < 0.05$ based on χ^2 analysis, and neurons were isolated from $n = 6$ –10 ganglia.

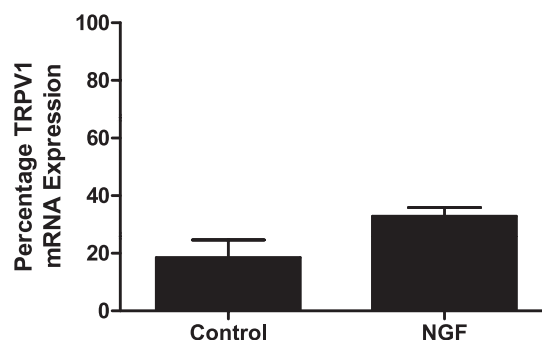


Fig. 4. Lack of nerve growth factor (NGF)-induced TRPV1 mRNA expression in tracheal-specific nodose neurons. Percentage of tracheal-specific nodose neurons expressing TRPV1 mRNA in ganglia isolated from control guinea pigs ($n = 10$ ganglia) and guinea pigs that had been treated 24 h earlier with 200 ng/ml NGF instillation ($n = 6$ ganglia). Data are presented as means $\pm$ SE; there was no significant difference in the values based on a χ^2 analysis.

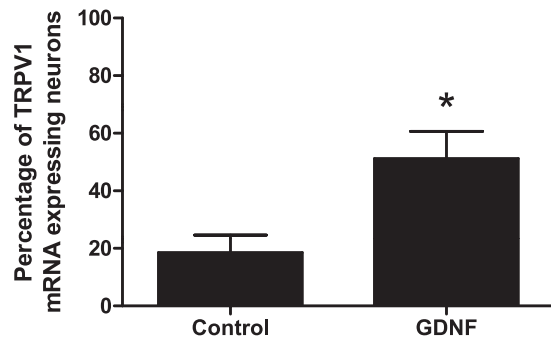


Fig. 5. Glial-derived neurotrophic factor (GDNF)-induced TRPV1 mRNA expression in tracheal-specific nodose neurons. Percentage of tracheal-specific nodose neurons that express TRPV1 mRNA in ganglia isolated from PBS (vehicle)-treated animals ($n = 10$ ganglia, control) and in ganglia isolated from guinea pigs treated 24 h previously with 200 ng/ml GDNF instillation. Data are presented as means $\pm$ SE, * $P < 0.01$ based on χ^2 analysis.

the TRPV1 mRNA expression data and demonstrate that exposure to BDNF can induce neuroplasticity at the functional level of TRPV1 ion channels in nodose A δ tracheal neurons.

Histology. Both BDNF and GDNF mimicked the OVA-induced TRPV1 induction in the nodose tracheal A δ neurons, but it is not known whether these growth factors are present at relevant sites in the allergically inflamed trachea. The nodose A δ -fibers terminate in defined structures just beneath the epithelium (17). After 3 days of allergen challenge, the tracheal epithelium was evaluated for the presence of immunoreactive BDNF and GDNF. Trachea isolated from naïve animals revealed trace amounts of BDNF present in the tracheal epithelium (Fig. 7A) and no evidence of GDNF in the mucosa of the control animals (Fig. 7E). By contrast, the tracheal mucosa of the OVA-challenged animals consistently stained strongly for both BDNF and GDNF (Fig. 7, B and F, and quantified in 7, D and H).

DISCUSSION

The results support the conclusion that allergic inflammation in guinea pig airways is associated with neuroplasticity in nodose A δ tracheal neurons with respect to TRPV1 expression. The data also support the hypothesis that allergen challenge induces the production of BDNF and GDNF in the airway mucosa, the region of the airway where the A δ -fibers terminate. Moreover, exogenously applied BDNF or GDNF mimics the effect of allergen challenge with respect to TRPV1 induction in the nodose A δ tracheal neurons.

In this study, we focused on nodose sensory neurons innervating the guinea pig trachea. Extensive functional studies have revealed that the nodose neurons innervating the extrapulmonary airways of guinea pigs comprise mainly one phenotype. These neurons do not express substance P or CGRP, are ~ 40 μ m in diameter, and project thin myelinated fibers to the trachea/bronchi that terminate just beneath the epithelium (17, 24). They have a very limited activation profile. They are exquisitely sensitive to punctate (touch) mechanical stimulation of the epithelium and to rapid, but not sustained, decreases in pH (12, 24). These neurons do not normally express TRPV1 and therefore are not activated by the many disparate stimuli capable of gating TRPV1. The induction of TRPV1 expression in the nodose tracheal A δ neurons therefore represents a

qualitative change in nerve phenotype similar to that observed previously with respect to neuropeptide induction (19). These effects predict that allergic inflammation may lead to situations in which activators of TRPV1 (e.g., acid, certain eicosanoids, heat, G protein-coupled receptor activation) cause qualitatively distinct central reflex activity. Moreover, the TRPV1-induced neurogenic inflammation due to local release of neuropeptides will not be limited to the tissue environment of C-fiber terminations but may also include regions of A-fiber innervation.

It is likely that the inhaled allergen-induced gene expression occurring in the neuronal cell body located in the distant vagal ganglion involves a neurotrophic factor(s). Neurotrophic factors are unique in that their ability to bind with high affinity to specific neurotrophic receptors in the membrane of the nerve terminal, and via axonal transport mechanisms, translocate to the cell body to influence gene expression (10). We have previously reported that the tracheal nodose A δ -fiber neurons express both neurotrophin receptors and GFR- α receptors (14). Among the neurotrophin receptors, guinea pig tracheal-specific nodose neurons nearly uniformly expressed TrkB (the receptor for BDNF and NT-4) with relatively few neurons expressing TrkA (the receptor for NGF) (14). We, therefore, predicted that BDNF, but not NGF, would mimic the allergen-induced neuroplasticity in these neurons. Indeed, BDNF induced TRPV1 expression in the majority of nodose tracheal A δ neurons,

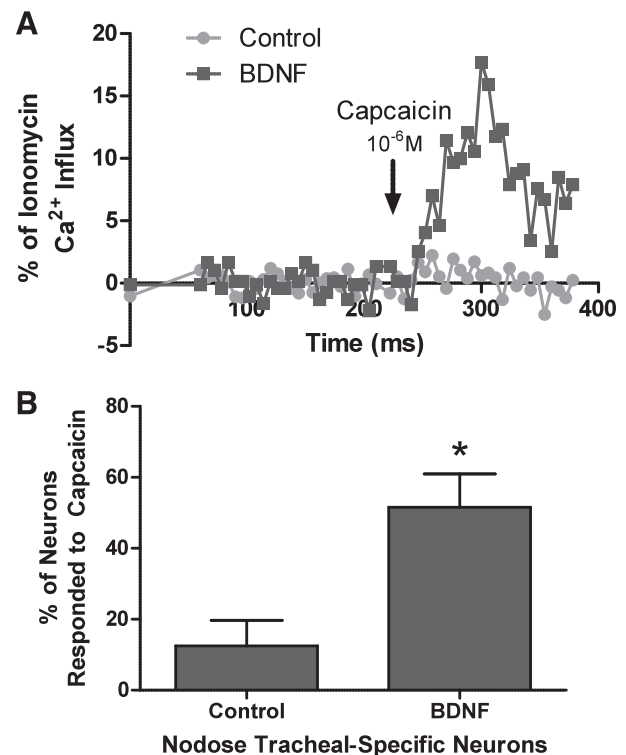


Fig. 6. Capsaicin-induced calcium responses in tracheal-specific nodose neurons in control and BDNF-treated guinea pigs. A: representative example of the Ca^{2+} responses, as measured by fura-2, in a tracheal-specific nodose neuron isolated from guinea pigs 2 wk following treatment with Matrigel alone (control, gray line) or treated with Matrigel containing 200 ng/ml BDNF (black line). Capsaicin (1 μ M) was applied for 60 s (black arrow). B: percentage of tracheal-specific nodose neurons from control animals ($n = 4$ ganglia) and BDNF-treated animals ($n = 4$ ganglia) that responded to capsaicin (as defined in MATERIALS AND METHODS). Data are presented as means $\pm$ SE, * $P < 0.05$ based on χ^2 analysis.

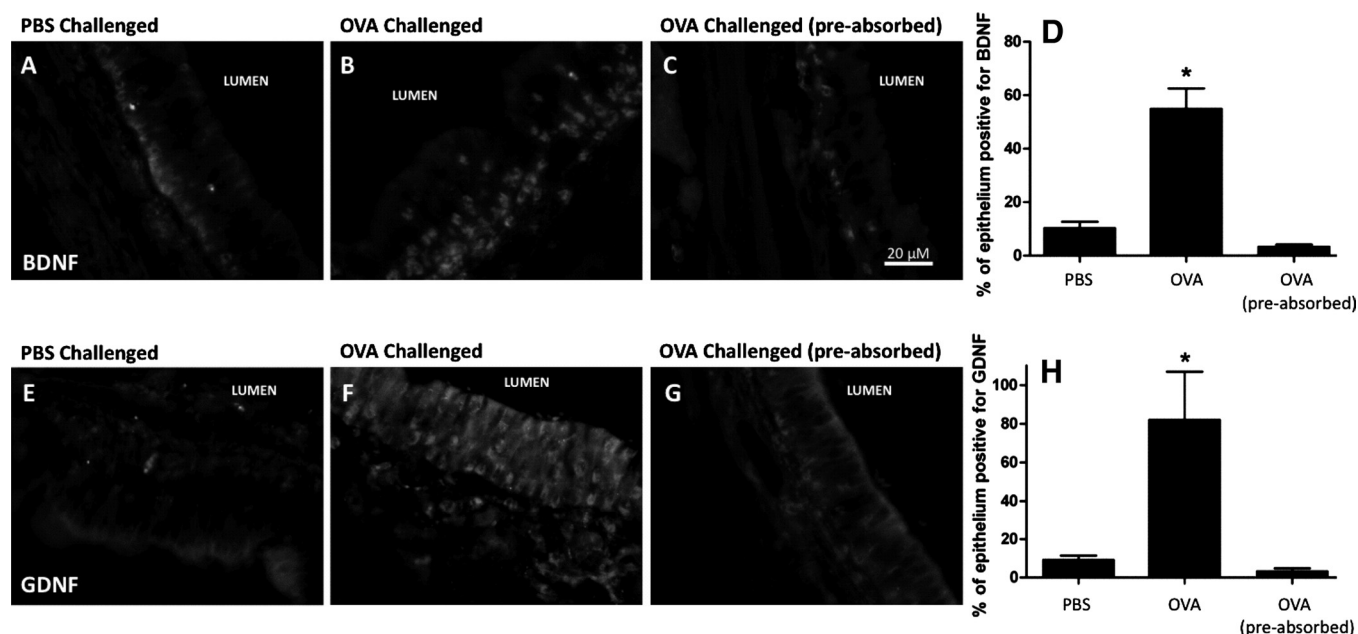


Fig. 7. Immunohistochemistry of allergen-induced BDNF/GDNF production in guinea pig tracheal sections. After active sensitization, animals were subjected to PBS or 0.3% OVA exposure for 3 days. The presence of BDNF (A–C) and GDNF (E and F) in the tracheal sections were determined immunohistologically. In C and G the OVA-challenged tracheal sections were also evaluated with preabsorbed antibodies as a negative control. Scale bar in C is for all images. The data for the percentage of epithelium stained, as described in MATERIALS AND METHODS, for BDNF and GDNF is quantified in D and H, respectively (* $P < 0.05$ relative to either control).

whereas NGF has little effect in this regard. This is consistent with the finding in rat isolated and cultured nodose neurons where BDNF but not NGF upregulated their capsaicin responsiveness (30). We have previously reported that NGF caused an induction of substance P expression in a modest number (~10%) of large diameter tracheal A δ neurons (11), a finding consistent with the fact that only about 10% of these neurons express TrkA. It is likely that BDNF would be a much stronger inducer of sensory neuropeptides (along with TRPV1) in these neurons than NGF; however, this hypothesis remains untested.

The majority of nodose tracheal A δ neurons also expresses GFR- α 1 and RET (14). GFR- α 1 is the high-affinity receptor for GDNF and along with RET can sensitize sensory neurons (16). It may therefore not be surprising that GDNF, when applied directly to the tracheal mucosa, like BDNF, mimicked the allergen-induced neuroplasticity we observed with respect to TRPV1 induction in tracheal-specific nodose neurons.

It is unlikely that the induction of TRPV1 in the nodose tracheal neurons was limited to expression of mRNA. Following BDNF treatment, not only was TRPV1 mRNA expression induced in >60% nodose tracheal neuron, but also about the same percentage of tracheal-specific neurons became responsive to capsaicin with brisk elevations in intracellular calcium. This indicates that the induction of mRNA led to the production of functional TRPV1 channels in the neuronal membrane.

Although we focused this study on nodose tracheal A δ neurons, it should be pointed out that nodose neurons also project faster A β -fibers to the intrapulmonary compartment, many of which are low-threshold stretch receptors, i.e., rapidly and slowly adapting receptors (RARs and SARs) (7, 13). These neurons also express TrkB and GFR- α 1/RET (14). Therefore, we hypothesize that allergen challenge may also induced TRPV1 in these neurons. This speculation is supported by an

elegant study in rats where allergen challenge induced TRPV1 in large diameter, presumably fast-conducting nodose A-fiber neurons (31). Importantly, this was accompanied by the induction of de novo functional responsiveness of intrapulmonary RAR and SAR nerve fibers to capsaicin (31). We have previously shown that allergen challenge also induced Substance P in intrapulmonary-presumed SAR/RAR neurons (8). Therefore, allergen-induced switching of vagal low-threshold mechanosensitive A-fibers to nerves with a quasi-nociceptor phenotype may be a general principle rather than mechanisms unique to nerves innervating the guinea pig trachea.

If BDNF and/or GDNF are involved with allergen-induced neuroplasticity in guinea pig tracheal A δ neurons, then these neurotrophic factors should be present in the allergically inflamed airway near the site of the A δ nerve terminations. In addition, selectively blocking TrkB and/or GFR- α 1 should inhibit the response. At present it is difficult to selectively antagonize GFR- α 1 and TrkB receptors in guinea pigs, but we were able to employ immunohistochemistry to show that allergen challenge induces the expression of immunoreactive BDNF and GDNF in the tracheal epithelium. If these factors are released from the epithelial cells, they would likely bind to receptors in the A δ -fibers that terminate just beneath the epithelial layer (17).

Although the present study is the first to report an allergen-induced production of GDNF in the airways, others have noted that allergic inflammation is associated with elevations in BDNF production (5, 6). This finding is not limited to laboratory animals. In humans, BDNF is increased in both upper and lower airways following allergen provocation (23, 29).

BDNF is a labile peptide, so it is likely to have a very limited half-life in the trachea. However, the TRPV1 induction caused by a single acute exposure of the trachea to BDNF persisted for

at least 7 days. It is therefore likely that allergic inflammation has both acute electrophysiological effects on airway sensory nerves: i.e., C-fiber activation by locally released autacoids, as well as more long-lived phenotypic changes such as those presented here. The former effects can explain the acute sneezing, coughing, and increased parasympathetic secretions and bronchoconstriction that may accompany acute allergen exposure. The neuroplasticity may provide some insights into the more persistent hypersensitive state that often accompanies allergic disease (18).

ACKNOWLEDGMENTS

Some of the preliminary data that are included in this study were presented as an abstract at the 6th International Symposium on Cough in London, 2010; the proceeding of this symposium is found in *Pulm Pharm Exp Ther* volume 24: issue 3.

GRANTS

This work was funded by a grant from the National Institutes of Health.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: T.L. and B.J.U. conception and design of research; T.L., A.C.M., and S.M. performed experiments; T.L. analyzed data; T.L. interpreted results of experiments; T.L. drafted manuscript; T.L., A.C.M., S.M., and B.J.U. approved final version of manuscript; A.C.M. prepared figures; B.J.U. edited and revised manuscript.

REFERENCES

- Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci* 3: 383–394, 2002.
- Barbacid M. The Trk family of neurotrophin receptors. *J Neurobiol* 25: 1386–1403, 1994.
- Bothwell M. Keeping track of neurotrophin receptors. *Cell* 65: 915–918, 1991.
- Bottomley KM, Lewis S, Rising TJ, Steward A. A new in vivo method for the measurement of repetitive anaphylactic responses in the guinea-pig. *Br J Pharmacol* 81: 195–199, 1984.
- Braun A, Lommatzsch M, Mannsfeldt A, Neuhaus-Steinmetz U, Fischer A, Schnoy N, Lewin GR, Renz H. Cellular sources of enhanced brain-derived neurotrophic factor production in a mouse model of allergic inflammation. *Am J Respir Cell Mol Biol* 21: 537–546, 1999.
- Braun A, Lommatzsch M, Neuhaus-Steinmetz U, Quarcoo D, Glaab T, McGregor GP, Fischer A, Renz H. Brain-derived neurotrophic factor (BDNF) contributes to neuronal dysfunction in a model of allergic airway inflammation. *Br J Pharmacol* 141: 431–440, 2004.
- Canning BJ, Mazzone SB, Meeker SN, Mori N, Reynolds SM, Undem BJ. Identification of the tracheal and laryngeal afferent neurones mediating cough in anaesthetized guinea-pigs. *J Physiol* 557: 543–558, 2004.
- Chuaychoo B, Hunter DD, Myers AC, Kollarik M, Undem BJ. Allergen-induced substance P synthesis in large-diameter sensory neurons innervating the lungs. *J Allergy Clin Immunol* 116: 325–331, 2005.
- Gao Y, Zhu S, Luo E, Li J, Feng G, Hu J. Basic fibroblast growth factor suspended in Matrigel improves titanium implant fixation in ovariectomized rats. *J Control Release* 139: 15–21, 2009.
- Ginty DD, Segal RA. Retrograde neurotrophin signaling: Trk-ing along the axon. *Curr Opin Neurobiol* 12: 268–274, 2002.
- Hunter DD, Myers AC, Undem BJ. Nerve growth factor-induced phenotypic switch in guinea pig airway sensory neurons. *Am J Respir Crit Care Med* 161: 1985–1990, 2000.
- Kollarik M, Undem BJ. Mechanisms of acid-induced activation of airway afferent nerve fibres in guinea-pig. *J Physiol* 543: 591–600, 2002.
- Kwong K, Carr MJ, Gibbard A, Savage TJ, Singh K, Jing J, Meeker S, Undem BJ. Voltage-gated sodium channels in nociceptive versus non-nociceptive nodose vagal sensory neurons innervating guinea pig lungs. *J Physiol* 586: 1321–1336, 2008.
- Lieu T, Kollarik M, Myers AC, Undem BJ. Neurotrophin- and GDNF family ligand-receptor expression in vagal sensory nerve subtypes innervating the adult guinea pig respiratory tract. *Am J Physiol Lung Cell Mol Physiol* 300: L790–L798, 2011.
- Lin LF, Doherty DH, Lile JD, Bektess S, Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 260: 1130–1132, 1993.
- Malin SA, Molliver DC, Koerber HR, Cornuet P, Frye R, Albers KM, Davis BM. Glial Cell Line-Derived Neurotrophic Factor Family Members Sensitize Nociceptors In Vitro and Produce Thermal Hyperalgesia In Vivo. *J Neurosci* 26: 8588–8599, 2006.
- Mazzone SB, Reynolds SM, Mori N, Kollarik M, Farmer DG, Myers AC, Canning BJ. Selective expression of a sodium pump isozyme by cough receptors and evidence for its essential role in regulating cough. *J Neurosci* 29: 13662–13671, 2009.
- Millqvist E. The airway sensory hyperreactivity syndrome. *Pulm Pharmacol Ther* 24: 263–266, 2011.
- Myers AC, Kajakar R, Undem BJ. Allergic inflammation-induced neuropeptide production in rapidly adapting afferent nerves in guinea pig airways. *Am J Physiol Lung Cell Mol Physiol* 282: L775–L781, 2002.
- Nassenstein C, Kerzel S, Braun A. Neurotrophins and neurotrophin receptors in allergic asthma. *Prog Brain Res* 146: 347–367, 2004.
- Neumann S, Doubell TP, Leslie T, Woolf CJ. Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. *Nature* 384: 360–364, 1996.
- Patapoutian A, Reichardt LF. Trk receptors: mediators of neurotrophin action. *Curr Opin Neurobiol* 11: 272–280, 2001.
- Raap U, Fokkens W, Bruder M, Hoogsteden H, Kapp A, Braunstahl GJ. Modulation of neurotrophin and neurotrophin receptor expression in nasal mucosa after nasal allergen provocation in allergic rhinitis. *Allergy* 63: 468–475, 2008.
- Riccio MM, Kummer W, Biglari B, Myers AC, Undem BJ. Interganglionic segregation of distinct vagal afferent fibre phenotypes in guinea-pig airways. *J Physiol* 496: 521–530, 1996.
- Rochlitzer S, Nassenstein C, Braun A. The contribution of neurotrophins to the pathogenesis of allergic asthma. *Biochem Soc Trans* 34: 594–599, 2006.
- Taylor-Clark TE, McAlexander MA, Nassenstein C, Sheardown SA, Wilson S, Thornton J, Carr MJ, Undem BJ. Relative contributions of TRPA1 and TRPV1 channels in the activation of vagal bronchopulmonary C-fibres by the endogenous autacoid 4-oxononanal. *J Physiol* 586: 3447–3459, 2008.
- Tomac A, Widenfalk J, Lin LF, Kohno T, Ebendal T, Hoffer BJ, Olson L. Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. *Proc Natl Acad Sci USA* 92: 8274–8278, 1995.
- Vellani V, Zachrisson O, McNaughton PA. Functional bradykinin B1 receptors are expressed in nociceptive neurones and are upregulated by the neurotrophin GDNF. *J Physiol* 560: 391–401, 2004.
- Virchow JC, Julius P, Lommatzsch M, Luttmann W, Renz H, Braun A. Neurotrophins are increased in bronchoalveolar lavage fluid after segmental allergen provocation. *Am J Respir Crit Care Med* 158: 2002–2005, 1998.
- Winter J. Brain derived neurotrophic factor, but not nerve growth factor, regulates capsaicin sensitivity of rat vagal ganglion neurones. *Neurosci Lett* 241: 21–24, 1998.
- Zhang G, Lin RL, Wiggers M, Snow DM, Lee LY. Altered expression of TRPV1 and sensitivity to capsaicin in pulmonary myelinated afferents following chronic airway inflammation in the rat. *J Physiol* 586: 5771–5786, 2008.
- Zweifel LS, Kuruvilla R, Ginty DD. Functions and mechanisms of retrograde neurotrophin signalling. *Nat Rev Neurosci* 6: 615–625, 2005.