Novel Expression of Vanilloid Receptor 1 on Capsaicin-Insensitive Fibers Accounts for the Analgesic Effect of Capsaicin Cream in Neuropathic Pain

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ABSTRACT

Here, we investigated the mechanism of the antihyperalgesic effect of capsaicin cream in the nerve injury-induced neuropathic pain model in mice. In naive mice, application of capsaicin cream onto footpad caused no significant changes in the thermal latency in contrast to the severe thermal hyperalgesia induced by a capsaicin ointment. On the other hand, application of the cream 3 h before test concentration dependently reversed both thermal and mechanical hyperalgesia observed after partial sciatic nerve injury in mice. In algogenic-induced nociceptive flexion (ANF) test, application of 0.1% capsaicin cream in naive mice blocked intraplantar (i.pl.) nociceptin- and ATP-induced flexion responses, whereas prostaglandin I2 (PGI2) agonist-induced responses were unaffected. After nerve injury, PGI2 agonist-induced flexion responses were also hypersensitized in such injured mice. Capsaicin cream completely reversed both i.pl. nociceptin- or i.pl. PGI2 agonist-induced hyperalgesia in neonatal capsaicin-treated injured mice. Finally, novel expression of VR1 receptors on neonatal capsaicin-insensitive neurons after nerve injury was confirmed by immunohistochemistry. The newly expressed VR1 receptors after nerve injury were mainly confined to A-fibers. Together, our results suggest that novel expression of capsaicin receptors in neuropathic condition contributes to the analgesic effects of the capsaicin cream.

Capsaicin [(E)-N-(4-hydroxy-3-methoxybenzyl)-8-methyl-6-nonenenamide], the pungent ingredient of hot pepper, has been recognized for almost 150 years to produce pain relief (Turnbull, 1850). Capsaicin stimulates the vanilloid receptor 1 (VR1) located mainly on polymodal C-fibers and initiates a complex cascade of events, including neuronal excitation and release of proinflammatory mediators, desensitization of the receptor, and neuronal toxicity (Caterina et al., 1997). Topical application of capsaicin has been shown to be effective in a variety of chronic painful conditions, including postherpetic neuralgia (Watson et al., 1988), painful diabetic neuropathy (The Capsaicin Study Group, 1991), chronic distal painful polyneuropathy (Low et al., 1995), and surgical neuropathic pain (Ellison et al., 1997). Desensitization of the capsaicin receptors has been demonstrated to play a major role in the analgesic actions of vanilooids (Jancsó and Jancsó, 1949; Holzer, 1991; Szallasi and Blumberg, 1999). On the other hand, it has been hypothesized that capsaicin exerts pain relief through reversible depletion of the neurotransmitter substance P (SP) from the sensory nerve endings (Fitzgerald, 1983). However, loss of SP immunoreactivity in small-diameter dorsal root ganglion (DRG) neurons and spinal dorsal horn has been reported in many experimental neuropathy models (Garrison et al., 1993; Malmberg and Basbaum, 1998). Moreover, peripheral axotomy caused down-regulation of neuropeptide SP at the nerve ending, which also explains why SP antagonists are ineffective in neuropathic pain.
pain (Hökfelt et al., 1994). Thus, the exact mechanism of the analgesic action of topical capsaicin in neuropathic pain state has not been fully understood.

Recently, we developed a new technique in mice to study the mechanism of signal transduction of different receptor ligands at the peripheral nerve endings (Ueda, 1999). This “peripheral nociception test or algogenic-induced nociceptive flexion (ANF) test” has been proved to be advantageous for the study of in vivo molecular mechanism of nociceptive transmission (Inoue et al., 1998; Ueda, 1999; Ueda and Inoue, 2000). With this technique, we also proposed the presence of at least three distinct types of nociceptive fibers depending on their pharmacological sensitivity to different peripheral receptor ligand stimuli and neonatal capsaicin treatment, which degenerates small-diameter primary afferents (Ueda et al., 2000). Utilizing the ANF test as well as immunohistochemical study of DRG neurons, in the present report, we studied the possible mechanism of the antihyperalgesic effect of topical capsaicin cream in mice partial sciatic nerve injury-induced neuropathic pain model.

Materials and Methods

Animals. Male ddY mice weighing between 20 and 25 g were used throughout the experiments. They were kept in a room maintained at 21 ± 2°C with free access to a standard laboratory diet and tap water. Procedures were approved by Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983).

Drugs. The following drugs were used: nociceptin/orphanin FQ (N/OFQ; Sawady Technology, Tokyo, Japan), ATP (Nacalai Tesque, Kyoto, Japan), and capsaicin (Nacalai Tesque). ONO-54918-07 [a stable prostaglandin I$_2$ (PGL$_2$) agonist; Iguchi et al., 1989] was a kind gift from Ono Pharmaceutical Co. Ltd. (Osaka, Japan). Capsaicin cream, base cream, and capsaicin ointment were prepared at the Central Research Laboratories of the Maruishi Pharmaceutical Co., Ltd. (Osaka, Japan). The capsaicin cream labeled 0.01, 0.025, 0.05, and 1% contained 0.1, 0.25, 0.5, and 1 mg of capsaicin in 1 g of hydrophilic cream base, respectively (Minami et al., 2001). The capsaicin ointment (0.1%) was prepared in a conventional hydrophilic ointment base. N/OFQ, ATP, and ONO-54918-07 were dissolved in physiological saline. Capsaicin solution for intraplantar application (i.pl., 1 ng/2 μl) was prepared by dilution with physiological saline of a 5 mg/ml capsaicin stock solution dissolved in 10% ethanol and 10% Tween 80 in physiological saline. The vehicle was found to be innocuous in ANF test. The cream was applied in a volume of 0.1 ml/10 g and then gently rubbed over the mouse footpad skin. The footpad was then covered with adhesive tape to prevent the mice from licking off the cream.

Partial Ligation of Sciatic Nerve. Partial ligation of the sciatic nerve of the mice was performed under pentobarbital (50 mg/kg i.p.) anesthesia, following the methods of Malmberg and Basbaum (1998). Briefly, the common sciatic nerve of right hind limb was exposed at high thigh level through a small incision. The nerve was carefully cleared off the surrounding connective tissues. A silk suture was inserted into the nerve with a 3/8 curved, reversed-cutting mini-needle and tightly ligated so that the dorsal 1/3 to 1/2 of the nerve thickness was held within the ligature. The wound was closed with a single muscle silk suture and antibiotic powder was dusted over the wound area after surgery. Sham operation was performed similarly except without touching the sciatic nerve. Immediately after surgery, the animals were kept in a soft bed cage with some food inside so that the animals could feed themselves without difficulty in standing. The wound healed within 1 to 2 days and the animals behaved normally. Experiments were carried out at 7 days postligation.

Thermal and Mechanical Nociception Tests. In the thermal paw withdrawal test, antinociception or analgesia was measured from the latency to withdrawal. Evoked by exposing the right hind (10°C) to a thermal stimulus (Hargraves et al., 1988). Tail-immunized animals were placed in Plexiglas cages on top of a glass sheet and an adaptation period of 1 h was allowed. The thermal stimulus (ITTC, Inc., Woodland Hills, CA) was positioned under the glass sheet to focus the projection bulb exactly on the middle of plantar surface of the animals. A mirror attached to the stimulus permitted visualization of the undersurface of the paw. A cut-off time of 20 s was set to prevent tissue damage. The paw pressure test was performed as described previously (Rashid and Ueda, 2002). Briefly, mice were placed into a Plexiglas chamber on a 6 x 6-mm wire mesh grid floor and were allowed to accommodate for a period of 1 h. The mechanical stimulus was then delivered onto the middle of the plantar surface of the right hind paw using a transducer indicator (model 1601; ITTC, Inc.). With this apparatus, a control response of 10 g was earlier adjusted for naive mice. A cut-off pressure of 20 g was set to avoid tissue damage.

ANF Test. Experiments were performed essentially as described previously (Inoue et al., 1998; Ueda, 1999; Ueda and Inoue, 2000; Dobolyi et al., 2002). All experiments were performed in compliance with the relevant laws and institutional guidelines. Briefly, mice were lightly anesthetized with ether and held in a cloth sling with their four limbs hanging free through holes. The sling was suspended on a metal bar. All limbs were tied with soft thread strings. Then three limbs were fixed to the floor, while the other one (right hind limb) was connected to an isotonic transducer and recorder. A polyethylene cannula (0.61 mm outer diameter) filled with drug solution was connected to a microsyringe and then carefully inserted into the undersurface of the right hind paw. Nociceptor flexor responses induced by intraplantar injection (2 μl) of algogenic substances (N/OFQ, ATP, ONO-54918-07, or capsaicin) were evaluated. All experiments were started after complete recovery from the light ether anesthesia and when i.pl. injection of saline did not show any significant flexor response. The nociceptive activity was represented as percentage of maximal reflex. The biggest response among the spontaneous and nonspecific flexor responses that occurred immediately after cannulation was considered as the maximal reflex.

Neonatal Capsaicin Treatment. For the degeneration of small-diameter afferent sensory neurons, capsaicin solution was injected subcutaneously into newborn (P4) ddY mice at a dose of 50 mg/kg (Hiura and Ishizuka, 1989; Inoue et al., 1999). As a control, vehicle (5% ethanol and 10% Tween 80 in 90% physiological saline) was injected. No gross behavioral changes were observed in such treated mice. Partial sciatic nerve injury in neonatal capsaicin-treated mice was performed as described in the previous section.

Immunohistochemistry. For immunohistochemical experiments, naive mice, neonatal capsaicin-treated mice, or neonatal capsaicin-treated nerve-injured mice at 7 days after nerve injury were used. Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg i.v.) and perfused transcardially with 50 ml of 0.1 M potassium free phosphate-buffered saline (K’-free PBS, pH 7.4), followed by 50 ml of 4% paraformaldehyde in K’-free PBS. The L4-L5 DRGs were removed, postfixed, and cryoprotected overnight in 25% sucrose in K’-free PBS. The DRGs were fast frozen in cryoembedding compound on a mixture of ethanol and dry ice and stored at −80°C until use. The DRGs were cut at 10 μm with a cryostat, thaw-mounted on silane-coated glass slide, and air-dried overnight at room temperature. For immunolabeling, DRG sections were first washed with K’-free PBS 3 times for 5 min each and then incubated with 50% methanol 10 min and 100% methanol for 10 min each, washed with K’-free PBS, and incubated with excess blocking buffer containing 2% bovine serum albumin in 2% NaCl, 0.1% Triton X-100 in K’-free PBS for 60 min. The sections were then reacted overnight at 4°C with goat polyclonal antibody raised against the C-terminal of vanilloid receptor 1 (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in blocking buffer containing 2% bovine serum albumin in 2% NaCl,
Results

New Type Nonirritating Capsaicin Cream. The new type capsaicin cream, when applied onto mouse footpad, was nonirritating compared with a capsaicin ointment, which produced some biting-licking behavior immediately after application (data not shown). Moreover, effects of the capsaicin cream on the thermal latency at different time points after application in naive mice was measured. A thermal latency of \(~10\) s was adjusted for naive mice before initiation of experiments. At 30 min after application, the capsaicin ointment (0.1%) induced a profound thermal hyperalgesia that persisted throughout all time points tested within 3 h after application. On the other hand, application of 0.1% capsaicin cream did not produce significant changes in the thermal latency at any time point within 3 h after application (Fig. 1).

Reversible and Concentration-Dependent Analgesic Action of the Capsaicin Cream against the Bradykinin-Induced Nociceptive Flexion Responses in Naive Mice. The effect of the cream was next examined with the more sensitive ANF test. When the nociceptive flexion responses induced by 2 pmol of bradykinin (BK) was measured at different time points after a single application of 0.1% capsaicin cream onto the footpad of naive mice, the BK-induced nociception was significantly blocked by the capsaicin cream at 1, 3, and 6 h after application compared with control BK response at 0 h before returning to baseline level at 12 h (Fig. 2A). The maximal blockade of BK nociception was observed at 3 h after application. Thus, the next experiments were performed at 3 h after the application of the capsaicin cream. On the other hand, capsaicin cream produced a concentration-dependent analgesic action from 0.01 to 0.1% against 2 pmol of BK-induced nociceptive flexion responses measured at 3 h after topical application (Fig. 2B). The maximal blockade of the BK nociception was observed with 0.1% capsaicin cream (Fig. 2B). Thus, this concentration has been used in most of the subsequent experiments.

Effects of the Capsaicin Cream on Different Peripheral Receptor Ligand-Induced Nociceptive Flexion Responses in Naive and Injured Condition. Using the ANF test, effect of the capsaicin cream on different peripheral receptor ligands-induced nociceptive flexion responses in naive and injured condition was next studied. With this test in naive mice, previously we have proposed the presence of three distinct types of nociceptors, depending on their stimulation by specific receptor ligands. The nociceptors called
neonatal capsaicin-sensitive type I, were stimulated by i.pl. injection of bradykinin, nociceptin/orphanin FQ, or substance P; the nociceptors called neonatal capsaicin-sensitive type II were stimulated by i.pl. P2X3 receptor agonists; the nociceptors called neonatal capsaicin-insensitive type III, were stimulated by i.pl. PGI2 agonist ONO-54918-07 (Ueda et al., 2000). In the present study, we observed the effect of the capsaicin cream on the nociceptive flexor responses induced by the above-mentioned receptor ligands in naive mice. Prior application of the cream (0.1%) onto mouse footpad 3 h before testing almost completely blocked the nociceptive responses induced by i.pl. injection of nociceptin/orphanin FQ (type I response) and ATP (type II response) (Fig. 3, A and B). The bradykinin and substance P-induced nociceptive (type I) responses were also blocked by capsaicin cream (Fig. 2, A and B; data not shown). However, the nociceptive flexor responses induced by i.pl. injection of PGI2 agonist ONO-54918-07 (type III response) were not affected by the cream in naive mice (Fig. 3C).

After peripheral nerve injury in mice, the type I nociceptive responses were found to be functionally lost and the type II nociceptive responses remained functionally unchanged (Fig. 4, A and B). However, PGI2 agonist-induced type III nociceptive responses were sensitized after nerve injury (Fig. 4C). Thus, experiments for the effect of capsaicin cream on nociceptin/orphanin FQ- or ATP-induced responses were not performed in injured mice. When the effect of the capsaicin cream on PGI2 agonist-induced type III hyper-responses in injured mice was examined, a concentration-dependent reversal of the hyperalgesic responses to the level in naive mice was observed (Fig. 4C).

**Effects of the Capsaicin Cream on Nerve Injury-Induced Thermal and Mechanical Hyperalgesia.** The effect of the capsaicin cream was also evaluated in nerve injury-induced thermal and mechanical hyperalgesia. The cream was applied 3 h before examining the thermal latency or pressure threshold. In the thermal paw withdrawal test, capsaicin cream concentration dependently reversed the hyperalgesia from 0.01 to 0.1% concentration (Fig. 5A). The cream also concentration dependently reversed the mechanical hyperalgesia in injured mice (Fig. 5B). The cream was slightly more potent in the mechanical than in the thermal test with an EC50 value of 0.042 and 0.066% in mechanical and thermal tests, respectively.

**Effects of Neonatal Capsaicin Treatment on Capsaicin Solution-Induced Nociceptive Responses.** Next, we utilized the neonatal capsaicin treatment technique to investigate the mechanism of the antihyperalgesic action of the capsaicin cream. Neonatal capsaicin treatment is reported to destroy the majority of the small-diameter C fiber primary afferents in rodents (Jancso et al., 1977; Hiura and Ishizuka, 1989; Inoue et al., 1999). Previously, we have reported that neonatal capsaicin treatment in mice abolished the type I and type II nociceptive responses in the ANF test (Ueda et al., 2000), indicating that these nociceptors falls under the category of small-diameter primary afferents. However, the type III response was insensitive to neonatal capsaicin treatment, indicating their presence on capsaicin-insensitive large-diameter afferents (Ueda et al., 2000). In the present experiment, we observed the effect of nerve injury on the sensitivity of i.pl. capsaicin solution (1 ng/2 μl) or i.pl. PGI2 agonist in such mice. Neonatal capsaicin treatment almost...
completely abolished the i.pl. capsaicin solution (1 ng/2 μl)-induced flexor responses in sham-operated mice, whereas the responses substantially increased after nerve injury in such mice, indicating novel expression of capsaicin receptors after injury (Fig. 6A). PGI₂ agonist-induced type III responses were also sensitized after nerve injury in neonatal capsaicin-treated mice (Fig. 6B). Application of 0.1% capsaicin cream completely blocked the hypersensitized responses to i.pl.

Fig. 4. Phenotypic changes in three types of nociceptive responses after nerve injury and effects of capsaicin cream on type III hyperalgesic responses. A, changes in the type I nociceptive responses of N/OFQ after partial sciatic nerve injury. The type I responses were functionally lost in nerve-injured mice (injured). *, p < 0.05 compared with the nociceptive responses in sham-operated mice (sham). B, effects of nerve injury operation on the type II nociceptive responses of ATP. The ATP-induced nociceptive responses were functionally unchanged in injured mice. C, hyperalgesia to the type III nociceptive responses after nerve injury and effects of capsaicin cream thereon. Partial sciatic nerve injury caused a selective hyperalgesia to i.pl. PGI₂ agonist ONO-54918-07-induced nociception. Prior topical application of capsaicin cream concentration dependently blocked the type III hyperalgesic responses (△, 0.01%; □, 0.05%; ■, 0.1% of capsaicin cream in injured mice, respectively; ◇, base cream). Each data point represents mean ± S.E.M. from six mice.

Fig. 5. Capsaicin cream blocked the thermal and mechanical hyperalgesia in nerve-injured mice. A, effects of capsaicin cream from doses 0.01 to 0.1% with the thermal paw withdrawal test in nerve-injured (injured) mice 3 h after topical application. The thermal hyperalgesia measured as lowering of paw withdrawal latency (PWT in seconds) in nerve-injured mice was concentration dependently blocked by prior application of capsaicin cream. B, effects of capsaicin cream on the paw withdrawal threshold (PWT in grams) in nerve-injured mice with the paw pressure test. The mechanical hyperalgesia in nerve-injured mice was concentration dependently blocked by capsaicin cream. *, statistically different from sham-operated (sham) mice. #, statistically different from base cream (0%)-treated group. Each data point represents mean ± S.E.M. from six to seven mice.

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PG12 agonist or i.pl. capsaicin solution observed after nerve injury in neonatal capsaicin-treated mice (Fig. 6, A and B).

**Novel Expression of VR1 on Neonatal Capsaicin-Insensitive Fibers after Nerve Injury.** To confirm our speculation that partial sciatic nerve injury caused novel expression of capsaicin receptors on neonatal capsaicin-insensitive fibers, immunohistochemical double-labeling was performed on DRG neurons with antibodies to VR1, the putative capsaicin receptor, and N-52, a marker of the myelinated A-fiber. As shown in Fig. 7A, in naive DRG VR1-immunoreactive neurons were not colocalized with N-52, indicating their presence on unmyelinated primary afferents. After neonatal capsaicin treatment, the VR1-immunoreactive neurons almost completely disappeared (Fig. 7B). However, after partial sciatic nerve injury in neonatal capsaicin-treated mice, large numbers of VR1-immunoreactive neurons were observed that were mainly colocalized with N-52 (Fig. 7C).

**Discussion**

Therapeutic value of topical capsaicin in various chronic pain states has always been under question. Moreover, its pungent and strong irritating nature makes it unacceptable to many patients in clinic. However, clinical evidence suggests its usefulness in chronic pain relief (Watson et al., 1988; The Capsaicin Study Group, 1991; Low et al., 1995; Ellison et al., 1997). In the present report, a new-type capsaicin cream was nonirritating in nature when applied to mouse footpad. Moreover, the cream did not produce thermal hyperalgesia in contrast to the capsaicin ointment (Fig. 1). We speculate that the water-based formulation of the cream may underlie such differences due to slower absorption of the cream than the ointment. Because the capsaicin cream contained a more hydrophilic-type cream base than the capsaicin ointment, the rate of absorption of the capsaicin ointment from skin surface is expected to be considerably faster than the capsaicin cream. This might cause a rapid and lasting sensitization of the nociceptors by the ointment, giving pronounced thermal hyperalgesia. However, a more detailed pharmacokinetic study for the apparent differences between the two formulations remains to be done. Another important finding of the present report is that although the cream was nonanalgesic to thermal stimulation in naive mice, it was analgesic to peripheral receptor ligand-induced nociception in ANF test (Figs. 2, A and B, and 3A). The analgesic action of the cream diminished by 12 h after application, indicating the reversible nature of the desensitizing effect (Fig. 2A). Moreover, the effect of the cream was not due to tachyphylaxis, which represents gradually diminishing effects due to repeated stimulations by an agonist (Liu and Simon, 1996). Although the type I (BK and N/OFQ) and type II (ATP) nociceptive responses were blocked by the cream in naive mice (Fig. 3B), the type III nociception induced by PGI2 agonist ONO-54918-07 could not be blocked, indicating that these responses were mediated through capsaicin-sensitive sensory neurons in naive conditions (Fig. 3C). On the other hand, PGI2-induced sensitization of capsaicin-sensitive sensory neurons has also been reported elsewhere (Pitchford and Levine, 1991; Hingtgen and Vasko, 1991).
Nevertheless, there has been very limited information on the ability of prostaglandin I\(_2\) receptor agonists to produce nociception by directly activating the sensory neurons. In our experiments, the PGI\(_2\) agonist ONO-54918-07 produced potent nociceptive flexion responses at extremely low doses (attomolar to picomolar ranges). These responses could not be abolished by neonatal capsaicin treatment, indicating their mediation through capsaicin-insensitive neurons (Ueda et al., 2000). Moreover, intrathecal pretreatment of mice with antisense oligodeoxynucleotide for prostaglandin I\(_2\) receptor completely abolished the nociceptive flexion responses of ONO-54918-07 (our unpublished data). This result clearly indicates that the nociceptive flexion responses of ONO-54918-07 were mediated through the prostaglandin I\(_2\) receptor at the peripheral nerve ending. We speculate that the sensitizing and the nociceptive effects of PGI\(_2\) might involve different mechanisms at the nerve endings. However, further biochemical and molecular experiments will only clarify the exact mechanism of the PGI\(_2\) agonist-induced nociception.

We examined the effect of the capsaicin cream under neuropathic pain condition. After partial sciatic nerve injury in mice, both the thermal and mechanical hyperalgesia were concentration dependently blocked by the capsaicin cream (Fig. 5, A and B). The potency of the cream was a little higher with the mechanical nociception test (mechanical test, EC\(_{50}\) value of 0.042%; thermal test, EC\(_{50}\) value of 0.066%). These
results indicate that the expression of putative capsaicin receptor, VR1 on neonatal capsaicin-insensitive fibers by immunohistochemistry. As shown in Fig. 7A, the VR1-positive neurons in naive mice were unlabeled by A-fiber marker N-52, indicating their presence on unmyelinated C-fibers. Consistent to the previous reports (Hiura and Ishizuka, 1989), neonatal capsaicin treatment caused almost complete loss of VR1-immunoreactive neurons (Fig. 7B). However, partial sciatic nerve injury in neonatal capsaicin-treated mice caused novel expression of VR1 receptors, which were mainly colocalized with A-fiber marker N-52 (Fig. 7C). These results confirm the speculation that capsaicin cream worked after nerve injury by desensitizing the newly expressed VR1 receptors mainly located on A-fibers. Recently, increase in VR1 protein expression on undamaged large-diameter DRG neurons has been reported after partial nerve injury in the rat (Hudson et al., 2001). Our results as well as these previous observations suggests that the analgesic effects of capsaicin cream in nerve injury-induced neuropathic pain might be due to desensitization of the newly expressed capsaicin receptors on previously capsaicin-insensitive fibers. On the other hand, our pharmacological classification of the nociceptive fibers into three distinct types does not exclude the presence of other functional types of fibers. Moreover, physiological role of these three types of fibers is yet to be studied. Nonetheless, the neonatal capsaicin sensitivity of type I and type II responses indicates their actions through small-diameter unmyelinated C-fibers, whereas the neonatal capsaicin insensitivity of the type III responses indicates these responses through large-diameter myelinated A-fibers. Moreover, our immunohistochemical results clearly support these propositions.

In conclusion, we demonstrate that the new-type capsaicin cream could be a better formulation than the currently available preparations to treat neuropathic pain because of its nonirritating and nonhyperalgesic effects. Our results indicate that the novel expression of VR1 receptors on previously capsaicin-insensitive fibers after peripheral nerve injury may account for the analgesic action of capsaicin cream in neuropathic pain.

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References


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