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Decreased Pain Sensitivity of Capsaicin-Treated Rats Results from Decreased VR1 Expression

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We investigated the neurotoxic effects of capsaicin (CAP) on pain sensitivity and on the expression of capsaicin receptor, the vanilloid receptor (VR1), in rats. High-dose application of CAP has been known to degenerate a large fraction of the sensory neurons. Although the neurotoxic effects of CAP are well documented, the effects of CAP on the vanilloid receptor (VR1) are not yet known. In this paper, we investigated the effects of high-dose application of CAP on the expression of VR1 in rats. Thermal and mechanical pain sensitivity was reduced when neonatal rats were treated with a high dose of CAP. This reduction of pain sensitivity was significantly decreased after initiating carrageenan-induced inflammation. The expression of VR1 in dorsal root ganglia (DRG) isolated from the CAP-treated rats was reduced compared to that from the vehicle-treated rats. Therefore, we can conclude that the neurotoxic effect of CAP is related to the decrease of VR1 expression.

Key words: Capsaicin, Capsaicin ion channel, Rat, Dorsal root ganglion (DRG), Pain behavioral test, Vanilloid receptor (VR1) expression

INTRODUCTION

Capsaicin (CAP) is a main ingredient of hot peppers and it is known to activate sensory neurons by opening an ion channel that is activated by CAP (Oh *et al.*, 1996). The high dose application or repeated application of CAP shows a strong analgesic effect; therefore, CAP is currently being used as an analgesic. This effect is known to result from the neurotoxicity of CAP that degenerates a large fraction of unmyelinated axons as well as the cell bodies of sensory neurons. The neurotoxic effect leads to deficits in nociception and other physiological reflexes as well (McDougal *et al.*, 1985; Arvidsson and Ygge, 1986; Hylden *et al.*, 1992). This CAP neurotoxicity is known to be confined to the unmyelinated fibers. Therefore, CAP is used to remove unmyelinated neurons and to remove specific neuropeptides from those unmyelinated neurons like substance P and calcitonin gene-regulated peptide (CGRP) (Hammond and Ruda, 1989).

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The effect of CAP on neurons is known to result from activation of CAP-activated ion channels on the dorsal root ganglion (DRG). The cDNA of the CAP-activated channel has been cloned and it is known as the vanilloid receptor 1, VR1 or TRPV1; it is characterized as a nonselective ion channel that is activated by noxious temperature, acid and vanilloids (Caterina *et al.*, 1997). Disruption of the VR1 gene reduces the thermal hyperalgesia induced by inflammation (Caterina *et al.*, 2000; Davis *et al.*, 2000), suggesting that VR1 is essential for the thermal hyperalgesia.

In this study, we investigated the effect of a high-dose application of capsaicin on pain sensitivity, and we examined the VR1 expression level. We found that the CAP treatment of neonatal rats reduced their pain sensitivity to mechanical and chemical stimuli and this seems to result from the decreased expression of VR1 protein in the DRG.

MATERIALS AND METHODS

Capsaicin (CAP) treatment

Capsaicin (CAP) was subcutaneously injected into Sprague-Dawley neonatal rats at the dose of 50 mg/kg (Tween 80: ethanol: saline=10: 10: 80) as previously

described (Gamse *et al.*, 1980; Kwan *et al.*, 1996). The rats were then raised with their mother. After weaning, four of these rats were kept in a cage under the conditions of a day/night cycle, and food and water was made freely available (Gao *et al.*, 2000).

Pain behavioral test

For the thermal pain test we used the plantar (Hargreaves) and tail flick tests. Briefly, we applied a beam of infrared (I.R.) light of intensity 60 and then we measured the time for the rats to move to avoid the noxious temperature. In order to get an average paw withdrawal latency time (PWL) and tail flick latency time (TFL), we measured the response at least twice. In order to test for thermal pain after inflammation, we induced inflammation by carrageenan (CAR) administration into the sole of the foot (50 μL of 20-40 mg/mL). Four hours after the CAR administration, we tested for thermal sensitivity by using the plantar test.

For the chemical sensitivity test, we performed the wiping test after an application of CAP 0.01% (w/v, Tween 80: 10% ethanol: saline=10:10:80, total volume of 50 μL) into the right eye, and we then measured the number of wipes or blinks the rat performed (Laszlo *et al.*, 2000). Each animal was used only once for each experiment.

For the mechanical pain test, we performed the Randall Selitto test 30 min after the thermal pain test. We positioned the foot of the rat between stylus and platform of the Randall Selitto system (analgesy-meter, UGO/ BASIL, 21025 Comerio, Varese, Italy). After stabilization, we put on weight to apply mechanical pressure onto the foot. We measured the paw withdrawal times when the animal tried to draw away its foot or make a sound, and we used the average of the responses. For CAR-induced peripheral hyperalgesia, we performed the mechanical test five hours after the CAR administration.

Western blotting

We isolated the dorsal root ganglia (DRG) neurons from the control and treatment rats after the pain behavioral test as previously described (Oh *et al.*, 1996). Briefly, the dorsal root ganglia from all the levels of the thoracic and lumbar spinal cord of the rats were excised and next washed twice in a cold phosphate buffered saline (PBS). The DRG were resuspended in 100 μL of 1X RIPA buffer (50 mM Tris pH 7.5, 0.15 M NaCl, 2 mM EDTA, 1% deoxycholic acid and 1% NP-40 with protease inhibitors) and homogenized. Twenty five, 50 and 100 μg/lane of the mixture were loaded for 8% SDS-PAGE, and after this the proteins were transferred to a nitrocellulose membrane and immunoblotted as previously described using an ECL Kit (Amersham Pharmacia). For the control, we used anti-actin. We analyzed the immunoblotting data using a Tina 2.0 program (Raytest).

Statistical analysis

The paired Students t test was used for comparing two means. To compare multiple means, two-way ANOVA testing was used followed by a Tukeys post hoc test. The results were considered as significant when the P values < 0.05 (Gao *et al.*, 2000).

RESULTS

We treated the neonatal rats with CAP (50 mg/kg) 48 hours after they were born via subcutaneous injection. We then examined their appearance and performed the behavioral tests including application of thermal and mechanical pain-evoking stimuli at 10 days and at two, three, four, five and six weeks after the CAP treatment.

Appearance

We examined the changes in the appearance of the rats from the two groups; the vehicle-treated rats and the CAP-treated rats. When we compare the two groups of rats, there is no difference between the two groups two and three weeks after CAP-treatment. However, after three weeks, the CAP-treated rats gradually lost the hair from their neck to the belly (Fig. 1). On the other hand, there was no change of hair for the control group. This phenomenon continues up to five weeks and then the hair loss slowly decreased thereafter. The hair loss was observed for all the CAP-treated rats.

Pain behavioral test

Thermal pain test

In order to examine whether the CAP-treated rats had

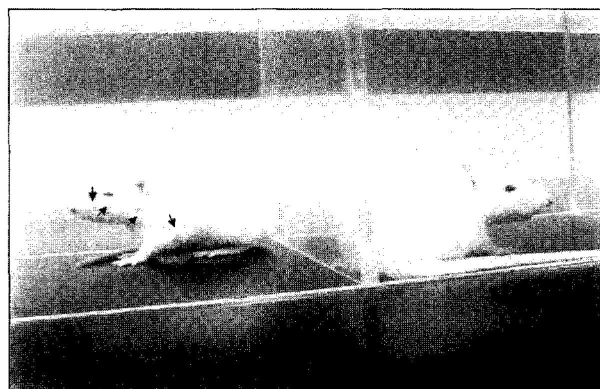

Fig. 1. Changes in appearance after high-dose CAP administration in neonatal rats. Changes in appearance after high-dose CAP administraion in neonatal rats occurred at four-weeks; the rats lost hair around the neck and face (arrow, left rat). This hair loss at four weeks, five weeks and six weeks was observed for all the CAP-treated rats. Left, CAP-treated; Right, vehicle-treated rats.

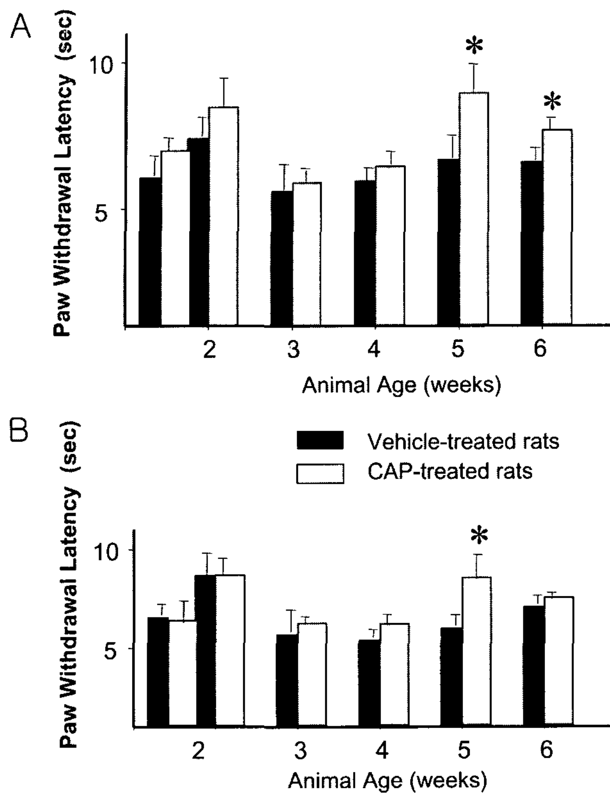

Fig. 2. Changes of paw withdrawal latency (PWL) in neonatally CAP-treated rats without CAR-induced inflammation. Changes in thermal pain sensitivity measured by the plantar test as a function of age after neonatal administration of 50 mg/kg S.C. CAP-treated rats are indicated as the gray bar and vehicle-treated rats are indicated as the black bar (*, P<0.05). A, left hindpaw; B, right hindpaw.

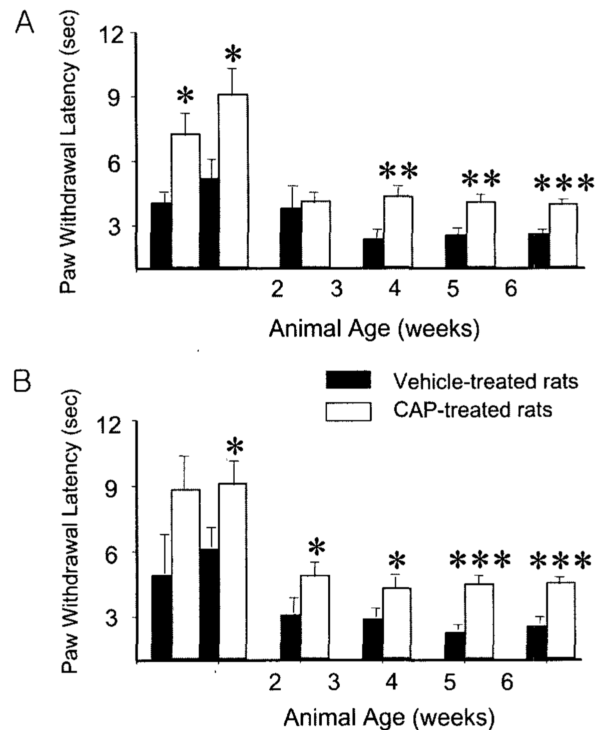

Fig. 3. Changes of paw withdrawal latency (PWL) in neonatally CAP-treated rats with CAR-induced inflammation. Changes in thermal pain sensitivity measured by plantar test as a function of age after neonatal administration of 50 mg/kg S.C. CAP-treated rats are indicated as the gray bar and vehicle-treated rats are indicated as the black bar (*, P<0.05). A. left hindpaw; B, right hindpaw.

different pain recognition compared to the control group, we performed the pain behavioral test. We first performed the thermal pain test. We measured the paw withdrawal latency time (PWL) and tail flick latency time (TFL). Fig. 2 shows that at the fifth week to sixth week, the CAP-treated rats showed a somewhat longer latency time compared to the control group on the paw withdrawal latency test. However, there is no observed difference at before the fifth week. Notably, five week-old CAP-treated rats show the longest PWL time.

Next, we examined the thermal pain perception of the CAP-treated rats after the inflammation has been induced (Fig. 3). We injected carrageenan (CAR) into the hindpaw of the rats to induce inflammation, and then we tested the thermal response by measuring the PWL time. As shown in Fig. 3, we found that CAP-treated rats showed a much more significance PWL time compared to vehicle-treated rats at all the ages we tested, and this was especially noticeable after four weeks. Therefore, the CAP-treated rats showed a much lower perception of the pain from thermal stimulus when the inflammation was induced.

We also examined the thermal pain perception by tail flick test (Fig. 4). Interestingly, the treatment group rats show a longer latency time only at the age of about two-week old. For the rest of the ages, there was no difference between the control and CAP-treated rats.

CAP sensitivity test

In order to examine whether the CAP-treated rats show different sensitivity to CAP compared to the control group rats, we performed the CAP sensitivity test (Fig. 5). When we applied CAP to the eye of the rats, the CAP-treated rats show a significant decrease in the number of wipes and winks compared to the control group, suggesting that the CAP-treated rats showed a much decreased CAP sensitivity compared to the control group.

Mechanical pain test

We performed Randall Selitto (analgesy-meter) test in order to examine the response of the two groups of rats to the mechanical stimulus. As shown in Fig. 6, the CAP-treated rats show a higher MPW (mechanical pain weight), which means the CAP treatment rats felt much less mechanical pain compared to the control rats starting from 4 weeks of age. Especially, at the age of four-week old, the CAP treated rats showed a significant difference

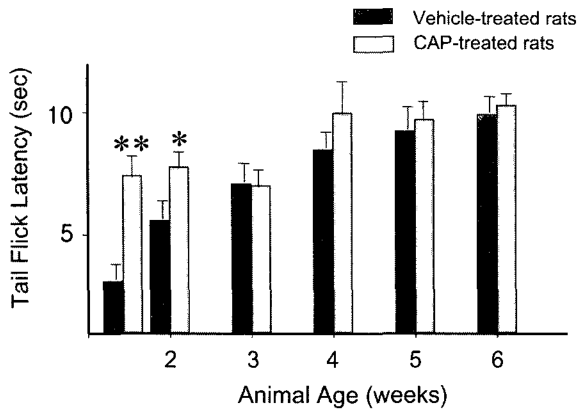

Fig. 4. Changes of tail flick latency in neonatally CAP-treated. Changes in thermal pain sensitivity were measured by the tail flick test (TFL). CAP-treated rats are indicated as the gray bar and vehicle-treated rats are indicated as the black bar (*, P<0.05; **, P<0.01).

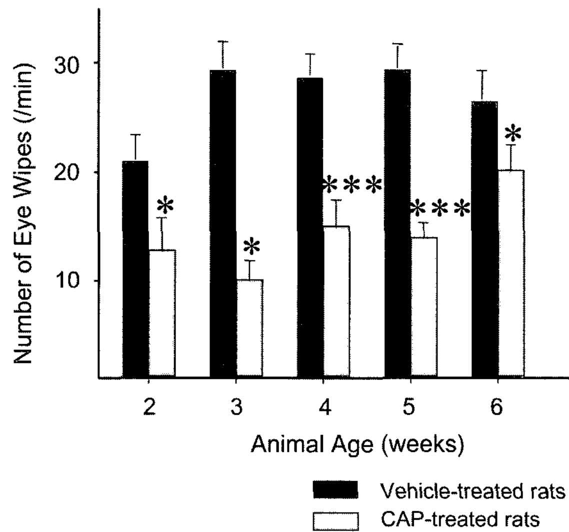

Fig. 5. Changes of capsaicin sensitivity in neonatally CAP-treated rats. Changes in CAP sensitivity by ophthalmic instillation of capsaicin were measured by number of eye wipes. CAP-treated rats are indicated as the gray bar and vehicle-treated rats are indicated as black bar. Number of wipes includes the number of blinks. Asterisks indicate values that are significantly different from the values in the corresponding vehicle-treated animals (*, P<0.05; **, P<0.01; ***, P<0.001).

to the mechanical stimulus.

When inflammation was induced by carrageenan (CAR), the difference for the MPW of the treatment rats and control rats increased significantly compared to when the inflammation was not induced.

Therefore, we can conclude that CAP-treated rats showed a much reduced perception to the pain of the mechanical stimulus as well to the thermal stimulus when *inflammation* was induced.

VR1 expression

In order to examine whether the different sensitivity of the CAP-treated rats to the pain resulted from changes in the VR1 expression, we examined the VR1 protein level in the DRG of both groups of rats by western blotting. The decrease in VR1 was a common phenomenon in both groups as time went by. As shown in Fig. 7, at the age of around two week-old, there was not much difference between the two groups. However, after three weeks, VR1 expression at the protein level of the CAP-treated rats was more decreased compared to the control group whereas the protein level of actin was not changed over time.

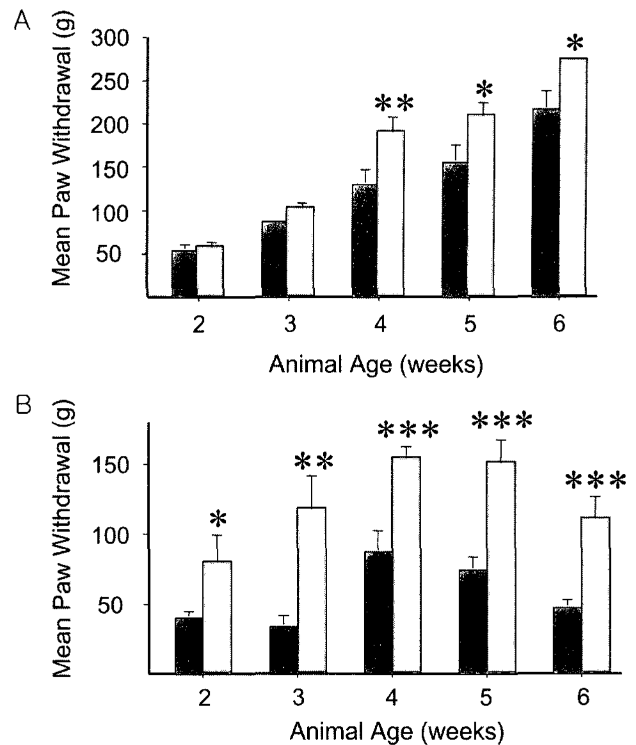

Fig. 6. Changes of mechanical pain sensitivity in neonatally CAP-treated rats without or with CAR-induced inflammation. Changes in mechanical pain sensitivity were measured by paw pinch (analgesymeter). The pressure, in grams, at which the animal struggled to remove its paw was termed the mean paw withdrawal. CAP-treated rats (gray bar) and vehicle treated rats (black bar). Asterisks (*, P<0.05; **, P<0.01; ***, P<0.001). A. without CAR-induced inflammation. B. with CAR-induced inflammation.

DISCUSSION

It is generally accepted that treatment with a high dose of CAP (50 mg/kg) on neonatal rats induces the destruction of C-fibers (Ren *et al.*, 1994; Hammond and Ruda, 1991; Holzer, 1991; Buck and Burks, 1986; Fitzgerald, 1983). In addition, a high dose of CAP shows persistent neurotoxicity; for example, a subcutaneous high dose injection of CAP gradually degenerates the C-fibers and Aδ-fibers that transfer pain signals (Jancso *et al.*, 1977).

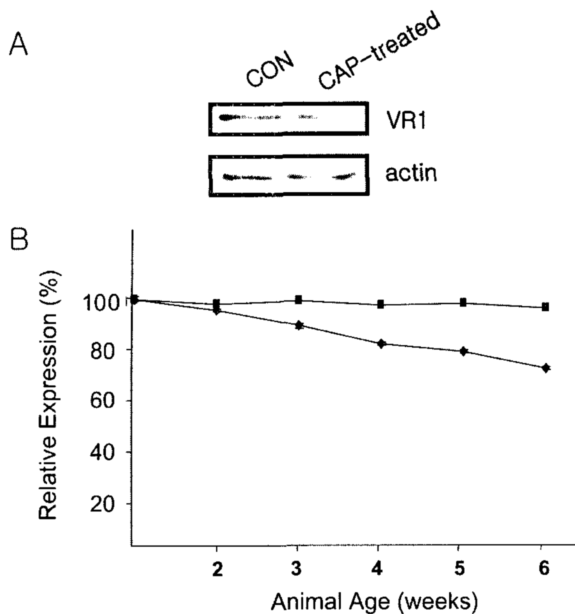

Fig. 7. Expression of VR1 protein in neonatally CAP-treated rats. Dorsal root ganglia were collected from the rats (five week-old, CAP-treated and vehicle-treated) and the expression of VR1 was analyzed by western blotting analysis. Vehicle-treated rats are indicated as CON, and CAP-treated rats are indicated as CAP-treated. Actin was used as an internal control. A. A representative Western blot. B. The relative expression levels of VR1 (◆) and actin (■) are indicated depending on the time after CAP-treatment. The expression level of each protein in the vehicle-treated rats was defined as 100% respectively. We performed all the experiments twice.

Even adult rats show degeneration of the sensory neurons within 1 h and the decreased function of sensory neurons 1 h after the high dose CAP treatment. This phenomenon was known to happen only at the primary afferent nerves (Gamse *et al.*, 1982; Gamse, 1982), and the CAP-activated ion channels have been reported to exist in the dorsal root ganglion (Oh *et al.*, 1996).

The gene encoding the CAP-activated ion channel is called VR1 (now known as TRPV1); it has been cloned and it is activated by CAP, noxious hot temperature and acid (Caterina *et al.*, 1997). The endogenous activators have recently been suggested to be lipoxygenase products, anandamide and unsaturated fatty acids (Zygmunt *et al.*, 1999; Smart *et al.*, 2000; Hwang *et al.*, 2000). These lipids are known to activate the CAP-channel at a higher concentration compared to CAP. Therefore, whether these are real endogenous activators or not is still controversial. However, these are still thought to be VR1 agonists in the physiological environment. These candidate activators are presumed to be produced after tissue damage from the pro-inflammatory agents (bradykinin, nerve growth factor or protons), and they open the VR1 channel to increase the thermal hypersensitivity.

Inflammation is a primary defense mechanism that is accompanied with fever, swelling, reddishness, and pain when tissue is damaged or exposed to noxious chemicals. Pain is caused by the chemicals that excite the sensory neurons during the inflammatory process. One characteristic of inflammation is hyperalgesia. We reported that the selective antagonist to the capsaicin (CAP) ion channel, capsazepine (CZP), decreased the hyperalgesia during inflammation (Kwak *et al.*, 1998). This supports that the CAP channel is the mediator of inflammatory pain and the chemicals produced during inflammation activate the CAP channel (Shin *et al.*, 2002).

Here in this study, we examined changes of the rats response to thermal and mechanical stimulus after a high dose injection of CAP in the presence or absence of the induction of inflammation by CAR. We then tested the response of the rats to various stimuli like heat, mechanical, and chemical pain-producing stimuli, and we examined the expression level of VR1.

At first, there was largely no difference in the rats appearance between control and treatment groups until 3 weeks after CAP treatment. However, 4 weeks after CAP treatment the rats lost their hair around the neck. This phenomenon can be explained by the report showing the effect of CAP as a neurotoxin on the primary sensory neurons, so that the neurons to the skin also affected the hair loss. Therefore, the loss of hair shows the destruction of the primary sensory neurons including the C-fibers and Aδ-fibers.

Pain behavioral testing by the plantar test and tail flick test showed that CAP treated rats had a longer latency time, suggesting they were less sensitive to pain. This effect was more significant when inflammation was induced by CAR. In order to explain the result, we can think that inflammation produces several pro-inflammatory materials and these sensitize CAP channels to open at a lower temperature and lower concentration compared to the non-inflammatory condition. When the VR1-containing C-fibers and Aδ-fibers are destroyed by a treatment of high dose CAP, then there is a reduced opening of the channels compared to the control group and the control group rats showed much less sensitivity to pain-producing stimuli.

However, the thermal pain sensitivities between the paw withdrawal latency test and tail flick latency test showed some discrepancy. In Fig. 4, CAP treatment showed a significant effect for increasing the tail flick latency time only at 14 days as compared to control; however, CAP-treatment increased the paw withdrawal latency time, as shown in Fig. 2 and 3, at 21, 28, 35, and 42 days. This discrepancy between tail flick and paw withdrawal latency time might result from the differences of the pain signaling pathways of sensing the stimuli on the tail and the foot.

In addition, the sensitivity to CAP as well as mechanical pain was also much decreased. Again, the difference between the two stimuli got larger when inflammation was induced by CAR.

This reduced sensitivity to pain-producing signals seems to result from the reduced expression of VR1 because the level of VR1 protein in the DRG was less when the rats were treated with a high dose of CAP. The decrease of VR1 expression was common between the control and treatment groups, but the degree of the decreased VR1 expression was much more significant for the treatment group. Therefore, we can conclude that the analgesic effect of CAP-treatment on rats resulted from the decrease in VR1 expression.

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