

Phenotypic alteration of neuropeptide-containing nerve fibres in seasonal intermittent allergic rhinitis

W. Heppt*, Q. Thai Dinh†‡, A. Cryer*, M. Zweng§, O. Noga‡, C. Peiser†, M. Melvan‡, C. Witt‡, A. Fischer† and D. A. Groneberg†‡

*Department of Otorhinolaryngology, Karlsruhe Teaching Hospital, University of Freiburg, Karlsruhe, Germany, †Division of Allergy Research, Department of Pediatric Pneumology and Immunology, Charité, Humboldt-University, Berlin, Germany, ‡Department of Medicine, Charité, Humboldt-University, Berlin, Germany and §Department of Otorhinolaryngology, Hannover School of Medicine, Hannover, Germany

Summary

Background Allergic rhinitis (AR) is the most common allergic disease affecting the respiratory tract. Next to inflammatory changes, the airway innervation plays an important modulatory role in the pathogenesis of the disease.

Objective To examine the participation of different neuropeptides in the human nasal mucosa of intermittent (seasonal) AR tissues in the allergic season.

Methods Immunohistochemistry for substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP) and neuropeptide tyrosine (NPY) was related to the characterization of inflammatory cells in tissues of patients with seasonal AR ($n = 18$).

Results While there was a significant increase in the number of eosinophils present if compared with a control group, no changes occurred in mast cell numbers. Immunostaining was abundantly found in different nerve fibre populations of both groups. SP expression was significantly increased in mucosal nerve fibres of patients with intermittent (seasonal) AR. Also, significantly increased numbers of VIP- and NPY-immunoreactive nerve fibres were found in biopsies of rhinitis patients in comparison with sections of normal human nasal mucosa. In contrast, CGRP expression did not change significantly.

Conclusion The increase of neuropeptide expression in mucosal nerve fibres indicates a major role of the autonomous mucosal innervation in the pathophysiology of intermittent (seasonal) AR.

Keywords allergy, human, mucosa, nasal, neuropeptides, rhinitis, seasonal

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Introduction

Intermittent (seasonal) allergic rhinitis (AR) is one of the most common allergic diseases with a high prevalence in Europe and the United States. It is commonly caused by pollinosis with representative plants for the different months: willow, hazel and alder from January to March, birch in April and May, grasses and rye from June to August, mugwort and plantain from August to September. The peak age of AR is between 10 and 25 years of age for the intermittent (seasonal) AR and approximately 20 years of age for the perennial AR, without difference between the genders [1].

AR is an IgE-mediated immediate allergic reaction (type I according to Combs and Gell [2]) with the main symptoms of nasal obstruction, pruritus, sneezing and rhinorrhea. Histamine is the most important mediator with effects such as sneezing, pruritus and nasal obstruction [3]. Following nasal

challenge the histamine level in nasal lavage fluid is increased in allergic patients but not in non-allergic patients [4]. These effects are mediated by H1 receptors on sensory nerve fibres and vascular smooth muscle [5]. Rhinorrhea results from increased vascular permeability and glandular secretion [6]. In addition, there are many other mediators such as leukotrienes, prostaglandins, bradykinine, platelet-activating factor (PAF), ILs and neuropeptides [7–10].

A previous study in patients with AR reported an increase of concentration of the neuropeptides substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP) in nasal secretions after nasal provocation and suggested a major modulatory role for the upper airway innervation in the development of the disease [11] but detailed studies on the expression of neuropeptides in the mucosa in AR have not been carried out so far.

The nasal mucosa is innervated by sensory, sympathetic and parasympathetic nerve fibres that lead to a co-transmission of classic transmitters (noradrenalin (NA) and acetylcholine (ACh)) and of neuropeptides (e.g. neuropeptide Y (NPY), VIP, CGRP and SP) after stimulation [12]. While sympathetic neurons contain NA and NPY, the transmitter

Correspondence: David Groneberg, Division of Allergy Research, Biomedical Research Center, Charité, Humboldt-University, BMFZ OR-3, Augustenburger Platz 1, D-13353 Berlin, Germany.
E-mail: david.groneberg@charite.de

profile of parasympathetic neurons is represented by ACh and VIP [12, 13].

As the sensory airway innervation has recently been demonstrated to participate in airway inflammatory changes [14, 15], the present study was carried out to assess differences in neuropeptide expression in human mucosal airway nerve fibres of patients with allergic intermittent (seasonal) rhinitis.

Methods

Patients

In total 30 subjects who underwent rhinosurgical intervention were included in the study. After approval by the local Ethics Committee and informed consent of each individual was obtained, two groups of study subjects were recruited (Table 1): A reference collective and a group of intermittent (seasonal) AR patients. The diagnosis was made after rhinological and allergological history taking, nasal examination, rhinomanometric test, prick test, nasal smear and saccharin test were performed. Also, nasal provocation or a serum IgE counts by radio immunosorbens assay completed the diagnostic procedures. All protocols were performed according to the Helsinki declaration.

Tissue sampling

Surgical biopsies of the lower nasal conchae were obtained during rhinosurgical interventions due to airflow limitations with causes such as deviation of the septum or conchal hyperplasia. During surgery, biopsies of the lower nasal conchae were taken, and processed as described previously [16]. The samples were immersed for 4 h in a Stefanini/Zamboni solution [17], washed twice in phosphate-buffered saline (PBS) and left overnight in 18% saccharose (Riedel-de Haen AG, Seelze, Germany) for cryoprotection. The samples were then snap-frozen in liquid nitrogen and stored in cryotubes (Nunc, Wiesbaden, Germany) at -80°C before cryostat cutting to 6–8 μm sections.

Eosinophil and mast cell quantification

Cryostat-cut sections were stained with haematoxylin–eosin according to a standardized [18] protocol to examine the tissue morphology. For differential counts, sections were stained with toluidine–giemsa dye to differentiate between toluidine blue mast cells and giemsa stained eosinophils.

Alternatively, sections were incubated with human eosinophil cationic protein (ECP) clone EG1 and EG2 antibodies to mark the eosinophils, and anti-human mast cell tryptase antibody, and anti-IgE antibody to stain mast cells (Table 2). Primary antibodies were detected using biotinylated secondary antibodies and a streptavidin–biotin–peroxidase complex which transforms chromogen 3-amino-9-ethylcarbazol (AEC) to a red colour. Endogenous tissue peroxidase was blocked by initial incubation with 0.5% H_2O_2 –methanol. For negative controls the tissue slices were incubated either without primary or secondary antibodies.

Eosinophils and mast cells were quantified using 250-fold magnification and a grating (Olympus, Hamburg, Germany), which was used to quantify the number of eosinophils and mast cells of 1000 lamina propria-associated nucleated cells by two independent observers. After a normal distribution was found using the Kolmogorov–Smirnov test, the groups were tested for significant differences ($P < 0.05$) using the Student *t*-test. Results were verified by the Bonferroni–Holm procedure.

Neuropeptide immunohistochemistry

Immunohistochemistry was carried out as described previously [19, 20] using a modified immunohistochemical method [21]. In brief, 8 μm cryostat sections were mounted onto chromalaun pre-coated slides, washed and air-dried for 1 h. Pre-incubation with 2% low fat milk powder in PBS pH 7.4 for 1 h at room temperature was followed by several washes in PBS and incubation with different primary antibodies (Table 3) overnight. For detection of the primary antisera, different solutions of secondary antibodies were used (Table 4). Control incubations were performed by omitting the primary or secondary antibodies. The slides were

Table 1. Patient characteristics

Patient collective	Total	Male/female	Age range (years)	Allergies	Number of smokers	Total IgE (IU/L)
Reference collective	12	6/6	17–66	0	0	8.84 ± 1.04
Seasonal allergic rhinitis	18	10/8	9–68	18: seasonal 0: perennial	4	57.43 ± 16.8 ($P = 0.02996$)

Table 2. Antibodies used to mark eosinophils and mast cells by indirect streptavidin–biotin-binding methods

Antibody	Dilution	Order number	Company	Host
Eosinophil cationic protein (ECP) clone EG1	1:100	10-9195-01	Kabi Pharmacia AB (Uppsala, Sweden)	Monoclonal mouse
ECP clone EG2	1:100	10-9196-01	Kabi Pharmacia Diagnostics AB	Monoclonal mouse
Mast cell tryptase	1:1500	MAB 1222	Chemicon International Inc. (Terneucula, CA, USA)	Monoclonal mouse
IgE	1:30	010195 A	Bio-Science Products AG (Emmenbrücke, Switzerland)	Monoclonal mouse

Table 3. Primary antibodies used for neuropeptide immunohistochemistry

Antibody	Dilution	Order number	Company	Host
Protein gene-product 9.5 (PGP 9.5)	1:800	RA95101	Ultraclone (Wellow, UK)	Polyclonal rabbit
Substance P (SP)	1:40	YMC1021	Accurate Chemical & Scientific Corp. (Westbury, NY, USA)	Polyclonal rabbit
Calcitonin gene-related peptide (CGRP)	1:400	RAS6009N	Peninsula (Belmont, CA, USA)	Polyclonal rabbit
Vasoactive intestinal peptide (VIP)	1:400	RAS7161N	Peninsula	Polyclonal rabbit
Neuropeptide tyrosine (NPY)	1:400	CA08295	CRB (Cambridge, UK)	Polyclonal rabbit

Table 4. Secondary and tertiary reagents

Antibody	Dilution	Order number	Company	Host
Anti-rabbit IgG, FITC conjugated	1:25	4271-1	BioMakor (Rehovot, Israel)	Goat
Anti-rabbit IgG, biotin conjugated	1:50	1004	Amersham (Little Chalfont, UK)	Goat
Anti-streptavidin texas red	1:50	RPN1233	Amersham	—

finally mounted in carbonate-buffered glycerol (pH 8.6) and viewed using epifluorescence microscopy.

For semi-quantitative evaluation of the mucosal nerve neuropeptide expression a 90-fold magnification was used and the staining intensity was evaluated using a previously established score [22–24]: grade 0 represents no detectable staining, grade 1 represents occasional (focal) staining, grade 2 represents few nerve fibre profiles (diffuse weak) detectable, grade 3 represents many nerve fibre profiles, and grade 4 densely innervated mucosa.

Results

Distribution of the eosinophils and mast cells

The eosinophils were found predominantly in the subepithelial layer and around glands and small blood vessels (Fig. 1). Immunohistochemical analysis with EG1 and EG2 antibodies revealed a similar distribution as giemsa staining. Toluidine- and tryptase/IgE-positive mast cells were found in the same regions as eosinophils. There was no major difference in staining of toluidine or IgE-marked mast cells between both groups.

Eosinophil and mast cell quantification

Percentages of cells positive for eosinophil markers in the control group were $1.3 \pm 0.9\%$ (SD) for giemsa, $2.6 \pm 1.3\%$ for EG1 and $2.7 \pm 1.2\%$ for EG2 of 1000 lamina propria-associated nucleated cells. For mast cell markers, $6.6 \pm 3.2\%$ for toluidine, $6.5 \pm 3.5\%$ for IgE and $6.8 \pm 3.2\%$ for tryptase of 1000 lamina propria-associated nucleated cells were found in the control group. In the intermittent (seasonal) allergy group there was a marked increase in the amount of eosinophils compared with the reference group. The differences were highly significant: $12.3 \pm 5.0\%$ for EG1 and $12.7 \pm 5.2\%$ for EG2 in the allergy group compared with $2.6 \pm 1.3\%$ and $2.7 \pm 1.2\%$ in the control group ($P < 0.001$). The percentage of mast cells varied between $7.1 \pm 3.5\%$

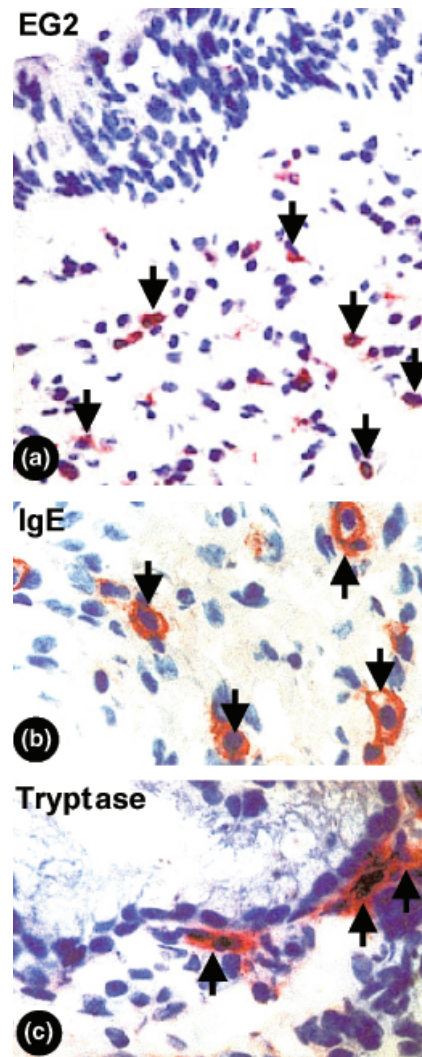


Fig. 1. Mast cell and eosinophil distribution. High-power photomicrographs of nasal mucosal sections that were cryostat cut and submitted to immunohistochemistry for eosinophil (a) and mast cell (b, c) identification. An abundant presence of eosinophils and mast cells in the mucosa was revealed using EG2, IgE and tryptase antibodies (arrows). Original magnification $\times 125$ (a) $\times 250$ (b, c).

(toluidine), $8.2 \pm 3.2\%$ (tryptase) and $8.3 \pm 3.0\%$ (IgE) for the different methods in the allergy group without a significant difference.

Cellular expression of neuropeptides

Immunohistochemistry for protein gene product 9.5 (PGP 9.5) led to an abundant staining of nerve fibres that were

present in the lamina propria around blood vessels, sinusoids and glandular structures. There was also a network of PGP 9.5-positive fibres below the basal membrane with a few nerves reaching into the epithelium. CGRP immunohistochemistry resulted in staining for CGRP-positive nerve fibres around blood vessels, sinusoids and glands.

SP immunoreactivity was found predominantly in fibres that were localized below the epithelium and around glands. Immunostaining for VIP-reactive nerve fibres was present around glands and blood vessels whereas NPY immunoreactivity was predominantly found around blood vessels (Fig. 2).

Quantification of neuropeptide expression in mucosal nerve fibres

In the control group, the immunoreactivity scoring was 1.75 ± 0.37 for SP, 1.58 ± 0.36 for CGRP, 1.17 ± 0.44 for VIP and 1.00 ± 0.35 for NPY.

In the group with intermittent (seasonal) AR there were significant increases found for SP (2.84 ± 0.16 , $P < 0.005$), VIP (2.63 ± 0.26 , $P < 0.005$) and NPY (2.37 ± 0.35 , $P < 0.02$) (Fig. 3) while CGRP did not increase significantly (1.80 ± 0.28 , $P = 0.68641$).

Discussion

The human nasal mucosa is abundantly innervated by sensory nerve fibres that originate from the trigeminal ganglion

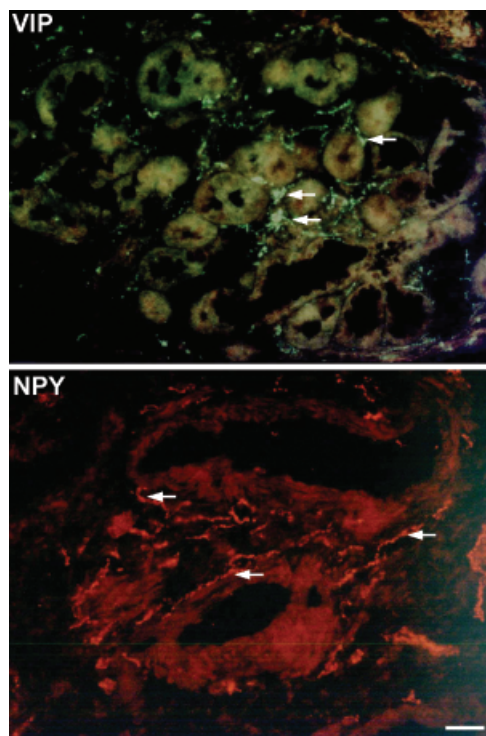


Fig. 2. Localization of nasal mucosal nerves using immunohistochemistry. Surgical specimen of inferior nasal turbinates from rhinitic patients were processed to cryostat sections and submitted to immunohistochemical protocols using neuropeptide antibodies. Vasoactive intestinal polypeptide (VIP)- and neuropeptide tyrosine (NPY)-positive nerve fibres (arrows) were abundantly present as branching networks underneath the epithelium. Scale bar represents 45 μ m.

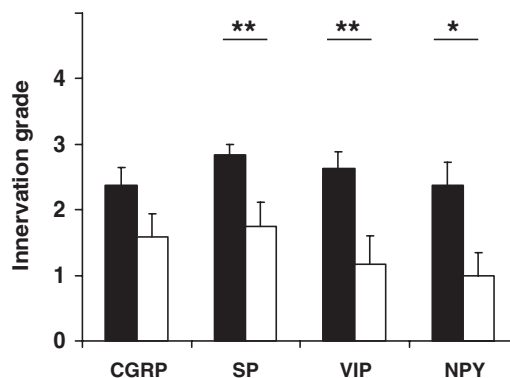


Fig. 3. Semi-quantitative immunohistochemistry of neuropeptide-containing nerve fibres. The scores of calcitonin gene-related peptide (CGRP), substance P (SP), vasoactive intestinal polypeptide (VIP), neuropeptide tyrosine (NPY) immunoreactive nerve fibres were examined by two independent observers in the nasal mucosa of randomized control (white bars) and intermittent (seasonal) allergic rhinitis (black bars) patients.

and contain SP, CGRP and neurokinin A (NKA) [25, 26]. They can be divided into unmyelinated group C fibres that are specifically activated by capsaicin and fibres that are resistant to capsaicin but can be activated by nicotine [27–29]. Sensory fibres may also be activated by other substances, i.e. histamine, serotonin or bradykinin [30] and the resulting secretion of neuropeptides like SP and CGRP leads to glandular hypersecretion and vasodilation. These features are pathologically altered in intermittent (seasonal) AR.

Here we established experiments on the alteration of the neuropeptide innervation of the nasal mucosa and found significant differences for mediators such as SP. This peptide belongs to the group of tachykinins that consist of 10–11 amino acids [31]. SP-containing nerve fibres have been previously reported to be present in the upper respiratory tract around blood vessels, glands, between muscle fibres and in the epithelium [32]. SP leads to vasodilation, the extravasation of proteins, increased mucociliary clearance and glandular secretion [33, 34]. Tachykinins such as SP can also induce degranulation of mast cells with consecutive synthesis of cytokines and leucocyte chemotaxis and to a production of inflammatory cytokines by monocytes [35]. CGRP is a peptide consisting of 37 amino acids which is often co-localized with tachykinins in sensory neurons [25, 27, 36].

A correlation between nerves and inflammatory cells has been discussed for a long time and peptides such as CGRP can produce delayed erythema accompanied by infiltration of polymorphonuclear leucocytes [37, 38]. It has also been previously shown that nasal challenges performed with SP in patients suffering from AR lead to an increase of nasal airway resistance (NAR) [39] and a protein extravasation.

The mucosal eosinophil numbers are increased in the lamina propria in subjects with AR after nasal allergen challenge [40, 41]. The present findings on elevated eosinophils support these findings but do not show significantly increased numbers of mast cells.

In previous experiments using allergen-sensitized guinea-pigs, a decreased histamine content in the nasal mucosa and increased SP and CGRP in nerve endings in the nasal mucosa were found after intranasal provocation [42] which is in accordance with the present data on human nasal mucosa.

Similarly, in the acute phase of nasal provocation of patients with AR, significantly elevated levels of SP and VIP were found in nasal lavages [11]. For the lower respiratory tract it was also earlier reported that the SP concentration in bronchoalveolar lavage increases significantly (P less than 0.05) after bronchial allergen provocation [39]. The present results demonstrate a significant elevation of neuronal SP and VIP expression. This phenomenon of neuropeptide up-regulation in two distinct nerve fibre subpopulations may be explained by an activation of sensory nerves (SP), which results in an axon reflex that leads to a consecutive activation of VIP-containing nerve fibres that project to numerous effector cell types [16, 18, 43].

Next to SP and VIP, a significant increase in NPY expression was found in mucosal nerves. Sympathetic nerve fibres frequently contain both NA and the neuropeptide NPY, which consists of 36 amino acids and belongs to the group of pancreatic polypeptides [44, 45]. It leads to vasoconstriction, raising the effect of NA [12, 46]. It was previously suggested that NPY plays an important physiological role in nasal mucosa autonomous innervation and that loss of NPY could lead to non-allergic rhinopathies [12]. Here we found increased levels of NPY expression in AR, which may be explained by an allergy-dependent stimulation.

We have previously demonstrated that neuropeptide-like immunoreactivity also changes in other rhinitis forms. In this respect, changes were found in biopsies of patients with aspirin-sensitive rhinitis [47], hyper-reflectoric rhinitis [23] and toxic rhinitis [24]. Toxic rhinitis patients had significantly elevated expression scores for VIP (2.83 ± 0.31 vs. 1.27 ± 0.47 control group) and NPY (3.17 ± 0.31 vs. 0.91 ± 0.37 control group) revealing an increase of mediators in distinct subpopulations of airway nerves [24]. In contrast, patients with hyper-reflectoric rhinitis had significant increases for SP (3.00 ± 0.37 vs. 1.64 ± 0.34 control group staining intensity) and VIP (2.33 ± 0.42 vs. 0.82 ± 0.33) [23]. In relation to these previous findings for non-AR, the present study indicates that there are differences in the innervation pattern between different rhinitis types since VIP, NPY and SP are commonly not co-stored in airway nerve fibres and therefore belong to different populations of airway nerves. However, while the distribution of single neuropeptides may vary, the overall finding of an increased neuronal activation in allergic and non-AR is clearly the same. A mechanistic link between allergy and the presently observed neuronal changes may be related to the induction of neurotrophin (NT) expression in intermittent AR. In this respect, the NTs nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3 and NT-4 have been shown to play a pivotal role in the development of neuronal changes underlying allergic inflammation [48]. Interestingly, it has been demonstrated that patients with allergic diseases have increased levels of NTs in the blood as well as locally in the airways and that NT release from immune cells is triggered by allergen contact [50]. NTs can then modulate afferent nerve function by stimulating the production of neuropeptides within airway afferent neurons. Such an increase was observed in the present study. The neuropeptides may be released from the central terminals of airway afferent neurons, which leads to heightened autonomic reflex activity, and typical symptoms of rhinitis as observed in the present group of intermittent seasonal AR.

In conclusion, the present study describes the induction of neuropeptide expression in different mucosal nerve subpopulations in AR. These data indicate a direct relationship between human allergic intermittent (seasonal) rhinitis and neuronal plasticity, which has previously only been directly shown for the lower airway innervation in an animal model of allergic asthma [49].

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