

Nerve growth factor-induced substance P in capsaicin-insensitive vagal neurons innervating the lower mouse airway

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Summary

Background Nerve growth factor (NGF) is elevated in allergic diseases such as bronchial asthma and can lead to an induction of substance P (SP) and related neuropeptides in guinea-pigs large-diameter, neurofilament-positive airway neurons.

Objective In the present study, the effect of NGF on tyrosine kinase receptor trkA and the capsaicin receptor TRPV1 expression in airway-specific vagal sensory neurons located in the jugular–nodose ganglia complex (JNC) of mice was investigated.

Methods Using retrograde neuronal tracing in combination with double-labelling immunohistochemistry, SP, trkA- and TRPV1-receptor expression was examined in airway-specific sensory neurons of BALB/c mice before and after NGF treatment.

Results NGF injected into the lower airway was able to induce SP ($13.0 \pm 2.03\%$ vs. $5.9 \pm 0.33\%$) and trkA expression ($78 \pm 2.66\%$ vs. $60 \pm 2.11\%$) in larger diameter ($> 25 \mu\text{m}$), capsaicin-insensitive and trkA-positive vagal sensory neurons that were retrograde-labelled with Fast Blue dye from the main stem bronchi.

Conclusion Based on the extent of SP and trkA co-expression in airway-specific neurons by NGF treatment, the present study suggests that, following a peripheral activation of trkA receptor on SP afferent by NGF which is elevated in allergic inflammation, there may be trkA-mediated SP induction to mediate neurogenic airway inflammation.

Keywords airway innervation, jugular–nodose ganglia, mouse, nerve growth factor, neurotrophin, tachykinin, tyrosine kinase receptor trkA, vanilloid receptor-1 (TRPV1 or VR1)

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Introduction

Airway sensory nerves have the capacity to respond to a broad range of stimuli including capsaicin, bradykinin, hyperosmolar saline, tobacco smoke and cold dry air with the release of the peptide substance P (SP) and related peptides such as calcitonin gene-related peptide (CGRP) [1–4]. SP has been so far proposed to be an important neuromodulator of the respiratory system [5, 6]. SP-positive nerve fibres innervating the lower airway derive mainly from cell bodies which are located in the jugular–nodose ganglia whereas the stem bronchi and intrapulmonary airways are additionally innervated by thoracic spinal afferents from dorsal root ganglia [7, 8]. SP is known to be axonally transported to central and peripheral nerve endings to be released [9–11].

The biosynthesis and release of SP are reported to be mediated by the activation of the capsaicin receptor TRPV1

(previously known as VR1), a polymodal receptor which is stimulated by heat, protons or lipids [12–14]. As nerve growth factor (NGF) which is elevated in allergic inflammation [15, 16] has been implicated to be a key regulatory molecule of neuronal development and responsiveness [17] and to be involved in regulating the expression of tachykinin genes in sensory neurons [18], it is also discussed to mediate the SP production in the airways. NGF effects are known to be mediated by the tyrosine kinase receptors trkA. Other members of neurotrophins such as brain-derived neurotrophic factor (BDNF) binds to trkB and neurotrophin (NT)-3 to trkC [19].

Recently, some studies have shown that allergic inflammation was able to induce SP synthesis in large-diameter and neurofilament-immunoreactive airway-specific nodose neurons in guinea-pigs [20–22] and the SP induction has been reported to mimic these effects. In recent years, the mouse was commonly used to investigate immunological, genetic and neuro-immunological mechanisms underlying upper airway diseases such as allergic and non-allergic diseases. Little is known about the mouse airway neurobiology and the present study therefore aimed to study the effects of NGF on the airway neurobiology of mice.

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Materials and methods

Animals

In total, 10 adult female BALB/c mice weighing 15–20 g were used. The animals were kept under clean laboratory conditions. All protocols were performed in accordance with the Animal Welfare Act and the National Institutes of Health 'Guidelines for the care and Use of Laboratory Animals' (NIH publication 85–23, revised 1985) and approved by the state animal committee (0059/01). The animals were divided into two groups with five mice in each group. All animals received an instillation of Fast Blue dye into the main stem bronchi. In one group, next to the Fast Blue injection, 5- μ L of NGF was instilled into the main stem bronchi and control group received, instead of NGF, 5- μ L injection of saline.

Retrograde neuronal tracing and tissue preparation

The animals were anaesthetized with intraperitoneal injections of ketamine hydrochloride (Ketanest, Parke-Davies, Freiburg, Germany, 10 mg/kg body weight) and xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany, 10 mg/kg). The tip of a 10- μ L syringe (Hamilton, Bonaduz, Switzerland) was inserted between the fourth and fifth tracheal cartilage via a glass micropipette and a 5- μ L injection of Fast Blue tracing dye (2% aqueous solution containing 1% dimethyl sulphoxide; Sigma Chemical Co., St Louis, MO, USA) was made into the main stem bronchi. After the injection procedure animals were placed on their ridges with heightening head for about 5 min. With this technique we have previously demonstrated that this small amount of substance is limited to the lower airways [20, 23]. All animals recovered undisturbed for a post-operative period of 7 days until they were killed by CO₂ asphyxiation. Directly after asphyxiation, the animals were perfusion-fixed retrogradely through the abdominal aorta with freshly prepared Zamboini's solution (2% paraformaldehyde, 15% picric acid, 0.1 M PBS, pH 7.4 for 5 min) as described previously [24]. This routine protocol was performed to ensure optimal conditions for immunohistochemistry and published previously for immunohistochemical analysis of ganglionic neuropeptide expression [20, 23]. Dissection of the jugular–nodose ganglion complex and the lung samples were followed by rinses in 0.1 M phosphate buffer (pH 7.4) and cryoprotection with 18% sucrose in 0.1 M phosphate buffer overnight. Serial sections of the ganglia and lung samples were cut on a cryostat (Leica CM 1900, Bensheim, Germany) to 7 μ m and air-dried for 30 min.

Treatment with nerve growth factor

Ten adult female BALB/c mice were anaesthetized and mid-cervical tracheae were surgically exposed as described above; in 5 animals, solution of 5 μ L NGF (Genentech, Inc., San Francisco, CA, USA, 0.1 mg/mL) or the diluent (5 μ L NaCl (0.9%)) was injected 24 h before the analysis between the fourth and fifth tracheal cartilage via a glass micropipette Hamilton syringe (Hamilton) into the main stem bronchi [25].

Immunohistochemistry for SP, trkA- and TRPV1-IR

Ganglia and lung samples sections were first incubated for 1 h with phosphate-buffered saline (PBS) containing 10% normal swine serum to block non-specific protein binding sites and 0.5% Tween 20 to enhance penetration of immunoreagents. Then, the sections were incubated overnight with a monoclonal antibody from rat raised against SP (Boehringer Ingelheim, Heidelberg, Germany, dilution 1:400) in combination with a polyclonal antiserum to trkA from rabbit (Santa Cruz Biotech Inc., CA, USA, dilution 1:200) or with a polyclonal antiserum to TRPV1 from rabbit (Chemicon Inc., Temecula, CA, USA, dilution 1:1000). After rinsing in PBS, a biotinylated anti-rat-IgG (Amersham, Freiburg, Germany, dilution 1:100) was used for 1 h. After several washing steps, the tissues were incubated with the secondary reagents Streptavidin–texas-red-conjugate (Amersham, dilution 1:200) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit-IgG (Cappel, ICN Pharmaceuticals, OH, USA, dilution 1:400) for 1 h. Finally, the slices were washed again in PBS and mounted in carbonate-buffered glycerol at pH 8.6. [20, 23]. For immunohistochemical analysis, 20 randomly chosen sections were selected from the serial sections of every ganglia (20 ganglia of 10 animals) and all neurons with an apparent nucleus were analysed by using an epifluorescence microscopy (Leica, DMRA2, Bensheim, Germany) and appropriate filter combinations. Fast Blue was detected with the filter module U1 (excitation filter: band-pass 330–380 nm, barrier filter long-pass 418), texas red with the filter G1 module (band-pass 546/10 nm, long-pass 590 nm) and FITC with the B2 module (band-pass 455–490 nm, long-pass 515–560 nm). The sections were photographed with a camera (Visitron-System, Imagine Microscopy, Puchheim, Germany).

Next to the analysis of the expression pattern of SP, trkA and TRPV1, for the determination of neuronal diameters, we have used an established method to measure neuronal diameter. The neuronal diameter was calculated as the maximum and minimum diameters of cell bodies and only cell bodies with visible nucleus were analysed in the present study. Previous analysis of the vagal action potential and neuronal morphology revealed that airway-specific neurons, with conduction velocities ranging from 0.3 to 1.5 m/s that are consistent with the C-fibre cell bodies, have diameters shorter than 20 μ m, whereas neurons with the diameter longer than 20 μ m are very likely medium and large neuronal populations [4].

To verify the immunoreactive specificity, pre-absorption studies of the polyclonal anti-trkA and anti-TRPV1 antiserum, as well as the monoclonal anti-SP antiserum with corresponding antigens were undertaken resulting in an absence of the labelling. Specific immunohistochemical signals also disappeared after replacement of the primary antisera by PBS or pre-immune serum.

Statistical analysis

The immunohistochemical analysis was carried out in a blinded fashion. The differences between the groups were analysed statistically using a χ^2 test.

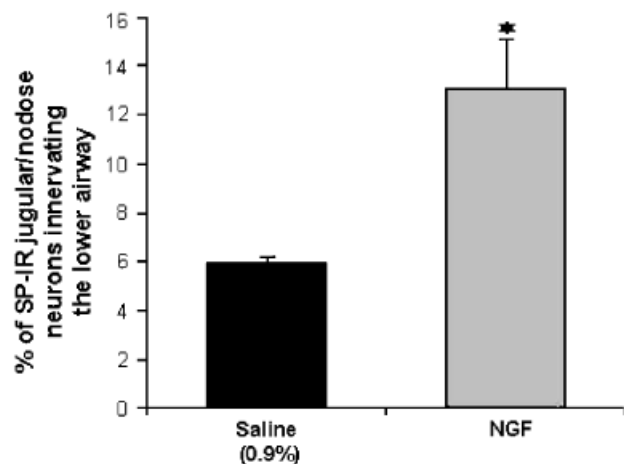


Fig. 1. The percentage of substance P positive and Fast Blue-labelled sensory neurons of the jugular–nodose ganglia 24 h after saline (0.9%) or nerve growth factor (NGF) treatment. A significant change from the saline as control to NGF-treated mice ($P \leq 0.05$). In NGF-treated airways, 1680 neurons were retrograde-labelled from the lower airways and for the control 865 neurons were investigated. Each value is a mean of 5 animals $\pm$ SEM.

Table 1. Percentage of substance P (SP)- and trkA-immunoreactive sensory neurons in the jugular–nodose ganglia innervating saline- and nerve growth factor (NGF)-treated airways

(Neuronal diameter)	FB+/trkA+ (10–40 μ m)*	FB+/trkA+/SP+ (<20 μ m)†	FB+/trkA+/SP+ (>20 μ m)‡
Ganglion			
NGF diluent,			
NaCl (0.9%)§			
Jugular–nodose (n = 478)	54.0 $\pm$ 2.08	6.0 $\pm$ 0.33	0
NGF (100 μ g/mL)¶			
Jugular–nodose (n = 1221)	65.0 $\pm$ 2.35	6.1 $\pm$ 1.5	6.9 $\pm$ 1.02

Percentage of retrograde-labelled neurons that expressed:

*FB+/trkA+ were neurons with diameters ranging from 10 to 40 μ m.

†FB+/trkA+/SP+ were neurons with diameters shorter than 20 μ m.

‡FB+/trkA+/SP+ were neurons with diameters longer than 20 μ m.

§Each value is a mean of the percentage obtained from 478 Fast Blue-labelled neurons of five animals $\pm$ SEM.

¶Each value is a mean of the percentage obtained from 1221 Fast Blue-labelled neurons of five animals $\pm$ SEM after NGF treatment.

Results

Neuronal retrograde tracing

Fast Blue dye was found in the epithelium of the main stem bronchi but not in the nasal mucosa or in the epithelium of the proximal trachea of the animals. The dye was detected in the basement membrane and underneath the lamina propria of the bronchial epithelium. Sensory neurons of all cell sizes in the jugular–nodose ganglia complex (JNC) were labelled with the tracer injected in the main stem bronchi; labelled neurons were found in both sides of the ganglia.

The effects of the nerve growth factor on SP and trkA, TRPV1 expression in labelled sensory neurons of the jugular–nodose ganglion

In five NGF-treated mice, 1680 neurons, which were retrograde-labelled from the lower airways were investigated;

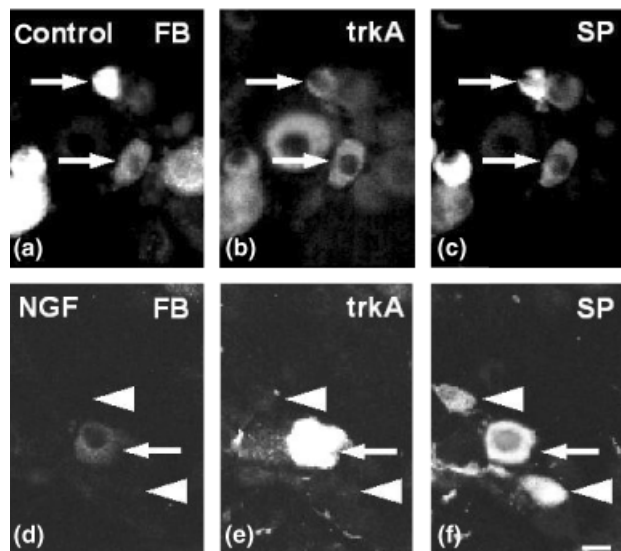


Fig. 2. Co-expression of substance P (SP) and trkA immunoreactivities in retrograde-labelled neurons of saline- and nerve growth factor (NGF)-treated jugular–nodose ganglia. Airway-projecting murine neurons were identified by retrograde tracing (control: a, NGF treatment: d) and examined for trkA (control: b, NGF treatment: e) and SP (control: c, NGF treatment: f) by double immunohistochemistry. SP was co-expressed with trkA: airway-projecting neurons simultaneously immunoreactive for trkA (control: arrow in b, NGF treatment: arrow in e) and SP (control: arrow in c, NGF treatment: arrow in f). The neighbouring neurons were not retrograde labelled from the airway (arrowhead in d), these neurons were not immunoreactive for trkA (arrowhead in e) and immunoreactive for SP (arrowhead in f). Scale bar represents 20 μ m.

13.0 $\pm$ 2.03% of retrograde-labelled neurons from the jugular–nodose ganglia were positive for SP immunoreactivity, whereas only 5.9 $\pm$ 0.33% of the control neurons (865 neurons from five animals) were found to show SP expression (Fig. 1). In contrast to the control ganglia where SP staining was found mainly in small neurons with a diameters shorter than 20 μ m, in NGF-treated ganglia, however, SP immunoreactivity was also detected in neurons with a diameter ranging from 20 to 40 μ m (largely in neurons with diameters longer than 25 μ m). After NGF treatment, next to the increased number of SP producing airway neurons, SP was observed to be co-localized to a great extent with trkA receptor in Fast Blue retrograde-labelled neurons. TrkA-immunoreactive airway-specific neurons were significantly increased after treatment with NGF; 78 $\pm$ 2.66% vs. 60 $\pm$ 2.11% of Fast Blue-labelled neurons were found to be trkA positive and a substantial number of these neurons did not express SP immunoreactivity. With respect to their cell size, neuronal profiles expressing trkA but not SP immunoreactivity were small to large neurons with a diameter ranging from 10 to 40 μ m (Table 1, Fig. 2).

Concerning TRPV1 expression, TRPV1 was found in 23.1 $\pm$ 2.01% neuronal profiles that were retrograde-labelled with Fast Blue and the number of these neurons did not significantly change after NGF application. TRPV1 expression was detected in Fast Blue-labelled neurons with a neuronal diameter ranging from 10 to 25 μ m. SP was generally co-localized with TRPV1; 6.1 $\pm$ 1.08% of Fast Blue-labelled neurons in the jugular–nodose ganglia were found to express SP immunoreactivity, while the other SP-positive airway-specific neurons found after NGF treatment

Table 2. Percentage of substance P (SP)- and TRPV1-immunoreactive sensory neurons in the jugular-nodose ganglion innervating saline- and nerve growth factor (NGF)-treated airways

Neuronal diameter	FB+/TRPV1+/SP- (10–25 µm)*	FB+/TRPV1+/SP+ (<20 µm)†	FB+/TRPV1- /SP+ (>20 µm)‡
Ganglion			
NGF diluent, NaCl (0.9%)§			
Jugular-nodose (n = 387)	14.0 ± 1.9	5.8 ± 1.1	0
NGF (100 µg/mL)¶			
Jugular-nodose (n = 459)	17.0 ± 2.28	6.1 ± 1.08	5.8 ± 1.3

Percentage of retrograde-labelled neurons that expressed:

*FB+/TRPV1+/SP- were neurons with the diameter ranging from 10 to 25 µm.

†FB+/TRPV1+/SP+ were neurons with diameters shorter than 20 µm.

‡FB+/TRPV1- /SP+ were neurons with diameters longer than 20 µm.

§Each value is a mean of the percentage obtained from 387 Fast Blue-labelled neurons of five animals ± SEM.

¶Each value is a mean of the percentage obtained from 459 Fast Blue-labelled neurons of five animals ± SEM after NGF treatment.

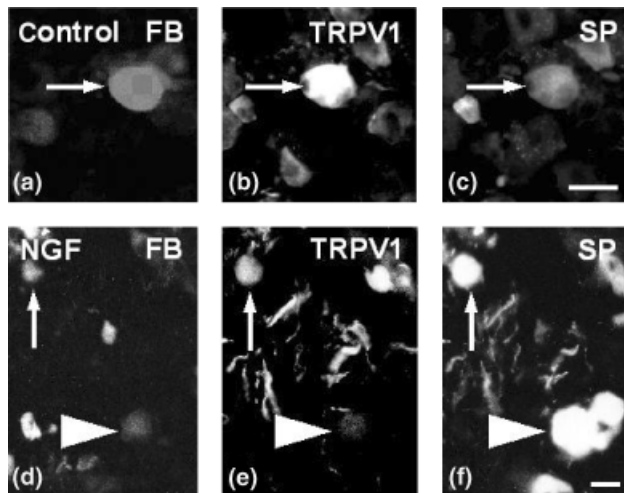


Fig. 3. Co-expression of substance P (SP) and TRPV1 immunoreactivities in retrograde-labelled neurons of saline- and nerve growth factor (NGF)-treated jugular-nodose ganglia. Airway-projecting murine neurons were identified by retrograde tracing (control: a, NGF treatment: d) and examined for TRPV1 (control: b, NGF treatment: e) and SP (control: c, NGF treatment: f) by double immunohistochemistry. SP was totally co-expressed with TRPV1: Airway-projecting neurons simultaneously immunoreactive for TRPV1 (control: arrow in b, NGF treatment: arrow in e) and SP (control: arrow in c, NGF treatment: arrow in f). SP was also present (NGF treatment: arrowhead in f) in large Fast Blue-labelled neurons (NGF treatment: arrowhead in d) which were negative for TRPV1 (NGF treatment: arrowhead in e) 24 h after NGF treatment. Scale bar represents: (a–c) 20 µm, (d–f) 10 µm.

did not exhibit TRPV1. Their number was about $5.8 \pm 1.3\%$ of Fast Blue-labelled neuronal profiles. SP producing and TRPV1-positive neurons had small diameters (<20 µm), and those negative for TRPV1 were only neurons with diameters longer than 20 µm (Table 2, Fig. 3).

Discussion

Neuronal reflexes play a key role in the initiation and modulation of airway hyper-responsiveness and inflammation

[4, 26, 27]. Next to this property, a direct interaction between the nervous and immune system has been proposed recently and a number of studies have addressed these questions using murine models since there is the ability to readily manipulate the murine genome. However, there is very little known about the sensory airway innervation in this species. We therefore explored the afferent innervation of the respiratory tract of the mouse using tracing techniques and quantitative immunohistochemistry to provide information about the expression and distribution pattern of substance P, trkA and TRPV1 receptor in the JNC. Consistent with the results of the previous experiment, airway afferent nerve fibres appear to uniformly originate from the JNC in some species including guinea-pigs [23]. It is also known that stem bronchi and intrapulmonary airways of rat and guinea-pig receive additionally spinal sensory supply from the dorsal root ganglia (segmental levels; C6–T8) [8, 23], therefore it is interesting to know whether thoracic spinal afferents from dorsal root ganglia contribute to the lower airway innervation of the mouse. To our best knowledge, however, spinal sensory airway innervation has not, so far, been studied for the mouse airways.

In the present study, SP expression was examined in neurons of the JNC in untreated, healthy, adult control BALB/c mice housed in a highly clean environment, since neuronal plasticity has been reported to be induced by a wide range of intrinsic and extrinsic factors such as infectious airway inflammation, allergic airway inflammation or exogenously administered NGF [20, 25, 28].

In control mice, only ~6% neurons labelled with Fast Blue were positive for SP immunoreactive. The percentage of airway-specific SP immunoreactive neurons in controls and even in allergen-treated mice is relatively low if compared with the high percentage (60–67%) of SP-positive neurons innervating the respiratory tract of the guinea-pigs, as published previously [23]. The discrepancy in the SP-expression pattern may arise from the species-specific difference as reported before for sensory ganglia of different species such as the rat, guinea-pig and mouse [29].

Under normal conditions, SP was extendedly co-localized with the receptor TRPV1 in airway-specific sensory neurons. This finding led to the suggestion that SP bio-synthesis and release in these neurons may be regulated by TRPV1 receptors as previously reported [4, 14, 30]. Like the control ganglia, TRPV1 was also expressed in non-airway as well as in airway-specific neurons with small-to-medium-sized diameters 24 h after NGF treatment. Interestingly, NGF was able to induce SP biosynthesis in large-diameter neurons that belong to the TRPV1-negative (capsaicin-insensitive) neuronal population. These present findings suggest that SP induction by NGF may not be associated with the TRPV1 receptor as NGF-induced SP expression was only found in large, neurofilament-positive and capsaicin-insensitive (TRPV1-negative) airway-specific neurons.

NGF, which is elevated in serum and nasal fluids in allergic rhinitis [31], in asthmatic bronchoalveolar lavage (BAL) [32, 33] and in inflamed tissues in other allergic diseases including rhinoconjunctivitis and atopic eczema [32] may have the capacity to act on trkA receptors to mediate SP biosynthesis and release from afferent neurons. In the present study, we found an extensive co-localization of SP and trkA receptor in neurons innervating the lung. These neuronal populations

were predominantly small-to-medium-sized neurons in control ganglia, whereas in NGF-treated ganglia, additionally to the small-diameter SP- and trkA-positive neurons SP was induced in neurons with large diameters ranging from 25 to 28 µm. These neurons always expressed the trkA receptor. Furthermore, we also found that SP and trkA expression in airway-specific sensory neurons were simultaneously up-regulated 24 h after NGF treatment. Concerning changes in receptor expression, the plasticity of receptors such as trkA in NGF-induced inflammatory process or TRPV1 after axotomy has been previously demonstrated in dorsal root ganglia and nodose neurons [34, 35]. The extensive co-localization between SP and trkA immunoreactivity in airway-specific sensory neurons and the induction of SP in airway TRPV1-negative neurons in NGF-treated mice indicate that SP synthesis and release may be partly trkA-dependent.

In the airway, released from afferent nerve terminals, SP acts on NK1 receptors and mediates neurogenic inflammation that can be described as a complex response consisting of increased vascular permeability, plasma extravasation, glandular secretion and pro-inflammatory cell influx [2]. Previous experiments using neurokinin-1 receptor antagonists have been shown to completely block the effects of NGF [36]. Furthermore, NGF treatment in guinea-pigs has also been demonstrated to induce SP expression in airway neurofilament-positive and non-peptidergic neurons [21]. Here, we were able to demonstrate that for the murine airway innervation, SP is induced in large-diameter, TRPV1-negative (capsaicin-insensitive) and trkA-positive airway-specific neurons by NGF.

The physiological role of these vagal sensory airway neurons in the mouse has not been studied so far. As in guinea-pig airways, the presently identified large-diameter neurons innervating the mouse airways are highly likely cell bodies of A-fibres with high conduction velocities and low mechanical thresholds, as some vagal afferent nerve fibres were also previously found to be mechanically sensitive and have high conduction velocities averaging about 4 m/s [4]. These afferents are activated by mechanical forces occurring during lung inflation and/or deflation [37]. The findings that mouse dorsal root ganglion neurons with response to low-mechanical thresholds have large diameters may support our suggestion that large neurons identified in the present study could be cell bodies of A-fibres [38]. However, the exact physiological role of these mouse vagal airway neurons remains to be determined in future studies.

In conclusion, based on the extent of SP and trkA co-localization, and the simultaneous induction of SP and the receptor trkA in afferent neurons innervating the lung after NGF treatment, the present study suggests that, following a peripheral activation of the trkA receptor on afferent neurons by NGF, tachykinin induction may be at least partly dependent on trkA-mediated neurotrophins effects.

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