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ORIGINAL PAPER

Mechanism of interaction between ocular and nasal neurogenic inflammation in allergic rhinoconjunctivitis

Xiao-Wei Gao · Xiao-Min Zhang · Hai-Yan Liu · Shan-Shan Wang · Hua-Jiang Dong

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Abstract

Purpose The mechanisms of naso-ocular interaction in allergic rhinoconjunctivitis are not well understood. Neurogenic inflammation affects both eyes and nose via the same neurogenic factors. The purpose of this study was to investigate the effects of neurogenic inflammation on conjunctival inflammation following nasal allergen provocation.

Methods Sensitized rats were exposed to ovalbumin (OVA) via the nose. Parts of the nasal mucosa and conjunctivae were sliced and used for hematoxylin–eosin staining, immunohistochemical analysis, western blotting, and real-time polymerase chain reaction. The slides were observed under a light microscope, and the acquired images were analyzed. The levels of substance P (SP), vasoactive intestinal peptide (VIP), and nerve growth factor (NGF) were detected.

Results The levels of SP, VIP, and NGF were increased in both nasal mucosa and conjunctivae 1 h and 24 h after OVA administration ($p < 0.05$). Higher levels of SP, VIP, and NGF expression were observed in the nasal mucosa and conjunctivae 24 h after OVA administration ($p < 0.05$). Following damage of the nasal sensory nerves by capsaicin, the protein and mRNA levels of SP, VIP, and NGF were reduced.

Conclusion In conclusion, the increased levels of VIP, SP, and NGF might be responsible for the ocular reaction following nasal challenge with allergen in rats.

Keywords Allergic rhinoconjunctivitis · Conjunctiva · Nerve growth factor · Substance P · Vasoactive intestinal peptide

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Introduction

The allergic conjunctivitis commonly accompanies allergic rhinitis (AR); consequently, the World Allergy Organization proposed that the disorder be appropriately termed allergic rhinoconjunctivitis (ARC) [1]. ARC is characterized by type I immunoglobulin E (IgE)-mediated hypersensitivity reaction to environmental allergens. It is a common allergic condition, affecting 10–40% of the general

population [2, 3]. Nevertheless, due to the high prevalence and the influence on patients' quality of life, it possesses clinical and socioeconomic relevance. Since ocular allergy is closely linked to AR, demonstrating similarities in epidemiology, pathophysiology, and treatment, the pathophysiologic mechanisms thought to be involved in the generation of ocular symptoms with nasal challenge deserve special attention.

Ocular symptoms in patients with ARC are thought to have three possible underlying mechanisms. Most evidently, direct exposure of the conjunctivae to allergens leads to topical IgE-mediated mast cell activation, which parallels the nasal immune reaction. Another possible mechanism is: various types of cells or factors released from nasal mucosa during allergic reactions reach the conjunctivae through the blood vessels, lymphatic system, and nasolacrimal duct, causing ocular symptoms. Finally, neurogenic inflammation may contribute to the generation of conjunctivitis in AR, as several clinical and experimental observations point in the direction of a naso-ocular reflex. The naso-ocular reflex has been theorized based on several studies [4–9].

Neurogenic inflammation is the process by which inflammatory mediators, including inflammatory neuropeptides and neurotrophins, are released from afferent nerve terminals in a target tissue and trigger inflammation with vasodilatation, increased vascular permeability, and hypersensitivity [10, 11]. The nasal mucosa and conjunctivae have a common embryonic origin and innervations (sensory nerves, and sympathetic and parasympathetic fibers). Parasympathetic nerves richly innervate the eyes, entering them after traveling in conjunction with the parasympathetic input to the nasal cavity. Parasympathetic innervations governing the tear film and nasal secretion intersect at the pterygopalatine ganglion. Several studies have concluded that ocular symptoms following nasal challenge occur secondary to the release of acetylcholine (ACh) from the parasympathetic nerve endings [12–14], called cholinergically mediated central reflex.

In addition to ACh, there are other neurogenic messengers that complete the signal transmission inside the neurogenic system. These neurotransmitters are neuropeptides and neurotrophins. Neuropeptides associated with ocular and nasal neurogenic inflammation include substance P (SP), calcitonin gene-related peptide (CGRP), neurokinin A (NKA), vasoactive intestinal peptide (VIP), and neuropeptide Y (NPY). Of these, SP, CGRP, and NKA are sensory neuropeptides, released from the sensory nerve fibers of the C category; VIP is a neuropeptide released from the nasal mucosa and the postganglionic parasympathetic fibers of the conjunctivae [15]; NPY is released from sympathetic fibers and has no effect on the secretion of mucous glands [16, 17]. Nerve growth factor (NGF), a neurotrophin that regulates neuronal development, enhances the production of neuropeptides and is associated with ocular and nasal neurogenic inflammation [15, 18, 19]. There are no studies on the role of neuropeptides, except SP [17, 20, 21], and the naso-ocular interaction in ARC.

The aim of this study was to illustrate the mechanism of interaction of ocular and nasal neurogenic inflammation in ARC. Therefore, we investigated the expression of SP, VIP, and NGF in nasal mucosa and conjunctivae at different stages following nasal allergic challenge in a rat model of ARC. Further, we examined the effects of topical capsaicin on the mechanisms of the neuropeptides and neurotrophin released following the allergic challenge.

Method

Animals

Sixty healthy male Sprague–Dawley rats (300–350 g, 10–12 weeks of age) were obtained from Beijing Hua Fukang Biological Polytron Technologies Inc. (Beijing, China) and bred in the laboratory of Institute of Biomedical Engineering, Chinese Academy of Medical Sciences and Peking Union Medical College. All animals were allowed to acclimate for 1 week before experimentation. The rats were housed at 19–25 °C, with 40–60% humidity and 12-h light/dark cycle (lights on at 7 am), and had free access to food and water. All experiments were conducted during the light phase of the light/dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Rat model of ARC

The basic sensitization was performed with intraperitoneal (i.p.) injection of 0.8 mL allergen suspension containing 0.3 mg ovalbumin (OVA; Grade V; Sigma, St. Louis, MO, USA) and 30 mg $Al(OH)_3$ gel (Xi'an Heart Biological Technology Co., Ltd., Xian Shi, Shaanxi, China) in 40 rats: a total of 7 times, once every 48 h. Further, 100 μL 2% OVA suspension drops (1 mg 50 μL^{-1} $nostril^{-1}$) were administered into nasal cavities of the rats bilaterally using a micropipette for endonasal (e.n.) challenge: a total of 7 times, once every 24 h. Twenty sensitized rats were administered 1 mM of capsaicin (N-vanillylnonanamide; Sigma) e.n. on day 10 [20]. All the rats were conscious and fixed appropriately during the procedures to prevent them from rubbing the solution from nose to eyes.

Study design

Animals were randomly allocated into 3 groups: (1) the non-capsaicin group (NC, $n = 20$) consisted of rats that were sensitized and intranasally challenged via administration of OVA and were administered saline e.n. instead of capsaicin on day 10; (2) the capsaicin group (C, $n = 20$) consisted of rats that were challenged similarly with OVA and administered capsaicin e.n. on day 10; and (3) the normal control group (N, $n = 20$) consisted of rats that were repeatedly sensitized and challenged with saline at the same dose. Half of the rats in each group were killed by cervical dislocation 1 h after the last challenge (NC1, C1, and N1), and the other half were killed after 24 h (NC24, C24, and N24).

Real-time polymerase chain reaction

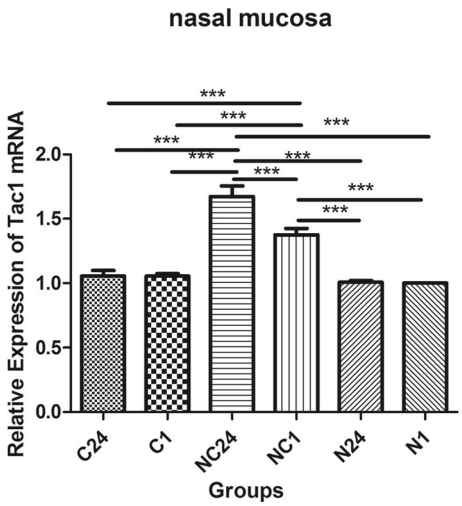
The right nasal mucosa and conjunctivae were dissected and prepared for real-time polymerase chain reaction (RT-PCR) to detect the level of *Tac1* (encoding SP), *Vip*, and *Ngf* mRNAs. Total mRNA was extracted using the Ultrapure RNA Kit (code: D9108B, TaKaRa Bio Inc., Japan). Each sample was reverse-transcribed to cDNA for 15 min at 37 °C using a reverse transcription kit (code: DRRO47A, TaKaRa Bio Inc.). The sequences of the primers used were as follows: *Tac1* forward 5′TGGCGGTCTTTTTTCTCGTT3′, reverse 5′GCATTGCCTCCTTGATTTGG3′; *Vip* forward 5′ACGCCAAACTTACAAAGA3′, reverse 5′GGAGTAGATAGAGCCCCA3′; *Ngf* forward 5′CGGTCTTCCCGCCCTAGCCTG3′, reverse 5′ATTTGCACGCCGCTCCTTTGC3′; *Gapdh* forward 5′CGTATCGGACGCCTGGTT3′, reverse 5′GTGCCGTTGAACTTGCCG3′. The levels of *Tac1*, *Vip*, *Ngf*, and *Gapdh* expression were detected by RT-PCR, using the SYBR Green and SYBR Premix Ex Taq (code: DRR420A, TaKaRa Bio Inc.).

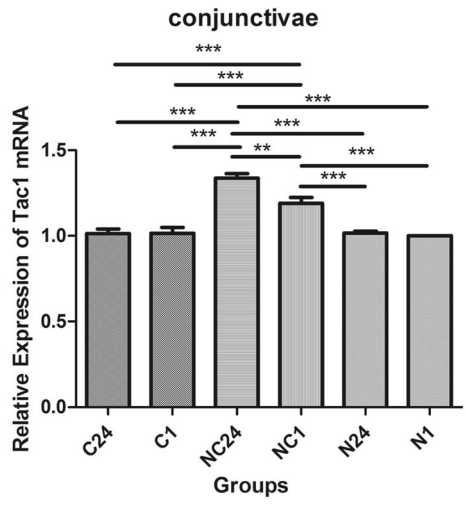
Western blotting

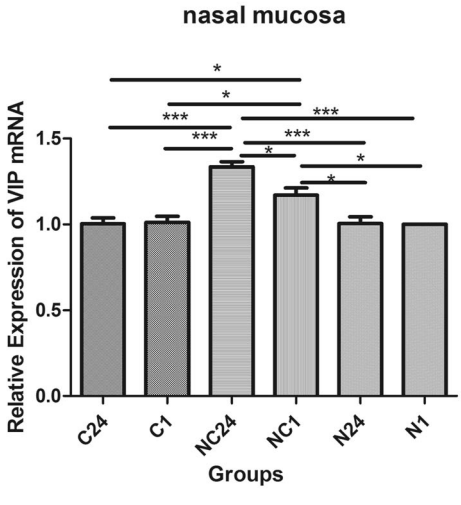
The remaining conjunctival tissues of the left eye were stored in liquid nitrogen for subsequent assays. Total proteins were extracted from the homogenate of the frozen tissues, and the concentrations of proteins were measured using a bicinchoninic acid protein assay kit (Sigma and Abcam, Cambridge, UK). The proteins were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then electrotransferred onto polyvinylidene difluoride membranes. After blocking with 5% skim milk powder in phosphate-buffered saline, the membranes were probed with rabbit anti-SP polyclonal antibody (Bioworld Technology, Inc., St. Louis Park, MN, USA) for 1 h or overnight, followed by incubation with 1:800 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody (Bioworld Technology, Inc., St. Louis Park, MN, USA). For the detection of VIP and NGF, membranes were probed with rabbit anti-VIP polyclonal antibody and rabbit anti-NGF polyclonal antibody (Sigma and Abcam, Cambridge, UK), followed by incubation with 1:1000 dilution of HRP-conjugated secondary antibody. β-Actin was used as the loading control.

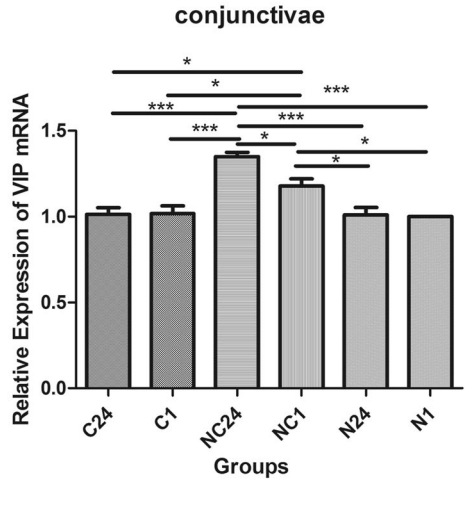
Immunohistochemistry

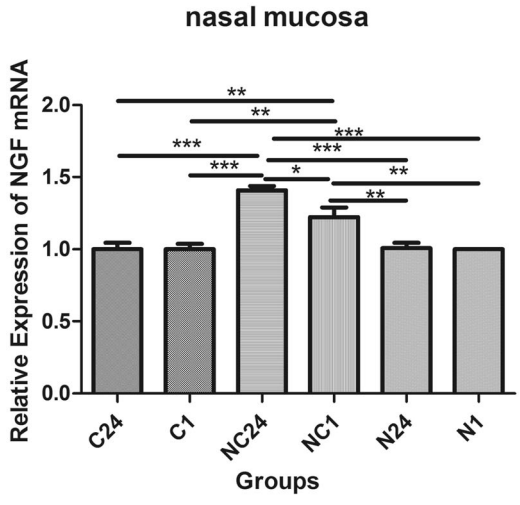
The nasal septum mucosa of left nasal cavity and the upper palpebral conjunctiva of the left eye were dissected and immediately fixed in 4% paraformaldehyde at 4 °C for 48 h. The tissues were decalcified with 10% ethylenediaminetetraacetic acid solution (pH = 7.2) for 7 consecutive days at room

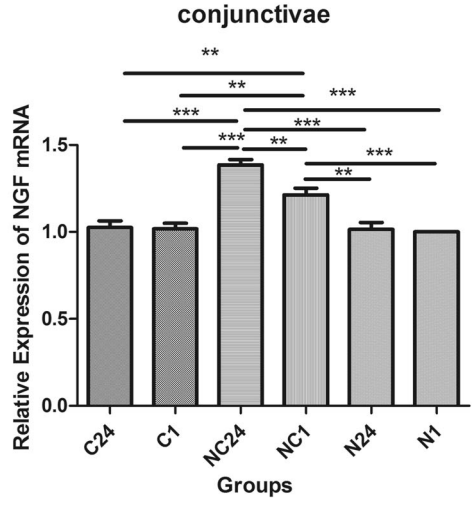

nasal mucosa
Relative Expression of Tac1 mRNA
2.0
1.5
1.0
0.5
0.0
C24
C1
NC24
NC1
N24
N1
Groups


conjunctivae
Relative Expression of Tac1 mRNA
1.5
1.0
0.5
0.0
C24
C1
NC24
NC1
N24
N1
Groups


nasal mucosa
Relative Expression of VIP mRNA
1.5
1.0
0.5
0.0
C24
C1
NC24
NC1
N24
N1
Groups


conjunctivae
Relative Expression of VIP mRNA
1.5
1.0
0.5
0.0
C24
C1
NC24
NC1
N24
N1
Groups


nasal mucosa
Relative Expression of NGF mRNA
2.0
1.5
1.0
0.5
0.0
C24
C1
NC24
NC1
N24
N1
Groups


conjunctivae
Relative Expression of NGF mRNA
1.5
1.0
0.5
0.0
C24
C1
NC24
NC1
N24
N1
Groups

◀**Fig. 1** Comparison of expression levels of *Tac1*, *Vip*, and *Ngf* mRNAs in the nasal mucosa and conjunctivae of allergic and control rats. *Indicates $p < 0.05$; **indicates $p < 0.01$; ***indicates $p < 0.001$. N1, part of the normal control group killed 1 h after the last challenge; N24, part of the normal control group killed 24 h after the last challenge; NC1, part of the non-capsaicin group killed 1 h after the last challenge; NC24, part of the non-capsaicin group killed 24 h after the last challenge; C1, part of the capsaicin group killed 1 h after the last challenge; and C24, part of the capsaicin group killed 24 h after the last challenge

temperature, with the solution being replaced daily. Following decalcification, tissues were embedded in paraffin; 5-μm-thick sections were prepared for hematoxylin and eosin staining, SP (Bioworld Technology, Inc., St. Louis Park, MN, USA), VIP (Sigma and Abcam, Cambridge, UK), and NGF (Sigma and Abcam) immunohistochemical staining. The results were expressed semiquantitatively, based on the estimated percentage of stained cells and the staining intensity. The immunohistochemical score was calculated by multiplying the percentage of stained cells and the staining intensity [22].

Statistical analysis

Data were imported to a statistical software program (SAS, version 9.4, SAS Institute Inc., Cary, NC, USA) for analysis. The data were analyzed using one-way analysis of variance, or a nonparametric test (Kruskal–Wallis test) when conditions of normality and homogeneity of variance were not satisfied. A p value < 0.05 was considered statistically significant. The Bonferroni correction was used in case of multiple comparisons, with the significance level set at $p < 0.01$.

Results

Real-time polymerase chain reaction analysis

RT-PCR analysis confirmed that the expression of *Tac1*, *Vip*, and *Ngf* increased in a time-dependent manner in both nasal mucosa and conjunctivae of rats after the e.n. OVA challenge (Fig. 1). In contrast, the expression of *Tac1*, *Vip*, and *Ngf* were markedly lower in both nasal mucosa and conjunctivae of rats from C1 and C24 groups than in those from NC1 and NC24 groups (Fig. 1).

Western blotting

Since no difference in mRNA expression of Tac1, VIP, and NGF was found between C1 and C24, and N1 and N24, respectively, the expression of the proteins detected by western blotting analysis was only compared in C24, N24, NC1, and NC24 groups. One hour after the e.n. OVA challenge, higher levels of SP were detected in conjunctival homogenates from the NC1 group than from the N24 group (Fig. 2). The VIP and NGF levels in conjunctivae showed similar trends between the NC1 and N24 groups (Figs. 3 and 4). 24 h after the e.n. OVA challenge, higher levels of SP, VIP, and NGF were detected in conjunctival homogenates from the NC24 group than from the N24 group (Figs. 2, 3, and 4). Conjunctivae of rats from C24 group showed significantly lower levels of SP, VIP, and NGF than those of rats from NC1 and NC24 groups (Figs. 2, 3, and 4).

Histological and immunohistochemical analyses

Immunohistochemical staining showed the localization of SP, VIP, and NGF in the cytoplasm of the glandular and the pseudostratified ciliated columnar epithelial cells, distributed in both nasal mucosa and conjunctival tissues (Figs. 5 and 6). Analysis of the images showed greater expression of SP, VIP, and NGF in the nasal mucosa and conjunctivae of rats from NC1 and NC24 groups than in those from N24 group (Fig. 7). The expression of SP, VIP, and NGF was significantly reduced by pretreatment with capsaicin (C24 group, Fig. 7).

Discussion

In recent years, there has been a steady increase in the prevalence of ARC [23, 24]. Despite not being a life-threatening disease, ARC has a profound influence on the patients' quality of life. To understand the disease better, the mechanisms underlying the naso-ocular interaction in ARC have been studied in depth. However, the precise mechanisms remain unclear. Although, of late, more studies are being conducted on the neurogenic systems between the eyes and nose,

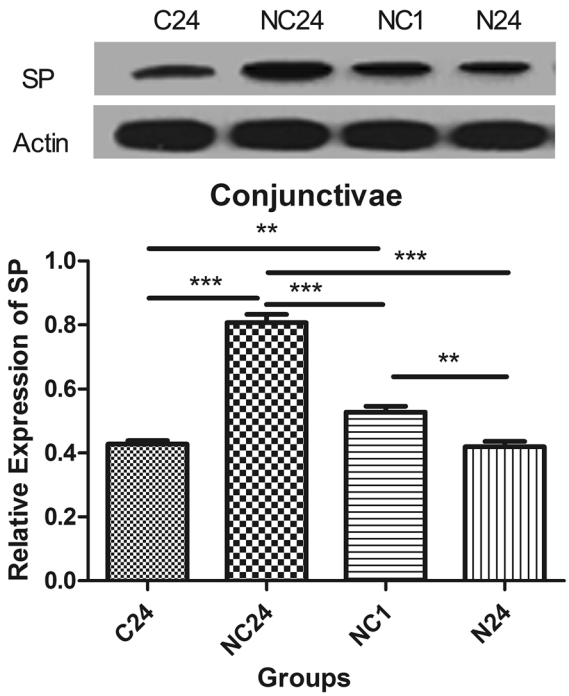


Fig. 2 Representative blot and quantification of substance P (SP) in the conjunctival tissues of allergic rats. **Indicates $p < 0.01$; ***indicates $p < 0.001$. N24, part of the normal control group killed 24 h after the last challenge; NC1, part of the non-capsaicin group killed 1 h after the last challenge; NC24, part of the non-capsaicin group killed 24 h after the last challenge; and C24, part of the capsaicin group killed 24 h after the last challenge

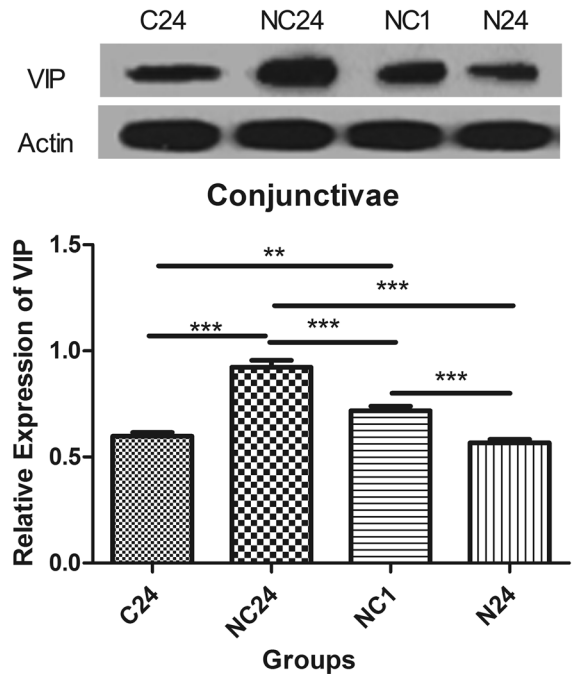


Fig. 3 Representative blot and quantification of vasoactive intestinal peptide (VIP) the in conjunctival tissues of allergic rats. **Indicates $p < 0.01$; ***indicates $p < 0.001$. N24, part of the normal control group killed 24 h after the last challenge; NC1, part of the non-capsaicin group killed 1 h after the last challenge; NC24, part of the non-capsaicin group killed 24 h after the last challenge; and C24, part of the capsaicin group killed 24 h after the last challenge

which play an important role in the mechanisms underlying the naso-ocular interaction in ARC [5, 12, 25].

The nasal mucosa and conjunctivae have common innervations: sensory nerves and sympathetic and parasympathetic fibers. Therefore, nasal challenge with OVA can activate the sensory nerve fibers in the nasal cavity, releasing SP. Besides this orthodromic pathway, the sensory nerve fibers in nasal mucosa and conjunctivae can also be antidromically stimulated by action potentials originating at different terminals of the same neuron, leading to the local release of neuropeptides such as SP. This phenomenon is called axon reflex [26]. The result of antidromic stimulation is the so-called neurogenic inflammation. This sensory nerve pathway is also called the non-adrenergic non-cholinergic nerve pathway.

Our study demonstrates that nasal challenge with OVA not only increases the levels of neuropeptides and neurotrophin in nasal mucosa, but also in conjunctivae. In addition, following sensory nasal nerve damage by capsaicin, protein and mRNA levels of neuropeptides and a neurotrophin were reduced in both nasal mucosa and conjunctivae of OVA-sensitized rats. Our findings are in accordance with those of Callebaut et al. [20], which showed that nasal challenge with OVA increased SP levels in the conjunctiva, and damage of the nasal sensory nerves by capsaicin reduced the OVA-induced conjunctival levels of SP. In another study, Callebaut et al. [21] found that nasal corticosteroid treatment of patients with ARC reduced SP levels in tear fluid. Similar

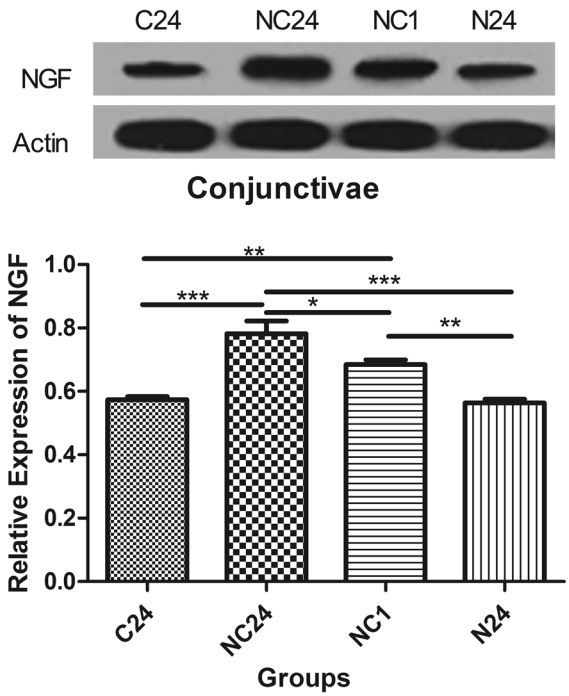


Fig. 4 Representative blot and quantification of nerve growth factor (NGF) in the conjunctival tissues of allergic rats. *Indicates $p < 0.05$; ** indicates $p < 0.01$; ***indicates $p < 0.001$. N24, part of the normal control group killed 24 h after the last challenge; NC1, part of the non-capsaicin group killed 1 h after the last challenge; NC24, part of the non-capsaicin group killed 24 h after the last challenge; and C24, part of the capsaicin group killed 24 h after the last challenge

results from our study suggest a potential role of SP in the naso-ocular interaction in ARC.

In this study, we also showed that, besides SP, VIP and NGF are also involved in the naso-ocular interaction in ARC. This interaction between the eyes and nose is thought to be a centrally mediated reflex, the signal for which originates in the afferent sensory nerve fibers from the nasal mucosa, reaching the efferent parasympathetic fibers terminating in the conjunctiva via the trigeminal nerve [27]. The neurotransmitters released by the parasympathetic nerves are ACh and VIP. The conjunctivitis caused by ACh involves the adrenergic-cholinergic nerve pathway. Consequently, the upregulation of *Tac1*, *Vip*, and *Ngf* provides evidence of neurogenic inflammation in ARC.

In contrast, the expression of neurotransmitters was lower in both nasal mucosa and conjunctivae of rats pretreated with capsaicin than the untreated rats. Capsaicin, an agonist of transient receptor potential cation channel receptor 1, is known to damage sensory nerve endings [28]. Our findings show that the damage of the afferent nasal nerve endings by capsaicin reduces protein and mRNA levels of SP, VIP, and NGF in nasal mucosa and conjunctivae.

Our findings show that the levels of neuropeptides and neurotrophin in the nasal mucosa and conjunctiva 24 h after OVA administration are higher than those after 1 h of the challenge. In contrast to our results, a study showed that there were no differences in the SP levels in tear fluid between different time points, following nasal pollen provocation [7]. Generally, the classical neurotransmitters are suitable for the rapid and accurate regulation of nerve, and neuropeptides are more suitable for the adjustment of slow and prolonged functional changes. This may explain the time-dependent increase in the expression of neuropeptides and neurotrophin observed in this study.

Study limitations

Further studies are needed to confirm the correlation between neuropeptides and serological allergy markers, such as mast cells and eosinophils.

Conclusion

In conclusion, interaction between ocular and nasal neurogenic inflammation has two main aspects. Nasal challenge with OVA can activate the sensory nerve fibers in the nasal cavity, releasing SP. At the same time, the sensory nerve fibers in nasal mucosa and conjunctivae can also be antidromically stimulated, leading to the local release of SP through axonal reflex. On the other hand, nasal sensory nerves, such as the afferent nerve, will spread the impulse to central pathways, leading to nasal and conjunctival efferent parasympathetic responses that release VIP. Moreover, NGF regulates neuronal development and enhances the production of neuropeptides. Following the damage of nasal sensory nerves by capsaicin, the protein and mRNA levels of SP, VIP, and NGF were reduced in the nasal mucosa and conjunctivae of

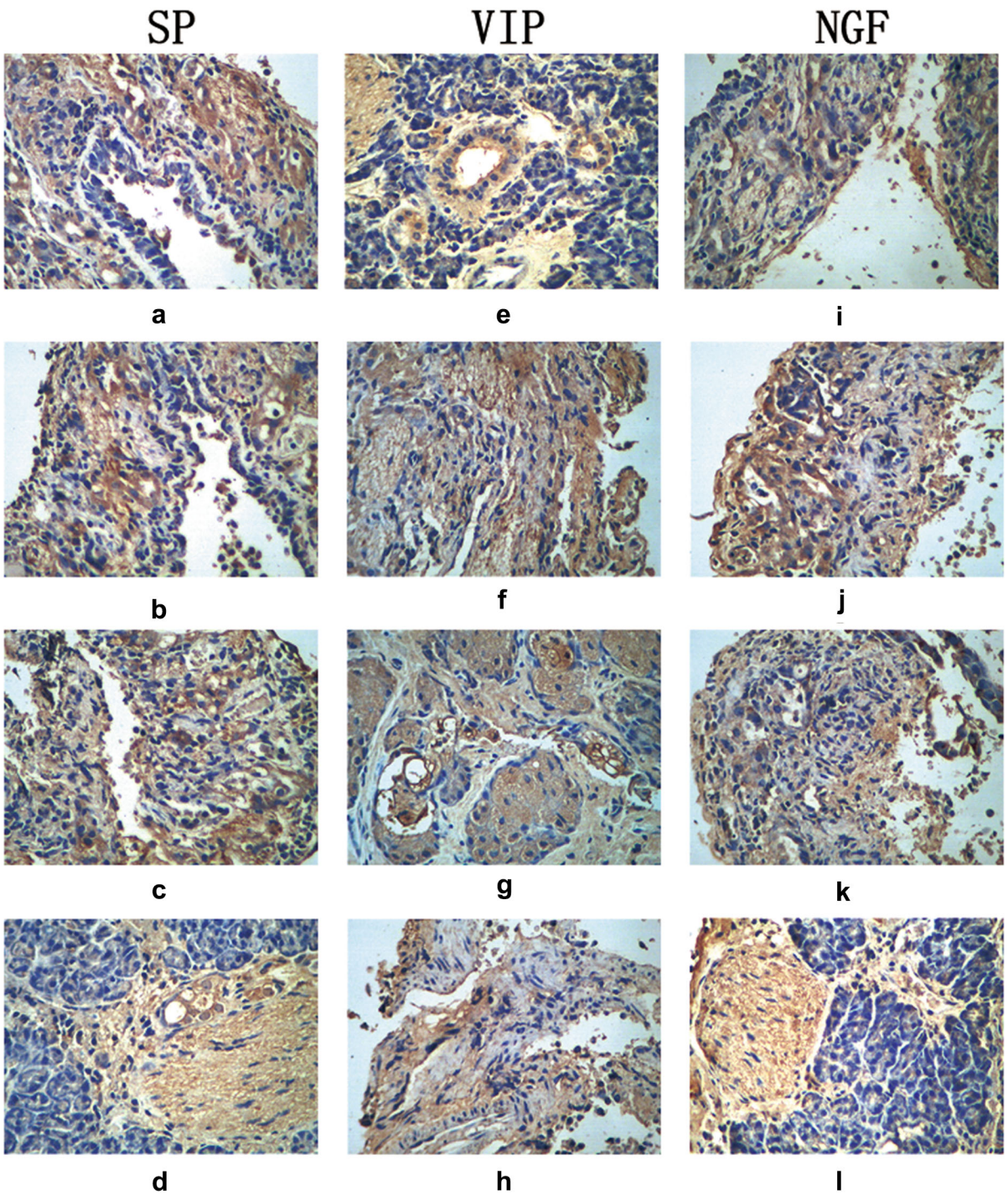


Fig. 5 Representative images for immunohistochemistry performed to assess substance P (SP), vasoactive intestinal peptide (VIP), and nerve growth factor (NGF) levels in the nasal mucosa (brown signal; ×400). **a–d** Expression of SP in N24, NC1, NC24, and C24 groups. **e–h** Expression of VIP in N24, NC1, NC24, and C24 groups. **i–l** Expression of NGF in N24, NC1, NC24, and C24 groups. N24, part of the normal control group killed 24 h after the last challenge; NC1, part of the non-capsaicin group killed 1 h after the last challenge; NC24, part of the non-capsaicin group killed 24 h after the last challenge; and C24, part of the capsaicin group killed 24 h after the last challenge

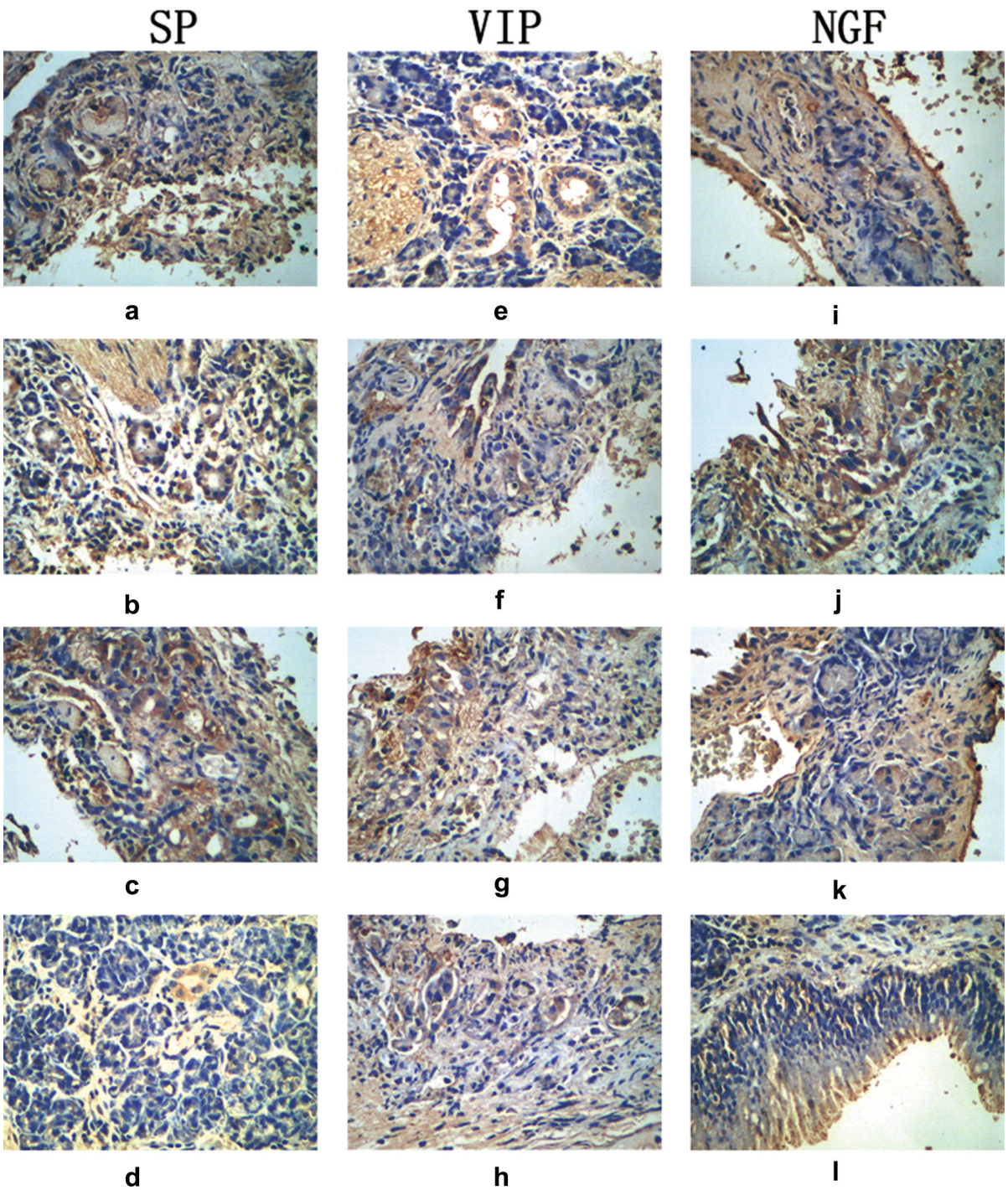
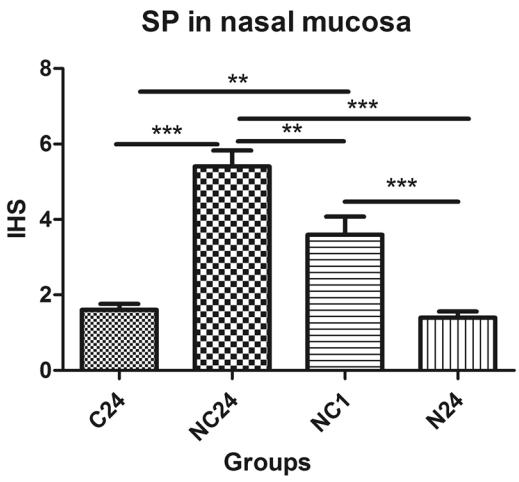


Fig. 6 Representative images for immunohistochemistry performed to assess levels of substance P (SP), vasoactive intestinal peptide (VIP), and nerve growth factor (NGF) in the conjunctivae (brown signal; ×400). **a–d** Expression of SP in N24, NC1, NC24, and C24 groups. **e–h** Expression of VIP in N24, NC1, NC24, and C24 groups. **i–l** Expression of NGF in N24, NC1, NC24, and C24 groups. N24, part of the normal control group killed 24 h after the last challenge; NC1, part of the non-capsaicin group killed 1 h after the last challenge; NC24, part of the non-capsaicin group killed 24 h after the last challenge; and C24, part of the capsaicin group killed 24 h after the last challenge


SP in nasal mucosa
IHS
Groups
C24
NC24
NC1
N24
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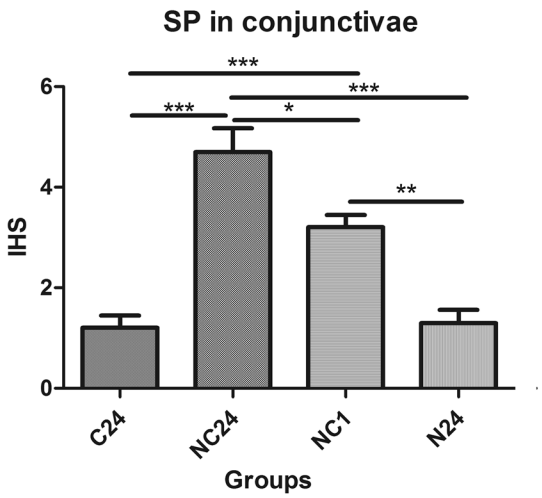
SP in conjunctivae
IHS
Groups
C24
NC24
NC1
N24
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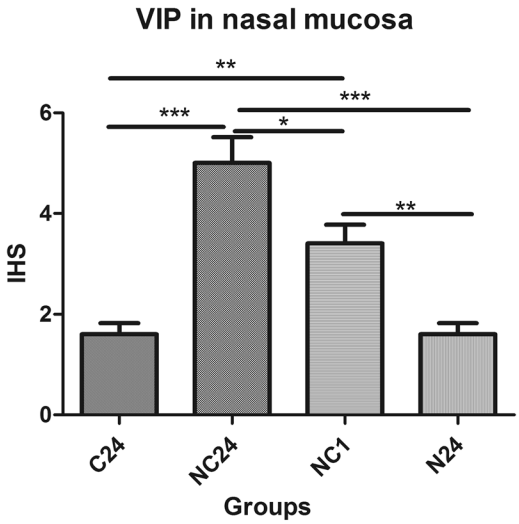
VIP in nasal mucosa
IHS
Groups
C24
NC24
NC1
N24
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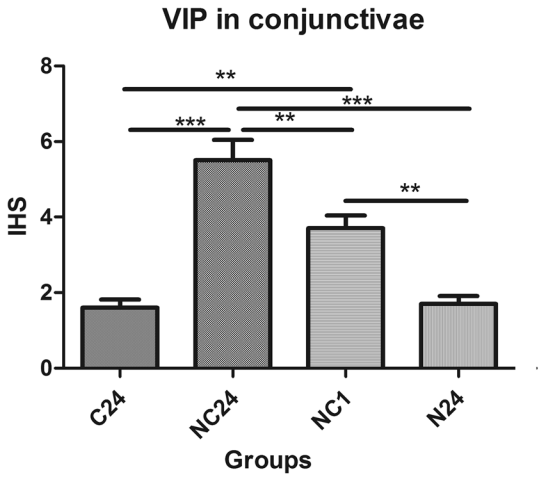
VIP in conjunctivae
IHS
Groups
C24
NC24
NC1
N24
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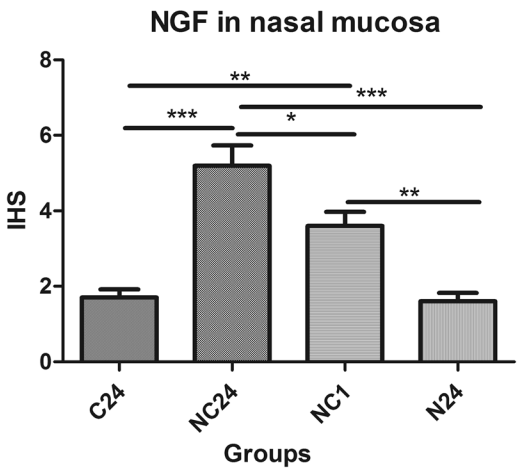
NGF in nasal mucosa
IHS
Groups
C24
NC24
NC1
N24
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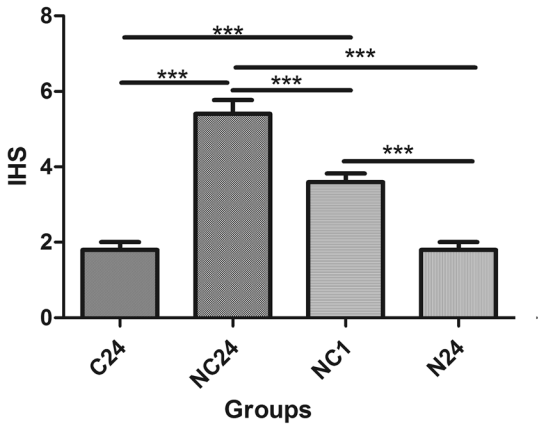
NGF in conjunctivae
IHS
Groups
C24
NC24
NC1
N24
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◀**Fig. 7** The immunohistochemical score (IHS) of substance P (SP), vasoactive intestinal peptide (VIP), and nerve growth factor (NGF) in the nasal mucosa and conjunctivae of allergic and control rats. *Indicates $p < 0.05$; **indicates $p < 0.01$; ***indicates $p < 0.001$. N24, part of the normal control group killed 24 h after the last challenge; NC1, part of the non-capsaicin group killed 1 h after the last challenge; NC24, part of the non-capsaicin group killed 24 h after the last challenge; and C24, part of the capsaicin group killed 24 h after the last challenge

OVA-sensitized rats. Therefore, neurogenic inflammation may be responsible for the naso-ocular relationship in ARC.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

Ethical approval All procedures were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Human and animal rights All the ethical standards for animal handling and experimentation were followed in this study.

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