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Chronically relapsing pruritic dermatitis in the rats treated as neonate with capsaicin; a potential rat model of human atopic dermatitis

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ABSTRACT

Background: The pathophysiological mechanisms underlying chronic pruritic skin diseases, e.g. atopic dermatitis (AD), and effective therapies remain elusive due to the paucity of animal models. Recently, we rediscovered that injection of capsaicin into rat pups resulted in vigorous scratching behavior and chronically relapsing AD-like cutaneous lesions well into adulthood.

Objectives: To characterize the chronic pruritic dermatitis induced by neonatal capsaicin treatment.

Methods: Capsaicin (50 mg/kg) was given to rat pups subcutaneously within 48 h after birth, and then scratching behavior, dermatitis and pathophysiological changes of rat skin were investigated chronologically.

Results: Neonatal capsaicin treatment led to not only severe scratching and cutaneous lesions but also a large number of pathophysiological changes in the skin, such as histopathological changes including the deficiency of epidermal filaggrin expression, increases in the number of mast cells, levels of tissue NGF and Th2 cytokine mRNA, impaired skin barrier function and colonization with *S. aureus*. In addition, we observed the hyperproduction of serum IgE, which is clinically similar to the pathophysiology seen in the patients with atopic dermatitis. During the follow-up observation, the rats showed the alternative periods of relapsing and remitting skin lesions.

Conclusion: Injection of capsaicin into rat pups results in chronically relapsing pruritic dermatitis, similar to human AD. Therefore, we think neonatal capsaicin treatment could be a useful model for studying human AD and for the development of novel therapeutic drugs.

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1. Introduction

Capsaicin is the main ingredient of hot chili peppers responsible for their spicy flavor and pungent taste. When subcutaneous injection into newborn rat pups, capsaicin acts as a neurotoxin inducing a highly specific destruction of a subset of small-diameter primary afferents of the rats [1]. For this reason, neonatal capsaicin treatment has been long considered as a useful tool in investigating the functions of nociceptive afferent fibers.

In our recent research into the effect of neonatal capsaicin treatment, we consistently found that all treated animals had long-lasting, occasionally relapsing, severe cutaneous lesions on their head, neck and back, and that lesions were closely related to vigorous scratching—a behavioral correlated of pruritus. A careful search of the literature revealed that this effect already has been

recognized by some investigators [2–4]. It has been speculated that dysfunction in the nervous system, due to the ablation of capsaicin-sensitive nerve fibers, might be related to the induction of pruritus and scratches, leading to the transient cutaneous lesions [3,4]. However, it is interesting that even in rats wearing a plastic collar to protect themselves from scratching-induced damage, cutaneous lesions were still readily detectable [4], indicating that the scratch itself may not be critical in the manifestation of cutaneous lesion in these rat. Considering recent evidence that the pathogenesis of chronic pruritus involves various resident cells of the skin [5], it is possible that chronic pruritic cutaneous lesion seen in the rats treated with capsaicin is not a simple neurologic disorder but rather a more complicated skin disease associated with dermatological as well as neurological problems. Chronic pruritus is a common symptom of many cutaneous and extracutaneous diseases [6], and when associated with a pathological skin condition, such as atopic dermatitis (AD), leads to itch-scratch cycles that injure the skin and exacerbate the problem [6]. Furthermore, it is often resistant to current therapeutic treatments. Although our understanding about chronic pruritus or dermatitis has improved significantly in recent years, its pathogenesis remains still unclear and controversial. Thus, we

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speculate that multidisciplinary approaches on the pruritic cutaneous lesion induced in the rat by neonatal capsaicin treatment may provide us with more information about refractory and idiopathic chronic pruritus or dermatitis.

In the present study, we first investigated the chronological characteristics of scratching behaviors and dermatitis followed by the pathophysiological changes in the skin of rats treated with capsaicin as neonate. We report that pruritic cutaneous lesioning in these animals is similar to human atopic dermatitis.

2. Materials and methods

2.1. Animals

All experiments were approved by the Korea University College of Medicine Animal Research Policies Committee (KUIACUC-2009-134). Pregnant Sprague-Dawley rats were obtained 1-week before parturition and allowed to deliver. Pups in each litter were randomly assigned to the experimental groups and weaned at postnatal day 21, at which time they were separated sexually. Only the male was used in the present study. All animals were raised in a room maintained at a 12 h light/dark cycle (light on at 07:00 h) and at 22–25 °C, with free access to food and water.

2.2. Neonatal treatment of capsaicin

As described previously [7], the newborn rat pups within 48 h of birth were subcutaneously administrated with capsaicin (50 mg/kg, Sigma) or vehicle (saline containing 10% Tween 80 and 10% ethanol) of a volume of 10 μ l/g body weight.

2.3. Scratching behavior

Rats were placed into separate plastic chambers (20 cm $\times$ 30 cm $\times$ 20 cm) equipped with a mirror placed behind the chamber to allow an unobstructed view for a 30 min habituation period. Following habituation, rat behaviors were recorded in an isolation room using a digital video camera (DCR-SR300, Sony, Japan) for an hour. The video files were played back on a computer and the number of scratches was counted by experimenters who were not aware of which rats had been treated with capsaicin. A bout of consecutive scratching strokes by hind paw was regarded as one scratch.

To determine the ability of preweaning rats to scratch, serotonin hydrochloride (10 mg/ml, 50 μ l, Sigma, MO, USA), dissolved in sterile saline, was intradermally injected into the interscapular area of the back skin using 31G insulin syringes. Immediately after the injection, scratching behaviors were recorded for 30 min.

2.4. Evaluation of cutaneous lesion

Cutaneous lesions were carefully inspected and assessed by scoring as described previously [8]. The skin condition in the three regions (the face, ears and back) was subjected to score, respectively, as follows;

Region	Score	Skin condition
Face	0	Normal
	1	Wispy fur
	2	Alopecia and flare
	3	Bleeding or ulcerative lesion
Ears	0	Normal
	1	Flare
	2	Bleeding
	3	Loss of part of the ear

(Continued)

Region	Score	Skin condition
Back	0	Normal
	1	Wispy fur
	2	Alopecia and flare
	3	Bleeding or ulcerative lesion

The sum of scores in the three regions was adopted as the dermatitis score for each rat.

2.5. Histology and immunohistochemistry

Skin tissue was sectioned at 12 μ m on cryostat and stained with hematoxylin and eosin (H&E) and toluidine blue (TB) for histological examination. Mast cells in a microscopic field at 400 $\times$ magnification were counted. For immunohistochemistry, sections were blocked with 1% BSA and 10% NGS in 0.05 M PBS for 1 h at RT and then reacted with rabbit anti-filaggrin (1:1000, #bs-1462R, Bioss Inc., MA, USA) or rabbit anti-PGP9.5 (protein gene product 9.5) (1:1000, #AB9724, Chemicon, CA, USA) at 4 °C two overnights. ABC staining system was used to visualize filaggrin or PGP9.5 in the sections (Vector Laboratories, CA, USA).

2.6. Transepidermal water loss (TEWL) and skin hydration

In a temperature (22–24 °C)- and humidity (40–50%)-controlled room, animals were slightly anesthetized by inhalation of isoflurane (0.5–2%) and TEWL was then measured at rostral back skin using Tewameter TM 210 (Courage+Khazaka GmbH, Cologne, Germany). Skin hydration was evaluated by measuring electrical capacitance with a Corneometer CM825 (Courage+Khazaka Electronic GmbH, Cologne, Germany). Measurement was repeated 3 times to give rise to an average value for each rat.

2.7. *Staphylococcus aureus* (*S. aureus*) colonization on the skin

Skin samples (each 2 mm in diameter) were obtained by punching biopsy. Bacterial cultures were grown using the standard laboratory techniques. The number of colonies was counted and expressed as colony-forming units (CFU) per cm². Bacterial identification was checked using an automated identification system, Vitek2 (BioMerieux, NC, USA). Methicillin resistance was monitored by Clinical and Laboratory Standards Institute (CLSI) antimicrobial susceptibility method. Briefly, after inoculation of testing isolate, a cefoxitin disk (30 μ g) was placed on Muller–Hinton agar and an inhibition zone diameter of $\leq$ 21 mm was considered as methicillin resistant.

2.8. Reverse transcription-PCR

Total RNA was extracted from skin tissues, purified with RNeasy[®] Fibrous Tissue mini kit (Qiagen[®], Texas, USA) and used for the synthesis of cDNA with the Reverse Transcription system (Promega, Wisconsin, USA). The cDNA of 1 μ g/ μ l was used for PCR amplification. Primer pairs for rat cytokines were designed by Primer 2 v0.6 (Bioneer, Daejeon, Korea) based on published sequence in GeneBank. Primer pairs are listed in [Supplementary Table S1](#). The amount of PCR product of each sample was normalized to the amount of GAPDH.

2.9. Enzyme linked immune sorbent assay (ELISA)

NGF in the skin samples and Serum IgE were quantified using NGF sandwich ELISA kit, ChemiKine[™] (EMD Millipore, MA, USA) and a rat IgE ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, USA), respectively, according to the manufacturer's protocols.

2.10. Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical significances were analyzed using Student's *t*-test or the Mann–Whitney rank sum test, depending on normality test. A difference of $P < 0.05$ was considered to be statistically significant. All statistical analyses were carried out using Sigma Stat (ver. 3.5, Systat Software Inc., IL, USA).

3. Results

3.1. Long-lasting, occasionally relapsing, pruritic dermatitis following neonatal capsaicin treatment

Although vehicle-treated control rats showed sporadic scratching behaviors, no cutaneous lesions were observed over the entire experimental period. In contrast, all adolescent rats treated with capsaicin as neonates began to show quite frequent scratching behaviors from PW3 (Fig. 1A). The number of scratches remained at a constantly low level for the first 3-weeks; however, from PW6 the number of scratches increased about two-fold. This increased activity was maintained for 3-weeks and then progressively declined by 12 postnatal weeks. Scratching was mainly targeted on the face, ears, neck and rostral back regions including the shoulders and upper forelimbs (Supplementary video 1).

Cutaneous lesions in capsaicin (Cap)-treated rats first appeared one week after the onset of spontaneous scratching, peaked on PW5–7 and then gradually healed over time (Fig. 1B). Cutaneous lesions were characterized by representative dermatitis such as alopecia, dryness, superficial erosion, deep excoriation, hemorrhage and scars. From about PW12, most lesions were observed as scars or alopecia. Despite the clear remission of symptoms at that time, the

dermatitis score of the Cap-treated rats remained high up to PW15. Cutaneous lesions were detected primarily around the head, neck, upper forelimbs and rostral back regions, which are accessible to scratching with the hind paws (Fig. 1D). Monthly observations of five Cap-treated rats were carried out for one year. Interestingly, new cutaneous lesions characterized by superficial erosion or deep excoriation with bleeding were occasionally observed in the rats during this time (Fig. 1E), indicating that neonatal capsaicin treatment leads to chronically relapsing cutaneous lesions.

Based on these observations, and to clarify the time course of behavioral and dermatological changes after neonatal capsaicin treatment, we will refer to Cap-treated rats up to 3-weeks old as 'non-lesional', and to rats from 4-weeks old with evidence of severe dermatitis as 'lesional'.

3.2. Serotonin-induced scratching

To determine whether non-lesional rat pups are able to scratch their body in response to pruritic stimuli, we assessed the scratching behaviors following the injection of serotonin (10 mg/ml, 50 μ l) into the rostral back of 2-week-old rats. As shown in Fig. 1C, serotonin elicited excessive scratching at the site of injection in both the Cap-treated and control rats, although the number of scratches was significantly lower in the Cap-treated. This result indicates that even young rats under the age of 3-weeks have an ability to scratch in response to pruritic stimuli.

3.3. Histopathological features of back skin

Histological examination was carried out on both the lesional skin of the 6-week-old rats and pruritic, but non-lesional skin of

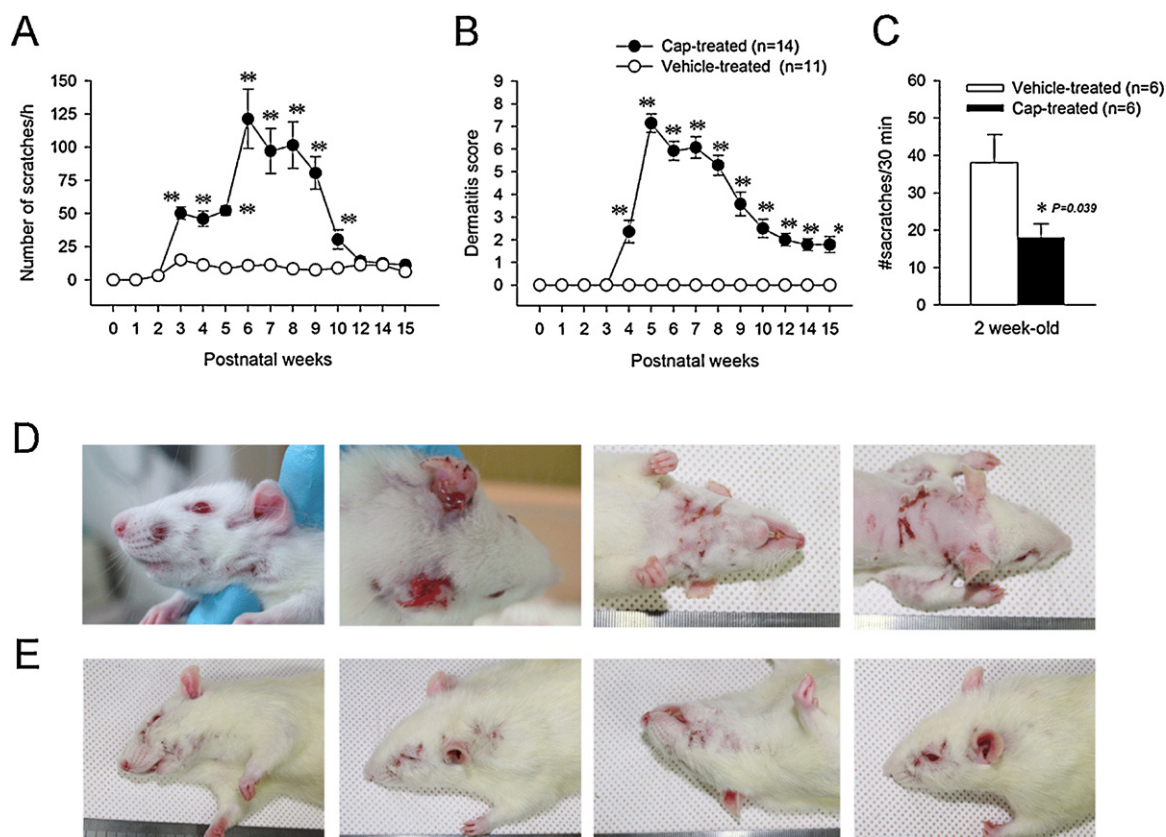


Fig. 1. Chronically relapsing pruritic cutaneous lesions in the rats treated with capsaicin as neonate. (A) and (B) The chronological characteristics of spontaneous scratching behaviors and skin conditions expressed as dermatitis score. (C) Scratching behaviors of the 2-week-old rat pups induced by an intradermal injection of serotonin (10 mg/ml, 50 μ l). (D) Cutaneous lesions first appeared in the rats treated with capsaicin as neonate at 6-weeks of age. (E) Relapsing cutaneous lesions characterized by new ulceration with bleeding in 20-week-old capsaicin-treated rats. * $P < 0.05$, ** $P < 0.001$ (*t*-test).

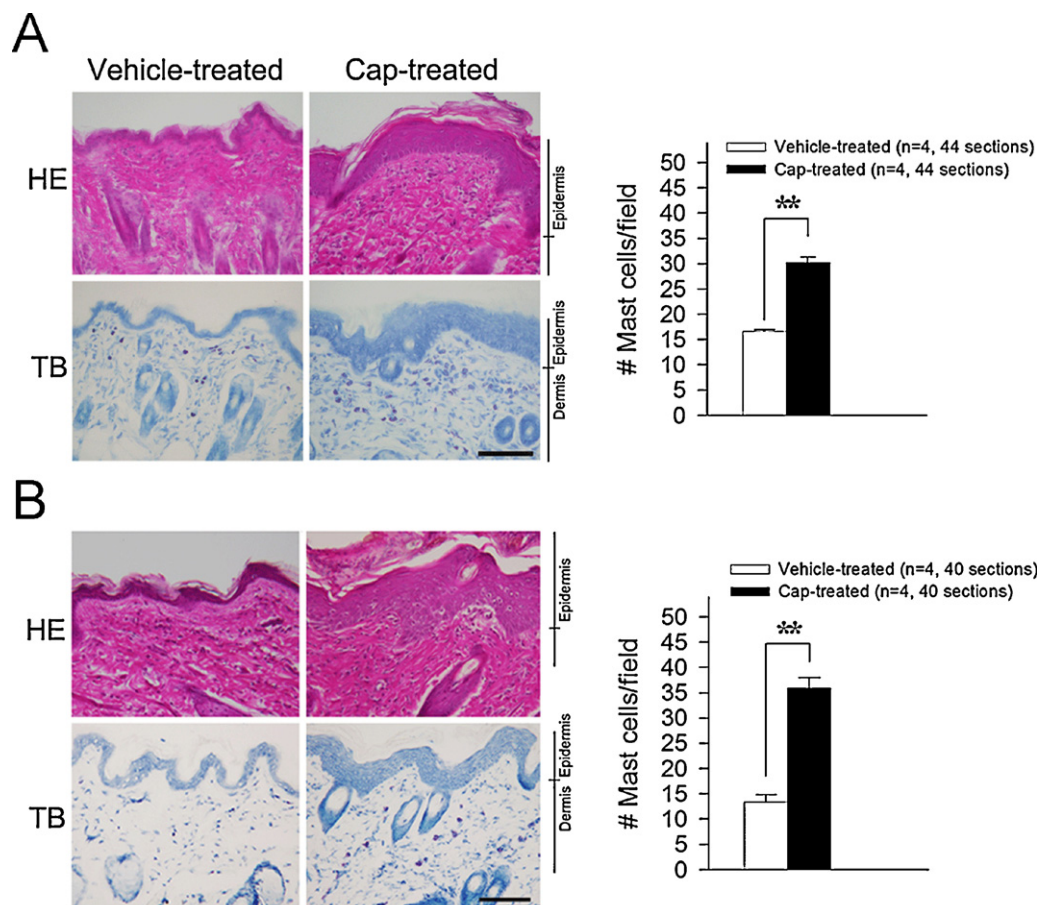


Fig. 2. Histological characteristics of rostral back skin in 3-week old (A) and 6-week old (B) rats treated with capsaicin as neonates. (A) Compared to control rats (Vehicle-treated), apparent histopathological changes such as hyperplasia and hyperkeratosis in the epidermis and increased number and hypertrophy of mast cells in the dermis were present in clinically non-lesional but pruritic skin regions of 3-week-old rats treated with capsaicin as neonate (Cap-treated). (B) Photographs showing the histopathological characteristics of lesional skin of the 6-week-old rats treated with capsaicin. Skin samples were stained with hematoxylin and eosin (HE) and toluidine blue (TB). Mast cells in a microscopic field at 400 $\times$ magnification were counted. $^{**}P < 0.001$ (t -test) (scale bar = 100 μ m).

3-week-old rats. Significant histopathological changes, such as epidermal hyperplasia, hyperkeratosis and increased number of infiltrating mast cells, were observed in the lesional skin of the 6-week-old rats (Fig. 2B). Interestingly, these changes of non-lesional 3-week-old Cap-treated rats were already presented in the pruritic skin regions (Fig. 2A). In addition, toluidine blue staining revealed a significantly increased number and hypertrophy of mast cells in the dermis of both the lesional and non-lesional skins (Fig. 2). The vehicle-treated control rats showed no similar changes histologically.

3.4. Serum IgE and skin NGF

As illustrated in Fig. 3A, the level of serum IgE in Cap-treated rats was remarkably higher than that of control rats at all experimental time points, except for the first postnatal week. Serum IgE levels were highest on PW3. Although no difference in serum NGF level was observed between the groups at any time point (data not shown), the tissue levels of NGF in back skin samples were found to be significantly higher in Cap-treated rats than in control animals at all the time points (Fig. 3B). Serum IgE and tissue NGF levels in the control rats remained at low levels.

3.5. Cytokine mRNA expression

Skin mRNA expression of IL-4 and IL-13 cytokines was analyzed on 1, 2, 3, 6, 8 and 10-weeks following neonatal

capsaicin treatment (Fig. 3C and D). Compared to the expression level in the control animals, IL-4 mRNA expression in Cap-treated rats was significantly increased at all experimental time points, regardless of the presence of cutaneous lesions (Fig. 3C), whereas the increase in IL-13 mRNA expression was limited to lesional skin (Fig. 3D).

3.6. Transepidermal water loss (TEWL) and hydration

TEWL (g/m²/h) measured at the lesional skin of the Cap-treated rats on 6 and 8 postnatal weeks was significantly increased compared with that of corresponding skin region of the control animals (Fig. 4A). Even on PW3 when no apparent outer cutaneous lesions were observed, TEWL was remarkably higher in Cap-treated rats than in controls (Fig. 4A), indicating that impairment of skin barrier function precedes dermatitis in these animals. No difference in TEWL between the two groups was observed by PW11. In line with TEWL changes, skin hydration was significantly lower in the Cap-treated rats than control animals (Fig. 4B).

3.7. Filaggrin-immunohistochemistry

Filaggrin is a key structure protein of the epidermis in skin barrier function. Recently, a vast body of accumulating evidence indicates that decreased expression of filaggrin in the skin predisposes patients to be susceptible for developing AD [9,10].

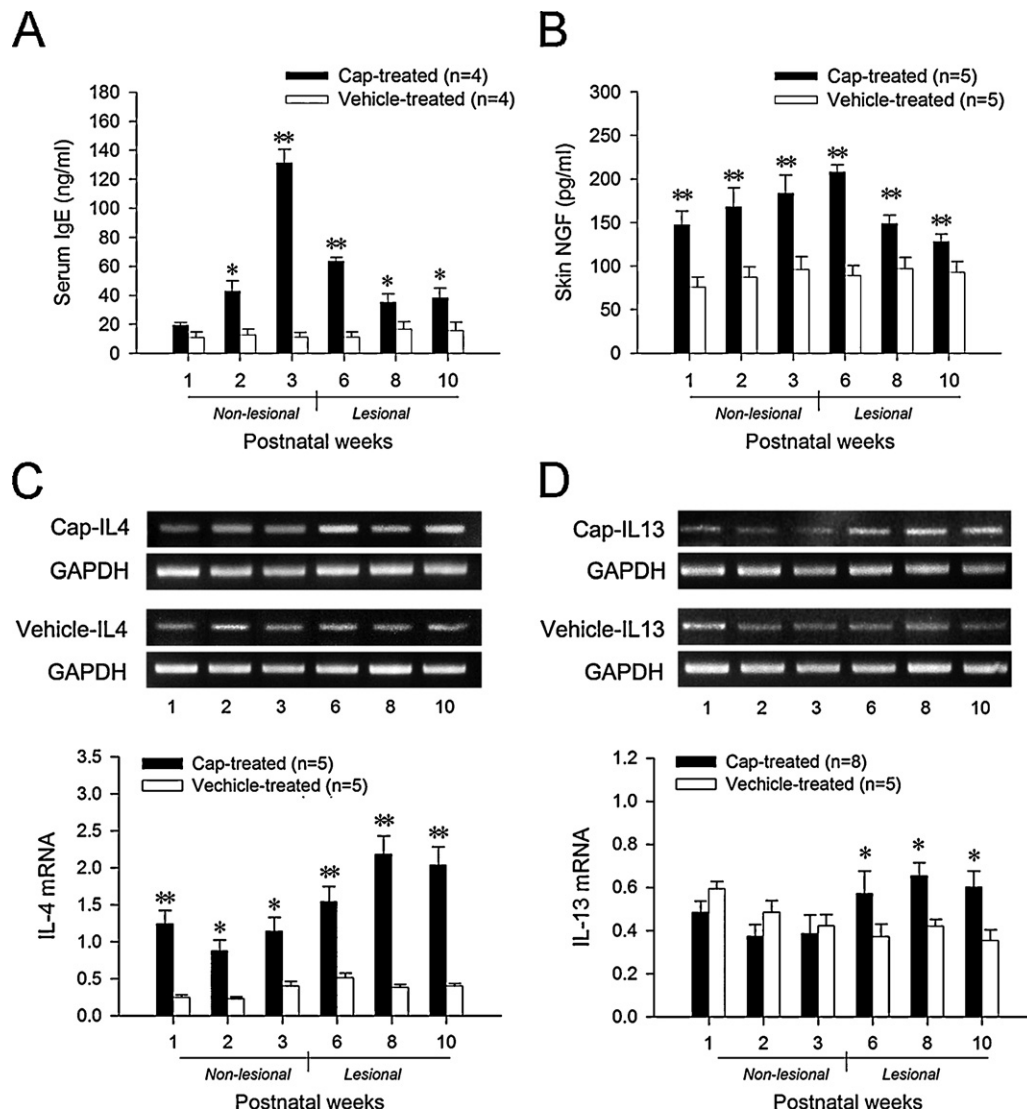


Fig. 3. Upregulation of serum IgE, skin NGF and cytokine mRNA expression following neonatal capsaicin treatment. Hyperproduction of serum IgE (A) was observed not only in the adult rats suffering from severe dermatitis (lesional) but also in rat pups with no developed cutaneous lesions (non-lesional). NGF (B) and IL-4 mRNA expression (C) in the skin was observed to be upregulated chronically, regardless of the presence of cutaneous lesions following capsaicin treatment, whereas upregulation of IL-13 mRNA expression (D) was limited to lesional skins after the capsaicin treatment. * $P < 0.05$, ** $P < 0.001$ (t-test).

As illustrated in Fig. 4C, filaggrin-immunoreactivity (ir) was completely abolished in both the non-lesional and lesional skin of rats treated with capsaicin as neonate, despite of the clear presence of epidermal hyperplasia. An intensive filaggrin-ir was observed in the corresponding skin region of the age-matched control animals.

3.8. Skin colonization with *Staphylococcus aureus* (*S. aureus*)

Skin colonization with *S. aureus*, a characteristic feature of atopic dermatitis [11,12], was examined in two different age groups; non-lesional 3-week- and lesional 6-week-old rats. The number of colonies and methicillin susceptibility in each sample are shown in Table 1. All lesional skin samples taken from 6-week-old Cap-treated rats were found to be colonized with a large number of *S. aureus*, all of which were methicillin-sensitive. Despite the absence of apparent cutaneous lesions at PW3, colonization of *S. aureus* was also observed in 2 of 3 non-lesional skin samples obtained from Cap-treated rats. In all the samples of the vehicle-treated control animals, *S. aureus* was not detected.

3.9. Loss of cutaneous nerve fibers and delayed hair growth following neonatal capsaicin treatment

Distribution of cutaneous nerve fibers at the injection site of capsaicin was investigated weekly for 5-weeks after the treatment. As shown in Fig. 5A, the rat pups treated with capsaicin showed a problem in hair growth during the preweaning period. In contrast to control rats whose bodies, including heads and limbs, were already covered densely with hair at PW1.5, Cap-treated rat pups at this time showed only sparse hair growth. However, this difference in hair growth was no longer detectable by PW3.

Immunohistochemical staining for PGP9.5 revealed that cutaneous nerve fibers were significantly reduced in the Cap-treated rats compared to control animals over the entire experimental time points (Fig. 5B), indicating an irreversible loss of cutaneous nerve fibers by neonatal capsaicin treatment.

4. Discussion

In accordance with previous studies [2–4], subcutaneous injection of capsaicin into newborn rat pups in the present study

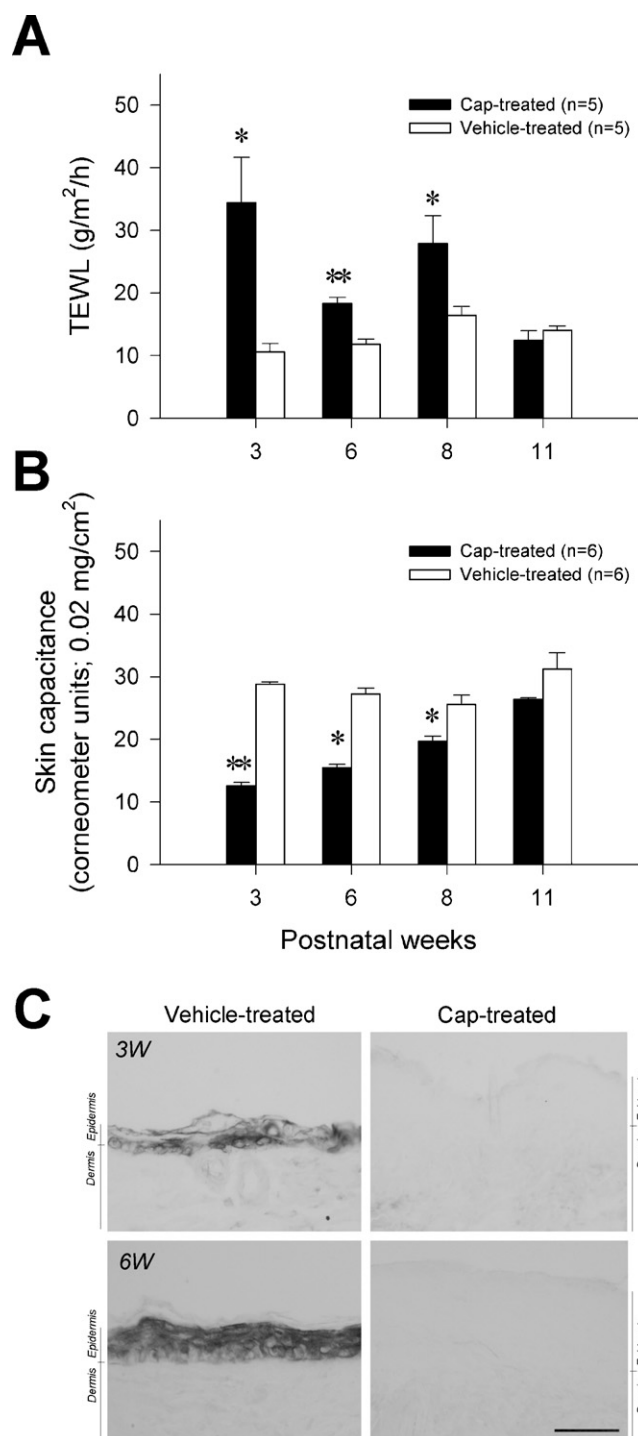


Fig. 4. Epidermal permeability barrier dysfunction following neonatal capsaicin treatment. (A) Greater transepidermal water loss (TEWL) was measured at non-lesional back skins of the 3-week-old rats, as well as at lesional skin of 6 and 8-week-old rats treated with capsaicin as neonate (Cap-treated), compared to the skin of control rats (Vehicle-treated). * $P < 0.05$, ** $P < 0.001$ (t -test). (B) Skin hydration was significantly lower in the Cap-treated rats while TEWL was higher than the control animals. (C) Immunohistochemistry of filaggrin. Compared to the age-matched control rats, filaggrin-immunoreactivity was completely abolished in both the non-lesional skin of 3-week-old rats and lesional skin of 6-week-old rats following neonatal capsaicin treatment. The numerals with "W" represent the postnatal weeks. * $P < 0.05$, ** $P < 0.001$ (t -test) (scale bar = 50 μ m).

led to long-lasting cutaneous lesions. Pioneering studies speculated that this pruritic skin disease might be a neurological problem because both pruritic sensation and scratching behavior themselves are fundamentally based on neuronal mechanisms [3,4].

There is known to be an antagonistic interaction between pruritus and pain [6]. Thus, it is likely that impaired nociception, due to loss of the TRPV1-expressing nociceptive afferents, leads to a loss of pruritic inhibition and may contribute to the induction of pruritus and resultant scratching behavior, which results in cutaneous lesions [6]. However, because itch responsive afferents do not display spontaneous activities [13], we must address the question of what triggers the pruritic signaling. In addition, previous studies in the development of pruritic cutaneous lesions by neonatal capsaicin treatment did not consider the potential role of skin, despite current evidence that chronic pruritus involves a pathophysiology of the skin [14]. Therefore, we conducted a dermatological assessment of lesional and non-lesional skin after neonatal capsaicin treatment.

In the present study, scratching and cutaneous lesions following neonatal treatment of capsaicin were rarely observed during the preweaning period. It has been suggested that the development or reorganization of the nervous system in preweaning rats treated with capsaicin might be not enough for the detection of pruritic sensation [3]. However, our results show that intradermal injection of serotonin elicited a vigorous scratching response in both the Cap-treated and control rats at 2-weeks of age indicating the ability to respond to pruritic sensation in the preweaning period. It is interesting to note that NC/Nga mice, an animal model of human AD, start showing the spontaneous dermatitis just around the time of weaning if the mice are delivered and housed in the conventional condition [15]. Considering that significant pathophysiological changes in the skin were already detectable even before the appearance of cutaneous lesions in the Cap-treated rats or NC/Nga mice, and that environmental factors have an influence on the onset of some allergic skin diseases [15], we hypothesize that the preweaning time of these animals may be a latent period during which the animals would be exposed to pathogenic environmental factors, which initiate the pathogenesis of spontaneous pruritus or cutaneous lesions.

Taken together with the chronicity, one of the most striking features of the cutaneous lesions caused by neonatal capsaicin treatment was that these lesions relapsed repeatedly. During our follow-up observations of five animals up to a year, we found the rats having the alternative periods of relapsing and remitting skin lesions. Given that human allergic skin diseases including AD are chronically relapsing inflammatory skin diseases characterized by highly pruritic eczematous skin lesions, chronicity and the recurrence of dermatitis seen in rats subjected to neonatal capsaicin treatment without any additional manipulations may provide some merits in the study of human allergic skin diseases.

Lesional back skins of 6-week-old rats were found to show histopathological changes, similar to the skin of AD patients. Interestingly, these epidermal changes were already present even in the non-lesional skin of 3-week-old rats treated with capsaicin, indicating that these histological changes precede macroscopic dermatitis. Consistently, a significant increase in the number of mast cells, an important factor in the pathophysiology of many atopic skin diseases, was observed in the non-lesional skin of 3-week-old rats. It is now clear that mast cells are closely involved in the pathogenesis of AD [16]. IgE-mediated activation of mast cells leads to the release of various kinds of chemical mediators, which results in recruitment of immune cells into the skin. Th2 cytokines such as IL-4 and IL-13 have been known to promote IgE synthesis [17]. Taken together with our histopathological evidence, the long-term increases in the number of mast cells, serum IgE and skin mRNA for IL-4 in the present study indicates that the cutaneous lesions seen in rats treated with capsaicin are clinically similar to that reported in AD patients. IL-4 mRNA was significantly increased throughout the experimental time points from the first

Table 1

Skin colonization with *S. aureus* and methicillin susceptibility. By punching biopsy (each 2 mm in diameter), the lesional skin samples were taken from the capsaicin-treated (Cap-treated) rats on 6 postnatal weeks, whereas the non-lesional samples were obtained from the areas targeted by frequent scratching on 3 postnatal weeks. Just one sample per animal was used to quantify the colonies in the present study. “–” represents “not detected”.

Age	Group (skin condition)	Animal	No. of colonies (CFU/cm ²)	Methicillin susceptibility
3 week	Cap-treated (non-lesional)	1	–	–
		2	>2000	Sensitive
		3	1500	Sensitive
	Vehicle-treated	1	–	–
		2	–	–
		3	–	–
6 week	Cap-treated (lesional)	1	450	Sensitive
		2	1200	Sensitive
		3	1200	Sensitive
	Vehicle-treated	1	–	–
		2	–	–
		3	–	–

week after the capsaicin treatment, whereas the increase in mRNA for IL-13 was limited to later time points. This result indicates that the onset of AD-like dermatitis following neonatal capsaicin treatment may be IL-4 dependent.

Significant increases in skin NGF levels were observed in rats treated with capsaicin. NGF is important for the maintenance and repair of cutaneous nerves [18]. Recently, there is growing evidence that NGF plays a role in the pathogenesis of AD. Previous studies reported that an increase in cutaneous nerve innervation, in connection with the elevation of NGF levels in the plasma or skins, correlated with the severity of AD [19–21]. However, it should be noted that despite consistently higher levels of skin NGF compared to control rats, one of characteristics of the rats treated with capsaicin is the significant reduction of cutaneous nerves. Considering the permanent reduction of cutaneous nerves and its known effects on the nervous system, the increased skin NGF is thought to play a role in the repair or survival of the nerves from the neurotoxicity of capsaicin. Therefore, it does not make sense that cutaneous hyperinnervation itself is directly related to pathogenesis of AD-like symptoms. NGF also has various biological effects on cells contributing to immune responses [5]. Previous studies have reported that immunosuppressants had therapeutic effects on AD in both humans and animals whose NGF levels were significantly increased [22–24]. Therefore, immune responses that would be expected by increased NGF may be more directly implicated in the pathogenesis of AD-like symptoms rather than the morphological changes. Increased NGF levels have been reported in plasma as well as skin of AD patients and animals [19–21,25]. However, in the present study, the increase of NGF level in plasma was not observed in rats treated with capsaicin over the entire experimental time, suggesting that the role of NGF on the AD-like symptoms is local and limited to specific cutaneous areas in these rats.

TEWL and skin hydration are believed to be objective and reliable biophysical indicators of epidermal permeability barrier function in both human and animal studies [26,27]. It is well known that TEWL increases in not only the lesional skin but also clinically unaffected skin of AD patients [28–30], indicating that skin barrier defects are associated with the pathogenesis of AD. In the present study, we measured TEWL and skin hydration at both the non-lesional skin of the 3-week-old rats and the lesional skin of the older ones, and found the increased TEWL and reversely proportional decrease in skin hydration at these areas, as compared to the age-matched control rats. It is surprising that the expression of filaggrin was completely abolished in the skin of rats treated with capsaicin, although we cannot provide clear mechanisms for loss of this protein in the present study. Given that

a deficiency of filaggrin in the skin is considered as a major factor compromising epidermal barrier functions in AD patients [9,10], lack of filaggrin in the skin of Cap-treated rats may be accountable for the increase of TEWL and the decrease of skin hydration. Our result implies that neonatal capsaicin treatment leads to barrier dysfunctions of the skin, which may in turn be related to the manifestation of AD-like symptoms. Impaired epidermal barrier function results in an increased risk of invasive infections by microbes and exposure to allergens. AD patients are known to be more susceptible to bacterial infection, commonly with *S. aureus* [31] producing various toxins and enzymes that may trigger inflammatory or immune responses in the skin [32]. Therefore, AD is often characterized by colonization with *S. aureus*. In the present study, we found that both the non-lesional and lesional skins of the rats treated with capsaicin were colonized with a large number of *S. aureus*. So, it is probable that impaired epidermal barrier function, caused by neonatal capsaicin treatment, results in infection by microbial pathogens including *S. aureus*, which in turn contributes to the pathogenesis of AD-like pruritic dermatitis in rats treated with capsaicin.

Neonatal capsaicin treatment led to the permanent loss of cutaneous nerve fibers, the majority of which are supposed to be peptidergic because most capsaicin-sensitive primary afferents contain neuropeptides such as substance P and CGRP [33–35]. It has been reported that CGRP-positive nerve fibers densely innervate the epidermis and upper dermis [36] where CGRP stimulation may contribute to epidermal reconstruction by inducing the proliferation of keratinocytes [37]. It is noteworthy that the rat pups treated with capsaicin showed a transient abnormality in hair growth in the present study and that partial depletion of CGRP by topical capsaicin treatment delayed wound healing [34]. Thus, the reduced epidermal barrier function may be a consequence of the loss of peptidergic fibers by capsaicin treatment. However, we cannot rule out the possibility that neurogenic inflammation by neuropeptide surges just after the capsaicin treatment may be implicated in an impairment of epidermal barrier function.

Lastly, it should be also noticed that, capsaicin treatment into mice completely abolishes or partially reduces pruritus associated with several pruritic diseases [8,38]. Consistently, we could not observe spontaneous scratching or cutaneous lesions in mice (C57BL/6J and ICR) treated with capsaicin as neonate (data not shown). Recently, it has been suggested that capsaicin-sensitive primary afferents are involved in pruritic sensation and behavioral responses to various pruritogens [39] as well as pain transmission. As shown in Fig. 1C, the rats treated with capsaicin were also less sensitive to serotonin-evoking pruritus compared to the control

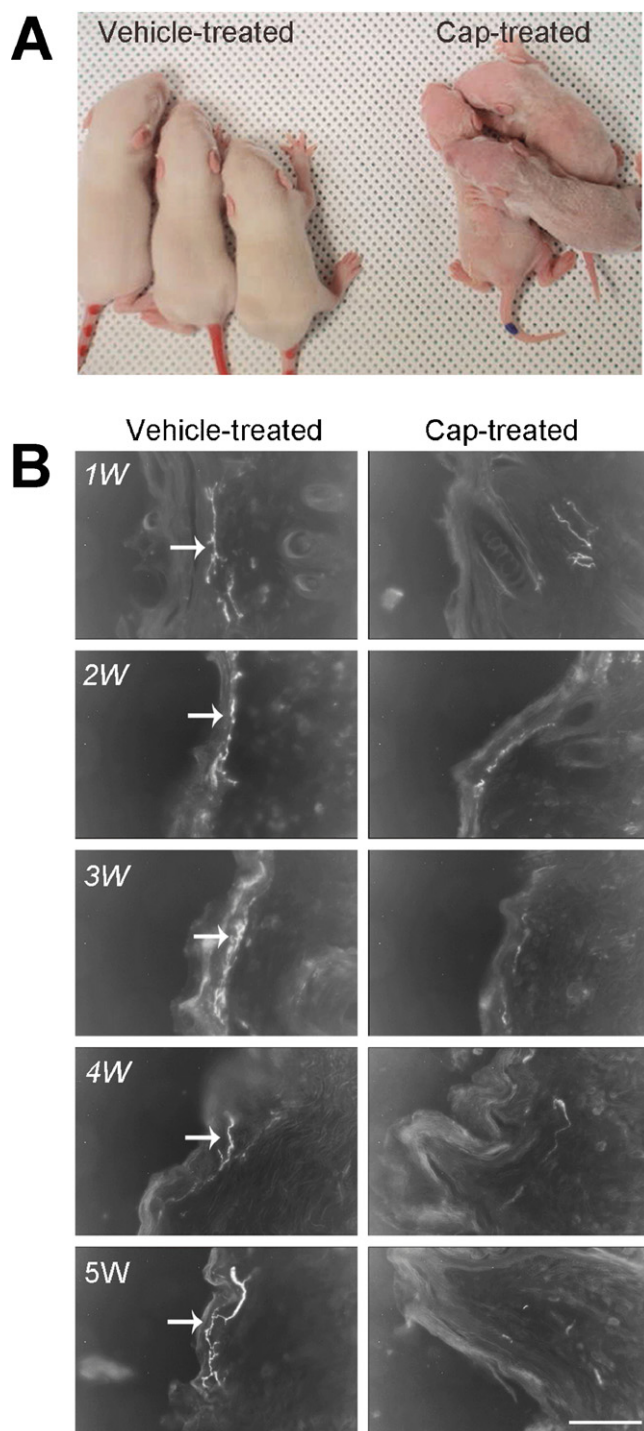


Fig. 5. Delay in hair growth and loss of cutaneous nerves following neonatal capsaicin treatment. (A) Delay in hair growth in rat pups treated with capsaicin at postnatal week 1.5. In comparison to vehicle-treated pups (left), Cap-treated rat pups showed sparse hair growth on their body (right). (B) Reduced cutaneous nerve innervation. PGP9.5-immunoreactive nerve fibers (arrows) are significantly reduced in the skin of the Cap-treated rats, compared to the control animals. The numerals with “W” represent the postnatal weeks (scale bar = 100 μ m).

rats, suggesting that the role of TRPV1-expressing primary afferents of rats in pruritic sensation is not different from that in mice. Unfortunately, we could not provide any clear explanations for why AD-like pruritic dermatitis develops in the rats, but not mice, following neonatal capsaicin treatment. We think that answering this question will be a good subject of further studies and helpful in enlarging our understanding about AD.

In conclusion, we provide evidence that neonatal capsaicin treatment results in chronically relapsing pruritic dermatitis of which pathogenesis is clinically similar to that of AD patients and propose it as a useful model for studying human AD and development of novel therapeutic drugs.

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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.jdermsci.2012.05.006>.

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Supplementary Data

Chronically relapsing pruritic dermatitis in the rats treated as neonate with capsaicin; a potential rat model of human atopic dermatitis

Supplementary Data include,

Supplementary Materials and methods

Supplementary Table 1

Supplementary Figure 1

Legend for Supplementary Figure

References

Supplementary Materials and methods

Observation of TRPV1 expression

Quantitative real time RT-PCR

The total RNA was extracted from the L5 DRG and purified with RNeasy[®] Micro kit (Qiagen[®], Texas, USA) according to manufacturer's instructions. The purified total RNA was reverse transcribed to cDNA using the Reverse Transcription System (Promega, USA). Primer pair for rat TRPV1 was designed by Primer express 2.0 program (Applied Biosystems, CA, USA) based on published sequence in GeneBank. Primer pairs for rat TRPV1 and GAPDH are listed in Table EI. The quantitative real time RT-PCR was performed with a total reaction volume of 20 μ l containing a SYBR Green PCR Master Mix (10 μ l, Applied Biosystems, CA, USA), primer pair (2 μ l, each 1 μ l of 10 pmol/ μ l primers) and diluted cDNA (8 μ l, 500 ng/ μ l), using a real-time PCR system (#7000, Applied Biosystems. CA, USA). Melting curve analysis was performed for all amplified products and gave rise to a single peak, indicating no contaminating products. Relative expression of TRPV1 was determined in comparison to a house keeping gene (GAPDH), using the $2^{-\Delta\Delta C_T}$ method. [1]

TRPV1 immunofluorescence

Animals were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused with heparinized saline followed by 4% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The S1 DRG was excised by laminectomy and then

cryoprotected within 30% sucrose in 0.1 M PB at 4 °C overnight. The DRG was sectioned at 14 μ m on cryostat. DRG sections from each group were mounted on the same slides and treated under the same condition during immunofluorescent staining procedure to minimize the inter-group variability. Sections were blocked with 1% bovine serum albumin (BSA), and 10% normal goat serum (NGS) in 0.05 M phosphate buffered saline (PBS) for 1 h at RT and then incubated with guinea pig polyclonal anti-TRPV1 (1:1000, #AB5566, Chemicon, CA, USA). Sections were sequentially rinsed with 0.05 M PBS, 3% and 1% NGS for 30 min each. They were then incubated with the mixture of FITC- -conjugated secondary antibodies at RT for 1 h. After PBS washing, the immunofluorescent sections were coverslipped with a mounting medium (Vector Laboratories, Burlingame, CA, USA). Fluorescent signals were detected by a fluorescence microscope (BX51, Olympus, Japan), and captured as digital images by a camera (EOS 60D, Canon, Japan). Six to ten sections were randomly selected for each rat and were used for counting a number of TRPV1-immunofluorescent cells. The proportion of TRPV1-expressing DRG neurons was determined by counting the neuronal profiles that exhibited distinctive fluorescent signal in the DRG sections. Only neurons with clearly visible nuclei were counted. A mean percentage of TRPV1-immunofluorescent neurons, relative to the total number of neurons, were calculated for each animal. Because a stereological approach was not adopted in this study, the data may represent a biased estimation of the actual number of neurons.

Evaluation of neonatal capsaicin treatment

Intake preference between the capsaicin-containing and vehicle waters

Capsaicin leads to irritation or pain when applied to oral mucosa [2] due to its pungent taste and animals are averse to taking food containing capsaicin. [3,4] We assessed the ability of rats to taste capsaicin in drinking water, using a paired preference paradigm. [2] In brief, two bottles containing either capsaicin water (1.65 μ M in 0.05% ethanol) or distilled water (0.05% ethanol) were suspended side by side on the front of each cage by wire loops. The rat was allowed to access *ad libitum* to both bottles. And then, intake volumes were measured on alternate days over 10 day period. Daily intake volume was determined and converted to a percentage of total volume consumed.

Physiological pain sensitivity to noxious heat stimulation

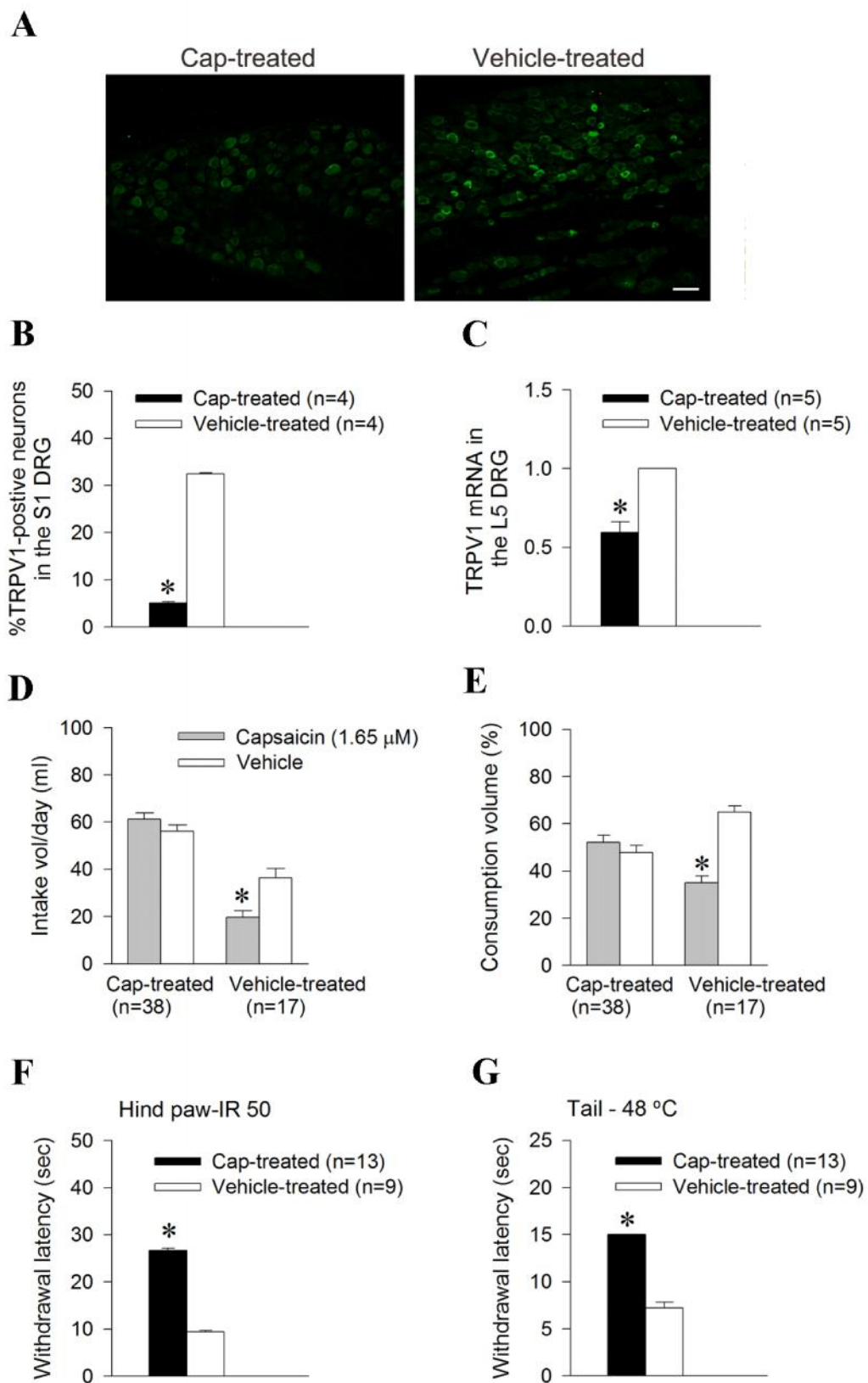
Capsaicin treatment has been reported to cause permanent thermal hypoalgesia, [5-7] which is now believed to be due to loss of TRPV1-expressing nociceptors. As described previously, [8] the paw-withdrawal latency to radiant infrared-heat stimulation was measured by using a plantar test apparatus (IR 50, 390, IITC Inc., Woodland Hills, CA, USA). The cut-off time was set at 20 s to prevent potential tissue damage. Heat stimulation was given three times for each hindpaw at an interval of 5-10 min. Mean withdrawal latency for each hindpaw was calculated. In addition, the tail-withdrawal latency to heat stimulation given by immersing the tail into the hot water (48 °C) bath was measured with a cut-off time of 15 s. This was repeated 3

times at a 15 min interval to give rise to an average value for each animal.

Supplementary Table 1. Primer pairs for PCR

Gene	Forward	Reverse
GAPDH	ACAACCACAACGATTCTTCTGTAA	CCTTTACAAGTTTTGCTGTGCTAA
IL-4	CTGCAGGGCTTCCAGGGTGC	AGACCGCTGACACCCTACAG
IL-13	GCCTCTGTAACCAAAAGGCCTC	CCCCTCAGTGGCCATAGCGG
TRPV1	TGAGTCCCCACCCCAAGAGAAC	CCAGCCCGCCTTCCTCATAAG

Supplementary Figure 1



Legends for Supplementary Figures

Supplementary Figure 1. Reduction of TRPV1-expressing DRG neurons and impaired nociception following neonatal capsaicin treatment. The number of TRPV1-expressing neurons in S1 DRG (A and B) and the expression level of TRPV1 mRNA in L5 DRG (C) were significantly reduced in adult (6 week-old) rats treated with capsaicin as neonate (Cap-treated), compared to the vehicle-treated control animals. Control rats clearly preferred to drink the vehicle water rather than capsaicin-containing water, whereas no preference between the two drinks was observed in the Cap-treated animals (D and E). The hindpaw- and tail-withdrawal latencies in response to noxious infrared radiant heat (IR 50) and 48 °C water stimuli, respectively, were remarkably longer in the Cap-treated rats than in the control animals (F and G). * $P < 0.01$ (t -test). Scale bar = 100 μm .

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