

## INVESTIGATIVE REPORT

# Different Roles of Capsaicin-sensitive and H<sub>1</sub> Histamine Receptor-expressing Sensory Neurones in Itch of Mosquito Allergy in Mice

Tasuku NAKANO<sup>1</sup>, Tsugunobu ANDOH<sup>1</sup>, Atsushi SASAKI<sup>1</sup>, Hiroshi NOJIMA<sup>2</sup> and Yasushi KURAIISHI<sup>1,3</sup>

<sup>1</sup>Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, <sup>2</sup>Department of Pharmacology, Faculty of Pharmaceutical Sciences, Ohu University, Koriyama, and <sup>3</sup>21st Century COE Program, University of Toyama, Toyama, Japan

**Although mosquito allergy induces the release of histamine, the itch-related response, scratching, is not effectively suppressed by blockade of H<sub>1</sub> histamine receptors. To address this question, we examined the effects of neonatal capsaicin treatment on allergic reactions and H<sub>1</sub> histamine receptor-expressing sensory neurones in mice. Neonatal capsaicin treatment almost completely abolished allergy-associated scratching, without effects on plasma extravasation or increase in serum concentrations of immunoglobulins E and G<sub>1</sub>. An injection of edema contents from an animal exhibiting allergic reaction elicited scratching in naive animals, suggesting the production of pruritogen(s) by allergic reaction; this production was not suppressed by neonatal capsaicin treatment. This treatment markedly decreased the number of sensory neurones immunoreactive for TRPV1 capsaicin receptor, with little effect on sensory neurones immunoreactive for neurofilament 200, a marker of myelinated A-fibre neurones. In addition, there was a trend towards a reduction in numbers of sensory neurones immunoreactive for H<sub>1</sub> histamine receptor. The results suggest that capsaicin-sensitive sensory neurones that lack H<sub>1</sub> histamine receptors play a key role in signalling of allergic itch. *Key words: mosquito allergy; itch; scratch; sensory neurone; capsaicin; TRPV1 receptor; H<sub>1</sub> histamine receptor.***

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Y. Kuraishi, Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. E-mail: kuraisiy@pha.u-toyama.ac.jp

Histamine is a well-known endogenous itch mediator. Although its role in itch of chronic pruritic dermatoses may not be pivotal, histamine plays an important role in itch of urticaria (1). In animal experiments, degranulation of mast cells in the localized skin elicits scratching of the affected region, which is inhibited by H<sub>1</sub> histamine receptor antagonists (2–4). Mosquito allergy induces plasma extravasation in the localized skin, which is markedly suppressed by H<sub>1</sub> histamine receptor antagonist (5), sug-

gesting the release of histamine in the skin. However, the non-sedative H<sub>1</sub> histamine receptor antagonist terfenadine does not inhibit scratching elicited by mosquito allergy, although it suppresses histamine-elicited scratching (5, 6). In mice sensitized by repeated mosquito bites, the serum concentration of immunoglobulin G<sub>1</sub> (IgG<sub>1</sub>) is markedly increased, and there is a tendency of increased IgE (5). Fc gamma receptor I, a high affinity IgG receptor, is expressed in primary sensory neurones, and the formation of immune complex there results in the excitation of primary sensory neurones (7). Therefore, it is possible that saliva antigen(s) bind to sensitized primary afferents to stimulate them. In addition, lipid mediator(s) other than histamine, produced in the skin by allergic reaction, may be also involved in scratching induced by mosquito allergy (8). Thus, lack of participation of histamine in itch may be partly due to the complex mechanisms of itch of mosquito allergy, but the precise reason remains unknown.

Histamine-induced itch may be mediated mainly by sensory C-fibres. Histamine-sensitive C-fibres respond to mustard oil, which causes burning pain (9). Mechano-heat-responsive “polymodal” C-fibres are thought to be involved in pain signalling, but C-fibres which are judged to convey histamine-evoked itch signal are mechano-insensitive (10), suggesting that a subpopulation of C-fibres is involved in histamine-induced itch. Capsaicin degenerates or desensitizes thin-fibre sensory neurones (11), which have mainly TRPV1 capsaicin receptor-expressing C-fibres and play a key role in pain signalling (12). Itch elicited by histamine has been shown to be suppressed by repeated topical application of capsaicin in healthy human subjects (13, 14). In contrast, capsaicin pre-treatment does not inhibit histamine-induced itch in patients with atopic eczema (14). In addition, it has been reported recently that repeated application of capsaicin does not significantly suppress histamine-induced itching, although it inhibits itch induced by itchy spicules from the plant *Mucuna pruriens*, cowhage (15). Therefore, the role of TRPV1 receptor-expressing C-fibres in signalling of histamine-evoked itch is unclear. In the present study, in order to ascertain the reason why the H<sub>1</sub> histamine receptor antagonist does not inhibit cutaneous allergic itch, we

investigated the effects of neonatal capsaicin treatment, which degenerates thin-fibre sensory neurones (11), on allergic reactions including itch, using a mouse model of itch of mosquito allergy (5).

## MATERIALS AND METHODS

### *Animals and neonatal capsaicin treatment*

Male ICR (Institute for Cancer Research) mice (5–10 weeks old, Japan SLC, Shizuoka, Japan) were used. They were housed in a room under controlled temperature ( $22 \pm 2^\circ\text{C}$ ), humidity ( $55 \pm 10\%$ ) and light (light on 07.00–19.00 h). Food and water were freely available. The study was approved by the Committee for Animal Experiments at the University of Toyama.

### *Neonatal capsaicin treatment*

The animals were given subcutaneous injections of capsaicin at a dose of 50 mg/kg or vehicle (10% ethanol and 10% Tween 80 in saline) twice on day 2 and 5 after birth (16). To verify the depletion of capsaicin-sensitive primary afferents, one drop (10  $\mu\text{l}$ ) of 0.1% capsaicin was applied to one cornea, and the number of wiping movements occurring in 30 sec was counted (17).

### *Sensitization*

Extract of salivary gland of mosquito (ESGM) was prepared from female adult mosquitoes, *Aedes albopictus* (5). The thorax, including the salivary glands, was isolated under a stereoscopic microscope (5). The isolated parts were homogenized and centrifuged at  $9000 \times g$  for 30 min. The supernatant was filtered through a cellulose acetate membrane with 0.45  $\mu\text{m}$  pores, lyophilized and kept at  $-80^\circ\text{C}$  until use. ESGM (10  $\mu\text{g}/\text{site}$ ) was injected intradermally into the caudal back of the animals (5 weeks old at the start of treatment) twice a week for 4 weeks.

### *Behavioural observation*

The observation of scratching behaviour was performed as described previously (18). The hair was clipped over the rostral part of the mouse back. The next day, saline or ESGM (10  $\mu\text{g}/\text{site}$ ) was injected intradermally in a volume of 50  $\mu\text{l}$  into the interscapular region using a 27-gauge needle connected to a microsyringe via a PE-10 tube. The animals were put individually into an acrylic cage (13 $\times$ 9 $\times$ 40 cm) for at least 1 h for acclimatisation. Immediately after intradermal injection, the animals were put back into the same cages and their behaviours were videotaped for 1 h with personnel kept out of the observation room. Counts of scratching bouts were made using video playback. The mice stretched either hind paw toward the injection site, leaned the head toward it, and rapidly scratched several times for about 1 sec. A series of these movements was counted as one bout of scratching; the scratch bout was considered to end when the mouse lowered its hind paw (19).

### *Collection of the ear oedema contents*

ESGM (10  $\mu\text{g}/\text{site}$ ) or saline was injected into the ears, which were cut off 10 min later under diethyl ether anaesthesia. The contents of the ear oedema were pushed out with a spatula into phosphate-buffered saline and were centrifuged at  $400 \times g$  for 5 min at  $4^\circ\text{C}$ . The supernatant was kept at  $-80^\circ\text{C}$  until use. It was injected intradermally into the rostral back of naive mice; each animal was given the contents collected from both ears of one mouse.

### *Plasma extravasation*

For the observation of plasma extravasation, 150  $\mu\text{l}$  of 1% Evans blue dissolved in saline was injected into the tail vein. Twenty minutes later, ESGM or saline was injected intradermally into the rostral back. The back skin was isolated 20 min after challenge and the bluish area of the skin (a circle of 17 mm diameter) was punched out. The skin sample was incubated in 2 ml of dimethyl sulphoxide overnight at room temperature to extract the dye, the concentration of which was determined spectrophotometrically at 620 nm.

### *Measurement of total immunoglobulins in the serum*

Blood was collected from the ophthalmic artery, incubated for 1 h at  $37^\circ\text{C}$  and centrifuged at  $1200 \times g$  for 15 min at  $4^\circ\text{C}$ . The supernatant was kept at  $-80^\circ\text{C}$  until assay. The serum levels of IgE and IgG<sub>1</sub> were determined using commercially available enzyme-linked immunosorbent assay kits (Bethyl Laboratories, Inc., Montgomery, TX, USA).

### *Immunohistochemistry*

The animals were anaesthetized with pentobarbital (80 mg/kg) and transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde in the same buffer. The dorsal root ganglion (DRG) was removed, post-fixed for 4 h in the same fixative and kept overnight in phosphate-buffered saline containing 30% sucrose at  $4^\circ\text{C}$ . The DRG was cut at 16  $\mu\text{m}$  with a cryostat and the sections were preserved at  $-80^\circ\text{C}$  until use. The sections were incubated overnight at  $4^\circ\text{C}$  with rabbit anti-TRPV1 vanilloid receptor antibody (1:100; Calbiochem, La Jolla, CA, USA), rabbit anti-neurofilament 200 (1:1000; Chemicon International, Temecula, CA, USA), or rabbit anti-H<sub>1</sub> receptor antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After being washed with phosphate-buffered saline containing 0.2% Tween 20, the sections treated with the first antibody were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG antibody (1:1000; Molecular Probes, Grand Island, NY, USA) for 2 h at room temperature. Fluorescence signals were observed using a confocal laser scanning microscope (Bio-Rad, Hercules, CA, USA). The number of cells positive for each marker was determined in the square area (151 $\times$ 151  $\mu\text{m}$ ). Two or three square areas were randomly selected from the cell-rich region of each section and three or four sections from each animal were used for analysis.

### *Data processing*

All data were presented as means and standard error of mean. Statistical significance was analysed using Student's *t*-test and one-way analysis of variance followed by Dunnett's multiple comparisons;  $p < 0.05$  was considered significant.

## RESULTS

### *Capsaicin sensitivity*

The mice were given neonatal capsaicin treatment to degenerate thin-fibre sensory neurones expressing TRPV1 receptors. A wiping test was performed to confirm the validity of the treatment. Obvious eye wiping was elicited by administration of capsaicin to the eye in mice given vehicle, but not capsaicin, as neonates; the number of wipes for 30 sec was  $18.8 \pm 0.9$

and  $0.4 \pm 0.1$  ( $p < 0.05$ , Student's *t*-test) in vehicle and capsaicin groups, respectively.

*Allergic itch-related response*

Although an intradermal injection of ESGM elicited slight scratching in naive mice, it elicited marked scratching in sensitized ones (Fig. 1A). In animals treated with vehicle as neonates, the first injection of ESGM did not elicit obvious scratching, but the repetition twice a week gradually increased scratching; a significant increase in scratching was observed following the eighth and eleventh injections (Fig. 1B). By contrast, the repetition did not increase scratching at least until the eleventh injection in mice given capsaicin as neonates (Fig. 1B).

*Pruritogen production in allergic oedema*

To show that the production of pruritogen(s) in the skin was due to an allergic reaction, we examined whether an

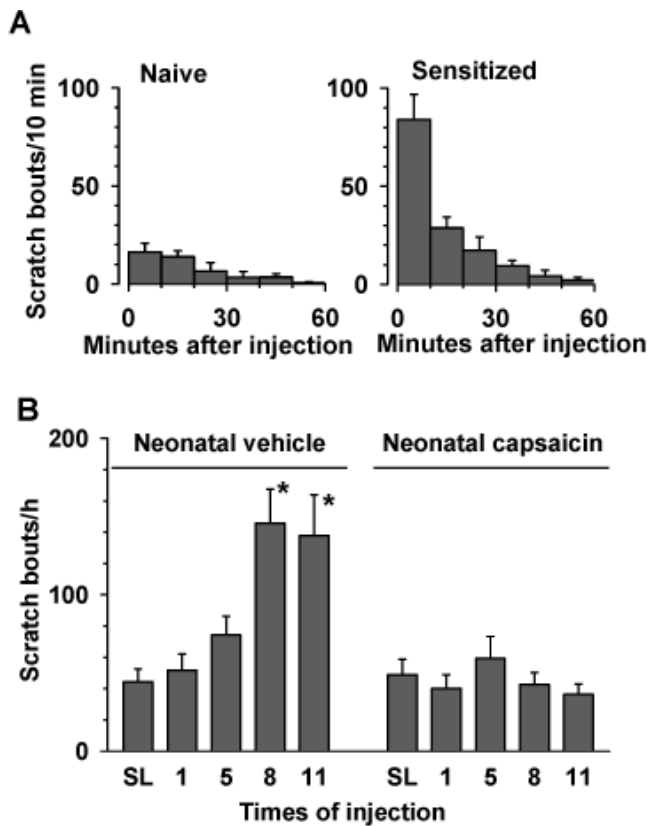


Fig. 1. Suppression by neonatal capsaisin treatment of scratching elicited by an injection of an extract from the salivary gland of mosquito (ESGM). The mice were given intradermal injections of ESGM into the caudal back for sensitization twice a week and challenge (the final injection) was done in the rostral back. (A) Time course of scratching following injection of ESGM in naive and sensitized animals. (B) Effect of neonatal capsaisin treatment on ESGM-induced scratching. The animals were treated with vehicle or capsaisin as neonates and were given ESGM. Data represent the number of episodes of scratching for 1 h after the injection of saline (SL) or ESGM. \* $p < 0.05$  compared with SL (Dunnett's multiple comparisons). Values are the means and standard error of the mean for 8 animals.

intradermal injection of crude contents of the challenged region of the ears would elicit scratching in other naive mice. Similar slight scratching was observed following intradermal injections of the contents of the ESGM-injected region of non-sensitized animals and of the saline-injected region of sensitized ones (Fig. 2A). On the other hand, extracts from the ESGM-challenged region of sensitized animals elicited marked scratching in naive mice (Fig. 2A).

In this series of experiments, in order to test the effect of neonatal capsaisin treatment, mice pre-treated with capsaisin as neonates were sensitized and challenged with ESGM. An intradermal injection of crude contents

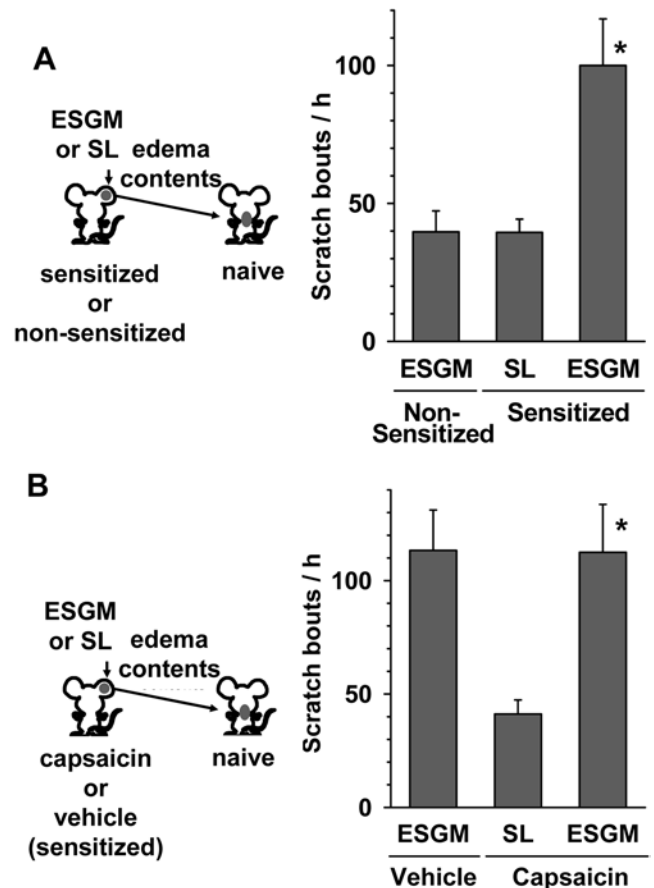


Fig. 2. Pruritogen production in allergic oedema through capsaisin-insensitive mechanisms. (A) Scratching elicited by the contents of allergic oedema. For sensitization, an extract of the salivary gland of mosquito (ESGM) was injected intradermally into the caudal back of the mice twice a week for 4 weeks. For challenge, ESGM or saline (SL) was injected intradermally into the ear. Ten minutes later, oedema contents were prepared (see Materials and Methods section). The oedema content was injected intradermally into the back of naive animals and scratching was counted for 1 h. (B) Neonatal capsaisin does not affect the production of pruritogen. The animals were treated with capsaisin or vehicle as neonates. From 5 weeks old, they were all sensitized with ESGM. For challenge, ESGM or SL was injected intradermally into the ear. Ten minutes later, oedema contents were prepared. The oedema content was injected intradermally into the back of naive animals and scratching was counted for 1 h. \* $p < 0.05$  when compared with SL (Dunnett's multiple comparisons). Values are the means and standard error of the mean for 6–8 animals.

from the ESGM-challenged ears of capsaicin-treated mice elicited scratching in naive mice (Fig. 2B). The effect of the contents from capsaicin-treated mice was similar to that of capsaicin-untreated ones (Fig. 2B).

#### Plasma extravasation and immunoglobulin production

An intradermal injection of ESGM produced a marked increase in plasma extravasation in the ESGM-sensitized mice. The increase in plasma extravasation was not suppressed by neonatal capsaicin treatment, but instead there was an increased tendency (Fig. 3). The serum concentrations of total IgE and IgG<sub>1</sub> were increased in ESGM-sensitized mice and these increases were not suppressed by neonatal capsaicin treatment (Fig. 4).

#### Dorsal root ganglion neurones

TRPV1-immunoreactive neurones in the DRG were mainly small ( $\leq 15 \mu\text{m}$  in diameter) or medium ( $> 15 \mu\text{m}$  and  $\leq 25 \mu\text{m}$ ) in size (Fig. 5A) and they were markedly and significantly decreased by neonatal capsaicin treatment (Fig. 5B and Table I). H<sub>1</sub> histamine receptor-immunoreactive neurones in the DRG were of various sizes (Fig. 5C). Neonatal capsaicin treatment produced a 14% decrease in H<sub>1</sub> histamine receptor-positive neurones, but the difference was not statistically significant (Table I). Capsaicin-resistant H<sub>1</sub> receptor-positive neurones were also of various sizes (Fig. 5D). Neurones immunoreactive for neurofilament 200, a marker of myelinated A-fibre neurones, were also of various sizes, many of which were large sized ( $> 25 \mu\text{m}$ ) (Fig. 5E). Neonatal capsaicin treatment did

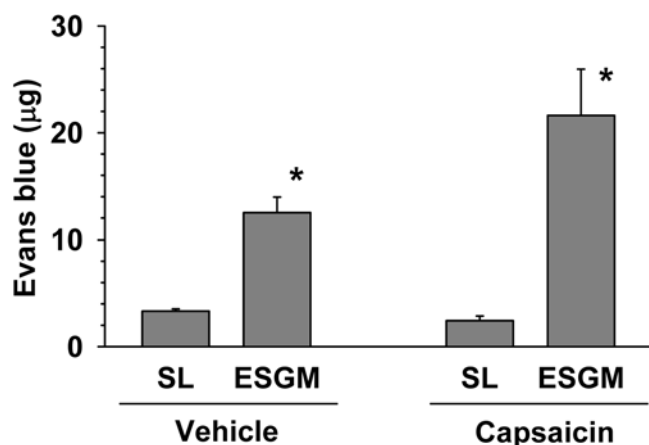


Fig. 3. Effects of neonatal capsaicin treatment on allergic plasma extravasation. Mice were treated with capsaicin or vehicle as neonates. Sensitization to an extract of the salivary gland of mosquito (ESGM) was performed as described in Fig. 2. Five weeks after the start of sensitization, Evans blue was injected intravenously and 20 min later ESGM or saline (SL) was injected intradermally. The amount of Evans blue in the challenged skin was determined. \* $p < 0.05$  when compared with SL (Student's *t*-test). Values are the means and standard error of the mean for 6 animals.

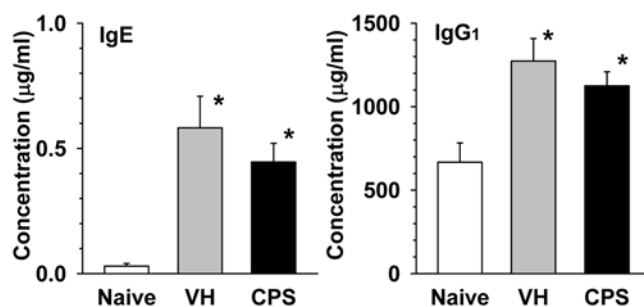


Fig. 4. Effects of neonatal capsaicin (CPS) treatment on the production of immunoglobulins. Mice were treated with CPS or vehicle (VH) as neonates. Sensitization to an extract of the salivary glands of mosquitoes was performed as described in Fig. 2. Five weeks after the start of sensitization, the blood was collected and the serum concentrations of IgE and IgG<sub>1</sub> were determined. \* $p < 0.05$  when compared with the naive groups (Dunnett's multiple comparisons). Values are the means and standard error of the mean for 6 animals.

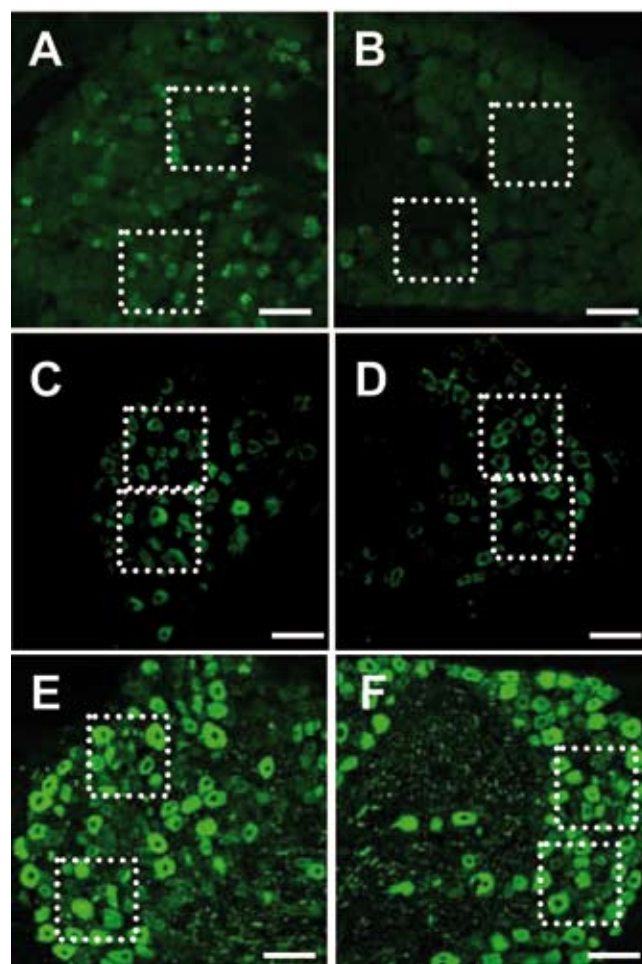


Fig. 5. Immunohistochemical analysis of the effects of neonatal capsaicin treatment on dorsal root ganglion neurones. The murine dorsal root ganglion neurones were immunostained with antibodies against TRPV1 receptor (A, B), H<sub>1</sub> histamine receptor (C, D), and NF200 (E, F). Mice were treated with vehicle (A, C, E) or capsaicin (B, D, F) as neonates. Scale bar = 100  $\mu\text{m}$ . The squares indicate the examples of assessment areas in Table I.

Table 1. Effects of neonatal capsaicin treatment on neurones immunoreactive for TRPV1 capsaicin receptor, neurofilament 200 and H<sub>1</sub> histamine receptor in the dorsal root ganglia of the mouse

Treatment	The number of positive neurones in an area of 151 × 151 μm <sup>2</sup> mean ± SEM			n
	TRPV1 receptor	H <sub>1</sub> histamine receptor	Neurofilament 200	
Control	8.3 ± 0.6	8.0 ± 0.5	9.6 ± 0.3	4
Capsaicin	0.3 ± 0.1*	6.9 ± 0.5	9.5 ± 0.4	4

\*Cells were counted in two cell-rich areas in each section, examples of which are shown in Fig. 5, and the average was used.

\**p* < 0.05 (Student's *t*-test).

SEM: standard error of the mean.

not exert evident effect on the number of neurofilament 200-immunoreactive neurones (Fig. 5F, Table I).

## DISCUSSION

Neonatal capsaicin treatment almost completely abolished scratching following antigen challenge. It markedly decreased the number of TRPV1-immunoreactive neurones in the DRG and the behavioural response to capsaicin stimulation, suggesting the marked degeneration of capsaicin-sensitive primary sensory neurones. Since it did not affect the number of DRG neurones positive for neurofilament 200, C-fibre neurones might be selectively degenerated. It might not affect allergy itself, because it did not suppress pruritogen production and the increase in the serum concentration of immunoglobulins and plasma extravasation. Taken together, these results suggest that TRPV1-positive primary afferents play a key role in signalling of cutaneous allergic itch.

Neonatal capsaicin treatment almost completely abolishes spontaneous scratching in mice with chronic dermatitis (17). Repeated topical application of capsaicin reduces itch of several pruritic diseases, including psoriasis (20, 21), prurigo nodularis (22), notalgia paraesthetica (23), aquagenic pruritus (24) and renal failure (25–27). Repeated application of capsaicin does not suppress histamine-induced itching in patients with atopic eczema, although it inhibits it in healthy subjects (14). These findings and the present results, taken together, suggest that capsaicin-sensitive primary afferents are involved in various types of itch, though not in all itch signals.

Neonatal capsaicin treatment did not produce a significant decrease in H<sub>1</sub> histamine receptor-positive DRG neurones. Since the same treatment almost totally abolished allergy-induced scratching, it is suggested that most H<sub>1</sub> histamine receptor-positive neurones are not involved in allergic itch. Though not significant, a 14% decrease in H<sub>1</sub> histamine receptor-positive neurones was observed. There were small neurones as well

as large ones in the DRG of the mouse treated with capsaicin as neonates, suggesting that there are small H<sub>1</sub> receptor-expressing neurones that are insensitive to capsaicin. In the guinea pig DRG, although H<sub>1</sub> histamine receptor mRNA-positive neurones are small in size, neonatal capsaicin treatment produced a 16% decrease in these neurones (28). The present results do not rule out the possibility that capsaicin-sensitive H<sub>1</sub> histamine receptor-expressing neurones are involved in itch of mosquito allergy. However, capsaicin-sensitive neurones are a small subpopulation of H<sub>1</sub> histamine receptor-expressing sensory neurones, which may be also a cause of resistance of allergic itch to H<sub>1</sub> histamine receptor antagonists.

An intradermal injection of the contents of the skin that had showed an allergic reaction elicited marked scratching in naive mice, suggesting the production of pruritogen(s) in the skin that had showed an allergic reaction. Pruritogen(s) may not be derived from mast cells and plasma, because scratching induced by mosquito allergy is not inhibited by deficiency in mast cells and suppression of plasma extravasation by H<sub>1</sub> histamine receptor antagonist. The scratching is inhibited by the 5-lipoxygenase inhibitor zileuton, the 5-lipoxygenase activating peptide inhibitor MK-886 and the glucocorticoid betamethasone, but not by the leukotriene B<sub>4</sub> antagonist ONO-4057, the cysteinyl leukotriene antagonist pranluceast, the leukotriene D<sub>4</sub> antagonist MK-571, the cyclo-oxygenase inhibitors indomethacin and ketoprofen, the TP prostanoid receptor antagonist SQ-29548, the platelet-activating factor antagonist CV-3988, and the nitric oxide synthase inhibitor N<sup>G</sup>-nitro-L-arginine methyl ester (8). It would therefore be interesting to determine 5-lipoxygenase metabolite(s) other than leukotriene B<sub>4</sub> and cysteinyl leukotrienes in contents of allergic oedema.

In summary, the present study shows an important role of capsaicin-sensitive sensory neurones in signalling of allergic itch. Since neonatal capsaicin treatment almost completely abolished allergy-induced scratching without a significant decrease in H<sub>1</sub> histamine receptor-expressing sensory neurones, it is suggested that capsaicin-sensitive sensory neurones that lack H<sub>1</sub> histamine receptors play a key role in signalling of allergic itch.

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