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Neuronal Fc-epsilon receptor I contributes to antigen-evoked pruritus in a murine model of ocular allergy

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ABSTRACT

Pruritus is the major symptom of ocular allergy but currently available treatments are often ineffective. Previous studies demonstrated that subpopulations of primary sensory neurons express Fc receptors and may contribute to antigen-specific pain. We investigated the role of neuronal Fc-epsilon Receptor I (FcεRI) in allergic ocular pruritus. Ovalbumin (OVA) was used as allergen together with alum adjuvant (OVA + alum) to produce a mouse model of ocular allergy with a significant elevation in the serum levels of both antigen-specific IgE and IgG. Mice sensitized by OVA without alum only induced elevation of serum IgG but not IgE. Scratching behavior toward the eyes with the hindlimb was used as an indicator of ocular itch. Topical OVA challenging to the eye dose-dependently induced scratching toward the eye in the OVA + alum sensitized mice, but not those sensitized by OVA only. The antigen-induced scratching was largely abolished by topical application of the blocking antibody to FcεRIα, but was only partially alleviated by pretreatment of mast cell stabilizer or histamine I receptor antagonist. The expression of FcεRI was detected in subpopulations of trigeminal ganglion (TG) neurons including those expressing pruriceptive markers and innervating the conjunctiva in the naïve mice. Moreover, FcεRI was found significantly upregulated in small-sized TG neurons in the OVA + alum sensitized mice. In acutely dissociated TG neurons, IgE-immune complex (IC), but not the antibody or antigen alone, induced intracellular calcium increase. The neuronal responses to IgE-IC could be specifically blocked by pre-application of a siRNA for FcεRIα. Our results indicate that FcεRI expressed on peripheral nociceptive neurons in the TG may be directly activated by IgE-IC and contribute to allergic ocular pruritus. This study may suggest a novel mechanism for the development of pathological itch in allergic diseases.

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1. Introduction

Itch is an unpleasant sensation that elicits the desire or reflex to scratch (Ikoma et al., 2006; Dhand and Aminoff, 2014). The sensation of itch shares a number of similar characteristics with pain and is likely transmitted by certain unmyelinated C fibers in the

peripheral nerve that was classified as nociceptor or, more specifically, pruriceptors (Steinhoff et al., 2006; LaMotte et al., 2014). Antigen-specific immune diseases such as atopic and allergic dermatitis are often accompanied with pathological itch as well as pain (Galli and Tsai, 2012; Greaves and Wall, 1996). Patients with allergic disorders usually have an elevated level of antigen-specific immunoglobulin (Ig), especially IgE in the serum (Galli and Tsai, 2012). IgE may bind with specific allergen forming immune complex (IC). IgE-IC can induce itch via three steps: IgE-IC activate immune cells via Fc epsilon receptor (FcεR); activated immune cells release pro-inflammatory mediators and pruritogens (such as histamine, tryptase, cytokines); pruritogens induce itch by activating peripheral sensory neurons (Turner and Kinet, 1999; Rivera, 2002; Stone et al., 2010). It has been well known that IgE may activate certain immune cells such as mast cells upon binding with

Abbreviations: Bam8-22, bovine adrenal medulla peptide 822; DAPI, 4', 6-diamidino-2-phenylindole; DRG, dorsal root ganglion; FcγRI, Fc-gamma-Receptor I; FcεRI, Fc-epsilon receptor I; FcεRII, Fc-epsilon receptor II; IC, immune complex; IgE, immunoglobulin E; IgG, immunoglobulin G; OVA, ovalbumin; TG, trigeminal ganglion.

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allergen and trigger a series of immune responses which can produce itch and/or pain. However, current therapeutics targeting histamine receptors or mast cells do not always effectively relieve the discomfort of itch or stop the destructive behavior which can produce tissue damage in certain pathological conditions such as allergic conjunctivitis or dermatitis.

FcεR type I (FcεRI) is the high-affinity activating receptor that binds to the Fc portion of IgE. FcεRI has been defined structurally as a trimeric form ($\alpha\gamma_2$) or a tetrameric form ($\alpha\beta\gamma_2$) (Turner and Kinet, 1999; Conner and Saini, 2005). The α -chain containing two immunoglobulin-type domains is the IgE binding chain. The β - and γ -chains contain conserved immunoreceptor tyrosine-based activation motifs (ITAMs) (Turner and Kinet, 1999; Rivera, 2002; Stone et al., 2010; Conner and Saini, 2005; Okayama et al., 2014). FcεRI typically expresses on immune cells (such as mast cell in skin and mucosa, or basophils in blood vessels). As suggested in a previous report (Andoh and Kuraishi, 2004; van der Kleij et al., 2010) and in our pilot studies (Liu et al., 2015, 2014), FcεRI may express in dissociated mouse dorsal root ganglia (DRG) neurons but the function of neuronal FcεRI was unknown. It was not clear whether the peripheral sensory neurons could be directly activated by IgE-IC.

We hypothesize that a subpopulation of peripheral nociceptive (including prurceptive) neurons that express FcεRI can be directly activated by IgE-IC inducing itch and/or pain. Furthermore, the expression of neuronal FcεRI and responses to IgE-IC may be modulated in allergic diseases therefore contributing to the exaggerated pruritus in such conditions. We tested these hypotheses in ovalbumin allergic mouse model of ocular itch. Our findings revealed a novel neural mechanism for the sensation of itch, and might suggest potential therapeutic strategies for the treatment of pruritus related to allergic diseases.

2. Materials and methods

2.1. Animals

Adult male C57BL/6 mice (20–25 g; 6–8 wk, National Institutes for Food and Drug Control, Beijing, China) were used in this study. The *Mrgprd* (Zylka et al., 2005) and *Mrgpra3* (Han et al., 2013) transgenic mice were generously provided by Dr. Xinzhong Dong (Johns Hopkins University School of Medicine). Mice were housed in temperature ($23 \pm 3^\circ\text{C}$) and 12 h light/dark cycle controlled rooms with standard rodent chow and water available. The experimental protocols were approved by the Institutional Animal Care and Use Committee in Chinese Academy of Medical Sciences, Institute of Basic Medical Sciences. Animals were randomly assigned to treatment or control groups.

2.2. Reagents

Capsaicin, BSA, BAM8-22, Cromolyn sodium, HEPES, ovalbumin (OVA), poly-D-lysine, laminin, terfenadine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Armenian hamster anti-FcεRI α antibody, Armenian Hamster monoclonal IgG Isotype control, and rabbit anti-FcεRI γ were from Abcam (Cambridge, UK). Rabbit anti-FcεRI α , mouse anti-FcεRI β , and goat anti-FcεRI γ were from Santa Cruz Biotechnology (Dallas, Texas, USA). Guinea pig anti-PGP9.5 was from LifeSpan Biosciences (Seattle, WA, USA). Mouse anti- β -actin was from ZSJQ (Beijing, China). Alexa Fluor 488-conjugated donkey anti-rabbit, Alexa Fluor 488-conjugated donkey anti-mouse, Alexa Fluor 488-conjugated donkey anti-goat, and Alexa Fluor 594-conjugated donkey anti-guinea pig were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Protease inhibitors and chemiluminescent reagents (eECL Kit) were

from CWBio (Beijing, China). Trypsin inhibitor, Liberase TL and Liberase TM were from Roche Diagnostics (Indianapolis, IN, USA). DMEM and F12 medium, FBS, Fura 2-acetoxymethyl ester and Imject Alum, TriZol Reagent and Lipofectamine[®] RNAi MAX Reagent were from Thermo Fisher Scientific (Waltham, MA, USA). Papain was from Worthington Biochemical (Lakewood, NJ, USA). RT Master Mix and SYBR premix Ex Taq[™] were from TAKARA (Dalian, China); VECTASHIELD Mounting Medium with DAPI was from Vector Lab (Burlingame, CA, USA).

IgE-IC was prepared by using the OVA as antigen and the affinity-purified mouse anti-OVA IgE (Acris Antibodies, Inc) as antibody. The storage buffer of all the antibodies (containing sodium azide as preservative) was exchanged to HEPES buffer using Zeba[™] spin desalting columns (Thermo Scientific) before application to avoid the possible toxic or non-specific effects of sodium azide. IgE-IC were formed by incubating 1 $\mu\text{g}/\text{ml}$ antigen and antibody at the ratio of 1:1 for 1 h at 25°C , and then diluting to the concentrations of 0.01, 0.1, 0.3, and 1 $\mu\text{g}/\text{ml}$. In control experiments, individual components of IC (i.e. mouse anti-OVA IgE, OVA) were applied.

2.3. Sensitization and challenging of mice

A mouse model of ovalbumin sensitization induced ocular itch was produced with the following procedures: on day 0, day 7 and day 14, i.p. injection of 100 μg OVA plus 100 μl Imject Alum dissolved in 400 μl of normal saline. On days 21, 10 μl of OVA (0.001–5% in normal saline) were applied topically to both eyes to induce ocular allergy (Groneberg et al., 2003).

2.4. Behavioral assessment for ocular itch and pain

Behavioral assessment for ocular itch and pain in the mice were preformed following the procedures as described in previous publications (Shimada and LaMotte, 2008; Huang et al., 2016). Briefly, mice were placed in an acrylic box ($13 \times 9 \times 40$ cm) in a sound-proof room without persons for 3 consecutive days before and 1 h on the day of recording to allow acclimation. They were returned to the same box immediately after drug administration, and were videotaped from the top of box with a high-resolution digital camera (SONY HANDYCAM HDR-PJ580E, Japan) for 1 h. Four mirrors were placed on the sides of the box for a better observation of the mouse behavior. The numbers of bout of scratching the treated eye with its hindpaw or wiping with the ipsilateral forelimb were counted during video playback.

2.5. Measurement of serum total and special IgG and IgE

The total IgG or IgE in the mouse serum were assessed using the mouse IgG or IgE ELISA kit (eBioscience), respectively. Briefly, Corning Costar 9018 ELISA plate was coated overnight at 4°C with capture antibody. Coated wells were blocked with Blocking Buffer at room temperature. Samples were diluted at 10,000-fold for IgG or 25-fold for IgE. Total IgG was detected using HRP-conjugated anti-mouse IgG monoclonal antibody; total IgE was detected using biotin-conjugated anti-mouse IgE and Streptavidin-HRP monoclonal antibody as a second-step reagent, according to the manufacturer's instructions. Similarly, the OVA-specific IgG₁ or IgE were measured using a Mouse OVA IgG₁ or IgE ELISA kit (Cayman, Maine, USA), respectively.

2.6. Cell dissociation and culture

TG neurons were dissociated and cultured from adult C57BL/6 mice. Briefly, TGs of both sides were harvested from the skull base of decapitated mice after quick removal of brain, and transferred

into oxygenated complete saline solution (CSS) for cleaning and mincing as previously reported (Qu et al., 2011). The TGs were then digested with Liberase TM for 20 min and for another 20 min with Liberase TL and papain in CSS containing 0.5 mM EDTA at 37 °C. After enzymatic digestion, the cells were dissociated by gentle trituration with a fire-polished Pasteur pipette in culture medium containing 0.5 mg/ml bovine serum albumin and 0.5 mg/ml trypsin inhibitor and placed on poly-D-lysine/laminin-coated 12-mm diameter circular glass coverslips (BioCoat, BD Biosciences). The culture medium contained equal amounts of DMEM and F12 with 10% FBS. The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ and were used within 24 h.

2.7. Immunohistochemistry

Immunohistochemistry for dissociated TG neurons: TG cells cultured for 18–24 h were post-fixed in cold 80% acetone for 0.5 h under 4 °C. After washing with PBS, the coverslips were incubated with 3% BSA for 1 h followed by overnight incubation at 4 °C with one of the primary antibodies (rabbit anti-FcεRIα, 1:150, Santa Cruz Biotechnology; mouse anti-FcεRIβ, 1:150, Santa Cruz Biotechnology; goat anti-FcεRIγ, 1:100, Santa Cruz Biotechnology; or guinea pig anti-PGP9.5, 1:100), and then with the proper secondary antibodies (Alexa Fluor 488-conjugated donkey anti-rabbit, 1:400; Alexa Fluor 488-conjugated donkey anti-mouse, 1:400; Alexa Fluor 488-conjugated donkey anti-goat, 1:400; or Alexa Fluor 594-conjugated donkey anti-guinea pig, 1:400, respectively) for 1 h at 25 °C before washing and mounting.

Immunohistochemistry for TG cryosections: TGs were harvested from adult mice transcardially perfused with PBS followed by 4% paraformaldehyde, post-fixed in the same fixative for 4 h, and then cryoprotected in 20% sucrose overnight. The tissue was frozen and sectioned at 10 μm thick on a cryostat. Tissue sections were permeabilized with 0.2% Triton X-100 in PBS for 15 min, then incubated with 10% normal horse serum for 1 h, followed by overnight incubation at 4 °C with one of the primary antibodies (rabbit anti-FcεRIα, 1:150; mouse anti-FcεRIβ, 1:150; or goat anti-FcεRIγ, 1:100), and then with the proper secondary antibodies (Alexa Fluor 488-conjugated donkey anti-rabbit, 1:400; Alexa Fluor 488-conjugated donkey anti-mouse, 1:500; or Alexa Fluor 488-conjugated donkey anti-goat, 1:500, respectively) for 1 h. The slides were then washed in PBS and cover-slipped with VECTASHIELD Mounting Medium with DAPI.

The cells were visualized and the images captured using a laser confocal microscopic imaging system (Olympus FV1000 and FluoView software). At least 12 fields of view (200X) from 3 sections on each ganglion were examined. Only neuronal somata (diameter > 10 μm, and with PGP9.5 in dissociated neurons) were included in the data analysis to avoid possible contamination from non-neuronal cells expressing FcεR. More than 100 neuronal somata with nucleus profiles for each condition were counted and the percentiles of immunopositive neurons were reported. Neurons were classified according to the cross-sectional diameter of soma as small- (< 25 μm), medium- (25–35 μm) and large-sized (> 35 μm).

2.8. Western blotting

Freshly harvested mouse TG tissue or dissociated TG neurons were homogenized in modified RIPA buffer with protease inhibitors. The homogenates (25 μg of protein) were separated by SDS-PAGE and transferred to a PVDF membrane. After incubation in 5% BSA solution for 1 h, the membranes were followed by overnight incubation at 4 °C with the one of the primary antibodies: hamster anti-FcεRIα (1:1000), mouse anti-FcεRIβ (1:100, Santa Cruz Biotechnology), rabbit anti-FcεRIγ (1:300) and mouse anti-

β-actin (1:800). Subsequently, the membrane was incubated with the corresponding HRP-conjugated anti-IgG antibody (goat anti-hamster IgG, 1:1500; goat anti-mouse IgG, 1:4000 and goat anti-rabbit IgG, 1:4000). Bands were determined using eECL Kit.

2.9. Quantitative RT-PCR

Total RNA was extracted from TG neurons with TriZol Reagent. Then cDNA was synthesized from 500 ng by using the prime script RT Master Mix according to the manufacturer's protocol. Approximately 100 ng RNA-equivalent cDNA template was used per well, and qRT-PCR was performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad) with SYBR premix Ex Taq™. The sequences of primers for FcεRIα, FcεRIβ, FcεRIγ and β-Actin control are presented in Table 1.

2.10. Small interfering RNA transfection

TG neurons were transfected with either a Stealth small interfering RNA (siRNA) or a Stealth RNAi negative control (Invitrogen) using the Lipofectamine®RNAi MAX Reagent according to the manufacturer's instructions. The target sequences of the Stealth siRNAs were 5'GCUAUGGGAACAAUACCUUCAAAU3' and 5'AUUUGAAGGUGAUUGUCCCAUAGC3' for FcεRIα; and Stealth™ RNAi Negative Control Duplexes (Medium GC) for the scrambled control of FcεRIα. After 6 h treatment with siRNA, the medium was changed with DMEM containing 10% FCS, and the cells were incubated for 24 h. The neurons without the addition of siRNA to the transfection reagents were used as an empty vector negative control.

2.11. Intracellular calcium imaging

Intracellular calcium imaging was performed as described before (Qu et al., 2011, 2012). Cultured mouse TG neurons were incubated with Fura 2-acetoxy-methyl ester (2 μM) in the dark for 45 min at 37 °C. After loading with calcium sensitive dye, TG neurons were washed twice in HEPES buffer to remove extracellular dye, and placed in a recording chamber continuously perfused with HEPES buffer at a flow rate of 1.5 ml/min at room temperature. Images were recorded using an upright NIKON ECLIPSE Ti microscope equipped with a ratiometric imaging system (Nikon NIS-Elements AR 4.00.00, Japan). The ratio of 340 nm/380 nm fluorescence intensity ($R_{340/380}$) within a certain region of interest after background subtraction was used as a relative measure of intracellular calcium concentration ($[Ca^{2+}]_i$). Calibration with external standards (Calcium Calibration Buffer Kit, Invitrogen) showed that $R_{340/380}$ increased linearly with $[Ca^{2+}]_i$ up to about 1 μM and $R_{340/380}$ of 0.7–1.25 corresponded to basal $[Ca^{2+}]_i$ of 90–180 nM. Therefore, neurons ($\leq 25 \mu m$) with $R_{340/380}$ at the range of 0.7–1.25 were included in this study.

All reagents were dissolved in HEPES buffer and applied locally to the neuronal cell bodies through a micropipette (with a tip diameter of 100 μm) and an 8-channel pressure-controlled drug application system (VC3-8PP, ALA Scientific). The interval between drug applications was at least 1 min. The viability of the dissociated TG neurons was confirmed by the increase in $[Ca^{2+}]_i$ produced by a 5-s application of HEPES containing 50 mM K⁺ at the end of each experiment. The proportion of TG neurons responsive to IC was calculated as the number of IC-responsive neurons divided by all the viable neurons tested. Neurons were considered capsaicin sensitive (CAP⁺) if a 10-s application of 1 μM capsaicin evoked an increase in $R_{340/380}$ that was equal or greater than 20% above baseline. Capsaicin sensitive and insensitive neurons were analyzed separately.

Table 1
List of primer sequences designed for qPCR.

Target gene		Primer sequence 5'-3'	Product length (bp)	GenBank No.
FcεRIα	FORWARD	GCTATGGGAACAATCACCTTCA	101	NM_010184.2
	REVERSE	GGCACTCACAATGACCAATGT		
FcεRIβ	FORWARD	GGGCAGAGGACCAGAACAT	214	NM_013516.2
	REVERSE	CCCTTGACACATGGAGAAC		
FcεRIγ	FORWARD	TCAAGATCCAGGTCCGAAAG	215	NM_010185.4
	REVERSE	AGCCAACCTTCAAAGCACAG		
β-Actin	FORWARD	GTCCTCACCCTCCCAAAAG	266	NM_007393
	REVERSE	GCTGCCTCAACACCTCAACCC		

2.12. Statistical analysis

Data values are presented as means with standard errors (mean ± SEM). Statistical analyses were performed using the SPSS software (version 17.0). A Student's *t* test was used to evaluate the statistical significance of a difference between mean responses of two groups. Statistical comparisons of differences among three or more groups were made with a one-way analysis of variance (ANOVA) followed by Scheffe's post hoc test. Chi-square tests were used to compare between two or more incidence of events. The criterion for statistical significance was a value of $P < 0.05$.

3. Results

3.1. Scratching with the hindlimb as a indicator for ocular itch in the mice

It has been reported that scratching with the hindlimb is a behavioral indicator of pruritic stimuli to the cheek of mice, and wiping with forelimb is an indicator of pain (Shimada and LaMotte, 2008). We first tested that such a behavior model also applies to the eyes in naïve mice. When capsaicin solution (5 μl, 5 μM freshly prepared in 0.1% Tween 20) was dropped to the eye, a stimulation that produces ocular irritation/pain in human, the mice demonstrated a significant increase of wiping with the forelimb toward the eye, but not a significant increase of scratching with the hindlimb as compared to the vehicle (Fig. 1A–B). In contrast, when a pruritogen Bam8-22 (5 μl, 10 μM in saline) (Han et al., 2013; Liu et al., 2009) was dropped to the eye, the mice showed significant increase of scratching but not wiping toward the eye (Fig. 1A–B). Therefore we concluded that scratching with the hindlimb is an indicator for ocular itch while wiping with hindlimb is an indicator for ocular pain in the mice.

3.2. A mouse model of antigen-specific ocular pruritus

We produced a mouse model of ocular pruritus via topical application of a specific antigen to the eyes of antigen-sensitized mice. Immunization with either OVA alone or OVA and alum adjuvant (OVA + alum) significantly increased the serum concentration of total IgG and allergen-specific IgG₁, although the IgG and IgG₁ concentrations were much less in the OVA-alum group (Fig. 1C–D). However, a significantly increased serum concentration of total IgE and allergen-specific IgE are found only in the mice sensitized with OVA + alum, but not in those sensitized with OVA alone (Fig. 1E–F).

Pruritic and painful responses to OVA allergen were examined in the mice according to the assay established above in 3.1. Topical application of allergen (OVA 0.1–5%, 5 μl dropped to the eye) dose-dependently induced a pruritic response, i.e. hind-paw scratching of the treated eye in OVA + alum sensitized mice but not in the naïve or OVA sensitized mice (Fig. 1G). There was no significant

change in pain-related behavior, i.e. wiping of the eyes in any of these groups (Fig. 1G). Among all doses tested, topical application of 1% OVA evoked the highest scratching responses (Fig. 1G–H), which started almost immediately after challenge, peaked during the first 10 min, and decreased gradually in 60 min (Fig. 1I). Compared to the naïve or OVA sensitized groups, the number of scratching bouts/hr was significantly higher in the OVA + alum sensitized mice immediately or at 24 h after OVA challenging, but not at 12 h after challenging (Fig. 1J).

In order to examine the role of mast cell degranulation, histamine release and FcεRI in the allergen-evoked ocular pruritus, we pre-administrated a mast cell stabilizer cromolyn sodium (6% in NS, 5 μl eye drop t.i.d. for 3 days), an antihistamine terfenadine (30 mg/kg i.p., 1 h before challenge), or a blocking antibody of FcεRI (Armenian Hamster monoclonal anti-Fc epsilon RI antibody, 1 μg/μl in NS, 5 μl eye drop t.i.d. for 3 days) before challenging with allergen in the naïve and OVA and alum sensitized mice. All three treatments significantly reduced the number of scratching bouts (Fig. 1K–M). The blocking antibody of FcεRI demonstrated the strongest effect by almost completely abolished the scratching evoked by OVA, as compared to the effects of mast cell stabilizer or antihistamine (Fig. 1K–M).

3.3. Expression of neuronal FcεRI in the TG

FcεRI was known to express on proinflammatory cells (such as in mast cells or basophils) in tetrameric form ($\alpha\beta\gamma_2$) (Turner and Kinet, 1999; Rivera, 2002; Okayama et al., 2014; Matsuda et al., 2008). Using double immunofluorescent staining, we found that the FcεRIα, β and γ were expressed on the surface of dissociated TG neurons that were immunopositive for neuron specific marker protein PGP9.5 (Fig. 2A–I). We also found that the conjunctival mucosa was innervated by FcεRIα positive neurons, as indicated by Dil tracer injected into the conjunctiva (Supplementary Fig. S1A–C). In two transgenic mice lines with GFP expressed in tentative pruriceptive neurons (MrgprA3⁺ or MrgprD⁺) (Zylka et al., 2005; Han et al., 2013), we found that 23.1% FcεRIα immunopositive neurons co-express MrgprA3, and 27.3% FcεRIα immunopositive neurons co-express MrgprD (Supplementary Fig. S1D–I). Immunofluorescent staining using the polyclonal and monoclonal antibodies produced similar results on cryosections of TG (Supplementary Fig. S2). These findings suggested that functional FcεRI might express in pruriceptive TG neurons innervating the conjunctiva.

Next, qPCR and western blotting was used to detect the expression of all three subunits of FcεRI (α, β and γ) in the TG of naïve and sensitized mice. The results showed that mRNA and protein levels of FcεRIα in TG were significantly increased in OVA + alum sensitized mice. Only the mRNA, but not protein level of FcεRIα were found significantly increased in OVA sensitized mice (Fig. 3A–B). Immunofluorescent staining indicated that the increased FcεRIα was only detected in small-sized TG neurons (somal

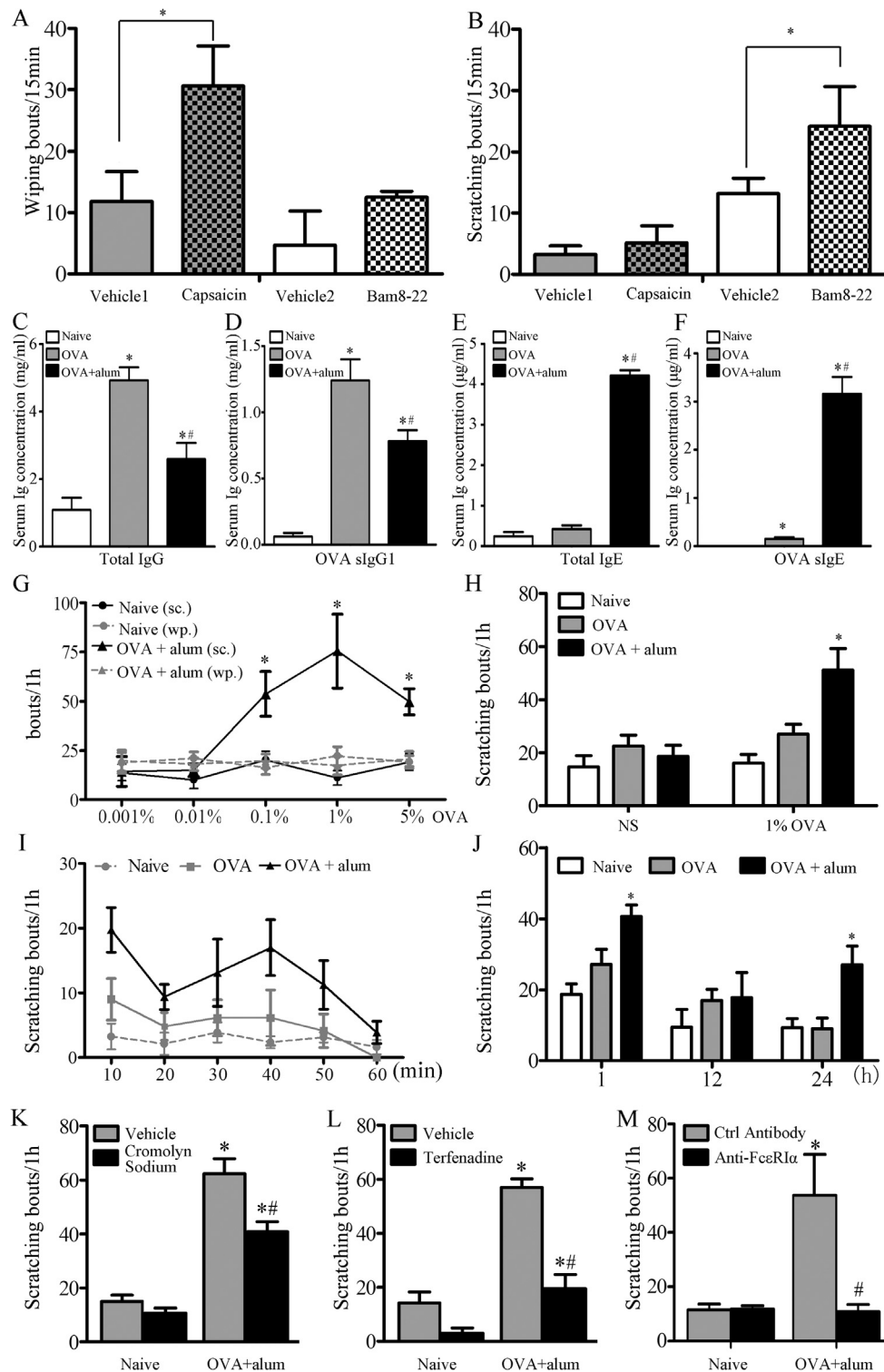


Fig. 1. OVA challenging induced ocular itch in the OVA + alum sensitized mice. **A**, Topical application of capsaicin (5 μ l, 5 μ M) induced wiping with the forelimb toward the eye. **B**, Topical application of a pruritogen Bam8-22 (5 μ l, 10 μ M in saline) induced mainly scratching with the hindlimb toward the eye. Vehicle1 and Vehicle2 are vehicles for capsaicin and Bam8-22, respectively. $P < 0.05$ vs. vehicle, t test, $n = 6$ per group (**A** and **B**). **C–D**, Injection of OVA alone or OVA with the adjuvant (OVA + alum) increased the levels of both total IgG (**C**) and OVA-specific IgG (**D**) in the serum. **E–F**, Injection of OVA + alum, but not OVA alone, increased the levels of both total IgE (**E**) and OVA-specific IgE (**F**) in the serum. $P < 0.01$ vs. naïve, $^{*}P < 0.05$ vs. OVA, one-way ANOVA, $n = 8$ per group (**C–F**). **G**, Topical application of OVA dose-dependently increased the scratching (sc.) but not wiping (wp.) behaviors toward the challenged eye in the OVA + alum sensitized (OVA + alum) mice. $P < 0.05$ vs. naïve, one-way ANOVA, $n = 8$ per group (**H** and **J**). **H**, Cumulative scratch bouts within 1 h after challenging with vehicle (saline, NS) or allergen (1% OVA) in the naïve, OVA sensitized and OVA + alum sensitized mice. **I**, Changes in the number of scratching bouts for each 10-min bin within the first hour after OVA challenging in the naïve, OVA sensitized or OVA + alum sensitized group. **J**, OVA allergen induced an acute (1 h) and chronic (24 h) pruritic response (scratching) in OVA + alum sensitized mice. $P < 0.05$ vs. naïve or OVA, one-way ANOVA, $n = 8$ per group (**H** and **J**). **K**, Effects of pretreatment of mast cell stabilizer cromolyn sodium (6% eye drop) or vehicle (saline) in Naïve and OVA + alum sensitized mice after allergen challenging. **L**, Effects of pretreatment with terfenadine (30 mg/kg i.p.) or vehicle (saline) in Naïve and OVA + alum sensitized mice after allergen challenging. **M**, Effects of pretreatment with hamster anti-FcεRIα antibody (10 μ g, 1 μ g/ μ l eye drop) or control antibody (Armenian Hamster monoclonal IgG Isotype control) in Naïve and OVA + alum sensitized mice after allergen challenging. $P < 0.05$ vs. naïve; $^{*}P < 0.05$ vs. vehicle or control antibody, two-way ANOVA, $n = 6$ per group (**I**, **J**, **K**).

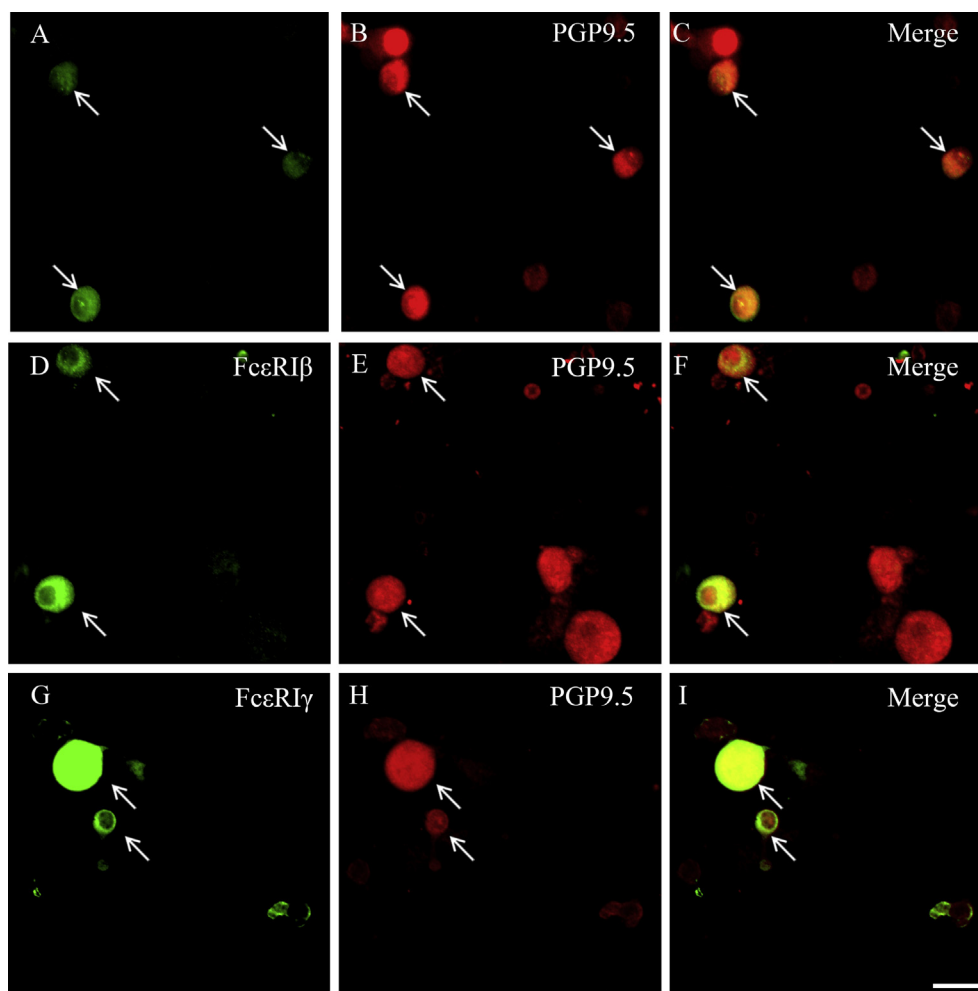


Fig. 2. Expression of FcεRI in mouse TG neurons. A–I, FcεRIα (A), β (D), and γ (G)-immunoreactivity was found co-localized with the neuronal marker PGP 9.5 (B, E, H) in dissociated TG neurons. C, F and I showed the merged images. Arrows indicated FcεRI-immunopositive neurons. Scale bar (in I): 25 μm.

diameter < 25 μm), but not in large- or medium-sized neurons in the TG (Fig. 3C–F). The mRNA and protein levels of FcεRIβ were found significantly increased in the OVA + alum sensitized mice (Fig. 4A–B). Immunofluorescent staining showed the increased immunoreactivity of FcεRIβ in the TG neurons (Fig. 4C–D). For FcεRIγ, significantly increased mRNA level was found in both the OVA and OVA + alum sensitized mice (Fig. 4F), but the western blotting (Fig. 4G) and immunofluorescent staining (Fig. 4H–J) did not reveal much difference between the sensitized and naïve mice. The low-affinity IgE receptor (CD23 or FcεRII) was significantly decreased in the TG of sensitized mice as revealed by immunofluorescent staining and western blotting (Supplementary Fig. S3).

3.4. IgE-IC induced $[Ca^{2+}]_i$ increase in the TG neurons via FcεRI

We further examined whether neuronal FcεRI could be directly activated by IgE-IC using calcium imaging. Different concentrations (0.01–1 μg/ml by antibody) of IgE-IC formed by mouse anti-OVA IgE (antibody) and OVA (antigen) were administrated to dissociated TG neurons via topical application. Antigen (OVA) or antibody (IgE) alone did not evoke any change in the $[Ca^{2+}]_i$ in TG neurons (Fig. 5A–B). Application of IgE-IC dose-dependently evoked $[Ca^{2+}]_i$ increase in TG neurons from both naïve and OVA + alum sensitized mice (Fig. 5A–C). The $[Ca^{2+}]_i$ returned slowly to baseline at least 3 min after washout of IC in most neurons tested. Interestingly, the IgE-IC (1 μg/ml) evoked calcium responses were

only observed in small-sized TG neurons (12.0% or 11/92), but not in large- (0/20) or medium-sized neurons (0/30) ($p < 0.05$, Chi-square test).

The changes in $[Ca^{2+}]_i$ as measured by $R_{(340/380)}$ were not significantly different between naïve and OVA + alum mice across all the doses applied (Fig. 5C). Neither was the percentage of IgE-IC responsive neurons significantly different between naïve and OVA + alum sensitized mice for any of the doses tested (0.01–1 μg/ml), although the percentages are always higher in the sensitized group (Fig. 5D). The percentages of TG neurons responding to IgE-IC were: naïve: 4.3% (5/117) for 0.01 μg/ml, 7.1% (3/42) for 0.1 μg/ml, 9.0% (8/89) for 0.3 μg/ml, and 12.0% (11/92) for 1 μg/ml; OVA + alum sensitized: 4.3% (3/70) for 0.01 μg/ml, 9.5% (8/84) for 0.1 μg/ml, 13.3% (13/98) for 0.3 μg/ml, and 14.5% (19/131) for 1 μg/ml.

IgE-IC induced calcium responses were observed in both capsaicin-sensitive (15.2%, 10/66) and capsaicin-insensitive (9.0%, 7/112) TG neurons from naïve mice, and also in both capsaicin-sensitive (21.8%, 17/78) and capsaicin-insensitive (3.6%, 15/140) TG neurons from OVA + alum sensitized mice. On the other hand, the percentage of capsaicin-sensitivity in IC-responsive TG neurons was similar between naïve (58.8%, 10/17) and OVA + alum mice (53.1%, 17/32).

In order to determine the role of neuronal FcεRI in the IgE-IC induced $[Ca^{2+}]_i$ increase, FcεRIα (the IgE binding chain) was knocked down with a specific siRNA in dissociated TG neurons.

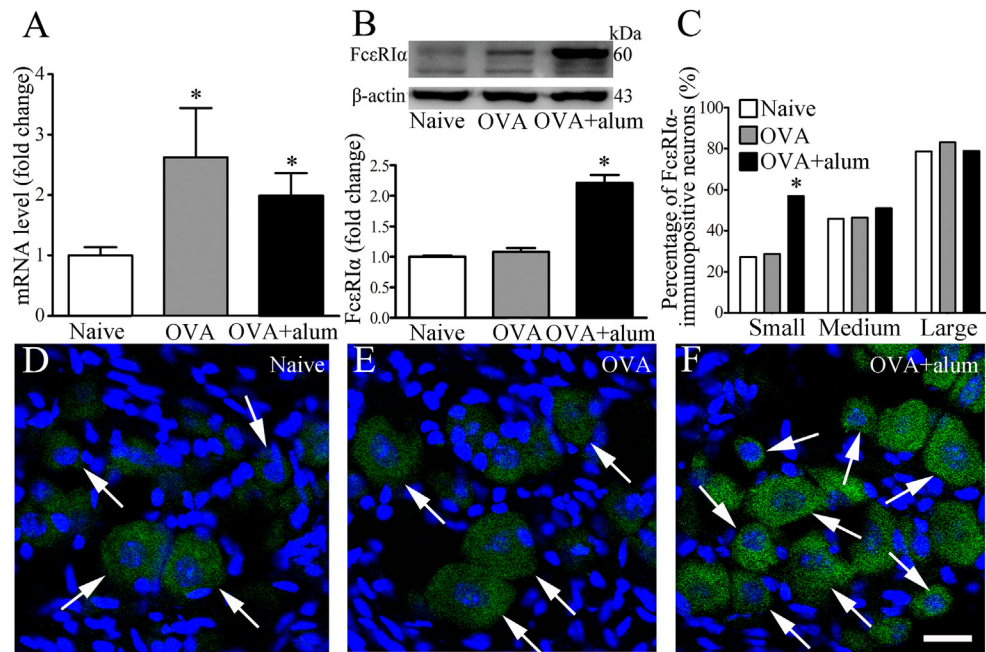


Fig. 3. Upregulation of FcεRIα subunit in the TG of sensitized mice. A, qPCR for the mRNA levels of FcεRIα subunit in TG of naïve, OVA, or OVA + alum sensitized mice. B, Western blot showing the protein levels of FcεRIα subunit in TG. Inset: example gel image for western blot. C, The percentage of FcεRIα-immunopositive neuronal somata in different size groups in the TG. D–F, Typical images for the immunofluorescence staining of FcεRIα in the naïve (D), OVA (E) or OVA + alum sensitized mice (F). Arrows indicated FcεRIα-immunopositive neurons (green-fluorescence) with nuclear profiles (blue-fluorescence). **P* < 0.05 vs. naïve, One-way ANOVA in A–B (*n* = 3 for each group) or χ^2 test in C (*n* > 100 neurons total for each group). Scale bar (in F): 25 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The significant reduction of protein expression of transfection was confirmed by Quantitative RT-PCR (Fig. 6A), western blotting (Fig. 6B) and immunofluorescent staining (Fig. 6C). Both the amount of IgE-IC evoked increase in $R_{(340/380)}$ and the percentage of responsive neurons were significantly reduced in FcεRIα siRNA-transfected TG neurons (*n* = 92) as compared to the negative control vector-transfected TG neurons (*n* = 109, transfected Stealth™ RNAi) (Fig. 6D–F). These findings indicate that IgE-IC induces $[Ca^{2+}]_i$ increase via neuronal FcεRI in the TG.

4. Discussion

Pruritus is the major symptoms of allergic conjunctivitis (Rosario and Bielory, 2011; Berdy and Hedqvist, 2000; Bielory, 2008), the most common form of ocular allergy that affects up to 40% population of the United States and 35% in Europe (Asher et al., 2006; Beasley, 1998; Petricek et al., 2006). Although treatments targeting inflammatory mediators or immune cells such as antihistamine, mast cell stabilizer and steroid have been widely used to treat allergic pruritus, the results are often partially effective and unsatisfying (Berdy and Hedqvist, 2000; Bielory, 2008; Fonacier et al., 2010; Steinhoff et al., 2012; Duffull and Begg, 1992; Buddenkotte et al., 2010; Simons and Simons, 2011; Kumar, 2009). The present study showed that a mouse model of allergic ocular pruritus elicited ocular scratching with the hind-paw, an itch- but not pain-specific behavior. We found that topical application of OVA antigen elicited ocular itch-related behaviors in the OVA + alum sensitized mice with an increased serum level of antigen-specific IgE as well as IgG, but not in the OVA alone sensitized mice with increased serum level of IgG only. Therefore the binding of IgE and antigen, i.e. IgE-IC was required to produce ocular pruritus. In accord with our findings, site-specific scratching behaviors were also reported in previous studies of allergen-elicited ocular pruritus (Shii et al., 2009; Andoh et al., 2012). The

scratching behavior could only be partially alleviated by pre-treatment with mast cell stabilizer or H_1 histamine receptor antagonist, but could be almost totally abolished by the FcεRI blocking antibody. These results are concordant with previous reports that cutaneous allergic itch in the mice could not be totally inhibited by H_1 histamine receptor antagonist and mast cell stabilizer (Inagaki et al., 1999; Bell et al., 2004), and indicate that allergic itch may involve factors other than inflammatory mediators and residential immune cells. Previous studies showed that mast cell that express the high-affinity IgE receptor FcεRI can be directly activated via allergen (antigen) with IgE (allergen-specific), and play a key role in allergic itch. The cross-linking of IgE-IC to FcεRI on mast cells can trigger mast-cell degranulation, releasing inflammatory mediators such as histamine, tryptase, platelet-activating factor, prostaglandin E2 and TNF and so on. These mediators can activate the sensory terminal of peripheral nociceptive neurons therefore inducing itch or pain (Fig. 7A). The present study revealed that FcεRI was expressed in mouse TG neurons, including those innervating the conjunctiva and those co-expressing pruriceptive neuronal markers MrgprA3 and MrgprD (Supplementary Fig. S1). We also found that IgE-IC induced calcium changes in certain small-sized TG neurons via neuronal FcεRI. Therefore, it is reasonable to propose that peripheral ocular endings of tentatively pruriceptive TG neurons expressing FcεRI can be directly activated by IgE-IC and contribute to allergen-induced ocular itch (Fig. 7B). This hypothesis was supported by our behavioral results that pre-treatment of anti-FcεRI antibody almost completely abolished allergen-induced scratching. However, due to the presence of FcεRI in both immune cells and sensory neurons, the exact role of neuronal FcεRI in allergen-induced itch will need to be further investigated. Moreover, antigen-induced immune responses may evoke an acute phase (Type I hypersensitivity mainly mediated by IgE immune complex), and a delayed phase (Type IV hypersensitivity, mainly mediated by T-cells). In this study, we focus on the acute phase of allergic ocular itch that may involve the neuronal

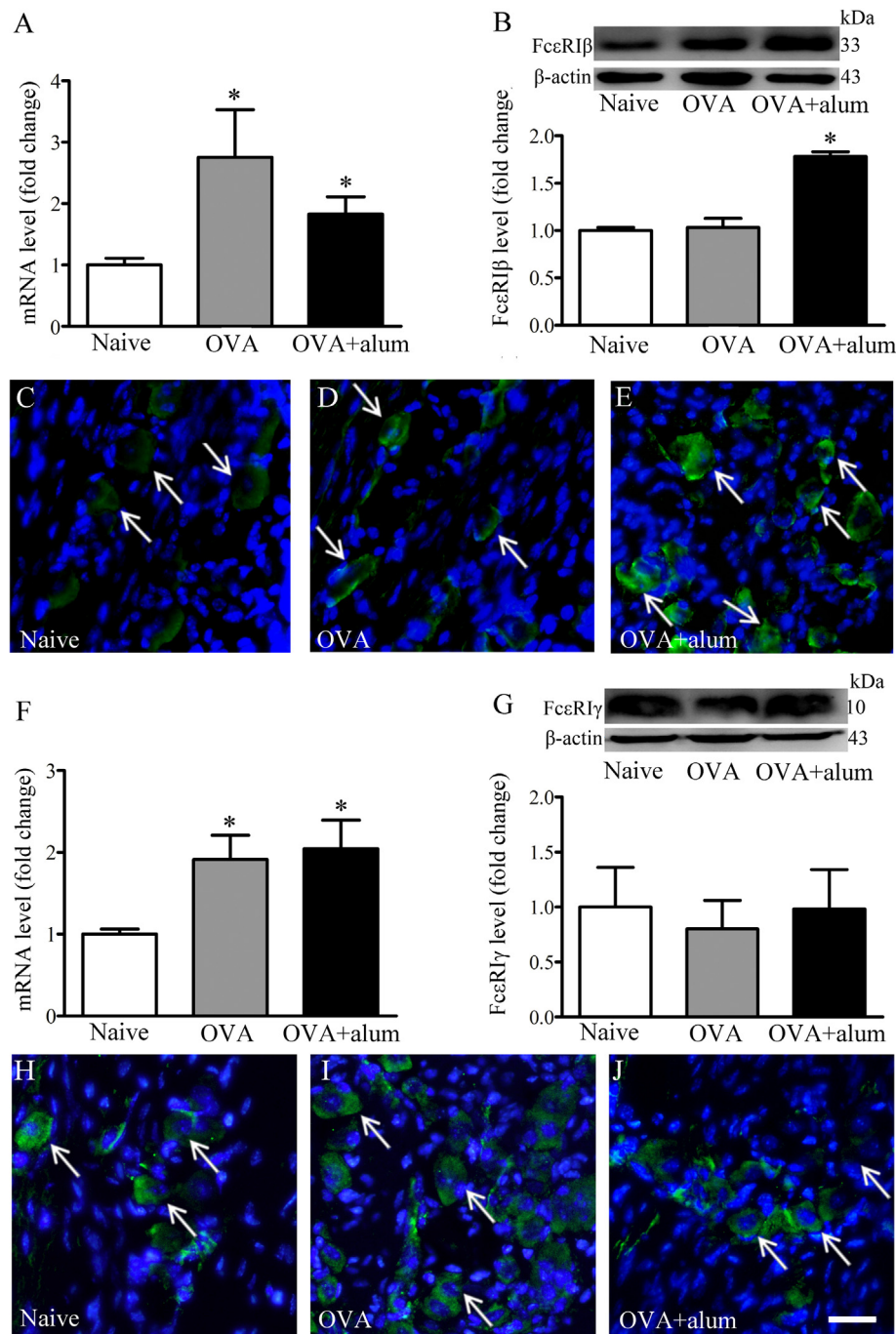


Fig. 4. Upregulation of FcεRIβ and γ subunits in the TG of sensitized mice. A, qPCR for the mRNA levels of FcεRIβ subunit in TG of naïve, OVA, or OVA + alum sensitized mice. B, Western blot for the protein levels of FcεRIβ subunit in TG. Inset: example of gel image for western blot. C–E, Immunofluorescent staining of FcεRIβ in TG neurons from naïve (C), OVA sensitized (D) or OVA + alum sensitized (E) mice. Arrows indicated immunopositive neurons. F, qPCR for the mRNA levels of FcεRIγ subunit in TG of naïve, OVA, or OVA + alum sensitized mice. G, Western blot for the protein levels of FcεRIγ subunit in TG. H–J, Immunofluorescent staining of FcεRIγ in TG neurons from naïve (H), OVA (I) or OVA + alum sensitized (G) mice. Arrows indicated immunopositive neurons. **P* < 0.05 vs. naïve, One-way ANOVA (in A, B, F and G, *n* = 3 for each group). Scale bar (in J): 25 μm.

FcεR, although the antigen did evoke a delayed phase of itch-related behavior (Fig. 1J). The potential role of FcεRI in the delayed phase will be explored in future studies.

The present study revealed that FcεRI (including α, β, and γ chains) was expressed in mouse TG neurons. It was reasonable to hypothesize that FcεRI was expressed on sensory neurons in tetrameric form (αβγ₂) as previously described in immune cells (Turner and Kinet, 1999; Rivera, 2002; Conner and Saini, 2005). We found that the IgE binding chain FcεRIα showed the most significant upregulation after sensitization, but the underlying

mechanisms and the role of each chain of neuronal FcεRI needs further investigation.

Recent studies showed the presence of Fc receptors including FcγR and FcεR in peripheral sensory neurons in the mice and rats (Andoh and Kuraishi, 2004; Liu et al., 2015, 2014; Qu et al., 2011, 2012; Andoh and Kuraishi, 2004; Harada et al., 2010). IgG-IC may directly act on neuronal FcγRI, evoking [Ca²⁺]_i increase and action potential discharges in nociceptive DRG neurons (Qu et al., 2011, 2012). In this study, we found that IgE-IC may directly act on neuronal FcεRI and evoke [Ca²⁺]_i increase in TG neurons from both

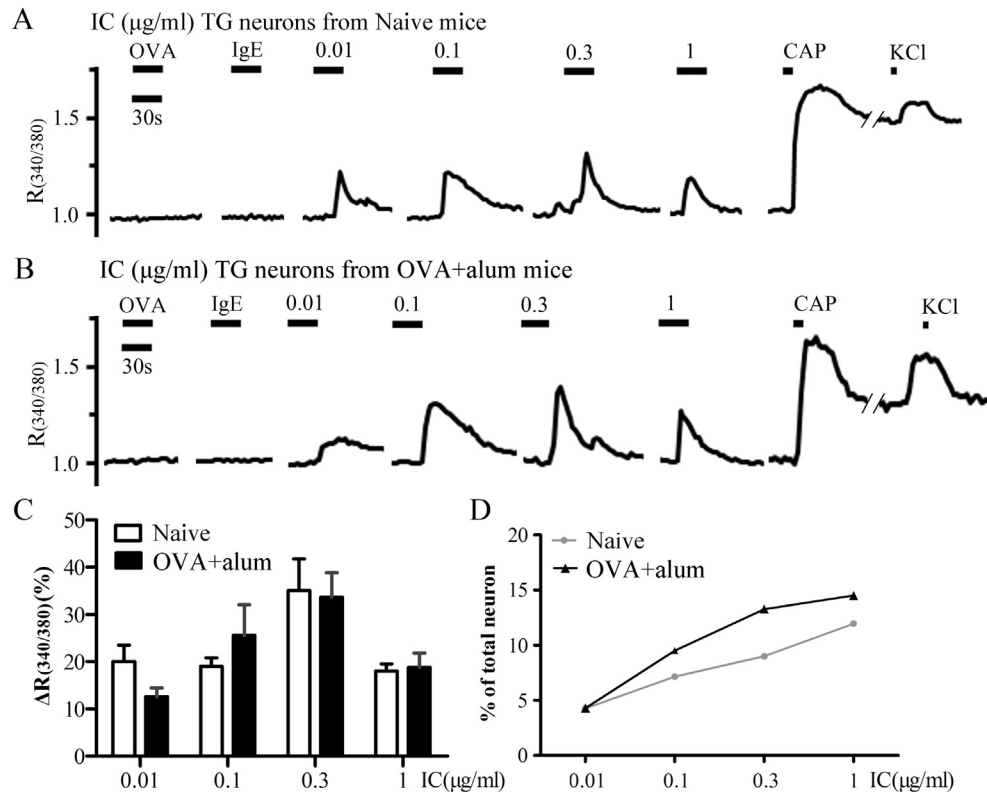


Fig. 5. IgE-IC evoke $[Ca^{2+}]_i$ increase in dissociated TG neurons. A–B, Typical examples of calcium responses ($R_{(340/380)}$) in dissociated TG neurons from naïve (A) or OVA + alum sensitized (B) mice by topical application of different doses of allergen only (OVA), antibody only (IgE), IgE-IC (IC, 0.01–1 μ g/ml, 30 s), capsaicin (CAP, 1 μ M, 10 s), and KCl (30 mM, 5 s). C, Summary of IgE-IC evoked $[Ca^{2+}]_i$ increase in dissociated TG neurons. D, Percentage of TG neurons responding to different doses of IgE-IC in naïve and sensitized mice.

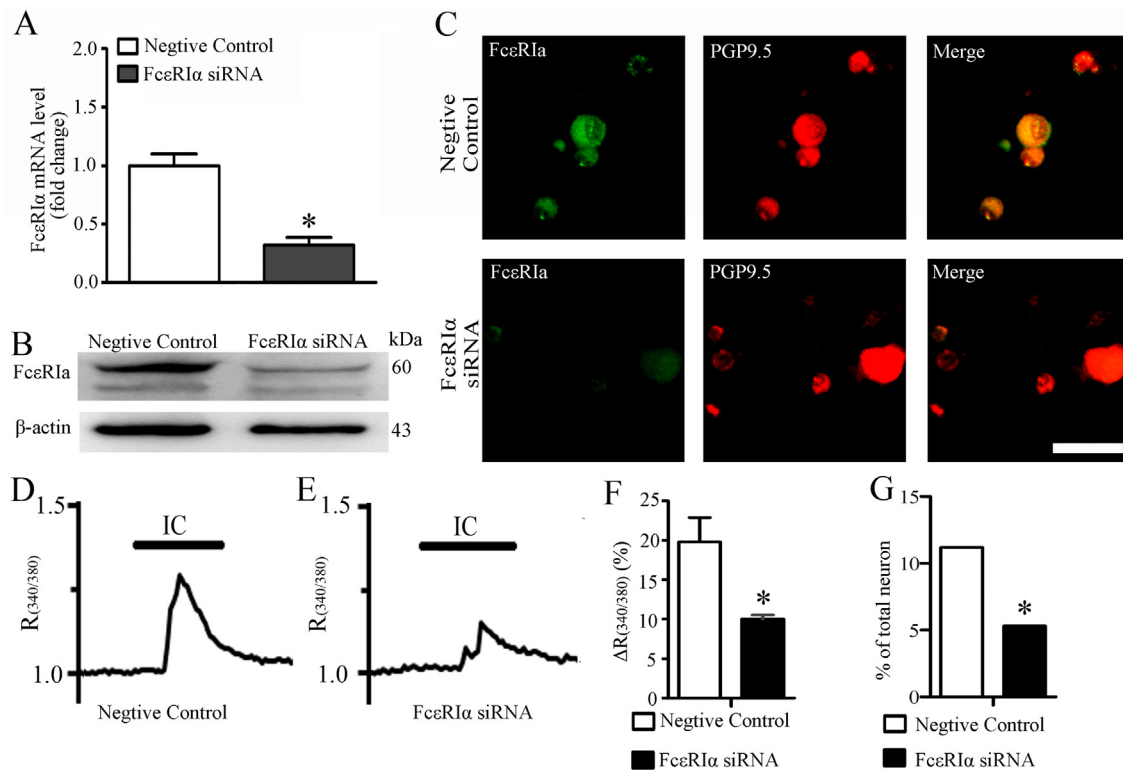


Fig. 6. Knockdown of FcεRIα with siRNA reduced the IgE-IC evoked neuronal responses. A–C, qPCR (A), Western blotting (B) and immunofluorescent staining (C) showed down-regulation of FcεRIα in dissociated TG neurons after incubation with specific siRNA but not Negative Control siRNA (Stealth™ RNAi). Scale bar: 50 μ m. D–E, Typical examples of IgE-IC evoked $[Ca^{2+}]_i$ responses in dissociated TG neurons after transfected with Negative Control siRNA (D) or FcεRIα-specific siRNA (E). F, The IgE-IC (0.3 μ g/ml, 30 s) evoked $[Ca^{2+}]_i$ response was significantly decreased in FcεRIα-specific siRNA-transfected TG neurons. G, The percentage of TG neurons responsive to IgE-IC in TG neurons transfected by FcεRIα-specific or Negative Control siRNA. * $P < 0.05$ vs. Negative Control siRNA.

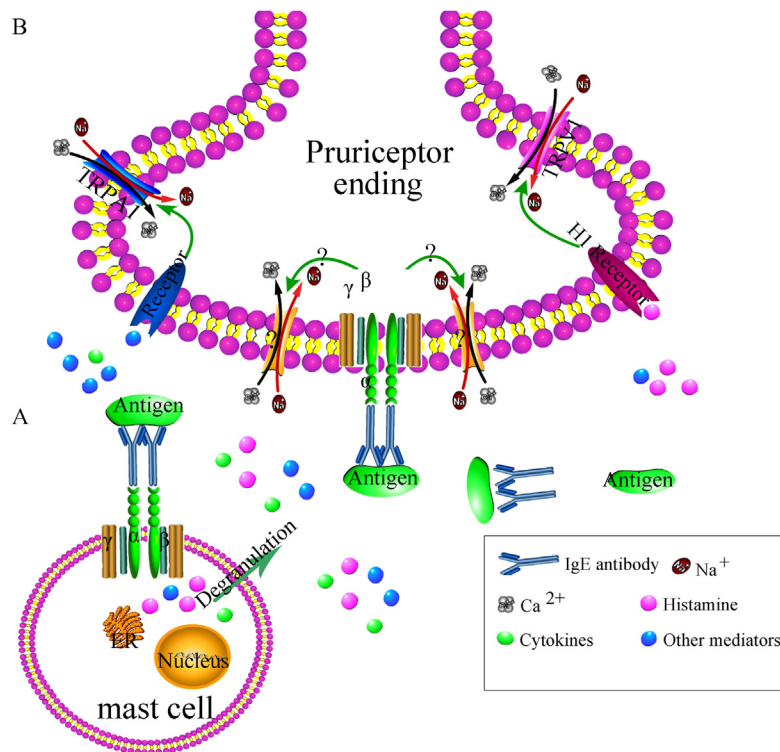


Fig. 7. Schematic illustration demonstrating the mechanism of IgE-IC induced itch in ocular allergy. IgE-IC may induce itch in ocular allergy via two mechanisms: A, Indirect mechanism: Mast cells were activated and degranulated, releasing cytokines and pruritic mediator such as histamine which can activate the nerve ending of pruriceptive neurons innervating conjunctiva and cause ocular itch. B, Direct mechanism: IgE-IC may directly bind to neuronal FcεRI, activating pruriceptive neurons innervating conjunctiva and cause ocular itch.

naïve and sensitized mice. Furthermore, knock-down of FcεRIα using siRNA in dissociated TG neurons significantly reduced the IgE-IC induced $[Ca^{2+}]_i$ increase, suggesting an essential role of the neuronal FcεRIα in IgE-IC induced calcium response (Fig. 6). Although FcεRI is expressed across all neuronal size categories, the upregulation of FcεRI in the TG of OVA + alum sensitized mice was only detected in small-sized TG neurons. Accordingly, the calcium responses to IgE immune complex were only observed in small- but not in large- or medium-sized TG neurons (See Results 3.4). These results imply that small-sized TG-neuron expressing FcεRI may contribute to allergic ocular pruritus. Moreover, IgE IC evoked calcium responses were found in both capsaicin-sensitive and capsaicin-insensitive neurons. Although topical application of capsaicin to the eye evoked mainly pain but not itch, previous publications indicate that most pruriceptive neurons express TRPV1 and were capsaicin-sensitive (Han et al., 2013; Ma et al., 2012). The roles of sub-populations of IgE-IC responsive TG neurons in itch, and the molecular mechanisms underlying the neuronal-type specificity will be studied in the future.

Despite a upregulation of FcεRI in the TG from OVA-alum sensitized mice, there was no significant difference between naïve and OVA-alum sensitized mice in the amplitude of change in $[Ca^{2+}]_i$ as measured by $R_{(340/380)}$, or the percentage of TG neurons responding to IgE-IC for all the doses tested. The lack of significant changes between naïve and sensitized mice in calcium responses of dissociated TG neurons to IgE-IC may due to insufficient sample size, possible changes induced by culturing, or other factors. Nevertheless, small-sized TG sensory neurons may be directly activated by IgE-IC in allergic conditions and contribute to ocular itch.

In summary, our results suggest that primary sensory neurons including pruriceptors in the TG express FcεRI and may be directly

activated by IgE-IC. Neuronal FcεRI, esp. FcεRIα may upregulate in sensitized mice with increased serum IgE, and may play a role in allergic ocular pruritus. Our findings revealed a novel mechanism of allergic ocular pruritus via direct activation of sensory neuronal FcεRI by IgE-IC. This study may suggest potential strategies for the treatment of pruritus in ocular allergy as well as other diseases with pathological itch.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbi.2016.11.017>.

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