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Activation of TRPM8 cold receptor triggers allodynia-like behavior in spinally injured rats

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ABSTRACT

Aims: Pain in response to innocuous cold stimulation (cold allodynia) is a common symptom in patients with neuropathic pain. Cold allodynia is difficult to treat and its mechanisms are poorly understood. Several transient receptor potential (TRP) channels have been shown to be the molecular sensors for cold stimulation in a temperature-dependent manner, but the contribution of various TRP channels in mediating cold allodynia in neuropathic pain is unclear. We have previously shown that spinally injured rats developed neuropathic pain-like behaviors, including marked cold allodynia. We now assessed the role of TRP channels in mediating cold allodynia in rats after ischemic spinal cord injury.

Methods: Spinal cord injury was produced using a photochemical method. The mechanical allodynia was assessed by examining the vocalization thresholds to graded mechanical touch/pressure applied with von Frey hairs. Temperature controlled cold stimulation was produced by a Peltier thermode (active surface 25 mm × 50 mm) connected to a MSA Thermal Simulator (Somedic, Sweden) with baseline temperature of 32 °C. The rate of temperature change was 0.5 °C/s. The temperature required to elicit cold allodynia was examined. The responses of the rats to topical application of icilin or menthol, agonists of transient receptor potential melastain 8 (TRPM8), were also studied.

Results: Normal rats did not exhibit nociceptive responses to cooling stimulation to the trunk and back area (minimal temperature +6 °C) and they also did not react aversively to topical application of icilin or menthol. After spinal cord injury, the rats developed mechanical allodynia at the trunk and back just rostral to the dermatome of the injured spinal segments. In the same area, rats exhibited significant nociceptive responses to cooling from day 1 after injury, lasting for at least 70 days which is the longest time of observation. For the first two weeks after injury, the majority of spinally injured rats had a nociceptive response to cooling above 17 °C. At day 70, about 50% of rats responded to cooling above 17 °C. Topical application of 400 μM icilin or 4 mM menthol also elicited pain-like responses in spinally injured rats and these two cold mimetics also significantly exacerbated existing mechanical allodynia.

Conclusions: Our results showed that activation of the TRPM8 channel by menthol or icilin triggers allodynia in spinally injured rats and increases, rather than decreases, mechanical allodynia. TRPM8 channels which respond to cooling above 17 °C may be involved at least in part in mediating cold allodynia in the rat model of neuropathic spinal cord injury pain.

Implications: The work introduced a method of quantitative testings of responses of rats to cold stimulation and may contribute to the understanding of mechanisms of cold allodynia after injury to the nervous system.

1. Introduction

Cold stimulation activates a small group of sensory neurons to generate distinct sensations that range from pleasantly cool to nociceptive aching [1,2]. The sensation of cool is mediated by Aδ and C fibers that respond to temperatures below 30 °C [3], while painful cold sensation is transmitted through C fibers with activation threshold below 20 °C [4]. Recent studies of the transient receptor potential (TRP) family of ion channels have identified two TRP channels, TRPM8 and TRPA1, as the primary target for sensing cool and noxious cold with the activation threshold below 28 °C and 17 °C, respectively [1,2,5,6]. TRPM8 are expressed in afferents of both nociceptors and non-nociceptors, while TRPA1 transcripts were almost exclusively found in nociceptive afferents [6].

Cold hypersensitivity (hyperalgesia/allodynia) is a common symptom in patients with neuropathic pain, including spinal cord

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injury pain [7–9]. Cold allodynia-like behaviors can also be readily observed in various rodent models of neuropathic pain [10–13]. The role of the two cold sensing TRP receptors (TRPA1 and TRPM8) in mediating cold hyperalgesia/allodynia after nerve injury has been studied in recent years. It has been shown that nerve injury induced a nerve growth factor (NGF) dependent up-regulation of TRPA1, but not TRPM8, in sensory neurons [14]. Furthermore, systemic application of the TRPA1 antagonist HC-030031 [15] or antisense knock down of TRPA1 [16] significantly reversed mechanical and cold hyperalgesia in rats after nerve injury.

Unlike TRPA1, the contribution of TRPM8 to nerve injury induced cold allodynia is controversial. Several studies have shown that pharmacological blockade or knocking out of TRPM8 attenuated cold hyperalgesia after nerve injury [17–20]. Katsura et al. [16], however, have shown that knock down of TRPM8 had no impact on the development of cold hypersensitivity after spinal nerve ligation. Moreover, it has also been suggested that activation of TRPM8 may produce analgesia in neuropathic pain [21,22].

We have previously reported that photochemically induced spinal cord injury in rats produced a chronic pain-like behavioral syndrome consisting of mechanical and cold hypersensitivity [10]. We also showed that cold hypersensitivity was mediated by capsaicin-sensitive afferents expressing TRPV1 [23]. In our previous work, the cold allodynia was induced by ethyl chloride spray which reduce the skin temperature to about 0 °C. In the current work, we assessed the cooling temperature required to elicit the cold allodynia in spinally injured rats. Furthermore, the response of spinally injured rats to topical application of the TRPM8 agonists icilin or menthol [6,24,25] was also studied.

2. Materials and methods

2.1. Animals

All experiments were approved by the Regional Research Ethics Committee. Female Sprague-Dawley rats (Möllegård, Denmark) weighing 250–300 g at the start of the experiments were used. Animals were housed 4 per cage at constant room temperature of 22 °C in a 12:12 h light–dark cycle with food and water *ad libitum*.

2.2. Photochemically induced spinal cord ischemic injury

The rats were anesthetized with Domitor (75 mg/kg ketamine + 1 mg/kg medetomidine in 1 ml/kg) and one jugular vein was cannulated. A midline incision was made in the skin overlying vertebral segments T12-L1. The animals were positioned beneath an argon laser beam and irradiated for 10 min with the beam directed toward vertebral segment T12 or T13 (spinal segments L3–5). Immediately prior to and 5 min after the start of the irradiation, erythrosin B (Red No. 3, Aldrich-Chemie, Steinheim, Germany) dissolved in 0.9% saline was injected i.v. at a dose of 32.5 mg/kg. A tunable argon ion laser (Innova model 70, Coherent Laser Product Division, Palo Alto, CA) operating at 514 nm was used. The average beam output power was 160 mW. During irradiation, the temperature of the rats was maintained 37–38 °C.

2.3. Determination of mechanical allodynia in spinally injured rats

The mechanical allodynia was assessed by examining the vocalization thresholds to graded mechanical touch/pressure applied with von Frey hairs. During testing the rats were gently restrained in a standing position and the von Frey hair was pushed onto the skin until the filament became bent. The area for testing of mechanical allodynia is lower back and flank area. The frequency of the stimulation was about 1/s and at each intensity 5–10 stimuli were applied. The intensity of stimulation which induced consistent vocalization (>75% response rate) was considered as pain threshold.

2.4. Cold stimulation using a Peltier thermode

Eight spinally injured rats which exhibited mechanical allodynia were used for experiments involving cold stimulation. Temperature controlled cold stimulation was produced by a fluid cooled Peltier thermode (active surface 25 mm × 50 mm) connected to a MSA Thermal Simulator (Somedic, Sweden) with baseline temperature of 32 °C. The rate of temperature change was 0.5 °C/s. The rats were held gently in a standing position and the thermode was pressed against shaved allodynic skin area. The temperature at which the animals vocalize was registered as cold pain threshold.

2.5. Topical application of icilin or menthol

In a separate group of spinally injured allodynic rats ($n = 12$), icilin or menthol at different concentrations was applied topically to the shaved allodynic skin using soaked cotton dressing (1.5 cm × 1.5 cm). Observation was made as to whether icilin or menthol treatment elicited vocalization and/or avoidance during 3 min. After removal of the dressing, mechanical response threshold was tested using von-Frey hairs for 60–90 min in the same area. The rats were used in multiple testings with at least three days in between.

2.6. Statistics

Experiments were conducted blindly as to the drugs applied. Data were presented as mean ± error of the mean (SEM) or median ± median absolute deviation (MAD), and were analyzed with ANOVA with repeated measurements followed by Dunnett's test or Wilcoxon signed rank test.

3. Results

3.1. The development of chronic mechanical hypersensitivities in spinally injured rats

The vocalization threshold of normal rats was 60–100 g. Similar to previously reported results [10,26], ischemic spinal cord injury in the present study induced marked mechanical hypersensitivity starting at day 1 with vocalization threshold of 2–6 g (Fig. 1). The low vocalization threshold was maintained at the same level for at

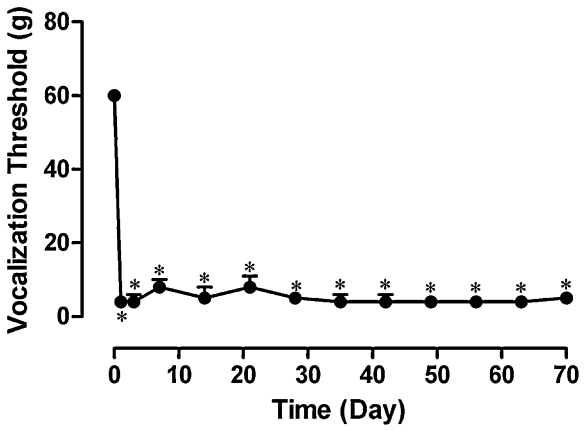

Fig. 1. The development of mechanical hypersensitivity measured as vocalization threshold to stimulation with von Frey hairs after spinal cord ischemic injury during 10 weeks ($N = 8$). Data were plotted as median ± MAD. $^*p < 0.05$ compared with baseline value at day 0 with Wilcoxon signed rank test.

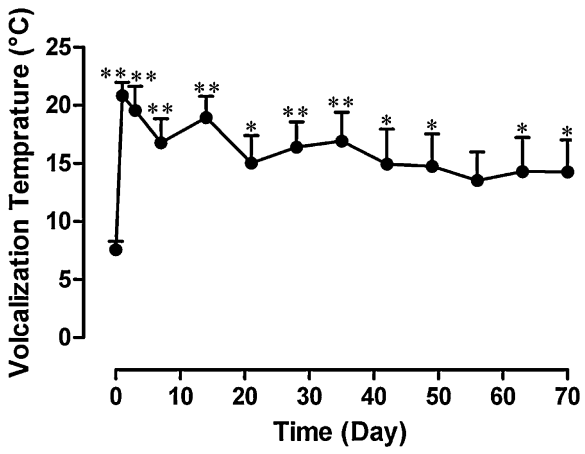

Fig. 2. The development of cold hypersensitivity after spinal cord ischemic injury during 10 weeks ($N = 8$). Data were presented as mean ± SEM. ANOVA with repeated measures suggested that there is a significant increase in response temperature to cold stimulation after spinal cord injury. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with baseline at day 0 with Dunnett test after ANOVA.

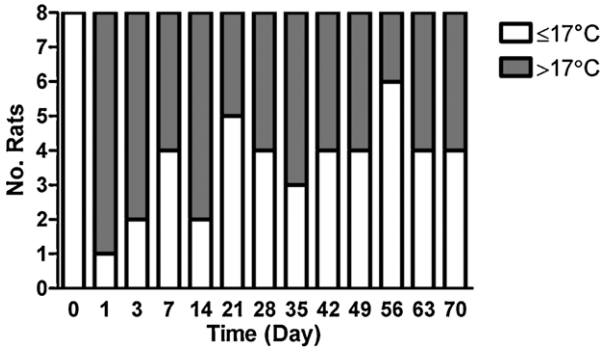

Fig. 3. Numbers of spinally injured rats that had pain response threshold to cooling with Peltier thermode > 17 °C (as shown in the shaded area in the column) and ≤17 °C (as shown as open area) after spinal cord ischemic injury for 10 weeks in 8 female rats.

least 70 days (Fig. 1). The bilateral area of mechanical allodynia was on the flank and lower back area.

3.2. Response threshold to cold stimulation by Peltier thermode

Normal rats had no aversive response to cooling by a Peltier thermode from 32 °C to 6 °C at the flank area. After spinal cord injury, all rats developed cold hypersensitivity as vocalization threshold was raised significantly from day 1 (Fig. 2), lasting for at least 70 days (Fig. 2). The cold hypersensitivity was most severe in the first two weeks with average vocalization threshold above 20 °C (Fig. 2).

Since it is generally accepted that temperature > 17 °C activate only TRPM8 whereas temperature ≤ 17 activate both TRPM8 and TRPA1 [2,27], we further illustrated the data using 17 °C as a dividing point (Fig. 3) and it can be seen that for most time points during the observation period, at least 50% of rats had vocalization threshold above 17 °C (Fig. 3).

3.3. Topically applied icilin or menthol elicited pain-like behaviors in spinally injured rats

In normal rats, topical application of up to 400 μM icilin or up to 4 mM menthol did not elicit pain-like behavior whereas in spinally injured rats (30 days after injury), the majority of rats tested showed vocalization threshold to topical application of 400 μM icilin or 4 mM menthol within 1–3 min (Fig. 4). No responses were

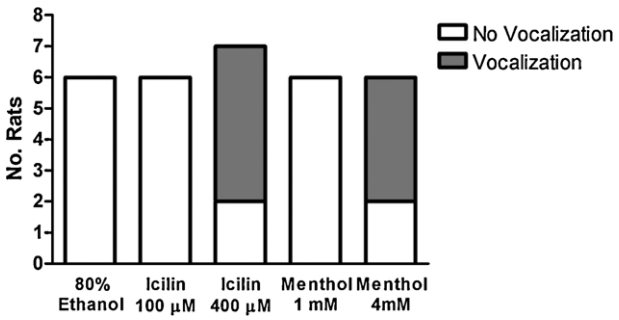

Fig. 4. Number of spinally injured rats that showed vocalization and other aversive pain-like responses to topical application of 80% ethanol (vehicle), icilin at 100 μM or 400 μM and menthol at 1 mM or 4 mM for 3 min. 6–7 female rats were included in each group.

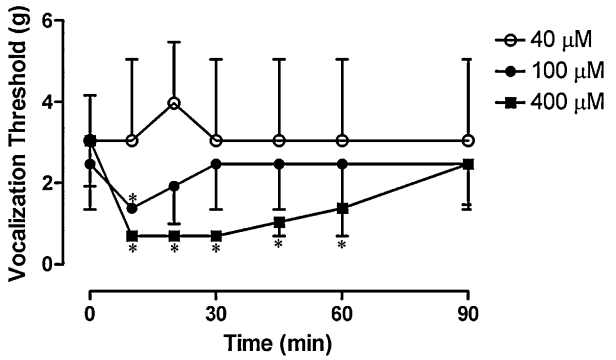

Fig. 5. The effect of 40, 100 and 400 μM topical icilin on mechanical response threshold in spinally injured rats. 6–7 female rats were included in each group and the data were shown as median ± MAD. ANOVA with repeated measures indicated a significant overall difference among the groups. $^{*}p < 0.05$ compared to with time 0 with Wilcoxon signed ranks test.

elicited by 80% ethanol (vehicle), 100 μM icilin or 1 mM menthol (Fig. 4).

3.4. Topical application of icilin or menthol enhanced mechanical allodynia in spinally injured rats

Topical application of icilin at 100 and 400 μM (Fig. 5) or menthol at 4 mM (Fig. 6) for 3 min significantly decreased vocalization threshold to mechanical stimulation in spinally injured rats, whereas lower doses of icilin or menthol and vehicle (80% ethanol) did not influence vocalization threshold to mechanical stimulation (Figs. 5 and 6).

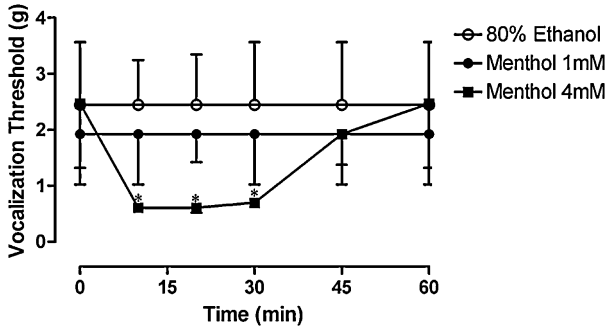

Fig. 6. The effect of topical application of 80% ethanol (vehicle) and menthol at 1 and 4 mM on mechanical response threshold in spinally injured rats. 6 female rats were include in each group and the data were shown as median ± MAD. ANOVA with repeated measures indicated a significant overall difference among the groups. $^{*}p < 0.05$ compared to with time 0 with Wilcoxon signed ranks test.

4. Discussion

We observed that cooling the skin to 6 °C by a Peltier thermode to the flank area did not elicit aversive responses in normal female Sprague-Dawley rats. Similarly, Allchorne et al. [28] reported that the threshold for eliciting a cold pain withdrawal response in rat hind paw is 5–9 °C using a Peltier-cooled device. After spinal cord injury and in accordance with the development of mechanical allodynia, the cold pain response threshold was also markedly decreased as the temperature required to elicit vocalization was significantly and persistently elevated. These results are in agreement with our previous finding using a non-quantitative cold spray [10] and support the notion that spinal cord injury in rats produced a localized cold allodynia at and rostral to the dermatome of injured spinal segments.

Almost all rats demonstrated a cold pain threshold above 17 °C immediately after injury. Although this rate decreased toward the end of the 10 week observation, around 50% of rats had consistent vocalization threshold above 17 °C, which is generally believed only to activate TRPM8 receptors [2,27]. Topical application of icilin or menthol at moderate doses also triggers pain-like responses in the majority of spinally injured, but not normal, rats. Menthol has long been thought to be a selective activator of the TRPM8 receptors [25] and icilin is a synthetic compound which is more potent and efficacious than menthol in this regard [24]. Although menthol and icilin have also been shown to activate TRPA1 receptors in some systems [29], the moderate doses we used suggest that TRPM8, but not TRPA1, receptors are likely to be involved [2,27]. Taken together, these results support a role for TRPM8 in mediating cold allodynia in central neuropathic pain after spinal cord injury. Our results are thus in agreement with some, but not all, previous studies using peripheral neuropathic models [18,19,21].

As indicated above, the doses of topically applied icilin or menthol that are required to trigger allodynia-like responses in spinally injured rats are moderate compared to those used in earlier studies [21] where these two cold-mimetics were shown to produce antinociception in a rat model of peripheral neuropathic pain. In contrast, our results suggest that at these dose ranges, icilin and menthol further increased existing mechanical allodynia in these rats. These results may reflect differences in the mechanisms of allodynia in central *vs.* peripheral neuropathic pain. In spinally injured rats, cold allodynia is mediated by increased responses of dorsal horn neurons, including both high threshold neurons and wide dynamic range neurons, to cold stimulation [30]. Treatment of spinally injured rats with a high dose of resiniferatoxin (RTX), which produces a substantial desensitization of capsaicin-sensitive afferents, abolished cold allodynia [23]. A sub-population of capsaicin sensitive afferents expresses TRPM8 receptors where they coexist with TRPV1 receptors [2,6]. It is thus likely that these receptors are those responsible for mediating cold allodynia in our model. Responses of dorsal horn neurons to inputs from these TRPM8 receptors may be inhibited under normal conditions and disinhibition after spinal cord injury may increases responses of dorsal horn neurons, resulting in cold allodynia [30,31].

Our previous studies have shown that mechanical allodynia in spinally injured rats is not affected by RTX treatment [23]. However, the present results suggested that mechanical allodynia is increased by exposure to icilin or menthol. The time course of the development of mechanical and cold allodynia after spinal cord injury is related, but some differences exist. There have been several studies on the effects of topical menthol on responses to mechanical stimulation in humans, but the results were also inconsistent [32,33].

In conclusion, our results provide strong evidence that activation of TRPM8 receptors by modest cooling or by topical icilin or menthol in rats with spinal cord ischemic injury triggered pain-like response and these receptors may play an important role in mediating cold allodynia in central neuropathic pain.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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