

## Allergic airway inflammation induces tachykinin peptides expression in vagal sensory neurons innervating mouse airways

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### Summary

**Background** Allergic airway inflammation has been shown to induce pro-inflammatory neuropeptides such as tachykinin peptides substance P (SP) and neurokinin A (NKA) together with related peptide like calcitonin gene-related peptide (CGRP) in nodose sensory neurons innervating guinea-pig airways.

**Objective** The present study was designed to examine the effects of allergen sensitization and challenge on the SP/NKA expression in the jugular–nodose ganglion neurons innervating the murine airways.

**Methods** Using retrograde neuronal tracing technique in combination with double-labelling immunohistochemistry, the expression of SP/NKA was investigated in a murine model of allergic airway inflammation.

**Results** Allergic airway inflammation was found to induce the expression of SP/NKA ( $13.2 \pm 1.43\%$  vs.  $5.8 \pm 0.37\%$ ,  $P < 0.01$ ) in large-diameter ( $> 20 \mu\text{m}$ ) vagal sensory neurons retrograde labelled with Fast blue dye from the main stem bronchi.

**Conclusion** Based on the induction of tachykinins in airway-specific large-sized jugular–nodose ganglia neurons by allergic airway inflammation, the present study suggests that allergen sensitization and challenge may lead to *de novo* induction of tachykinins in neurons. This may partly contribute to the pathogenesis of airways diseases such as allergic airway inflammation.

**Keywords** allergic airway inflammation, jugular and nodose ganglia, mouse, retrograde neuronal tracing, substance P, tachykinin

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### Introduction

The tachykinins are members of the family of closely related neuropeptides comprising substance P (SP), neurokinin A (NKA), neuropeptide  $\gamma$  (NP $\gamma$ ) and neuropeptide K (NPK). These peptides are encoded by the *PPT-A* gene and are widely but selectively expressed throughout the central and peripheral nervous system [1–3], where their functions were first identified as neurotransmitters. Tachykinins were also shown to be involved in the pathogenesis of pain [4], inflammatory bowel disease [5], rheumatoid arthritis and airway inflammatory diseases [6], such as asthma [7].

The tachykinin peptides, SP, NKA, and related neuropeptide, such as the calcitonin gene-related peptide (CGRP), are known to have potent pro-inflammatory effects in the airways. They can be released from sensory nerve endings mainly originating from the jugular ganglia to the guinea-pig and rat airways [8–11]. In contrast to the airway innervation of the guinea-pig and rat, there remains very little information

on the airway neurobiology in the mouse. With this aim, recent studies have investigated whether tachykinins are also involved in the pathogenesis of the allergic airway inflammation in mice. These studies found that treatment with a dual neurokinin (NK) receptor NK-1/NK-2 antagonist partly prevents allergic airway inflammation in a transgenic mouse model [12]. However, the expression and localization of tachykinins in airway sensory neurons of the jugular and nodose ganglia complex (JNC) have yet to be elucidated under pathological situations such as in allergic airway inflammation.

Despite mouse models of allergic airway inflammation having a number of dissimilarities to human asthma [13], the mouse was chosen for this study. This choice was because of the lack of literature detailing murine airway neurobiology combined with the advantages of utilizing mice with manipulated genomes such as transgenic, gene knockouts or knockin strains [14,15].

### Materials and methods

#### Animals

Twenty-one female BALB/c mice (8 weeks old, Harlan Winkelmann, Borcheln, Germany) weighing 15–20 g were

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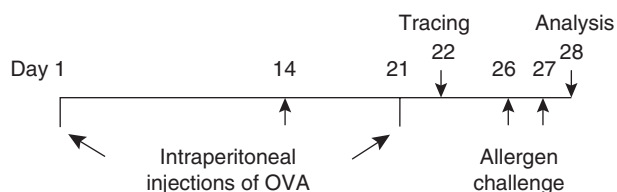


**Fig. 1.** Allergic sensitization and challenge protocol. The animals were systemically sensitized by means of intraperitoneal injections of ovalbumin (OVA) on days 1, 14, and 21 and were aerosol challenged twice on days 26 and 27. The tracing was carried out after allergen sensitization and before allergen challenge on day 22 and at day 28 the animals were sacrificed.

used for this study and housed in pathogen-free conditions for the duration of the investigation. 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). All protocols were approved by the state animal committee of Berlin, Germany (0059/01).

### Allergen sensitization

The protocol of allergic sensitization and challenge has been previously reported [16]. Seven animals were systemically sensitized by means of intraperitoneal injections of 10 µg ovalbumin (OVA, Grad VI, Sigma, Deisenhofen, Germany) adsorbed to 1.5 mg of aluminium hydroxide  $\text{Al}(\text{OH})_3$  (AlumImject, Pierce, Rockford, IL, USA) as adjuvant on days 1, 14, and 21. Before analysis on day 28, animals received two consecutive local aerosol challenges of 1% OVA. OVA was diluted in phosphate-buffered saline (PBS) and delivered by aerosol for a period of 20 min on days 26 and 27 ( $2 \times$  challenge) (Fig. 1). Non-immunized control mice ( $n = 7$ ) received intraperitoneal  $\text{Al}(\text{OH})_3$  plus PBS and a corresponding PBS challenge. A second group of control animals ( $n = 7$ ) was treated with intraperitoneal PBS and aerosol OVA challenge.

### Retrograde neuronal tracing

At day 22 of the sensitization and challenge protocol (Fig. 1), all 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 mid-cervical trachea was exposed and the tip of a 10-µL syringe (Hamilton, Bonaduz, Switzerland) was inserted between the fourth and fifth tracheal cartilage via a glass micropipette, and 5 µL of Fast blue tracing dye (2% aqueous solution containing 1% dimethyl sulfoxide, Sigma Chemical Co., St Louis, MO, USA) was infused into the right main stem bronchus. The resulting cut between the two cartilages was then sutured with a 7-0 Vicryl suture and the skin sutured with 4-0 Vicryl sutures (Ethicon, Norderstedt, Germany). No major bleeding was observed during or post-operatively. Following the dye injection, the animals were placed with an elevated head for approximately 5 min. We have previously demonstrated with this technique that the infused tracing substance is confined to the lower airways [8,17]. All animals recovered undisturbed post-operatively,

for a period of 7 days, when they were killed by  $\text{CO}_2$  asphyxiation. After asphyxiation and bronchoalveolar lavage (BAL) fluid recovery, the animals were retrogradely perfusion-fixed through the abdominal aorta with freshly prepared Zamboni's solution (2% paraformaldehyde, 15% picric acid, 0.1 M PBS, pH 7.4 for 5 min) as previously described [18]. This protocol was performed routinely to ensure optimal conditions for immunohistochemistry and has been previously published for use in the immunohistochemical analysis of ganglionic neurotransmitter expression.

### Determination of antibody titres

Blood was obtained from the lateral caudal veins on day 0 and on the days of the analysis. Serum concentrations of total IgE and allergen-specific IgE antibody titres were then determined by ELISA as previously described [16].

### Tissue preparation

Dissection of the Zamboni-fixed JNC and lungs was followed by rinsing in 0.1 M phosphate buffer (pH 7.4) and cryoprotected with 18% sucrose in 0.1 M phosphate buffer overnight. Serial 8 µm sections of the ganglia and lung tissues were then cut using a cryostat (Leica CM 1900, Bensheim, Germany) and air dried for 30 min.

### Histology of the lung

For the assessment of the airway inflammation, serial sections of Zamboni-fixed lung tissues of allergen sensitized/challenged and control animals were stained with haematoxylin and eosin.

### Bronchoalveolar lavage fluid

BAL was obtained after the animals were killed by  $\text{CO}_2$  asphyxiation and their tracheas cannulated. Airways were lavaged twice with 0.8 mL ice-cold PBS and the total number of cells was determined according to morphologic criteria of the immune cells as described previously [12].

### Immunohistochemistry

In total, 42 JNC (21 JNC of the right and 21 JNC of the left side) were investigated in the present study. Every fourth section from serial sections of the sensory ganglion complex (to avoid double counting, only neurons with nuclei were analysed) were first incubated for 1 h with PBS containing 10% normal swine serum to block non-specific protein-binding sites, and 0.5% tween 20 to enhance penetration of immunoreagents. Ganglia sections were incubated overnight with a monoclonal antibody from rat raised against SP/NKA (Boehringer Ingelheim, Heidelberg, Germany, dilution 1:400) overnight. The monoclonal antibody from rat raised against C-terminal of SP is classified as a pan-tachykinin-antibody as it was tested by Kummer et al. [19] as having cross-reactivity with NKA and NKB. Following several washing steps, all sections were incubated for 1 h with a Cy-3-conjugated donkey anti-rat-IgG (Jackson ImmunoResearch, Inc., Baltimore, MD, USA, dilution 1:200). Finally, the

sections were washed again in PBS and mounted in carbonate-buffered glycerol at pH 8.6 [17,20,21].

For immunohistochemical analysis, all neurons with an apparent nucleus were analysed using epifluorescence microscopy (DMRA2, Leica, 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) and Cy3 with the filter module (band-pass 525–560 nm, long-pass 570–650 nm). The sections were visualized with a camera system (Visitron-System, Imagine microscopy, Puchheim, Germany). With respect to the determination of the size of neurons, the neuronal diameter was calculated as the maximum and minimum diameters of cell bodies.

For quantification, every fourth section of each ganglion complex was selected from the serial sections for each ganglion complex (42 ganglia complex (two per animal = right and left JNC of 21 animals ( $2 \times 21 = 42$ )) – where each ganglion generated 65–100 sections ( $8 \mu\text{m}/\text{section}$ ). Thirty (30) to 50 sections were analysed per mouse. Pre-absorption studies showed no specific immunohistochemical signalling. Specific immunohistochemical signals also disappeared after replacement of primary antisera by PBS.

### Statistical analysis

Immunohistochemical analysis was carried out in a blinded fashion. The number of neutrophils, lymphocytes and eosinophils is expressed as the means  $\pm$  SD and the numbers of neurons are presented as means  $\pm$  SEM. Differences between the groups were analysed statistically using Student's *t*-test. *P*-values of  $<0.05$  were considered significant.

## Results

### Sensitization of mice to ovalbumin

All allergen sensitized and challenged BALB/c mice have developed signs of allergic airway inflammation. Additionally, animals sensitized to OVA by intraperitoneal injections expressed high total IgE levels and also developed high anti-OVA IgE titres. This provides strong evidence for allergic airway inflammation in these animals (Table 1). Histological study of the lung tissues of all sensitized and challenged animals showed infiltration of mononuclear cells in every compartment of the lung demonstrated by haematoxylin and eosin-stained lung tissue sections from allergen sensitized and challenged mouse. In contrast, the histology of the lung from control BALB/c mice did not contain inflammatory cell aggregates (Fig. 2).

**Table 1.** Serum Ig titres in OVA-sensitized and challenged and control BALB/c mice

| Ig                          | OVA-sensitization/challenge | Controls     |
|-----------------------------|-----------------------------|--------------|
| Total IgE (ng/mL serum)     | 1375 $\pm$ 269              | 139 $\pm$ 37 |
| Anti-OVA IgE (LU/mL serum)  | 948 $\pm$ 387               | 28 $\pm$ 13  |
| Anti-OVA IgG1 (LU/mL serum) | 2868 $\pm$ 658              | < 15         |

Serum levels of anti-OVA IgE, anti-OVA IgG1 and total IgE were analysed in mice sensitized and challenged to OVA or PBS by ELISA. Values are presented as means  $\pm$  SD; OVA, ovalbumin, LU, lab units; PBS, phosphate-buffered saline.

### Cell differentiation in bronchoalveolar lavage fluid

The differentiation of immune cells recovered from BALF showed an infiltration of the airways with leucocytes and eosinophils following the sensitization and airway allergen challenges. Total leucocytes, including numbers of lymphocytes and eosinophils were significantly increased in OVA-sensitized and challenged mice in comparison to controls (Fig. 3).

### Immunohistochemistry for tachykinins in JNC neurons innervating the airways

At the time of the tissue harvest, Fast blue dye was found in the epithelium of the right main stem bronchi, although the oesophagus wall, stomach and the main left bronchi of all analysed animals did not show any tracer substance. However, the present study cannot completely exclude the possibility that muco-ciliary activities could bring small amounts of Fast blue dye to the vicinity of the left bronchi and inhalation may cause some dye to enter the left small airways.

The percentage of tachykinin-immunoreactive neurons in allergen-sensitized and challenged JNC was found to be

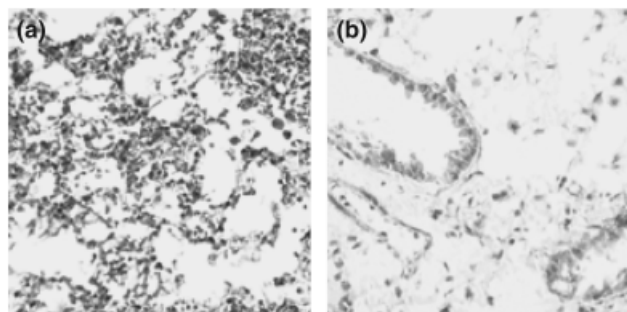


**Fig. 2.** Histological sections of the lungs from ovalbumin (OVA)-sensitized and challenged mice (a) showed infiltration in the airways with mononuclear cells which were stained with haematoxylin and eosin whereas the lungs from the control alum BALB/c mice (b) do not contain inflammatory cell aggregates ( $\times 240$ ).

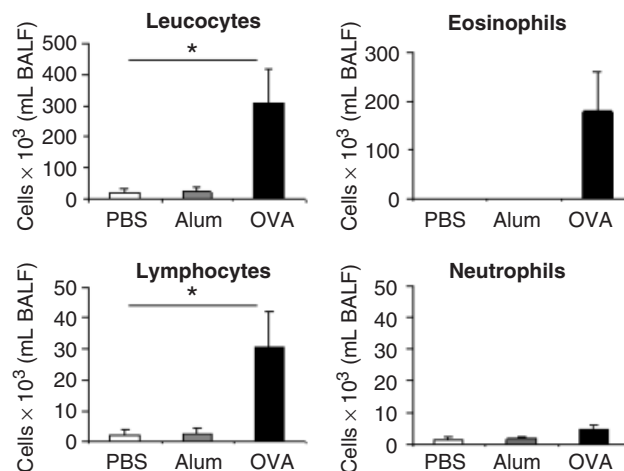


**Fig. 3.** Immune cells in bronchoalveolar lavage fluid (BALF) neutrophils, lymphocytes, eosinophils and total leucocytes in BALF of ovalbumin (OVA)-sensitized mice were differentiated according to morphologic criteria after allergen challenge. Results are expressed as mean  $\pm$  SD.

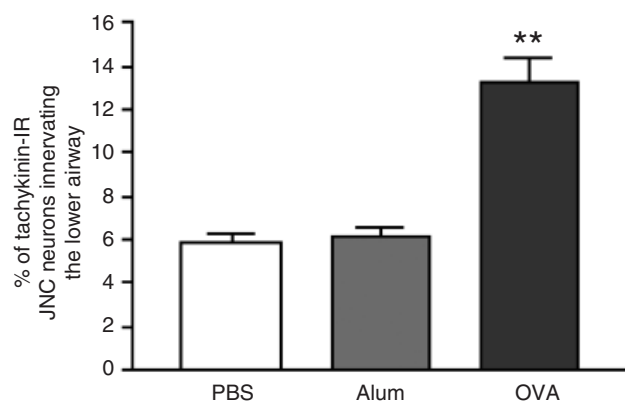


**Fig. 4.** The percentage of substance P/neurokinin A (SP/NKA)-immunoreactive airway-specific jugular and nodose ganglia complex (JNC) sensory neurons innervating the airways of alum-, phosphate-buffered saline (PBS)- and allergen-treated mice. A significant change of the airway-specific and tachykinin-immunoreactive neurons of JNC was found in ovalbumin (OVA)-treated mice in comparison to the controls with alum ( $P < 0.01$ ) and PBS ( $P < 0.01$ ). In OVA-sensitized and challenged airways, 4834 neurons which were retrograde labelled from the lower airway and for the controls (5627 neurons of the alum and 1450 neurons of the PBS group) neurons were investigated. Each value is a mean of seven animals  $\pm$  SEM.

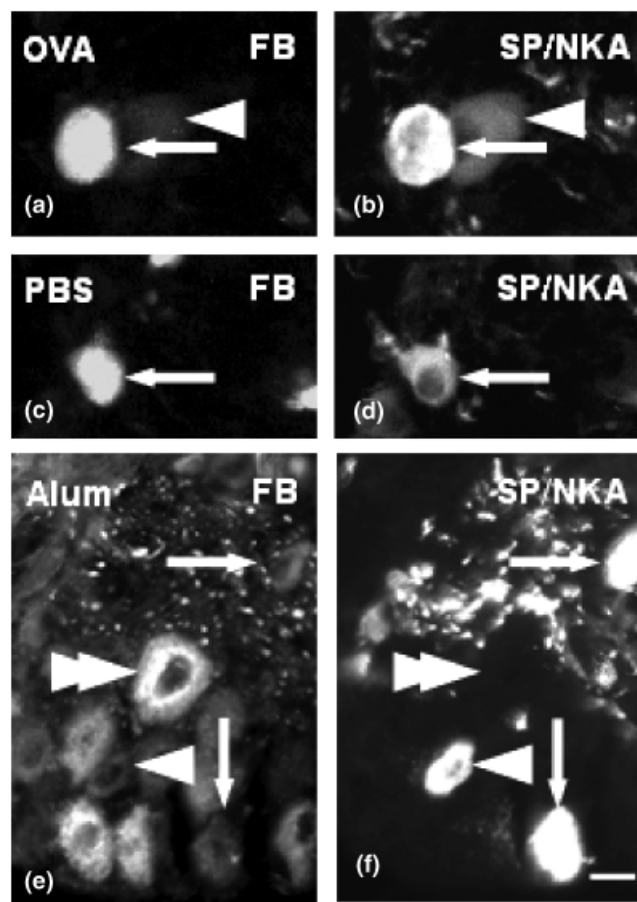


**Fig. 5.** Co-expression of substance P/neurokinin A (SP/NKA)-immunoreactivities (IR) in airway-specific jugular and nodose ganglia complex sensory neurons innervating the airways of alum-, phosphate-buffered saline (PBS)- and ovalbumin (OVA)-sensitized/challenged mice neuron that was labelled with Fast blue (FB) and immunoreactive for SP/NKA (OVA: arrows in (a) and (b); controls (PBS): arrows in (c) and (d); alum: arrows in (e) and (f)). Neuron was immunoreactive for SP/NKA, but was not labelled with FB (OVA: arrowheads in (a) and (b); alum: arrowheads in (e) and (f)). Neuron that was FB labelled, but was negative for SP/NKA was also observed (alum: double arrowheads in (e) and (f)). Scale bar represents 20  $\mu$ m.

**Table 2.** Percentage of SP/NKA-immunoreactive JNC sensory neurons innervating the control and allergen-treated airways

| Neuronal diameter                       | FB+/SP (NKA)+* ( $> 20 \mu$ m) | FB+/SP (NKA)+† ( $< 20 \mu$ m) |
|-----------------------------------------|--------------------------------|--------------------------------|
| Controls, Al(OH) <sub>3</sub> plus PBS‡ |                                |                                |
| JNC ( $n = 5627$ )                      | 1.2 $\pm$ 0.21                 | 5.8 $\pm$ 0.37                 |
| Allergic OVA IP/challenge§              |                                |                                |
| JNC ( $n = 4834$ )                      | 7.2 $\pm$ 1.35                 | 6.0 $\pm$ 0.43                 |

SP, substance P; NKA, neurokinin A; JNC, jugular nodose ganglia complex; FB, fast blue.

Percentage of retrogradely labelled neurons which express

\*FB+/SP(NKA)+ were neurons with diameters larger than 20  $\mu$ m.

†FB+/SP(NKA)+ were neurons with diameters smaller than 20  $\mu$ m.

‡Each value is a mean of the percentage obtained from 5627 FB labelled neurons of seven animals  $\pm$  SEM after alum treatment.

§Each value is a mean of the percentage obtained from 4834 FB labelled neurons of seven animals  $\pm$  SEM after allergen sensitization and challenge.

significantly increased from  $5.8 \pm 0.37\%$  to a maximum of  $13.2 \pm 1.43\%$ ,  $P < 0.01$  24 h after allergen challenge (Figs 4 and 5). In contrast to control JNC neurons, tachykinin-immunoreactive neurons of allergen-treated JNC were large in diameter. Tachykinin-immunoreactive neurons were seen in neurons with diameters larger than 20  $\mu$ m, 24 h after allergen challenge whereas control neurons expressing tachykinins were consistently found to be small to medium-sized with diameters smaller than 20  $\mu$ m (Table 2, Fig. 5).

We also observed that tachykinin-immunoreactive neurons were located in a preferring region of the immunohistochemical section. However, the exact location of such a neuronal cluster within the ganglion complex could not be determined in this study as the ganglion complex was not oriented before sectioning.

## Discussion

Airway nerves have the capacity to control airway functions via nervous reflexes and through neuropeptides such as tachykinins. Neuronal mechanisms are known to play a key role in the initiation and modulation of airway hyper-responsiveness and inflammation [22–24]. In this respect, tachykinins have been shown to mediate neurogenic allergic airway inflammation in guinea-pigs. In mouse airways, studies blocking tachykinin receptors using antagonists to NK-1 and NK-2 prevented allergic airway inflammation. This indicates the participation of tachykinins in the pathogenesis of allergic airway inflammation in the mouse [12].

However, the expression of tachykinins in airway-specific sensory neurons innervating the mouse airways has not been previously studied under pathological conditions such as allergic airway inflammation. The present study therefore aimed to investigate the effect of allergic airway inflammation on tachykinins expression in sensory airway nerves. Consistent with previous studies, airway sensory nerve fibres appear to uniformly originate from the JNC in species including guinea-pigs [8,9,17,19,25]. The present findings also revealed or demonstrated that tachykinin-immunoreactive neurons were located in a preferring region of the immunohistochemical section. However, the exact location of such a neuronal cluster within the ganglion complex could not be

determined in this study as the ganglion complex was not oriented before sectioning. Fast blue dye was only detected on the epithelium of the right main stem bronchus, whereas the epithelium of the oesophagus was free from the dye. This investigation was important to minimize the possibility that other non-airway neurons such as those with projection to the oesophagus were retrogradely labelled with Fast blue [26].

Despite the fact that murine models of allergic airway inflammation have a number of dissimilarities to human asthma [13], we selected the mouse for this study, as the use of transgenic, knockout or knockin mice was highly attractive. Furthermore, this species is commonly used to investigate immunological, genetic and neuro-immunological mechanisms underlying airway diseases such as bronchial asthma or chronic obstructive pulmonary diseases. In addition, contrary to the well-characterized immunological mechanisms of the mouse airways, mouse airway neurobiology remains poorly understood.

In the present study, tachykinins were extendedly expressed in small airway specific sensory neurons with diameters smaller than 20 µm under normal conditions. These findings suggest that expression of tachykinins was found in airway-specific sensory neurons, previously identified as capsaicin-sensitive and transient receptor potential variloid-1 (TRPV1) positive sensory neurons [27]. Similar results reported that tachykinergic neurons innervating the airways of guinea-pigs were also C-fibre profiles [9,25].

Interestingly, tachykinin expression was found to be induced in large-diameter airway-specific neurons in mice with allergic airway inflammation. This was confirmed by an increase in the number of leucocytes and eosinophils recovered from BALF in these animals [9,10]. Induction of tachykinins in vagal sensory neurons has already been described in guinea-pig airways by allergen and nerve growth factor (NGF) treatment [25,28] and murine airways by injection of NGF into the airways [17].

Previously, the induction of tachykinins was found in neurons with neurofilament-immunoreactivities that are known to be fast-conducting Aδ-fibres [25]. In the present study, induction of tachykinins was found in large-diameter neurons that may express neurofilament protein and probably belong to the cell bodies of Aδ-fibres. However, the exact physiological role of these airway-specific mouse neurons with phenotypic change remains to be investigated in the future.

Tachykinins such as SP/NKA and CGRP can be released to the site of inflammation by axonal transport [23,29–31] and by activation of the capsaicin (TRPV1) receptor [32–35]. In the airways released from afferent nerve terminals, SP acts on NK1-receptors and mediates neurogenic inflammation. Neurogenic inflammation can be described as a complex response consisting of increased vascular permeability, plasma extravasation, glandular secretion and pro-inflammatory cell influx and pulmonary vascular remodelling [7–18,20–24,19,25–36]. However, the effects of tachykinins have not, so far, been intensively studied in mouse airways and therefore, discussion of the role of tachykinergic neurons in mouse airway remains controversial. Previous experiments involving stimulation of tachykinergic afferent nerve fibres showed a relaxation of the airway smooth muscle in the mouse [37] whereas in guinea-pigs, tachykinins are known to cause bronchoconstriction [22].

In the present study, the possibility that allergic inflammation may lead to a higher neurotransmitter output in the airways cannot be excluded, as tachykinin concentration recovered from BALF was not investigated.

In conclusion, based on the induction of tachykinins by allergic airway inflammation, these data suggests that the effect of allergen challenge may contribute to the pathogenesis of airways diseases such as bronchial asthma.

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