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Stress induces substance P in vagal sensory neurons innervating the mouse airways

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Summary

Background Tachykinins-like substance P (SP) have been shown to play an important role in initiating and perpetuating airway inflammation. Furthermore, they are supposed to be released into tissues in response to stress.

Objective The aim of this study was to investigate the effects of stress alone or in combination with allergic airway inflammation on SP expression in sensory neurons innervating the mouse airways.

Methods Balb/c mice were systemically sensitized to ovalbumin (OVA), followed by allergen aerosol exposure, and compared with non-sensitized controls. Additionally, OVA-sensitized and -challenged and non-sensitized mice were exposed to sound stress. SP expression in airway-specific and overall vagal sensory neurons of the jugular and nodose ganglion complex was analysed using retrograde neuronal tracing in combination with immunohistochemistry. Preprotachykinin A (PPT-A) mRNA, the precursor for SP, was quantified in lung tissue by real-time PCR. Bronchoalveolar lavage (BAL) fluid was obtained, and cell numbers and differentiation were determined.

Results Stress and/or allergic airway inflammation significantly increased SP expression in retrograde-labelled vagal sensory neurons from the mouse lower airways compared with controls [stress: $15.7 \pm 0.8\%$ (% of retrograde-labelled neurons, mean $\pm$ SEM); allergen: $17.9 \pm 0.4\%$; allergen/stress: $13.1 \pm 0.7\%$ vs. controls: $6.3 \pm 0.3\%$]. Similarly, SP expression increased in overall vagal sensory neurons identified by the neuronal marker protein gene product (PGP) 9.5 [stress: $9.3 \pm 0.6\%$ (% of PGP 9.5-positive neurons, means $\pm$ SEM); allergen: $12.5 \pm 0.4\%$; allergen/stress: $10.2 \pm 0.4\%$ vs. controls: $5.1 \pm 0.3\%$]. Furthermore, stress significantly increased PPT-A mRNA expression in lung tissue from OVA-sensitized and -challenged animals, and immune cells were identified as an additional source of SP in the lung by immunohistochemistry. Associated with enhanced neuronal SP expression, a significantly higher number of leucocytes were found in the BAL following allergen exposure. Further, stress significantly increased allergen-induced airway inflammation identified by increased leucocyte numbers in BAL fluids.

Conclusion The central event of sound stress leads to the stimulation of SP expression in airway-specific neurons. However, in sensitized stressed mice an additional local source of SP (probably inflammatory cells) might enhance allergic airway inflammation.

Keywords airway inflammation, allergy, mouse, sensory neuron, sound stress, substance P

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Introduction

Bronchial asthma is considered a chronic inflammatory airway disease, characterized by reversal episodes of air-flow obstruction. Psychological factors like stress have been associated with the exacerbation of major asthma

symptoms [1]. Along these lines various clinical studies have found a strong correlation between stressful experiences and the onset of asthma symptoms [2–5].

Stress can be defined as a stimulus that activates both the hypothalamic pituitary adrenal (HPA) axis and the sympathetic nervous system. This activation induces

humoral and neuronal changes that may finally lead to an adaptation of the individual to the changed environment [6]. Recently, animal studies have revealed that stress further leads to the release of neuropeptides, such as the tachykinin substance P (SP), from sensory neurons, resulting in the induction or exacerbation of various inflammatory processes [7]. Thus, SP has been associated to stress-linked diseases such as abortion in mice [8, 9]. In rats, acute immobilization stress triggered mast cell activation and degranulation via SP released from primary afferent sensory nerve fibres in the skin [10] and the bladder [11]. In a murine model of allergic airway inflammation, we have recently demonstrated that exogenously applied stress enhanced the allergen-induced airway inflammation and airway hyper-reactivity [12]. This enhancement was abrogated by the application of the highly specific non-peptide neurokinin 1 (NK-1) receptor antagonist RP 68750, indicating an important role of SP in stress mediation [13].

Airway sensory innervation mainly originates from the jugular-nodose ganglia and thoracic dorsal root ganglia [14]. Sensory nerves are known to contain pro-inflammatory neuropeptides including the tachykinins SP and neurokinin A (NKA). Under physiological as well as pathophysiological conditions, tachykinins influence airway functions via neuronal reflexes and the release of a variety of mediators that cause bronchoconstriction [15–17], hyperaemia [18] and increased mucus secretion [19].

Tachykinins are released into the airways after exposure to allergen in allergic airway inflammation. Atopic subjects showed higher levels of SP in bronchoalveolar lavage (BAL) fluid compared with non-atopic individuals, and allergen provocation further increased this difference [20]. In a guinea-pig model of allergic airway inflammation, a significant increase in SP and NKA was detected in lung tissues after allergen challenge. A higher expression of the SP and NKA encoding preprotachykinin-I (PPT-I) mRNA and higher tachykinin protein levels were found in the nodose ganglia of allergen-challenged animals [21].

Although enhanced tachykinin expression in airway-specific sensory neurons after allergen exposure has been attributed to neuronal plasticity [21], the effect of stress on SP expression in neurons innervating the airways has not been investigated. Therefore, the present study aimed to explore the effect of stress on sensory airway innervation, addressing the following questions:

- (1) does stress increase tachykinin expression in retrograde-labelled airway-specific sensory neurons of the jugular and nodose ganglion complex (JNC) and
- (2) is there a synergistic effect of stress and allergen exposure on SP expression in sensory JNC neurons innervating the airways of mice.

Methods

Animals

Balb/C mice were purchased from Harlan-Winkelmann (Borchen, Germany) and maintained on a 12 h light/dark cycle. Animal care and experimental procedures were followed according to institutional guidelines and conformed to the requirement of the state authority for animal research conduct (LaGetSi, Berlin, Germany).

Protocol of sensitization and challenge

Balb/C mice were sensitized by intraperitoneal (i.p.) injection of 10 µg chicken ovalbumin (OVA, Grad VI, Sigma Chemie, Deisenhofen, Germany) emulsified in 1.5 mg Al(OH)₃ (Alum Imject Pierce, Rockford, IL, USA) on days 0, 14 and 21. Mice were challenged twice with OVA aerosol (1% OVA diluted in phosphate-buffered saline (PBS)) via the airways on days 26 and 27, as described previously [22]. Control animals were sham sensitized by Al(OH)₃ i.p. injections and PBS challenged (Fig. 1).

Stress treatment

After i.p. sensitization, mice were randomized into four different experimental groups (Fig. 1). At the time of the first aerosol challenge, two groups (PBS/stress, *n* = 6 and OVA/stress, *n* = 6) were exposed to sound stress for a 24 h period. The sound stress was emitted by a rodent-repellent device (Conrad Electronics, Berlin, Germany) at a frequency of 300 Hz (sound pressure level 75–80 dB) for 1 s each time, in irregular intervals of about 15 s. The stress device was placed in their home cage, so that the mice were not able to escape perception of the sound. Meanwhile, the other groups (PBS/no stress, *n* = 6 and OVA/no stress, *n* = 6) remained in a room undisturbed of sound.

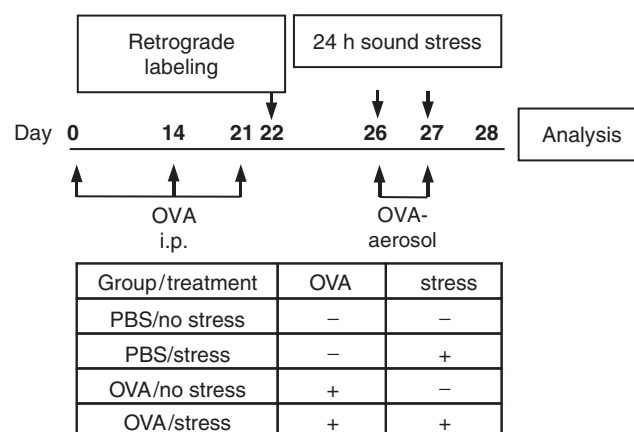


Fig. 1. Experimental design and group denomination.

Retrograde neuronal tracing and tissue preparation

On day 22, 6 days before the analysis, animals were anaesthetized with an i.p. injection of ketamine hydrochloride (Ketanest, Parke-Davies, Freiburg, Germany, 10 mg/kg, body weight) and xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany, 10 mg/kg). The trachea was exposed and incised to gain direct access to the tracheal lumen, to reach the right main stem bronchus, bypassing the larynx. The cartilage rings were visible and countable by using a stereoscope during the operation. The tip of a 10 µL syringe (Hamilton, Bonaduz, Switzerland) was inserted between the fourth and fifth tracheal cartilage via a glass micropipette and a 5 µL injection of FluoroGold tracing dye (hydroxystilbamidine; Biotium Inc., Hayward, CA, USA) dissolved in normal saline (0.9%) containing 1% dimethylsulphoxide (DMSO), was administered into the right main stem bronchus [23]. After the injection procedure, animals were kept in a vertical, head-up position for about 5 minutes. All animals recovered undisturbed for a post-operative period of 4 days until challenge and stress exposure. On day 28, 24 h after the last challenge, the animals were perfusion-fixed retrogradely through the left ventricle with freshly prepared Zamboni's solution (2% paraformaldehyde, 15% picric acid, 0.1 mol/L PBS, pH 7.4) for 5 min. Dissection of the Zamboni-fixed JNC and lung tissue was performed by rinses in 0.1 mol/L PBS (pH 7.4) and cryoprotection with 18% sucrose in 0.1 mol/L PBS overnight.

Bronchoalveolar lavage

In an additional set of animals ($n = 24$) treated the same way as described but not perfusion fixed, BAL fluid was obtained 24 h after the second airway challenge (day 28). Animals were killed by cervical dislocation and tracheas were cannulated. Airways were lavaged twice with 0.8 mL ice-cold PBS, and the total number of cells in BAL fluid was determined. Cytospins were prepared for each sample by centrifugation of 50 µL BAL fluid. After fixation, cytopins were stained with Diff Quik (Dade Behring, Marburg, Germany) and differential cell counts were performed. Cells were classified as neutrophils, eosinophils, lymphocytes or macrophages using standard morphological criteria and counting at least 100 cells per cytospin [12].

Immunohistochemical labelling for substance P and protein gene product 9.5

Immunohistochemistry for SP was carried out as described previously [23]. In brief, after blocking of non-specific protein binding sites, the ganglia and lung sections were subjected to single- or double-labelling immunohistochemistry by overnight incubation with a

monoclonal antibody from rat raised against SP (Chemicon Inc., Temecula, CA, USA, dilution 1 : 400) and the neuronal marker protein gene product (PGP) 9.5 (dilution 1 : 1000; Biotrend, Köln, Germany) to identify neurons within the ganglia. After rinsing in PBS, a biotinylated anti-rat-IgG (Amersham, Pharmacia Biotech, Freiburg, Germany, dilution 1 : 100) was used for 1 h. After several washing steps, the sections were incubated with the secondary reagent Streptavidin-Texas-Red conjugate (Amersham, dilution 1 : 200) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (dilution 1 : 100; Cappel, Aurora, OH, USA) for 1 h. Finally, the sections were washed again in PBS and mounted in carbonate-buffered glycerol at pH 8.6.

The ganglia and lung were cut in serial sections that were 8 µm thick. Each ganglion provides about 100 sections. Every eighth section of each ganglion (in total, about 1100–1800 neurons for each group in 24 sections for both left and right side JNC, 12 sections of each jugular-nodose ganglion) was selected to be analysed by immunohistochemistry. To avoid double counting of neurons, only neurons with an apparent nucleus were counted using an epifluorescence microscope (Leica, DMRA2, Weilburg, Germany) with appropriate filter combinations. Photo documentation and analysis was performed by a digital image analysis system (SPOT software, Visitron, Puchheim, Germany). For immunoreaction specificity testing, pre-absorption studies were carried out and no specific immunohistochemical signalling was detected. Specific immunohistochemical signals also disappeared after replacement of the primary antisera by PBS.

RNA isolation, reverse transcription and real-time polymerase chain reaction

Total RNA was extracted from whole lung tissue. Isolated tissue was frozen in liquid nitrogen and stored at -80°C until use. About 100 mg/mouse lung tissue were treated with 1 mL Trizol (Invitrogen, Karlsruhe, Germany) and RNA was then extracted [24]. The total RNA was treated with DNase I (1 U/µg RNA, Invitrogen), followed by inactivation with EDTA (Invitrogen). There was no detection of genomic DNA via PCR. First strain cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen) [24]. Real-time PCR was used to obtain quantitative data on differences of preprotachykinin A (PPT-A, precursor mRNA from which SP is synthesized) mRNA expression between OVA-sensitized and -challenged mice with and without stress (OVA/stress vs. OVA/no stress). This assay uses the 5' nuclease activity of AmpliTaq Platin DNA polymerase (Invitrogen) to cleave a fluorogenic Taq Man Probe designed for PPT-A during a PCR reaction. As endogenous reference for target gene mRNA, PCR for the constitutively expressed protein hypoxanthine guanine phosphoribosyl transferase (HPRT)

was performed for each sample. PCR primers and fluorogenic probes for target genes were purchased from Tib Molbiol (Berlin, Germany). The following sequences for primers and probes were used:

- mPPT1-F: 5'-CTA AAT TAT Tgg TCC gAC Tg
- mPPT1-R: 5'-TTC TgC ATT gCg CTT CTT TC
- mPPT1-TM: 5'-6FAM-ACC CAA gCC TCA gCA gTT CTT Tgg AT XT A p
- mHPRT-F: 5'-gTT ggA TAC Agg CCA gAC TTT gT
- mHPRT-R: 5'-CAC Agg ACT AgA ACA CCT gC
- mHPRT-TM: 6FAM-TTg CAg ATT CAA CTT gCg CTC A XT CTT PH

The real time PCR reactions were developed as published [24], except that we used 0.25 μ L probe at 20 μ M. Each analysis was normalized to HPRT, by calculating the difference between the 'threshold cycle' (C_T) for PPT-A and the C_T for HPRT as $\Delta C_T = C_T \text{ PPT-A} - C_T \text{ HPRT}$. The amount of mRNA was calculated using the formula $1/2^{\Delta C_T}$ (for details, see [24]). The mean of $1/2^{\Delta C_T}$ from non-stressed animals (OVA/no stress) was set as one and compared with the mean of stressed animals (OVA/stress).

Statistics

The results are presented as mean values $\pm$ SEM. Significance of differences between groups was determined using the non-parametric Kruskal–Wallis test. The Mann–Whitney *U*-test was used to compare two groups, respectively. Significance was set at $P \leq 0.05$.

Results

Airway inflammation

To evaluate airway inflammation, BAL fluids were analysed for the number and type of leucocytes. Significantly higher numbers of total leucocytes were found in OVA-sensitized and -challenged animals exposed to stress (OVA/stress, mean $416 \pm 58 \times 10^3/\text{mL}$) compared with OVA-sensitized and -challenged, non-stressed mice (OVA/no stress, $204 \pm 33 \times 10^3/\text{mL}$, $P \leq 0.01$, Fig. 2). Cell differentiation revealed that the difference between the two groups was predominantly based on an increased number of eosinophils (mean $237 \pm 52 \times 10^3/\text{mL}$ vs. $115 \pm 25 \times 10^3/\text{mL}$, $P \leq 0.05$).

The examination of sham-sensitized animals revealed no difference between stressed and non-stressed mice (PBS/stress, mean $33 \pm 11 \times 10^3/\text{mL}$ vs. PBS/no stress, mean $33 \pm 8 \times 10^3/\text{mL}$, Fig. 2). The dominating cell population in both groups was macrophages ($32 \pm 11 \times 10^3/\text{mL}$ vs. $31 \pm 7 \times 10^3/\text{mL}$).

OVA-sensitized and -challenged animals (OVA/no stress, OVA/stress) showed significantly higher total and

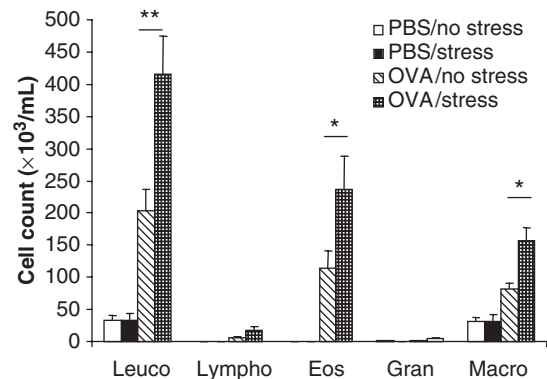


Fig. 2. Total leucocyte number and cell type identification in bronchoalveolar lavage (BAL) fluids. BAL was performed 24 h after last aerosol challenge. Columns represent mean and SEM. (leuco, leucocytes; lympho, lymphocytes; eos, eosinophil granulocytes; gran, neutrophil granulocytes; macro, macrophages).

differential cell counts than sham-treated animals (PBS/no stress, PBS/stress). To keep the figure concise, these results are not indicated in detail in Fig. 2.

Immunohistochemistry for substance P in airway-specific jugular–nodose ganglia neurons

To identify the share of SP expressing neurons from the airways, SP-positive cells were determined in the jugular–nodose ganglia from all retrograde-labelled neurons. In non-stressed non-sensitized animals (PBS/no stress), only $6.3 \pm 0.3\%$ of retrograde-labelled neurons from the jugular–nodose ganglia were positive for SP, whereas $15.7 \pm 0.8\%$ ($P < 0.01$ compared with PBS/no stress) of the neurons in non-sensitized stressed mice (PBS/stress) were found to express SP (Fig. 3a and b).

Non-stressed mice that were exposed to allergen sensitization/challenge (OVA/no stress) showed $17.9 \pm 0.4\%$ ($P < 0.01$ vs. PBS/no stress) of SP-positive retrograde-labelled neurons in the jugular–nodose ganglia. Animals exposed to both allergen sensitization/challenge and stress (OVA/stress) showed $13.1 \pm 0.7\%$ ($P < 0.01$ vs. PBS/no stress) of SP-positive neurons, a percentage that is significantly lower than the percentage found in animals only exposed to allergen sensitization/challenge (OVA/no stress, $P < 0.01$) (Fig. 3a and b).

Immunohistochemistry for substance P and protein gene product 9.5 in jugular and nodose ganglion complex ganglia neurons

Using antibody against PGP, overall neurons in JNC ganglia were identified. Double-labelling immunohistochemistry revealed that $5.1 \pm 0.3\%$ of PGP 9.5-positive neurons of the JNC ganglia in the PBS/no stress group displayed SP immunoreactivity. A percentage of

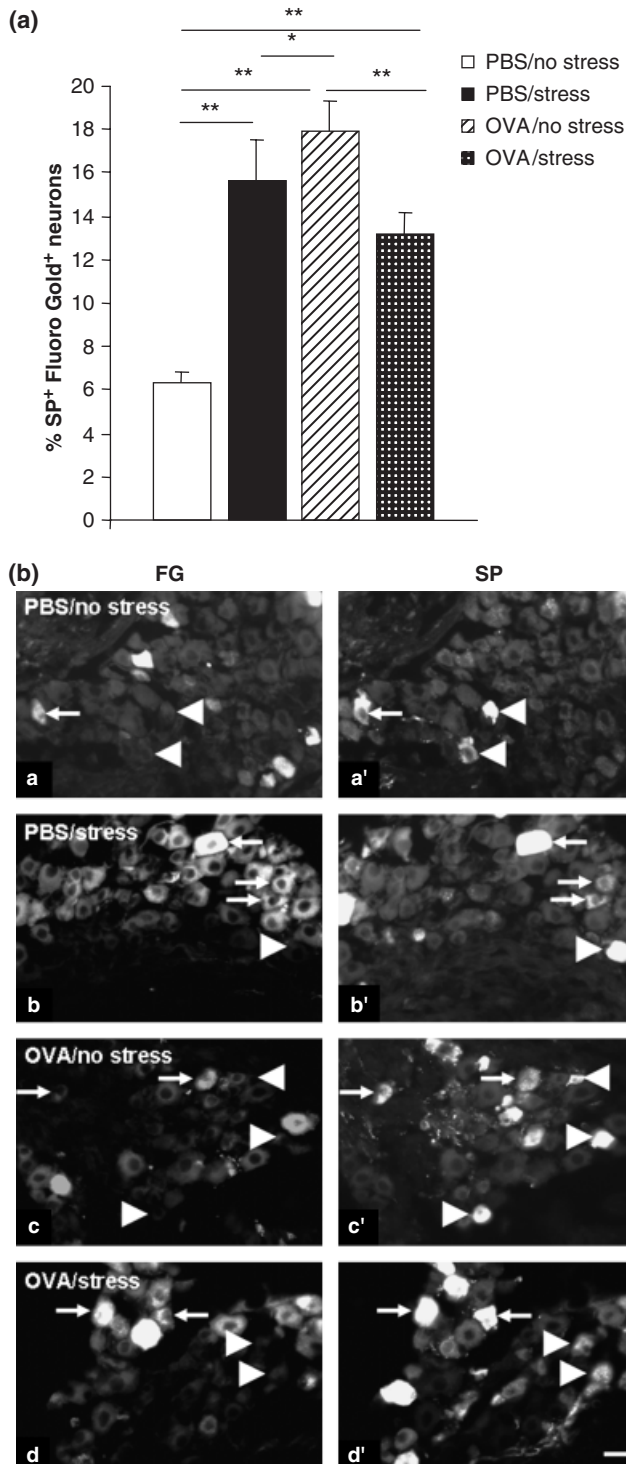


Fig. 3. (a) Expression of substance P (SP) immunoreactivity in jugular and nodose ganglion complex (JNC) neurons innervating the lower mouse airways. Columns represent mean and SEM. (b) JNC neurons of different groups (for group denomination, please see Fig. 1) were retrograde-labelled with FluoroGold (FG) and stained for SP immunoreactivity (phosphate-buffered saline (PBS)/no stress, arrows in (a) and (a'); PBS/stress, arrows in (b) and (b'); OVA/no stress, arrows in (c) and (c'); OVA/stress, arrows in (d) and (d')). Non-airway neurons expressing SP (arrowheads). Scale bar represents 15 μ m.

$9.3 \pm 0.6\%$ ($P < 0.01$ vs. PBS/no stress) neurons positive for PGP 9.5 was found to express SP in the PBS/stress group (Fig. 4a and b).

In OVA/no stress animals, $12.5 \pm 0.4\%$ ($P < 0.01$ vs. PBS/no stress) of all PGP 9.5-positive neurons were SP-positive and $10.2 \pm 0.4\%$ ($P < 0.01$ vs. PBS/no stress) in the OVA/stress group were found to be SP-positive. Thus, the percentage found in the OVA/stress group was significantly lower than in the OVA/no stress group ($P < 0.01$, Fig. 4a and b).

Immunohistochemistry for substance P in the lung

Qualitative immunohistochemical analysis for SP expression in the airways demonstrated that it was found mainly in nerve fibres innervating the vasculature of the airways from PBS/no stress, PBS/stress, OVA/no stress and OVA/stress mice. Immune cells from PBS/no stress, PBS/stress, OVA/no stress and OVA/stress mice were also found to express SP (Fig. 5).

Preprotachykinin-A messenger RNA in allergic airway inflammation

To determine non-neuronal sources of SP in allergic airway inflammation, we assessed PPT-A mRNA expression (encoding for SP) in lung tissues. Stress exposure significantly enhanced PPT-A expression in the OVA/stress group when compared with the OVA/no stress animals ($P < 0.05$, Fig. 6).

Discussion

In the present study, we showed for the first time that exogenously applied stress was associated with increased SP expression in JNC neurons, particularly in airway innervating sensory neurons.

After allergen sensitization and challenge, stressed animals showed a marked influx of leucocytes in the airways, reaching significantly higher levels than in non-stressed mice. In contrast, in non-sensitized animals, stress had no influence on cell numbers in BAL fluids. Stress-induced enhancement of allergic airway inflammation is in line with recently published data from Forsythe et al. [25], describing increased cell numbers in BAL fluids of mice with allergic airway inflammation after stress, and similar results have been observed in rats [26, 27]. In the literature, the classic effect of chronic stress on the immune response has been reported as suppression of cellular immunity [28, 29], while acute stress has been associated with enhanced immune responses [30–33]. Here, we found an enhancing effect of acute stress on allergic airway inflammation.

The pathways, by which sound stress influences airway pathophysiology, are largely unknown. In a previous

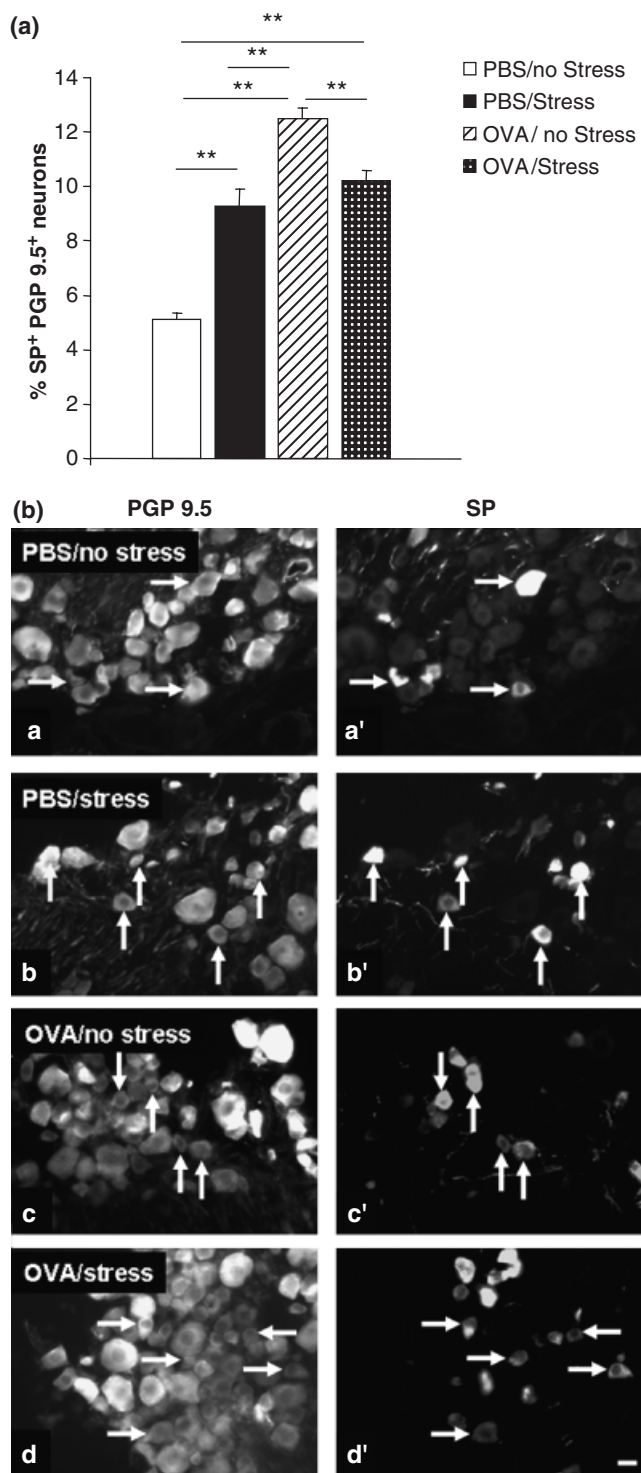


Fig. 4. (a) Expression of protein gene product (PGP) 9.5 and substance P (SP) immunoreactivity in overall jugular and nodose ganglion complex (JNC) neurons. Columns represent mean and SEM. (b) JNC neurons of different groups (for group denomination, please see Fig. 1) were double stained with a pan-neuronal marker PGP 9.5 (PGP 9.5) and for SP-immunoreactivity (phosphate-buffered saline (PBS)/no stress, arrows in (a) and (a'); PBS/stress, arrows in (b) and (b'); OVA/no stress, arrows in (c) and (c'); OVA/stress, arrows in (d) and (d')). Scale bar represents 15 μ m.

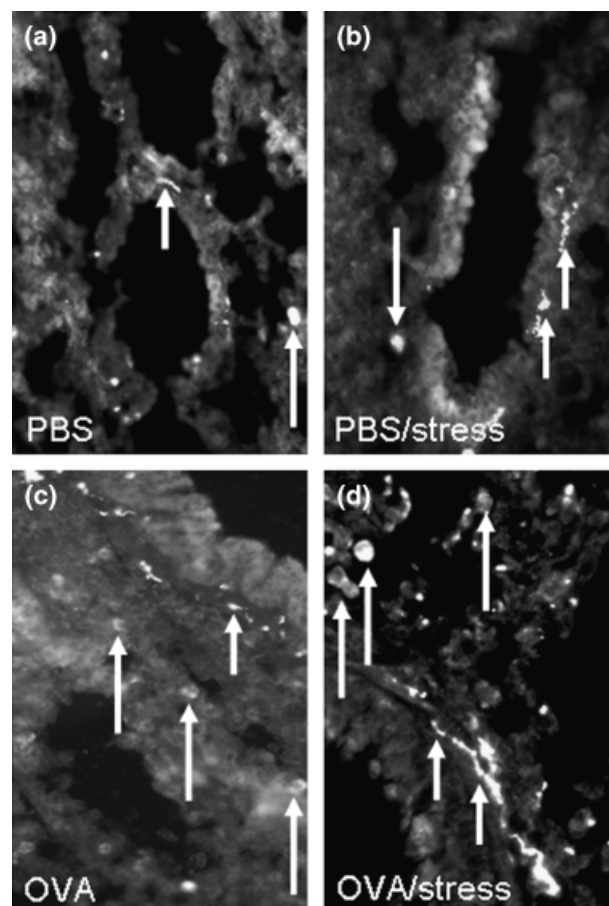


Fig. 5. Immunoreactivity of substance P (SP) in the lower airways (for group denomination, please see Fig. 1). SP expression was found mainly in nerve fibres (small arrows) innervating the vasculature of the airways from phosphate-buffered saline (PBS)/no stress (a), PBS/stress (b), OVA/no stress (c) and OVA/stress mice (d). Immune cells of all groups were found to express SP (large arrows) ($\times 240$).

study, we recently demonstrated a mediating role for the SP NK1 receptor [13]. A hard-wired neuroanatomical structure that is involved in the pathophysiology of airway inflammation is the system of sensory nerves. Airway sensory nerves are known to synthesize pro-inflammatory neuropeptides including SP in the nerve cell bodies, located in the ganglia. These mediators are transported to the synapses and released into the airways. Under physiological as well as pathophysiological conditions, the neuropeptides influence airway functions via neuronal reflexes and the release of a variety of subsequent mediators that cause bronchoconstriction [15–17], hyperaemia [18] and increased mucus secretion [19]. The importance of sensory neurons in airway inflammation might be appreciated by depletion studies, in which the elimination of airway sensory neurons by continued exposure to capsaicin abolished airway hyper-reactivity and attenuated airway inflammation in guinea-pigs [34].

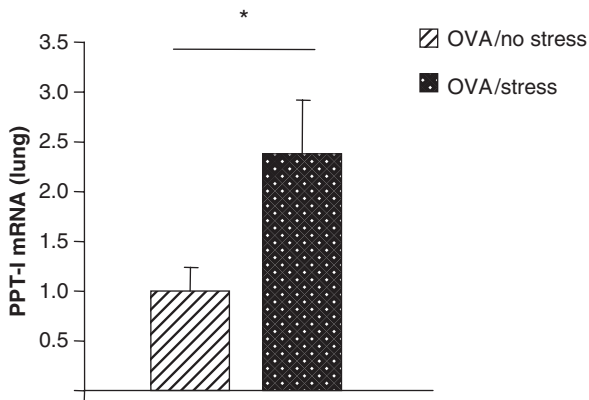


Fig. 6. Preprotachykinin-A mRNA expression in lung tissue from animals with allergic airway inflammation. The amount of mRNA was calculated using the formula $1/2^{\Delta C_T}$ (for details, please see 'Methods'). The mean of $1/2^{\Delta C_T}$ from non-stressed animals (OVA/no stress) was set as 1 and compared with the mean of stressed animals (OVA/stress). Columns represent mean and SEM.

A variety of local stimuli that induce SP expression and release from sensory neurons have been identified. In an animal model of adjuvant-induced paw inflammation increased content of SP in dorsal root ganglion (DRG) sensory nerves and axonal transport towards the paw has been demonstrated [35]. With respect to airway sensory nerves, several groups have recognized various substances, including low-dose capsaicin, bradykinin, tobacco smoke, dry air, allergen and NGF that elicited SP expression and local release [21, 23, 36–39]. Fischer et al. [21] have demonstrated for the first time that allergen sensitization and challenge induces SP in airway sensory nerves in guinea-pigs. SP expression in the nodose ganglia was associated with increased SP contents in lung tissues. In the guinea-pig model, non-sensitized animals showed a percentage of about 0.2%, whereas in OVA-sensitized and -challenged animals 10% of retrograde-labelled neurons were SP-positive. In the present study we also found a highly significant increase in SP-positive neurons in the JNC ganglia on comparing the respective groups (PBS/no stress, OVA/no stress). The higher percentages found in mice might be due to species-specific differences as reported before for sensory neurons of different species such as rat, guinea-pig and mouse [40–42]. Moreover, in our study, the entire JNC complex has been examined. SP expression in airway sensory neurons of non-stressed mice without (PBS/no stress) and with OVA treatment (OVA/no stress) corresponds to results found in earlier studies by Dinh et al. [23, 43].

In contrast to the above-mentioned examples, in which local stimuli led to the induction of SP locally or in sensory ganglia, we have found that a significant increase of SP expression in the JNC ganglia might also be caused

by a centrally processed event. In airway sensory neurons of stressed mice (PBS/stress), the SP expression was elevated as compared with non-stressed mice (PBS/no stress). This result suggests that the central event of exogenously applied stress might be a stimulus for the induction of SP in sensory neurons. Similarly, a higher percentage of SP-positive neurons was also found in PGP 9.5-positive, overall JNC neurons of stressed control (PBS/stress) and OVA-sensitized and -challenged animals (OVA/stress), further strengthening this hypothesis. Recent data from a murine model of stress-impaired hair growth support our findings. Sensory neurons in DRG innervating the skin were retrograde labelled with FluoroGold and stress was applied to the animals, which, 2 days after stress, showed a significantly higher percentage of SP-positive FluoroGold-labelled neurons in DRG than non-stressed control animals [44]. Future research will focus on identifying the remaining structures of the puzzle.

Interestingly, elevated SP expression in sensory airway nerves without the local stimulus of allergen exposure as seen in the PBS/stress group did not induce airway inflammation. As in the guinea-pig model [21], in the present model the SP expression in sensory airway neurons was significantly higher in allergic airway inflammation (OVA/no stress) compared with non-sensitized animals (PBS/no stress). Likewise, the combination of allergic airway inflammation and stress (OVA/stress) resulted in increased SP expression compared with non-stressed control animals (PBS/no stress). Surprisingly, compared with stress or OVA conditions alone, the combination of airway inflammation and stress did not lead to any further increase in SP-positive airway sensory neurons but to a significant decrease. We only can provide hypothetical explanations for these findings. The immunohistochemical analysis just demonstrates that these neurons contain SP. Following the activation of, for example TRPV1 receptor on these neurons, SP can be released into the airways to mediate neurogenic inflammation [45]. At the time point of examination, when differences in airway inflammation are already measurable, this release might have already occurred. Alternatively, these findings lead to the suggestion that combining conditions (OVA and stress) may act as a stimulus resulting in higher release of SP per neuron, as reflected by increased leucocyte numbers in BAL fluid, but not in a further increase of SP-expressing neurons. The mechanism of SP induction in sensory airway neurons may be mediated by nerve growth factor (NGF). Following external stress, NGF has been found to be increasingly expressed [46, 47]. Aggressive behaviour, a form of social stress, has been demonstrated to increase serum and hypothalamic NGF levels [48–50]. NGF has been shown to induce SP in airway sensory nerves [39]; thus, exposure to sound stress may lead to an up-regulation of SP in the airways via NGF. Future investigation will identify the

exact role that NGF plays in the transmission of stress-induced effects.

From earlier experiments, we knew that the increased accumulation of inflammatory cells in the BAL of stressed animals was effectively inhibited by an NK-1 receptor antagonist, which blocked the effect that stress had on inflammation, via the inhibition of SP [13]. However, the total amount of SP in the airways might not only stem from neuronal cells but might also originate from non-neuronal cells that are present during inflammatory conditions. It has been shown that inflammatory cells like macrophages, eosinophils, lymphocytes and dendritic cells express SP *in vitro* as well as *in vivo* [51–54]. Recently, Springer *et al.* [55] demonstrated that in chronic obstructive pulmonary disease (COPD), besides sensory neurons, inflammatory cells seem to be an important source of tachykinins. Furthermore, SP induces chemotaxis in neutrophils, eosinophils, monocytes and lymphocytes *in vitro* [56–60]. The release of SP from immune cells under stress might consequently amplify the inflammatory response. To uncover possible non-neuronal local sources of SP, we examined PPT-A mRNA expression in lung tissues from animals with allergic airway inflammation. A significantly higher expression of PPT-A mRNA was found in lung tissue samples from the OVA/stress group compared with the OVA/no stress group. Furthermore, immunohistochemistry for SP in lung sections revealed that the tachykinin is expressed in immune cells present in airway inflammation. Therefore, the increasing effect of stress on airway inflammation might in part be mediated by SP originating from immune cells that are present in lung tissue during allergic airway inflammation, synergizing with neuronal SP release.

In conclusion, the present data suggest an up-regulation of SP in airway-specific sensory neurons after the central event of sound stress via a yet unknown pathway. In OVA-sensitized and -challenged and challenged animals, stress-induced SP of neuronal origin adds to the locally synthesized SP, thus enhancing local allergic airway inflammation.

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