

Full Paper

Intranasal Administration of Semaphorin-3A Alleviates Sneezing and Nasal Rubbing in a Murine Model of Allergic Rhinitis

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Abstract. Sneezing and persistent itching of the nasal mucosa are distressing symptoms of allergic rhinitis (AR). Recent studies have revealed that hyperinnervation of sensory neurons in the nasal turbinate is one of the underlying causes of sneezing and itching. Since Semaphorin-3A (Sema3A) has been previously shown to restrict innervation of sensory neurons, it is presumed that reduced Sema3A expression in the nasal mucosa might contribute to the hypersensitivity. Analysis of the mouse model of ovalbumin-sensitized AR demonstrated a decreased expression of Sema3A in the nasal epithelium, which was accompanied by an increased nerve fiber density in the lamina propria of the turbinate. In rescue experiments, intranasal administration of recombinant Sema3A in the AR model mice alleviated sneezing and nasal rubbing symptoms. In addition, histological examinations also revealed that nerve fiber density was decreased in the lamina propria of the Sema3A-treated nasal turbinate. These results suggest that the nasal hypersensitivity of AR may be attributed to reduction of Sema3A expression and intranasal administration of Sema3A may provide a novel approach to alleviate the allergic symptoms for AR treatment.

Keywords: allergic rhinitis, itch, nasal mucosa, protein gene-product 9.5 (PGP9.5), Semaphorin-3A

Introduction

Allergic rhinitis (AR) is a common allergic disease that significantly impairs patients' quality of life. Due to increased exposure to allergens, such as pollens and house dust, the prevalence of AR has been increasing in recent decades (1, 2). The principal symptoms of AR include sneezing, rhinorrhea, and nasal obstruction, which are frequently accompanied by itching of the nasal mucosa. These symptoms are primarily induced by allergic mediators released from mast cells, basophils, eosinophils, lymphocytes, and epithelial cells (3). Such mediators, including histamine, prostanoids, and inflam-

matory cytokines, have been known to play a major role in the pathophysiology of AR (4).

Recent studies have shown that hyperinnervation of sensory neurons and/or increased expression of neuropeptides in nerve terminals might be associated with the itchy sensations and sneezing reflexes (5 – 7). Sensory nerve fibers in the nasal mucosa are afferent connections from the trigeminal nerve; they are classified into two categories, myelinated thick A δ -fibers and non-myelinated thin C-fibers. The A δ -fibers mediate the initial rapid sharp pain (8), and the C-fibers mediate delayed-onset but more prolonged burning pain. In AR, some polymodal nociceptive type C-fibers convey itching and sneezing stimuli from the nasal mucosa (9 – 11). Neuropeptides, such as Substance-P (SP) and calcitonin gene-related peptide (CGRP), are expressed in trigeminal sensory fibers and have been shown to induce allergic symptoms (12 – 14). In the rat model of AR, the number

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of SP-positive neurons increases in the nasal epithelial layer following allergen exposure (15).

C-fiber outgrowth is regulated by several extracellular molecules, including nerve growth factor (NGF), and Semaphorin-3A (Sema3A). Nasal NGF expression is significantly increased following nasal allergen exposure in AR patients, compared with healthy controls (16–18). Interestingly, nerve fiber density in the nasal epithelium, lamina propria (subepithelium), and glandular/vascular regions is also increased in AR patients (5, 19). The positive correlation between NGF expression and C-fiber density suggests that increased NGF expression in nasal mucosa might facilitate sensory nerve invasion and lead to the hypersensitive condition to itch (7, 20).

In contrast, C-fiber innervation is negatively regulated by Sema3A expression, which acts as a chemorepulsive guidance molecule for sensory and sympathetic neurons during developmental stages (21, 22). Recent studies suggest that downregulation of Sema3A expression correlates with increased itch sensation in some allergic diseases. For instance, in atopic dermatitis, decreased Sema3A expression and increased numbers of peripheral nerve terminals in the epidermis have been shown to exacerbate the itch sensation (23). We have reported that subcutaneous administration of Sema3A ameliorates scratching behavior and skin lesions in the mouse model of atopic dermatitis, suggesting that Sema3A suppresses hypersensitivity to itch by interrupting elongation of peripheral sensory nerve fibers in the epidermis. These results suggest a potential clinical application for Sema3A (24).

Current therapeutic approaches of AR treatment target the inhibition of inflammatory molecules and suppression of the immune reaction (25) through the use of antihistamines, leukotriene antagonists, and steroids. The neurological approach to AR treatment has not been fully investigated, although there is a considerable body of evidence indicating that hypersensitivity to itch in nasal mucosa of AR patients could be the result of nerve fiber hyperinnervation. The present study therefore analyzed whether Sema3A expression levels correlate with AR symptoms and C-fiber sprouting in nasal mucosa of AR model mice, as well as the pharmacological effects of Sema3A in AR treatment.

Materials and Methods

Animals

Female BALB/c mice (6–8-week-old) were purchased from Japan SLC (Shizuoka), housed in a standard mouse facility, and fed a sterile diet with water. Throughout all experimental procedures, efforts were made to minimize the number of animals used and their suffering.

The experimental procedures were approved by the ethical committee of Yokohama City University Graduate School of Medicine.

Establishment of the AR murine model and analysis of sneezing and nasal rubbing

AR model mice were established based on the previously described methods (26, 27), with minor modifications. The experimental timetable is provided in Fig. 1a. Mice were intraperitoneally administered 0.1 mg/ml ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO, USA) and 40 mg/ml $\text{Al}(\text{OH})_3$ (Thermo Scientific, Waltham, MA, USA) in saline at a dosage of 100 μl /mouse. Sensitization was repeated twice (days 0 and 7), followed by daily injections of 10 μl /mouse OVA solution (15 mg/ml in saline) into nostrils from day 14 to 20 (challenge). On day 21, immediately following final intranasal OVA administration, the numbers of nasal rubbing movements and sneezing were recorded by video for 10 min. One point was assigned to every five fast, consecutive rubbing movements or to one isolated, relatively slow rubbing movement, as well as to one sneezing movement (28, 29).

ELISA

Sera from the AR model mice were collected after 2 h of the last challenge on day 21. Concentrations of total IgE were evaluated using Mouse IgE ELISA kit (Shibayagi, Gunma) according to the manufacturer's instructions.

Recombinant Sema3A preparation and administration

Recombinant Sema3A was prepared as previously described (30, 31). Sema3A activity was assessed using the growth cone collapse assay in chick dorsal root ganglia neurons (30, 32). One unit was defined as the amount of Sema3A per milliliter that induced growth cone collapse by 50%. The Sema3A administration protocol is shown in Fig. 5a. Purified Sema3A was diluted with saline, and 300 U/10 μl of the solution was intranasally administered to OVA sensitized mice at 1.5 h prior to intranasal OVA administration, from day 15 to 21 once per day.

Total RNA preparation

Mice were sensitized with OVA by intraperitoneal injection on days 0 and 7. Next, intranasal OVA administration was performed from day 14 to 36. To enhance the change of NGF and Sema3A expression level, we employed long-term administration of OVA for quantitative RT-PCR experiments. On day 36, after the final intranasal administration, 10 nose samples from each non-treated and AR model mice were obtained. Nose

samples were clear of cheek muscle and skin, and nose sections were obtained by cutting coronal sections at the line of the incisor teeth and acral part of the nose for RNA preparation. Total RNA was isolated using the illustra RNA spin Mini RNA Isolation Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

Real-time PCR

The reverse transcription (RT) reaction was performed using random primers and ExScript RTase (Takara Bio, Kyoto). Equal amounts of RNA from each sample were converted to cDNA. Quantitative RT-PCR analysis was performed using the ABI PRISM 7500HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) based on the TaqMan[®] PCR manufacturer's protocol. The assay was performed in triplicate in optical 96-well reaction microtiter plates covered with optical caps in a volume of 20 μ l containing 1 μ l Taqman Gene Expression Assay 20X for *NGF* (Mm 00443039_ml assay), *Sema3A* (Mm00436469_ml assay), and *CGRP* (00801463_gl assay); 10 μ l Taqman Gene Expression Master Mix 2X; 100 ng template; and 5 μ l RNase-free water. The default ABI Prism 7500 amplification conditions were 2 min at 50°C, 10 min at 95°C, followed by 15 s at 95°C and 1 min at 60°C for 40 cycles. Quantitative RT-PCR analyses were performed at least three independent times. Relative expression was calculated using the Δ CT method and values were normalized to β -actin mRNA expression as the housekeeping gene (Mm01205647_gl).

Histology and immunohistochemistry

Nose samples were obtained on day 21, fixed in 10% formalin, embedded in paraffin, and cut into 4- μ m-thick coronal sections for Hematoxylin-Eosin staining, as well as 6- μ m-thick coronal sections for immunohistochemistry. For toluidine blue and giemsa staining, nose samples were obtained on day 21 and day 42, embedded in paraffin, and cut into 4- μ m-thick slices. The sections were deparaffinized with xylene and graded ethanol and then rehydrated and autoclaved at 121°C for 15 min for antigen retrieval.

Endogenous peroxidases were quenched by immersing in 0.3% hydrogen peroxide for 30 min at room temperature. The sections were then blocked with normal goat serum for 15 min at 37°C and incubated at 4°C overnight with polyclonal rabbit antibodies against protein gene-product 9.5 (PGP9.5) (1:12,500; Ultra Clone, Isle of Wight, UK) (33). The sections were subsequently incubated with biotinylated goat anti-rabbit IgG antibody for 30 min at 37°C, followed by avidin-biotinylated peroxidase complex using the histofine SAB-PO(R) kit accord-

ing to the manufacturer's instructions (Nichirei, Tokyo). Peroxidase activity was visualized with 3,3'-diaminobenzidine, and sections were counterstained with hematoxylin. The number of PGP9.5-positive nerve fibers in the nasal turbinate was quantified using ImageJ (<http://imagej.nih.gov/ij/>). The number of PGP9.5-positive nerve fibers in the turbinate area was scored as follows: Firstly, the number of nerve fibers in each side of the turbinate was counted. Secondly, the pixel number of the turbinate area was measured. Thirdly, the area size was divided with the pixel number in a unit area (250 μ m $\times$ 250 μ m square). Then, the number of nerve fibers in the turbinate was represented as that per 250² μ m².

Frozen sections were used for Sema3A immunohistochemistry. Nose samples were obtained on day 36 by using the same protocol as the PCR study. The sections were incubated with rabbit polyclonal antibodies against Sema3A (1:200; Abcam, Cambridge, UK), followed by biotinylated goat anti-rabbit IgG antibody and avidin-biotinylated peroxidase complex. Visualization of reaction products was performed with 3,3'-diaminobenzidine, and sections were counterstained with hematoxylin.

Statistics

Statistical differences were determined by the two-sided Mann-Whitney U-test. In Fig. 1, b and c, nasal symptom score between the control and challenge and the score between challenge and AR were compared. In these cases, $P < 0.025$ was considered statistically significant to account for the multiplicity of statistical testing. In the other cases, $P < 0.05$ was considered statistically significant. In Fig. 5, b and c, to detect the effect of Sema3A, the nasal symptom scores between AR and AR+Sema3A were compared.

Results

Nerve fiber density increases in the nasal turbinate lamina propria in a murine AR model

The experimental protocol is depicted in Fig. 1a. In addition to AR mice, non-treated mice (Control) and mice intranasally administered OVA without prior sensitization (Challenge) were prepared. On day 21, the allergic symptom score was counted. The control mice exhibited no sneezing and very little nasal rubbing. The AR mice exhibited significantly higher scores for sneezing ($P < 0.001$) and nasal rubbing ($P < 0.01$) than the challenged mice (Fig. 1: b and c). The challenged mice also presented with higher scores for sneezing ($P < 0.001$) and nasal rubbing ($P < 0.01$) than control mice, suggesting that repeated antigen administration into nasal cavity could induce priming and weaken the allergic response without prior sensitization. In the AR mice, distinct

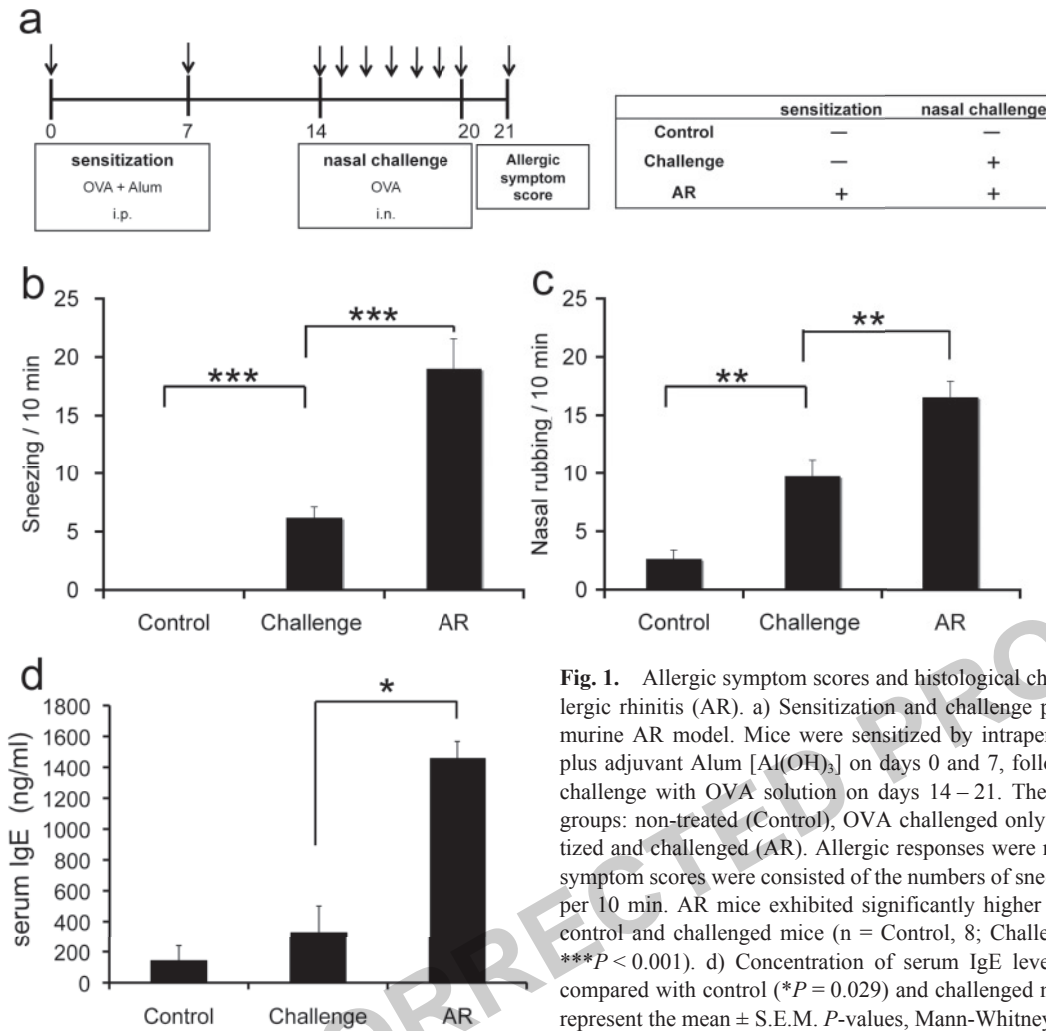


Fig. 1. Allergic symptom scores and histological changes in a mouse model of allergic rhinitis (AR). a) Sensitization and challenge protocols for the experimental murine AR model. Mice were sensitized by intraperitoneally (i.p.) injected OVA plus adjuvant Alum [Al(OH)₃] on days 0 and 7, followed by daily intranasal (i.n.) challenge with OVA solution on days 14–21. The mice were assigned to three groups: non-treated (Control), OVA challenged only (Challenge), and OVA sensitized and challenged (AR). Allergic responses were monitored on day 21. Allergic symptom scores were consisted of the numbers of sneezing (b) and nasal rubbing (c) per 10 min. AR mice exhibited significantly higher allergic symptom scores than control and challenged mice ($n = \text{Control}, 8; \text{Challenge}, 11; \text{AR}, 12$; $**P < 0.01$, $***P < 0.001$). d) Concentration of serum IgE level was increased in AR mice compared with control ($*P = 0.029$) and challenged mice ($n = 4$, $*P = 0.021$). Data represent the mean $\pm$ S.E.M. P -values, Mann-Whitney U-test.

sneezing and nasal rubbing behaviors were observed from day 15, which was the second day of OVA intranasal administration. Most of the rubbing movements were fast and continuous more than 5 times and slow movements were rare. Symptoms were gradually aggravated in a time-dependent fashion (data not shown). The AR mice showed a significantly higher level of total IgE in serum compared with the control ($P = 0.029$) and challenge ($P = 0.021$) (Fig. 1d). This indicates that our protocol of sensitization had succeeded in inducing allergic condition in the treated mice.

Histological alterations in nasal mucosa were also observed in the mice. Within the nasal tissue, swelling of the nasal turbinate generally reflected allergic inflammation (34). Histopathological evaluation revealed edema in the lamina propria and venous dilatation along with irregularity of the epithelial surface (arrows) in the AR group. Submucosal edema was indicated by loosening of the connective tissue (* in Fig. 2: a and b).

Since AR accompanies neural hypersensitivity, the distribution of nerve fibers in the nasal mucosa was determined by immunohistochemical examination using anti-PGP9.5 antibody, which labels peripheral neurons (33, 35). In the control mice, PGP9.5-positive signals were mainly localized in the lamina propria, with few nerve fibers observed in the epithelial layer (Fig. 2c). In the AR mice, nerve fiber density in the lamina propria was significantly increased ($P = 0.043$, Fig. 2: c – e). Furthermore, some PGP9.5-positive signals were apparent in the AR epithelial layer (arrowheads in Fig. 2d), suggesting increased innervation into the epithelial layer. These results suggest that nerve-ending alterations in this model may correlate with the increased allergic symptom score.

Despite the severe allergic nasal symptoms and increased innervations in nasal mucosa in the AR groups on day 21, apparent increase of infiltrating eosinophils and mast cells in the nasal mucosa was not detected in

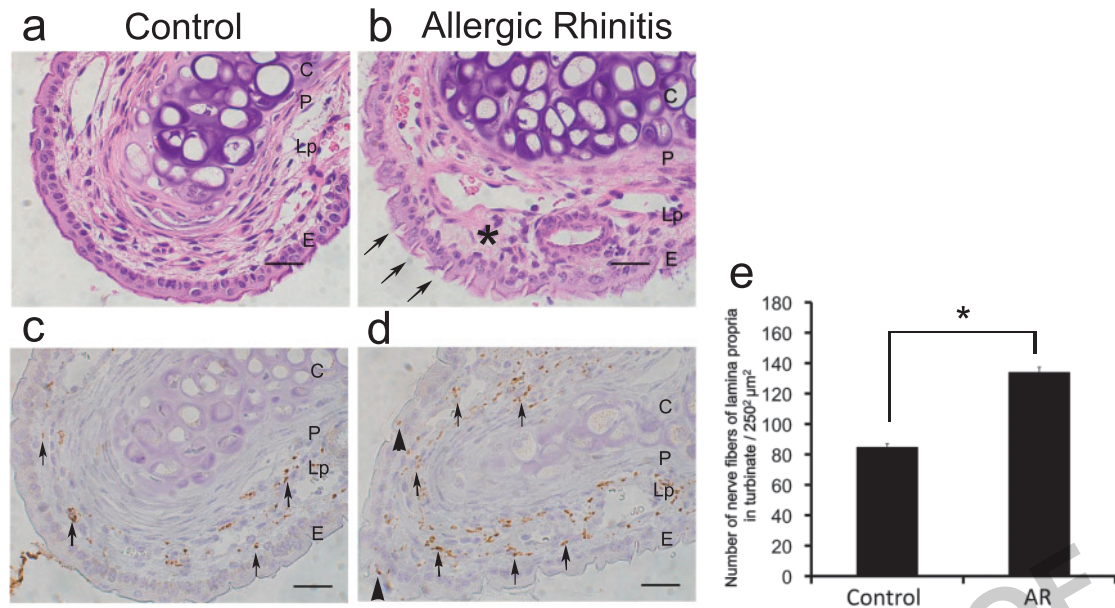


Fig. 2. Increased sensory afferents in turbinates of allergic rhinitis (AR) model mice. a, b) Histopathological evaluation revealed edema (*) and venous dilatation in lamina propria along with irregularity of the epithelial surface (arrows) in the AR group. Sub-mucosal edema was indicated by loosening of the submucosal connective tissue. c, d) Immunohistochemical evaluation using anti-PGP9.5 (peripheral nerve fiber) antibody revealed the increased numbers of positive fibers in the lamina propria of AR mice compared with control mice. Arrows indicate PGP9.5-positive nerve fibers. Arrowheads in panel d indicate invading nerve terminal in nasal epithelial layer. Abbreviations: E, epithelium; Lp, lamina propria; P, perichondrium; C, cartilage. Scale bars, 25 μ m. e) Numbers of nerve fibers per area in lamina propria were quantified using ImageJ (n = Control, 6; AR, 7, * P = 0.043). Data represent the mean $\pm$ S.E.M. P -values, Mann-Whitney U-test.

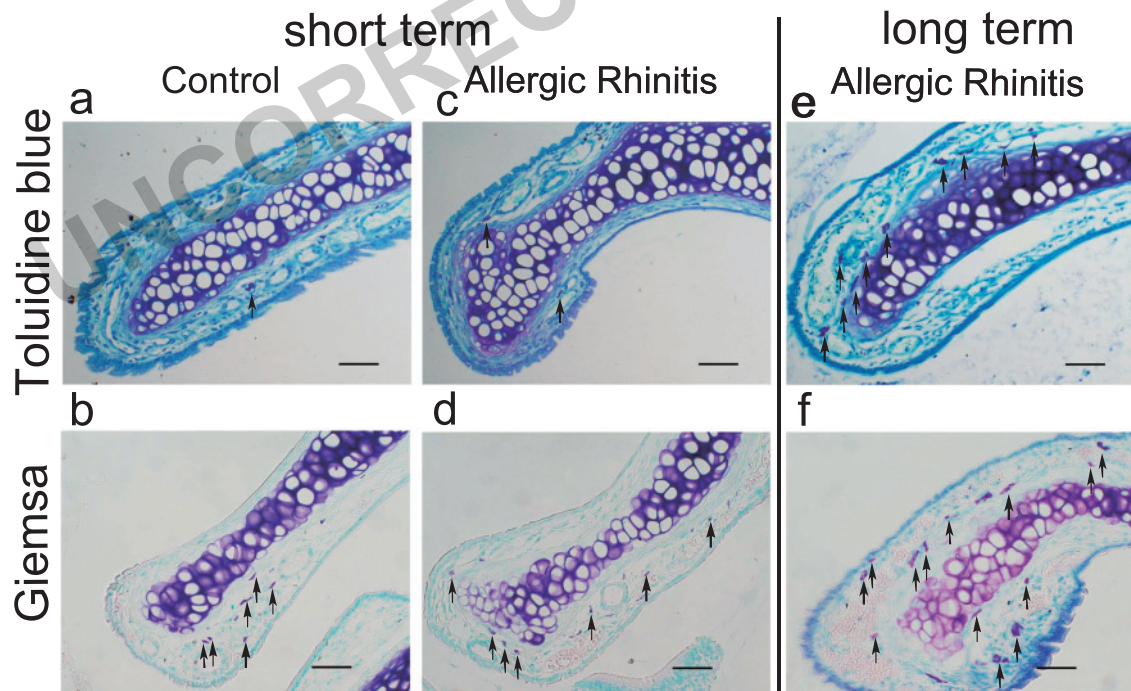


Fig. 3. Increased immune cells in nasal turbinates of chronic allergic rhinitis (AR) model mice. a – d) On day 21, small number of infiltrating mast cells (arrows in toluidine blue staining; a, c) and eosinophils (arrows in giemsa staining; b, d) in the nasal mucosa were observed in the AR model mice. Significant changes were not observed in each group. Chronic AR model mice showed apparent increase of mast cells (e) and eosinophils (f) in nasal turbinates. Chronic rhinitis model were given intranasal OVA administration for 4 weeks. Scale bars, 50 μ m.

the AR model mice (Fig. 3: a – d). In contrast, chronic AR model mice, in which intranasal administration of OVA lasted for 4 weeks, showed apparent increase of those inflammatory cells in nasal turbinates (Fig. 3: e and f). This suggests that neural hypersensitivity may precede the infiltration of immune cells in the AR model mice.

Decreased Sema3A expression level in the nasal epithelial layer of the AR murine model

NGF has been shown to stimulate outgrowth of sensory C-fibers, whereas Sema3A inhibits sprouting of C-fibers (36 – 38). Therefore, we asked whether the altered expression of these factors can be observed in the AR nasal turbinate and whether it might contribute to excessive invasion of C-fibers and allergic symptoms. Since the alteration of *Sema3A* mRNA expression in the AR

model mice (intranasal OVA administration for 7 days) was minimal (not shown), we treated the mice with the prolonged administration from day 14 to 36. Ten nose samples from each non-treated and AR model mice were obtained.

NGF and *Sema3A* mRNA expression levels were measured in the nose of AR model mice. Total RNA isolated from nose sections was subjected to quantitative RT-PCR analysis using specific primers for NGF and *Sema3A*. mRNA expression levels for these genes were calculated using the comparative Ct method according to β -actin expression. *Sema3A* expression level was significantly decreased in AR mice compared with controls ($P < 0.001$, Fig. 4a), while NGF expression remained unchanged (Fig. 4b). The reduction of Sema3A expression was also supported by immunostaining. In the con-

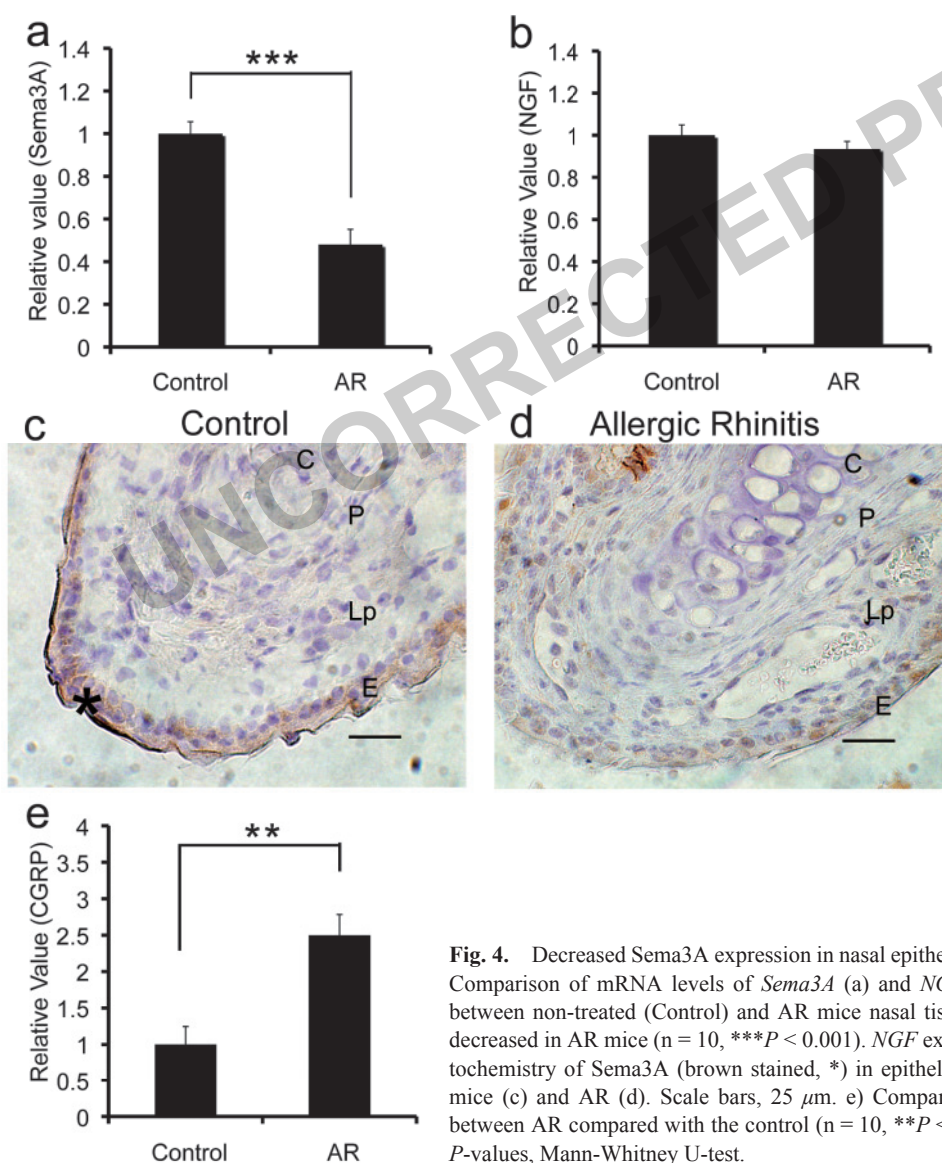


Fig. 4. Decreased Sema3A expression in nasal epithelium of allergic rhinitis (AR) model mice. Comparison of mRNA levels of *Sema3A* (a) and NGF (b) normalized by β -actin expression between non-treated (Control) and AR mice nasal tissues. *Sema3A* expression is significantly decreased in AR mice ($n = 10$, *** $P < 0.001$). NGF expression remains unchanged. Immunohistochemistry of Sema3A (brown stained, *) in epithelial layer of the nasal turbinate in control mice (c) and AR (d). Scale bars, 25 μ m. e) Comparison of CGRP mRNA expression levels between AR compared with the control ($n = 10$, ** $P < 0.01$). Data represent the mean $\pm$ S.E.M. P -values, Mann-Whitney U-test.

trol mice, Sema3A expression was observed in the epithelial layer of nasal mucosa (*), while the expression level was decreased in the AR mice (Fig. 4: c and d). Specificity of this immunostaining was confirmed using the absorption test with Sema3A antigen peptide (data not shown). We also examined *CGRP* mRNA expression levels in nose samples of AR model mice. *CGRP* mRNA was significantly increased in AR mice compared with controls ($P < 0.01$, Fig. 4e). These alternations, decreased Sema3A and increased CGRP, may contribute to the neural hypersensitivity in AR mice.

Intranasal administration of Sema3A alleviates allergic symptom scores of AR

The decreased Sema3A expression in the nasal mucosa of AR mice may bring the increased innervation of C-fibers in the epithelium and lamina propria, leading to nasal neurologic hypersensitivity including itch. Therefore, administration of Sema3A to the nasal mucosa was hypothesized to suppress hyperinnervation and alleviate symptoms such as sneezing and nasal rubbing behavior in AR mice.

Recombinant Sema3A or normal saline was intranasally administered to sensitized mice once/day at 1.5 h prior to OVA intranasal challenge from day 15 to 21. After the challenge, allergic symptom scores were recorded (Fig. 5a). On day 21, AR mice treated with Sema3A showed a significant decrease in the amount of sneezing ($P < 0.01$, Fig. 5b) and nasal rubbing ($P < 0.01$, Fig. 5c), compared with AR mice treated with saline. The Sema3A effect was observed from the second day of Sema3A administration. More than 7 days of Sema3A treatment did not further alleviate allergic symptoms compared with values on the 7th day.

The nasal turbinate of Sema3A treated AR model mice was then histologically examined. HE staining did not reveal any apparent reduction of edema in the lamina propria (not shown). However, immunohistochemical analysis of nasal turbinates revealed that Sema3A treatment decreased the number of PGP9.5-positive nerve fibers in the lamina propria of turbinate in AR mice. In contrast, saline administration did not reduce PGP9.5 expression in the lamina propria or nasal epithelium (Fig. 5: d – f). Quantitative analysis revealed significantly decreased numbers of PGP9.5-positive fibers in nasal mucosa of Sema3A-treated mice compared with AR mice ($P = 0.037$, Fig. 5g). These results suggest that Sema3A administration alleviated AR symptoms by reducing the number of sensory nerve terminals in the nasal mucosa rather than by reducing the allergic reactions such as edema.

Discussion

The present study revealed that Sema3A was expressed in the epithelial layer of nasal mucosa in control BALB/c mice. The combination of intraperitoneal OVA injection and repeated intranasal OVA administration induced AR symptoms in BALB/c mice. In this AR model, Sema3A expression level in the nasal epithelium was decreased and the peripheral nerve fiber density in the epithelial layer and lamina propria of turbinate was increased. Intranasal administration of recombinant Sema3A alleviated sneezing and nasal rubbing in the AR mice, as well as decreased nasal mucosa innervation. These findings suggest that intranasal Sema3A administration may compensate the reduced Sema3A in AR and this approach may become a novel therapeutic strategy for treating AR.

Previous studies have shown that allergen exposure in sensitized animals resulted in increased responsiveness of airways to nerve stimulation (39, 40). Indeed, AR patients exhibit significantly exaggerated symptomatic responsiveness following exposure to capsaicin, bradykinin, or hyperosmolar saline compared with non-AR or with healthy control subjects (41 – 43). In addition, the numbers of PGP9.5-, SP-, and CGRP-positive fibers invading the epithelial and subepithelial regions are increased in AR patients (5).

In our AR model mice, the combination of intraperitoneal OVA injection and intranasal OVA administration for 1 week induces allergic nasal symptoms such as sneezing and rubbing (Fig. 1: b and c). These signs are considered to be associated with itch sensation because the same signs are induced by the intranasal administration of histamine, a potent pruritogen (44). Histochemical analysis of this stage revealed hyperinnervation of PGP9.5-positive fibers in nasal mucosa (Fig. 2d), while there was minimal increase of mast cells and eosinophils in the nasal turbinates (Fig. 3: c and d). The serum IgE amount of this stage was significantly increased (Fig. 1d), indicating that the 1-week OVA administration had induced the allergic condition. When the intranasal OVA administration was prolonged for 4 weeks, infiltrating immune cells in nasal mucosa were significantly increased (Fig. 3: e and f). These results suggest that hyperinnervation and hypersensitivity of nasal mucosa may precede the invasion of immune cells during the formation of AR.

We also found the elevation of *CGRP* mRNA in the nose samples of AR model mice (Fig. 4e). The non-neuronal tissues in nasal turbinate may express *CGRP* mRNA upon allergic stimulation. Alternatively, this may reflect the hyperinnervation of sensory fibers in epithelium and lamina propria of the AR nasal specimen be-

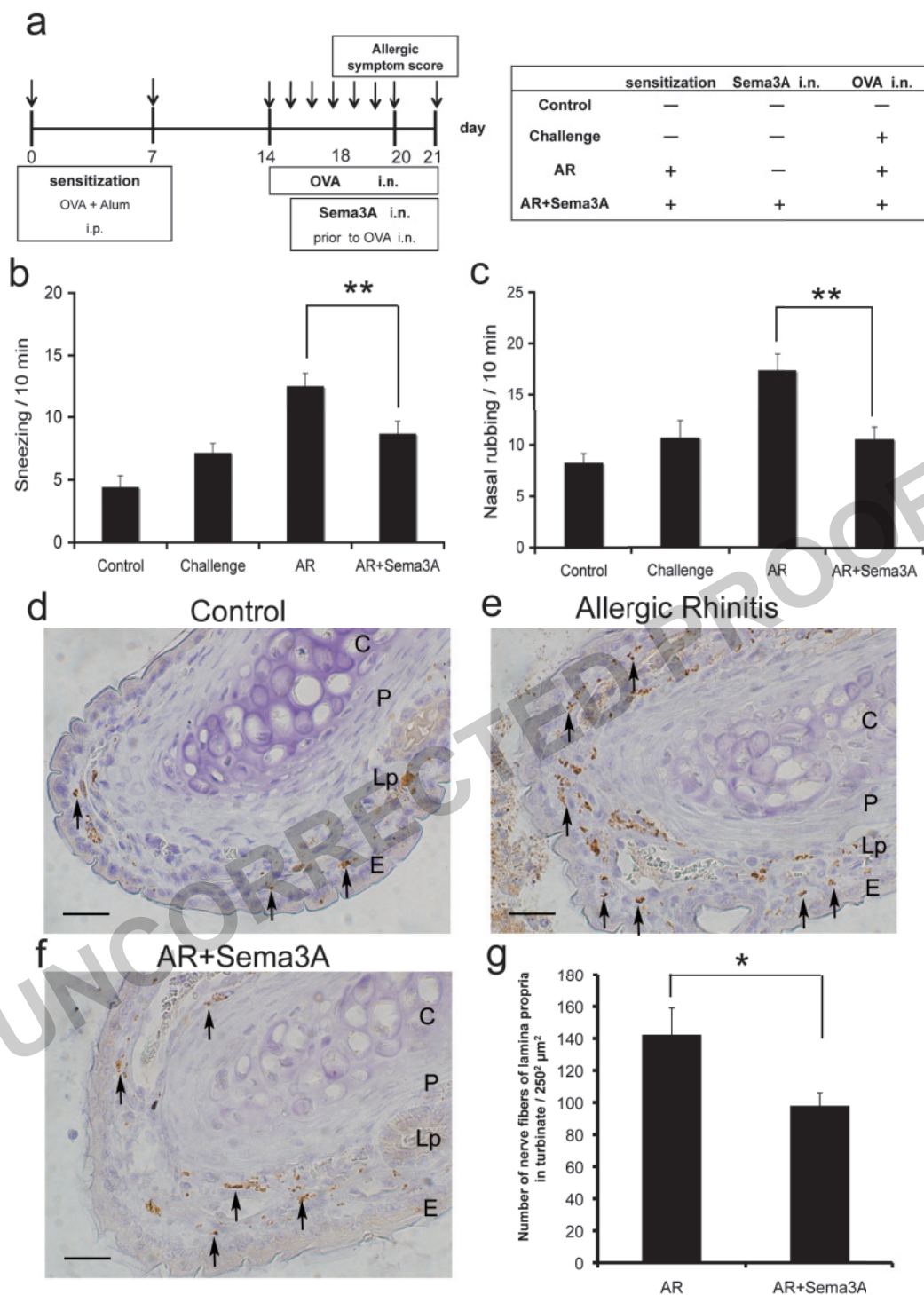


Fig. 5. Amelioration of clinical symptom scores of allergic rhinitis (AR) in Sema3A-treated mice. **a)** Protocol of intranasal Sema3A administration to AR model mice. Mice were sensitized with OVA on days 0 and 7. Sema3A (300 U) or normal saline (NS) was administered to the nostril of sensitized mice at 1.5 h prior to OVA intranasal challenge from day 15 to 21. Mice were assigned to four groups: NS challenged (Control), OVA challenged (Challenge), OVA sensitized and challenged (AR), and AR mice with Sema3A (AR+Sema3A). Allergic symptom scores were recorded on day 18 or 21 following OVA challenge. AR+Sema3A mice exhibit decreased amounts of sneezing (** $P < 0.01$, **b**) and rubbing (** $P < 0.01$, **c**), compared with AR mice. (n = Control, 12; Challenge, 7; AR, 26; AR+Sema3A, 30). **d – f)** Immunohistochemistry of PGP9.5 revealed decreased nerve fiber density in the lamina propria after Sema3A treatment. Arrows indicate PGP9.5 positive nerve fibers. Scale bars, 25 μ m. **g)** Numbers of nerve fibers per area in lamina propria were quantified using ImageJ (n = AR, 8; AR+Sema3A, 7, * $P = 0.037$). Data represent the mean $\pm$ S.E.M. P -values, Mann-Whitney U-test.

cause *CGRP* mRNA is predominantly expressed in neurons and the mRNA could be localized in the nerve endings by axonal transport (45).

Sema3A regulates axonal projection of peripheral neurons, including sensory, sympathetic, and motoneurons, during fetal stages (21, 22). Recent studies have shown that Sema3A also regulates sensory afferent sprouting during postnatal and adult stages (36, 46). The Sema3A receptor components, Neuropilin-1 and Plexin-A, are still expressed in sensory C-fibers in postnatal and adult stages (47–49). Here we demonstrate Sema3A expression in the epithelial layer of nasal mucosa (Fig. 4: c and d), as well as limited distribution of PGP9.5-positive signals in the lamina propria (Fig. 2: c and d). This suggests that Sema3A may restrict increasing invasion of C-fibers into the epithelial layer under normal conditions. In our AR model mice, Sema3A expression was decreased in the nasal epithelial layer (Fig. 4d), which possibly allowed hyperinnervation of C-fibers into the nasal epithelial layer and excess sprouting of nerve terminals in the lamina propria. Consequently, this could lead to hypersensitivity to itch. Similar correlations have been reported in atopic dermatitis (23, 50).

In addition to Sema3A, several reports have indicated that allergen challenge results in significant and acute increased NGF protein levels in nasal lavage fluids from human AR patients (16, 51). NGF induces sprouting of C-fibers (52) and increases neuropeptide expression including SP in sensory fibers (53), which in turn enhances sensitivity. Contrary to these reports, elevation of *NGF* mRNA expression was not detected in the nose of our AR model mice. NGF protein might be released upon allergic stimuli without accompanying the increase of *NGF* mRNA transcription. Indeed, incongruent trends in *NGF* mRNA and protein concentrations have been reported in AR patients (16).

We previously reported that subcutaneous administration of Sema3A was effective for alleviating skin lesions and scratching behavior in the mouse model of atopic dermatitis (24). The administered Sema3A repelled invasive nerve fibers in the epidermis and interrupted the itch-scratch cycle of atopic dermatitis. Here we demonstrate that nasal administration of Sema3A to the AR model mice ameliorated sneezing and rubbing behavior (Fig. 5: b and c). Immunohistochemical examinations revealed decreased nerve fiber density in the turbinate of Sema3A-treated AR mice (Fig. 5: d–g). Thus, the symptom severity may correlate with the density of nerve fibers in nasal mucosa. The inhibition of sensory fiber hyperinnervation in the epithelium could be an effective approach for suppressing hypersensitivity of allergic diseases. One notable advantage of nasal administration is that Sema3A is directly absorbed from the nasal mu-

cosa surface, suggesting that Sema3A could be used to treat other ailments such as allergic conjunctivitis or bronchial asthma.

Sema3A also modulates the immune system. Sema3A exhibits immunological regulatory functions of lymphocyte proliferation (54). We previously reported that subcutaneous injection of Sema3A in the mouse model of atopic dermatitis resulted in reduced infiltration of CD4⁺ T cells and IL-4 levels in the dermis (24). In this study, the infiltration of immune cells in nasal mucosa was limited in the early stage of AR model mice (Fig. 3: c and d). Therefore, the effect of Sema3A on immune cells in nasal turbinate could not be assessed. Nonetheless, considering the suppressive action of Sema3A on immune cells and the apparent immune cell invasion in the chronic stage of AR, intranasal Sema3A administration may attenuate immunological reactions in the nasal mucosa and may contribute to alleviate AR symptoms.

Although further investigation is required to assess the immunological effects of Sema3A on AR, Sema3A and its receptor components, Neuropilin-1 and Plexin-A, could serve as novel therapeutic targets for various allergic and immunologic diseases in the future.

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