

# Distinct Reactivity of Transient Receptor Potential Vanilloid Subtype 1 in a Murine Model of Atopic Dermatitis with Serious Scratching

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**Abstract:** *Background:* Abnormality in skin sensitivity may be responsible for unbearable itch in patients with atopic dermatitis (AD).

*Objectives:* We evaluated reactivity of NC/Tnd mice, a model for human AD, against various experimental stimulations.

*Methods:* Several behavioral tests were performed after external stimuli were applied to NC/Tnd mice. Transient receptor potential vanilloid subtype 1 (TRPV1) reactivity of neuronal cells collected from the dorsal root ganglions (DRG) was analyzed with a Ca<sup>++</sup> influx test. Finally, we evaluated suppressive effect of capsaicin on atopic itch of NC/Tnd mice.

*Results:* Pain responses to heat, acidic stimulation, and capsaicin injection, which are transduced through TRPV1, were decreased in NC/Tnd mice, when compared to two standard strains BALB/c and C57BL/6 mice. The reactivity of the primary neurons isolated from DRG to capsaicin was markedly reduced in NC/Tnd mice. Topical application of histamine evoked scratching in NC/Tnd mice as well as other two strains; however, the scratching intensities induced by non-histamine pruritogens were significantly lower in NC/Tnd mice comparing to the two strains. In conventional NC/Tnd mice with AD, topical application of capsaicin reduced the scratching behavior.

*Conclusion:* TRPV1 is associated with both pain and itch sensation; however, abnormalities in TRPV1 reactivity may involve in severe itch in NC/Tnd mice.

**Keywords:** Atopic dermatitis, itch, NC/Tnd mice, TRPV1.

## INTRODUCTION

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disorder [1]. One of the serious clinical symptoms is itch, which is difficult to control. The investigation of itch mechanisms has emerged as an important topic of current research. Despite its great clinical significance, little is known about itch, especially in AD. Itch can be induced by a variety of chemical stimuli generated within or applied to the skin, which produce an itch-scratch reflex [2]. Itch, which is readily evoked in the skin, is innervated by primary afferent neurons. Primary afferents respond directly to itch-producing stimuli or they are activated indirectly by itch-producing compounds released from keratinocytes, mast cells, and Langerhans cells [3]. Recurrent itch is the main symptom of AD accompanying eczematous skin lesions. The itch-scratch cycle is one of the characteristics of AD, and it contributes to the development and exacerbation of skin

lesions [4]. Thus, it is essential to control unbearable itch for improvement of AD patients' quality of life.

NC/Tnd mice develop spontaneous itchy dermatitis in the air-unfiltered (conventional) condition, and this spontaneous itchy dermatitis is similar to human AD [5]. Therefore, NC/Tnd mice are widely used for the study of itch to evaluate the effectiveness of new anti-pruritic drug candidates. In the affected skin of NC/Tnd mice as well as human subjects [6], hyperproduction of nerve growth factor (NGF) in proliferating keratinocytes has been demonstrated [7]. On the other hand, decreased production of semaphorin 3A, which inhibits NGF-induced sprouting of sensory neurons, was recently reported in the skin lesions of patients with AD and conventional NC/Nga mice [8, 9]. More recently, we have demonstrated that thymic stromal lymphopoietin (TSLP) released from keratinocytes of the skin lesions contributes to the early stage of AD in NC/Tnd mice, and peroxisome proliferator activated receptor gamma activation downregulates the onset of AD *via* inhibition of dendritic cell functions activated by TSLP [10]. However, skin sensitivity in NC/Tnd mice has been unclear.

Transient receptor potential vanilloid subtype 1 (TRPV1) is a member of the transient receptor potential

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family of cation channels that are highly expressed in primary afferent sensory neurons and dorsal root ganglion (DRG) neurons [11]. TRPV1 is a voltage-dependent cation channel, which can be activated at physiological membrane potentials by stimuli, including noxious heat ( $> 42^{\circ}\text{C}$ ), capsaicin, hydrogen ions, and endogenous vanilloids [12]. TRPV1 has been widely studied in inflammatory pain, and has been implicated the role in itch induction by histamine, cytokines, and other pruritogens [13-15]. Particularly, TRPV1 has been identified as one of co-activators that are responsible for itch sensation in IL-31-mediated itch [15].

Mechanisms of itch sensation are still complicated; however, recent findings indicate that some neuronal signals may exert an influence on both itch and pain sensation. A TRPV1 antagonist has been reported to display potential as a therapeutic agent for itch in both pruritogen-induced allergic dermatitis models and spontaneous NC/Nga mice [16, 17]. On the other hand, a TRPV1 agonist, capsaicin, has been reported to exert a suppressive effect on histamine-, substance P-, and Protease activated receptor (PAR-2) agonist-induced itch responses [18]. Currently, Roberson, DP., *et al.* [8] found that the fibers that mediate histamine and non-histamine itch are functionally separated, and activation of these itch-generating fibers was not required for eliciting responses to acute mechanical and thermal pain. Furthermore, they revealed that silencing of TRPV1 with transient receptor potential channel A1 (TRPA1) showed different effects on histamine and non-histamine evoking itch depending on their activity, suggesting that certain peripheral afferent neurons might normally indirectly inhibit algogens from eliciting itch [19]. TRP channels normally act as nociceptors in physiological condition; however, co-activation of TRPV1 and histamine receptor 1 produce histamine evoking itch, whereas non-histamine itch is induced by co-activation of TRPA1 with Mas-related G protein-coupled receptors (Mrgpr) possibly in the diseased skin [20, 21]. In the present study, we attempted to clarify sensitivity to various experimental stimuli and found the abnormality in TRPV1 reactivity in NC/Tnd mice.

## MATERIALS AND METHODOLOGY

### Mice

Six to eight-week-old specific pathogen-free (SPF) NC/Tnd mice without AD, and 8 to 10-week-old conventional NC/Tnd mice with AD were maintained as previously described and used for the study [22]. BALB/c mice and C57BL/6 mice were used as controls. In all animal experiments, the same numbers of male and female mice were used. All experiments with animals complied with the standards specified in the guidelines of the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

### Behavioral Tests

A Hot/Cold plate (Ugo Basile, Varese, Italy) was used to evaluate the reactivity to thermal pain [23]. Mice were habituated to the experimental cage for 30 min. Mice were placed on a hot plate ( $52 \pm 0.5^{\circ}\text{C}$ ), and the time until the mouse jumped or licked either of its hind paws was recorded

as hot plate latency. Following a response, the mouse was immediately removed from the plate. Each test was repeated three times with a 15-min interval between tests. Latencies from the three tests were averaged.

The Von Frey test was used to measure reactivity to mechanical pain. Mice were placed in a clear plexiglass compartment with a mesh floor and were allowed to habituate for 30 min. Mechanical pain was evaluated with a Dynamic Planter Aesthesiometer (Ugo Basile) in the ascending order of force (0–10 g) to the plantar surface of the hind paw [23, 24].

To evaluate acute and chronic inflammatory pain, formalin or capsaicin (Sigma-Aldrich) was injected as described [25-27]. Briefly, mice were acclimated for 30 min in a transparent plexiglass box. A diluted solution of formalin (2.5% formalin in saline) or capsaicin (dissolved in 100% DMSO followed by dilution with 0.9% saline to a concentration of  $0.08 \mu\text{g}/\mu\text{L}$ ) was injected into the plantar surface of the hind paw. The frequency of licking the hind paw was automatically counted using a SCLABA<sup>®</sup> system (Noveltec, Inc, Kobe, Japan). The frequency of licking behavior in 0-5 min after injection was calculated as the acute phase response, and those in 6-60 min after the injection were estimated as the inflammatory phase response [28-30].

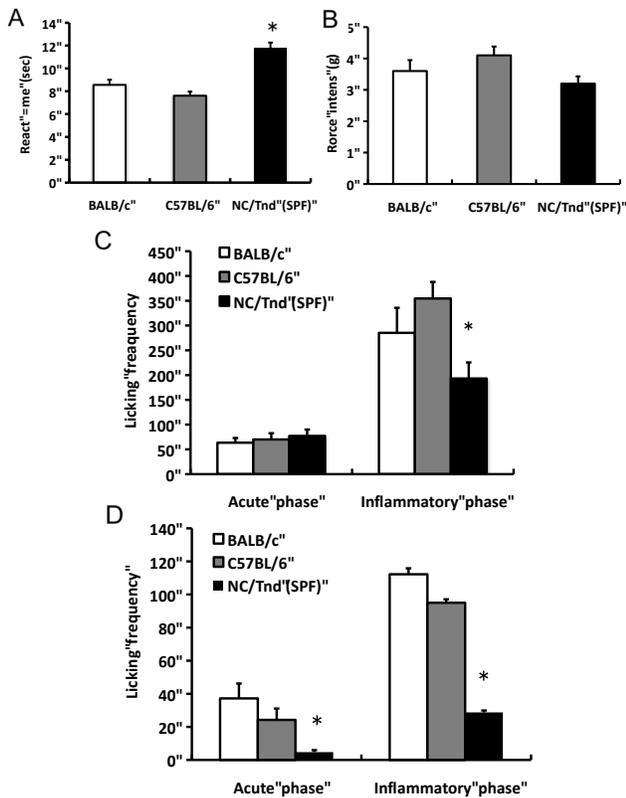
The itch behavior assay was performed using pruritogens. Pruritogens (histamine, serotonin, chloroquine (CQ), and SLIGRL-NH<sub>2</sub>; purchased from Sigma-Aldrich, Tokyo, Japan) were dissolved in 30  $\mu\text{L}$  of saline and applied to the surface of the nape of the neck. The scratching frequency and duration were counted automatically for 1 h using a SCLABA<sup>®</sup>-Real system (Noveltec, Inc) [31]. Before application of pruritogens, the skin barrier of each mouse was broken by acetone/ether (A/E) water treatment [32]. All mice used in the behavioral experiments were maintained in a SPF room until use.

### DRG Neuron Cell Culture

DRGs were dissected from all spinal levels of mice as described [33]. These were collected in cold DH10 medium, minced, and digested in enzyme solution containing 0.2% type III collagenase (Worthington Biochemical Corp., Lakewood, NJ) and 0.25% trypsin (Sigma-Aldrich) at  $37^{\circ}\text{C}$ . The suspensions were homogenized repeatedly with a Pasteur pipette (Fisher Scientific, Pittsburgh, Pa). Neuronal cells were isolated by using 30% Percoll density gradient centrifugation. The cell suspensions were filtered through a cell-strainer (40  $\mu\text{m}$ , BD Biosciences, Bedford, MA) and centrifuged at 250 g for 10 min. After washed twice, cells were resuspended in DH10, plated on glass coverslips coated with poly-D-lysine, cultured in an incubator at  $37^{\circ}\text{C}$ , and used within 24 h. All mice used in DRG isolation were maintained in a SPF room until use.

### Ca<sup>2+</sup> Imaging

Cells were loaded with fura-2-acetoxymethylester for 45 min at  $37^{\circ}\text{C}$ . Cells were washed and imaged at excitation levels of 340 and 380 nm to detect intracellular free Ca<sup>2+</sup> levels in a fluorescence spectrophotometer F2500 (Hitachi, Ltd., Tokyo, Japan) [34]. Neurons were tested for responses



**Fig. (1).** Reactivity to 4 individual pain stimuli. A hot plate test (A) indicated dull response to thermal pain of SPF NC/Tnd mice without AD. On the other hand, no significant difference was identified in each strain in a von Frey test (B). A formalin test (C), and a capsaicin test (D) showed low sensitivity to inflammatory pain of SPF NC/Tnd mice without AD compared with BALB/c mice and C57BL/6 mice. Each column represents mean  $\pm$  SE of 6 mice in each group. All mice used in the experiment were kept in a SPF room until use. \* $P < 0.05$ , when compared to BALB/c mice and C57BL/6 mice.

to capsaicin in at least four independent experiments, with a minimum of 100 cells analyzed each time.

### Capsaicin Treatment

For topical application, hydrophilic ointment with 0.025% capsaicin (100 mg/site) was applied onto the hair-clipped dorsal skin once a day for 7 consecutive days [35]. A day before the start of application, and on day 3 and day 7, the scratching behavior of conventional NC/Tnd mice was analyzed using a SCLABA<sup>®</sup>-Real (Noveltec, Inc.) according to the manufacturer's instructions [10]. For subcutaneous injection, mice were anesthetized with isoflurane and capsaicin (15 mg/kg), which was subcutaneously injected into the nape of the neck in a volume of 20  $\mu$ L using a 100  $\mu$ L Hamilton syringe (Reno, Nev, USA) with a 30-gauge needle [36]. After 24 h, the scratching behavior of the treated mice was analyzed with a SCLABA<sup>®</sup>-Real, as described.

### Trans Epidermal Water Loss (TEWL) Measurement

Stratum corneum (SC) hydration is important for its cosmetic properties and barrier function. TEWL is one of

key indexes used for SC characterization, which reflects barrier function of the skin. In patients with AD, TEWL is increased as the disease advances [37, 38]. TEWL of each mouse was measured before and after A/E water treatment by using Multi probe adopter (CK electronic GmbH, Germany). At the time of measurement, mice were adopted to the laboratory where temperature and humidity were set at 23°C and 40%, respectively, for 30 minutes prior to the measurement. Measurement for each mouse was performed 3 times and the average of 3 values was estimated as the data.

### Data Analysis

Data are expressed as mean  $\pm$  SE. Statistical comparisons were made using a Student's *t* test or a Fisher's multiple comparison. A statistically significant difference was defined as  $P < 0.05$ .

## RESULTS

### NC/Tnd Mice have Low Sensitivity to Thermal and Inflammatory Pain

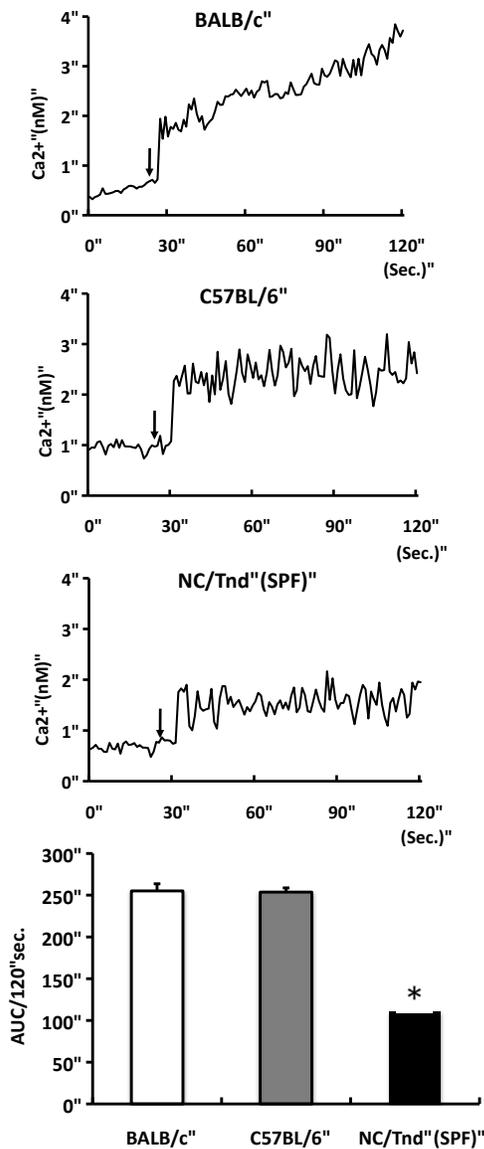
The altered reactivity of sensory neurons to external and internal stimuli in the skin of AD patients has been reported previously [39]. To investigate the reactivity to experimental stimulations in SPF NC/Tnd mice, we performed a hot plate test (thermal pain), a Von Frey's test (mechanical pain), and a formalin and capsaicin test (chemical pain). Reaction time of SPF NC/Tnd mice to thermal stimulation was significantly delayed when compared to control mice (Fig. 1A), though there was no statistical difference in the reactivity to mechanical stimulation among the 3 groups (Fig. 1B). After injection of formalin, the licking behavior of SPF NC/Tnd mice in the inflammatory phase was less than that of BALB/c mice and B6 mice (Fig. 1C). Especially with the capsaicin test, not only in the inflammatory phase but also in the acute phase, the reactivity of SPF NC/Tnd mice was significantly reduced (Fig. 1D).

### Reactivity of DRG to Capsaicin Stimulation

TRPV1 is strongly involved in the development of thermal pain [40, 41]. According to the behavioral results, we speculated that the reactivity of TRPV1 in NC/Tnd mice might differ from other mice. Using  $Ca^{2+}$  imaging, we found that the  $Ca^{2+}$  influx of cultured DRG neurons after the addition of capsaicin, a TRPV1 stimulus [42], was significantly lower in SPF NC/Tnd mice than in BALB/c mice and B6 mice (Fig. 2).

### Effects of Topical Capsaicin on Atopic Itch

Capsaicin has been shown to exert antipruritic effects by depleting and preventing reaccumulation of substance P [40, 43]. External application of capsaicin also exerted inhibitory effects on histamine-induced scratching [34]. Therefore, to investigate whether or not capsaicin affected atopic itch, we used NC/Tnd mice maintained in a conventional environment, in which atopic dermatitis was developed. With topical application of capsaicin ointment, the scratching behavior of

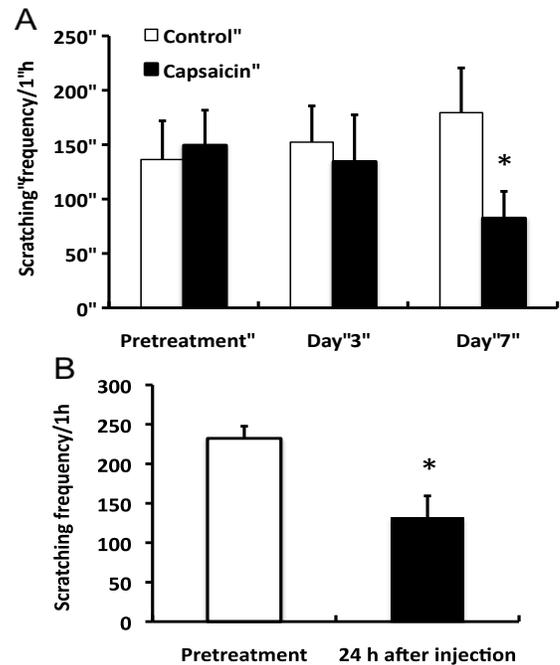


**Fig. (2).** Ca<sup>2+</sup> influx of DRG neuronal cells after capsaicin stimulation. DRG was isolated from each strain of mouse kept in SPF condition. The vertical axis represents the area under curve after the addition of capsaicin into the culture of cells isolated from DRG of each mouse strain. Each column represents mean  $\pm$  SE of 4 mice in each group. \*P < 0.05, when compared to BALB/c mice and C57BL/6 mice.

conventional NC/Tnd mice with established AD was gradually decreased during the experiment (Fig. 3A). At the end of the experiment (day 7), the scratching of NC/Tnd mice was significantly reduced when compared to that of control mice (Fig. 3A). When capsaicin was subcutaneously injected into the dorsal skin, both scratching frequency and total scratching duration were significantly decreased in NC/Tnd mice after 24 h (Fig. 3B).

#### NC/Tnd Mice have Low Sensitivity to Pruritogens

To define the sensitivity of NC/Tnd mice to itch sensation, we used 4 different pruritogens, which are generally

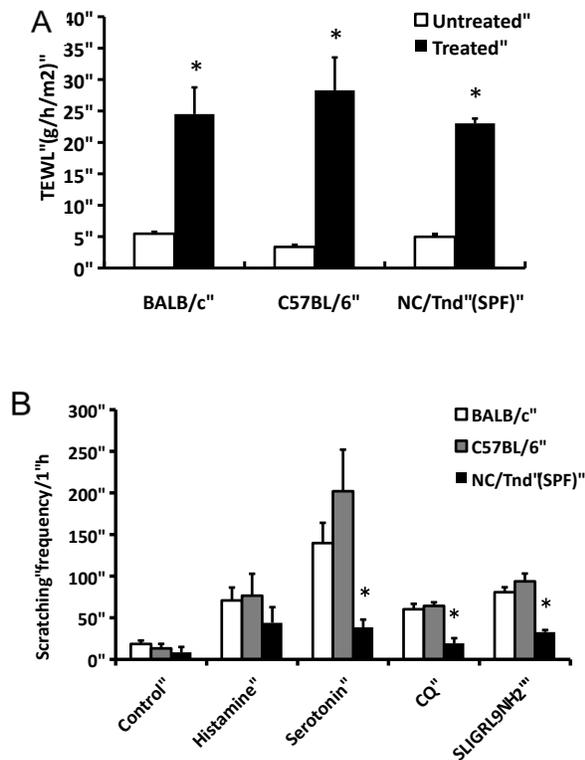


**Fig. (3).** Effect of capsaicin treatment on scratching behavior of conventional NC/Tnd mice with AD. (A) Topical application of 0.025% capsaicin hydrophobic ointment. Each column represents mean  $\pm$  SE of 6 mice in each group. \*P < 0.05, when compared to mice applied with ointment base alone (Control). (B) Subcutaneous injection of capsaicin (15 mg/kg). Each column represents mean  $\pm$  SE of 4 mice in each group. \*P < 0.05, when compared to pretreatment.

used for the induction of itch. Histamine is a well-known itch inducer [44], and serotonin-induced itch is dependent on phospholipase C $\beta$ 3 [13]. The antimalarial drug CQ is transduced in a histamine-independent manner by the G Protein-coupled receptor (GPCR) MrgprA3 [45], and SLIGRL-NH2 is a protease-activated receptor (PAR) 2 agonist [46]. First, we checked barrier disruption after A/E water treatment on SPF NC/Tnd mice without AD, BALB/c mice, and B6 mice by measuring TEWL. As indicated in (Fig. 4A), TEWL was markedly elevated at the affected skin site after A/E water treatment in 3 strains of mice used in this study. After disrupting the skin barrier by A/E water treatment, histamine (10  $\mu$ mol/mL), serotonin (10 nmol/ $\mu$ L), CQ (25 mg/mL), and SLIGRL-NH2 (1 mg/mL) were applied to the dorsal skin. Pruritogens significantly increased the scratching behavior in BALB/c mice and B6 mice (Figs. 4B and 4C). However, the scratching frequency and the total scratching duration of SPF NC/Tnd mice after pruritogen were lower than those of BALB/c mice and B6 mice (Figs. 4B and 4C).

#### DISCUSSION

It is reported that hyperesthesia or paresthesia of the skin may be one of the causes of serious itch in patients with AD [47, 48]. Similar to the itch sensation in human patients, scratching behavior is dramatically increased in NC/Tnd mice with advancement of AD [6]. According to the results from the pain sensation tests, NC/Tnd mice showed low sen-



**Fig. (4).** Pruritogen application onto the A/E water treated dorsal skin. **(A)** TEWL before and after A/E water treatment. Each column represents mean  $\pm$  SE of 6 mice in each group. \* $P < 0.05$ , when compared to untreated. **(B)** Scratching frequency and total scratching duration (sec) of SPF NC/Tnd mice without AD after topical application of 4 different pruritogens. Each column represents mean  $\pm$  SE of 6 mice in each group. All mice used in the experiment were kept in a SPF room until use. \* $P < 0.05$ , when compared to BALB/c mice or C57BL/6 mice.

sensitivity to thermal pain and chemical pain. These results allowed us to speculate that the long-lasting scratching behavior might be caused by the dull pain sensation in NC/Tnd mice. Since thermal and chemical pain is transmitted at least in part, through activation of TRPV1 [40, 41], we checked the reactivity of DRG neuronal cells isolated from NC/Tnd mice against TRPV1 stimulation. Cells obtained from DRGs of NC/Tnd mice showed markedly low sensitivity to capsaicin treatment, suggesting that abnormal reactivity of NC/Tnd mice to thermal and inflammatory pain may result from the impaired TRPV1 response. In previous studies, subcutaneous injection of capsaicin into newborn pups of mice and rats inactivated TRPV1, and led to long-lasting cutaneous lesions [49-51]. Therefore, we attempted to stimulate TRPV1 in NC/Tnd mice with established dermatitis by using topical application and subcutaneous injection of capsaicin. Topical application of low dose capsaicin has been implicated to reduce itch sensation induced by pruritogens [35]. Subcutaneous injection of low dose capsaicin has been reported to induce TRPV1 activation after 24 h [36]. In this study, repeated topical application of capsaicin reduced itch behavior in NC/Tnd mice with AD. Since repeated application of capsaicin desensitizes the TRPV1 receptors, reduc-

tion in reactivity after transient activation of TRPV1 may be associated with the relief of scratching behavior in NC/Tnd mice with AD. Topical capsaicin has been reported to relieve pruritus in AD patients to whom anti-histamine was not effective [51, 52]. Moreover, since neonatal injection of capsaicin depletes sensory neurons resulting in widespread neurodegeneration, a careful strategy must be plotted on TRPV1 targeting in AD. The scratch reactivity to various pruritogens was much lower in NC/Tnd mice than that of BALB/c and B6 mice, neglecting hypersensitivity to pruritogens in NC/Tnd mice. Recent studies have revealed that TRP channels may play a critical role in itch induction [13, 15, 20]. Moreover, interaction and activity balance between TRPV1 and TRPA1 may complicatedly regulate itch and pain [19]. Since involvement of TRP channels in itch sensation and development of skin regions in NC/Tnd mice has been still unclear, alterations in reactivity of these channels may play a certain role in pathogenesis of AD.

## CONCLUSION

Further investigation must take place to identify each involvement of TRPV1 and TRPA1 in atopic itch; however, our results indicated that TRPV1 in the skin might be one of important sensors on itch sensation, and NC/Tnd mice would be a precise animal model for a study of TRP channels in the skin.

## ABBREVIATIONS

AD	=	Atopic dermatitis
A/E water	=	Acetone/ether water
CQ	=	Chloroquine
SC	=	Stratum corneum
TRPA1	=	Transient receptor potential channel A1
Mrgpr	=	Mas-related G protein-coupled receptor
GpcR	=	G protein-coupled receptor
PAR	=	Protein activated receptor
DRG	=	Dorsal root ganglion
NGF	=	Nerve growth factor; RT, room temperature
SPF	=	Specific pathogen-free
TEWL	=	Trans-epidermal water loss
TRPV1	=	Transient receptor potential vanilloid 1
TSLP	=	Thymic stromal lymphopoietin

## CONFLICT OF INTEREST

The author confirms that this article content has no conflicts of interest.

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