

Physiological Research Pre-Press Article

Short title: TRPV1 and NGF expression in DRG after joint immobilization

Development of heat hyperalgesia and changes of TRPV1 and NGF expression in rat dorsal root ganglion following joint immobilization

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Summary

The aim of this study was to examine whether threshold to heat stimuli, and expression of transient receptor potential vanilloid1 (TRPV1) and nerve growth factor (NGF) in dorsal root ganglion (DRG) altered under conditions of long-term limb immobilization. A plastic cast was wrapped around the right limb from the forearm to the forepaw to keep wrist joint at 90° of flexion for 5 weeks. Heat hyperalgesia was tested using the plantar test at 6 hours after removing cast. The rats were perfused transcardially with 4% paraformaldehyde and DRGs were excised at 24 hours after removing cast. For size distributions of the TRPV1-IR and NGF-IR neuronal profile, the DRG area measurements over 1000 DRG neurons per animal were measured in each side, on both the immobilized (ipsilateral) and contralateral sides. Ipsilateral withdrawal latency was significantly shorter than contralateral sides. Ipsilateral percentage of immunoreactive neurons in the total DRG neurons was significantly higher than contralateral sides in TRPV1-IR and NGF-IR. Long-term casting induced heat hyperalgesia, and up-regulation and phenotypic change of TRPV1-IR and NGF-IR in DRGs on the immobilized side. These DRG alterations may involve heat hyperalgesia after long-term limb immobilization.

Keywords: long-term immobilization · pain · heat hyperalgesia · Transient receptor potential vanilloid1 · dorsal root ganglion

Joint immobilization by casting is commonly utilized for local resting after fracture. Allen and colleagues proposed that immobilization by cast or splint may play a role in the development of complex regional pain syndrome (CRPS), known as chronic pain (Allen *et al.* 1999). It has been reported that limb immobilization by casting alone (without a fracture) could induce similar symptoms and sign of CRPS in human and animal experimental study (Guo *et al.* 2004; Terkelsen *et al.* 2008). We previously developed a long-term joint immobilization model, which showed a joint contracture in the affected limb, and observed changes in electrophysiological responses of dorsal horn neurons to mechanical and motion stimuli (Ushida and Wills 2001). More recently, we detected phenotypic changes in calcitonin gene-related peptide (CGRP) expression both in dorsal root ganglion (DRG) and spinal deep layers in immobilized side (Nishigami *et al.* 2009). Our results suggested that long-term immobilization by casting changes function and chemical coding in DRG and spinal dorsal horn neurons, and these changes are partly responsible for pathological pain states in immobilized side.

Transient receptor potential vanilloid1 (TRPV1) and nerve growth factor (NGF) are implicated as key molecules in development and persistence of pain disorder particularly heat hyperalgesia. Heat hyperalgesia is largely absent in mice lacking TRPV1 (Caterina *et al.* 2000), and TRPV1 is up-regulated in DRG of inflammation and nerve injury models, which showed heat hyperalgesia (Fukuoka *et al.* 2002; Amaya *et al.* 2003). The level of NGF was elevated at inflammation site, retrograde transport of NGF to the DRG from the inflamed site up-regulated various pain-related molecules such as CGRP and

brain-derived neurotrophic factor (BDNF) (Donnerer *et al.* 1992; Cho *et al.* 1997), and led to hypersensitivity to heat. Therefore, we postulate that immobilization induces heat hyperalgesia as a result of TRPV1 and/or NGF up-regulation. The aim of the present study is to examine alternation of threshold to heat stimuli and expression of TRPV1 and NGF in DRG after long-term immobilization.

All procedures used in these experiments were approved by the Konan Woman's University Animal Care and Use Committee and were consistent with the guidelines of the International Association for the Study of Pain for the care and use of laboratory animals. Fourteen adult male Sprague-Dawley rats (250-300g; Japan SLC, Inc.) were used in this study. Long-term joint immobilization was maintained by using a plastic cast as described previously (Ushida and Wills 2001). The rats were anesthetized with tribromoethanol (200 mg/kg i.p.), and a plastic cast was wrapped around the right limb from the forearm to the forepaw to keep wrist joint at 90° of flexion for 5 weeks.

Heat hyperalgesia was tested using the plantar test at 6 hours after removing cast. Latency until the rat lifted the forepaw was recorded. The measurements of latency were taken at least fourth times in each forepaw and alternatively with longer than 3 min. intervals between consecutive tests. The measurements of latency per side were averaged.

At 24 hours after removing cast, the six rats were perfused transcardially with 4% paraformaldehyde and C7 to C8 levels DRGs, which innervate the wrist joint in the rat, were excised and used for

immunohistochemistry. All of the sections were cut on a cryostat at a thickness of 12 μm , incubated in the primary antibody to TRPV1 (1:1000, Alomone Lab, Israel) or NGF (1:1000, Abcam, UK) for 48 hours at 4° C, incubated in biotinated anti-rabbit IgG from goat (1:1000, Vector Labs, UK) for 2 hours at room temperature, and incubated in avidin-biotin complex (1:100, Vector Labs, UK). All of the sections were visualized using diaminobenzidine staining.

Ten sections in ipsilateral and contralateral DRGs were extracted per animal at random for analysis. The ratio of TRPV1-IR and NGF-IR neurons to total number of neurons were calculated in each side. For size distributions of the TRPV1-IR and NGF-IR neuronal profile, the DRG area measurements over 1000 DRG neurons per animal were measured in each side, on both the immobilized (ipsilateral) and contralateral sides, using imaging analysis software (NIH Image). Only immunoreactive neurons with visible nuclei were selected for measurement. The area measurements were performed blindly by using randomized sets of digital photos of DRGs

The data were expressed as median and inter-quartile range. The differences in latency of hot plantar test and in the number of immunoreactive neurons between the ipsilateral and contralateral sides were assessed using Mann-Whitney's U test. The differences in size distribution of immunoreactive neurons were assessed using Mann-Whitney's U test for each 300 μm^2 size. Statistically significant differences between groups were expressed as p values less than 0.05 ($p < 0.05$).

Ipsilateral withdrawal latency was significantly shorter than contralateral sides (4.9, 4.1-5.2 sec vs 6.0, 5.7-6.6 sec). Ipsilateral percentage of immunoreactive neurons in the total DRG neurons were significantly higher than contralateral sides in TRPV1-IR and NGF-IR (Fig.1 A and 2 A: TRPV1; 33.6, 30.6-35.8 % vs 23.5, 22.0-27.8 %: NGF; 34.0, 30.0-35.9 % vs 23.5, 21.7-24.5 %). The TRPV1-IR and the NGF-IR cell size in ipsilateral side significantly increased in 300-600 μm^2 compared to contralateral side (Fig.1 B and 2 B)

The present study revealed that long-term immobilization by casting induced heat hyperalgesia and an up-regulation and the shift in the size distribution of TRPV1-IR and NGF-IR neurons in DRGs.

Up-regulation of TRPV1 expression and phenotypic changes of TRPV1-IR neurons from small to medium-size lumbar DRG neurons which are myelinated A-delta fibers were observed after inflammation, suggesting that A-delta fibers in DRG neurons might contribute to the development and maintenance of inflammatory hyperalgesia (Amaya *et al.* 2003; Luo *et al.* 2008). In the present study, the ratio of TRPV1-IR neurons at the ipsilateral side reached about 1.5-fold the contralateral side and the 300-600 μm^2 size immunoreactive neurons were prominent. Although it is unclear which A-delta fibers and corresponding size of cervical DRG neurons are, long-term forelimb immobilization might up-regulate TRPV1 mainly in A-delta fibers like inflammation.

Similar changes (up-regulation and alternation in size distribution) were detected in the NGF expression in DRG neurons. NGF seems to have important functional significance in the development of heat hyperalgesia in conjunction with TRPV1, because heat hyperalgesia induced by injection of NGF is largely absent in TRPV1 null mice (Chuang *et al.* 2001). Therefore, TRPV1 expression might be induced by NGF expression. NGF is induced by inflammation and nerve injury in the rat (Donnerer *et al.* 1992; Obata *et al.* 2004). Previous studies have reported that immobilization induced marked inflammatory changes in synovium and primary inflammatory reaction (Michelsson and Hunneyball 1984; Kontinen *et al.* 1990). Considering these results, long-term casting might induce the inflammation and increase of NGF-IR neurons in the current immobilization model.

We have previously demonstrated that the population of wide dynamic range (WDR) neurons in the spinal deep layers on the immobilized side increased in this immobilized model (Ushida and Wills 2001). TRPV1 receptor antagonist inhibited heat hyperalgesia and electrical response of WDR neurons in the deep layers on day 1 and 14 after inflammation (Luo *et al.* 2008). Furthermore, systematic administration of TRPV1 receptor antagonist reduced the spontaneous firing of WDR neurons in inflammatory rat (McGaraughty *et al.* 2008). These results raise the possibility that an increase of TRPV1-IR in DRGs induce heat hyperalgesia through WDR neuronal activation after long-term immobilization.

In conclusion, long-term casting induced heat hyperalgesia, up-regulation and phenotypic change of

TRPV1-IR and NGF-IR in DRGs on the immobilized side. These DRG alterations may be involved in heat hyperalgesia after long-term limb immobilization.

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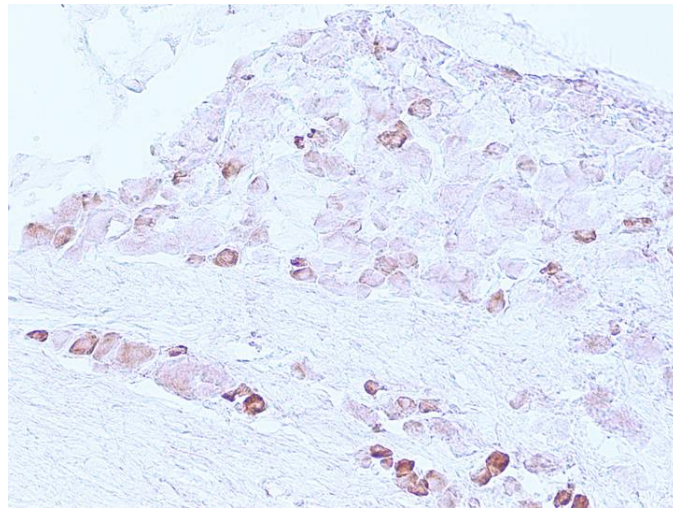
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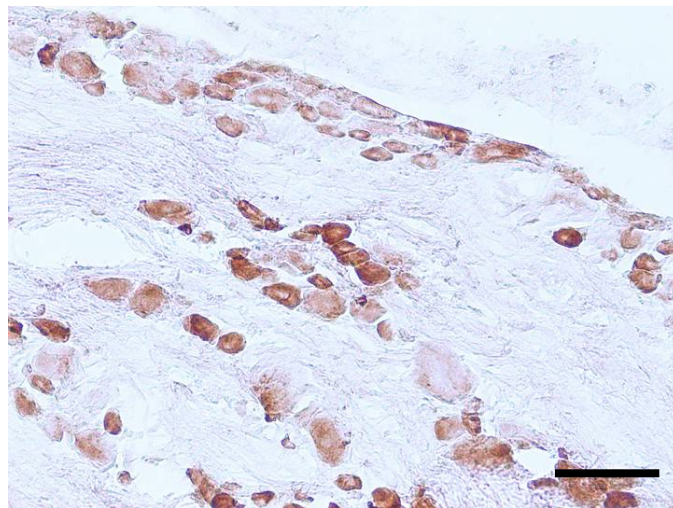
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Contralateral side



Ipsilateral side

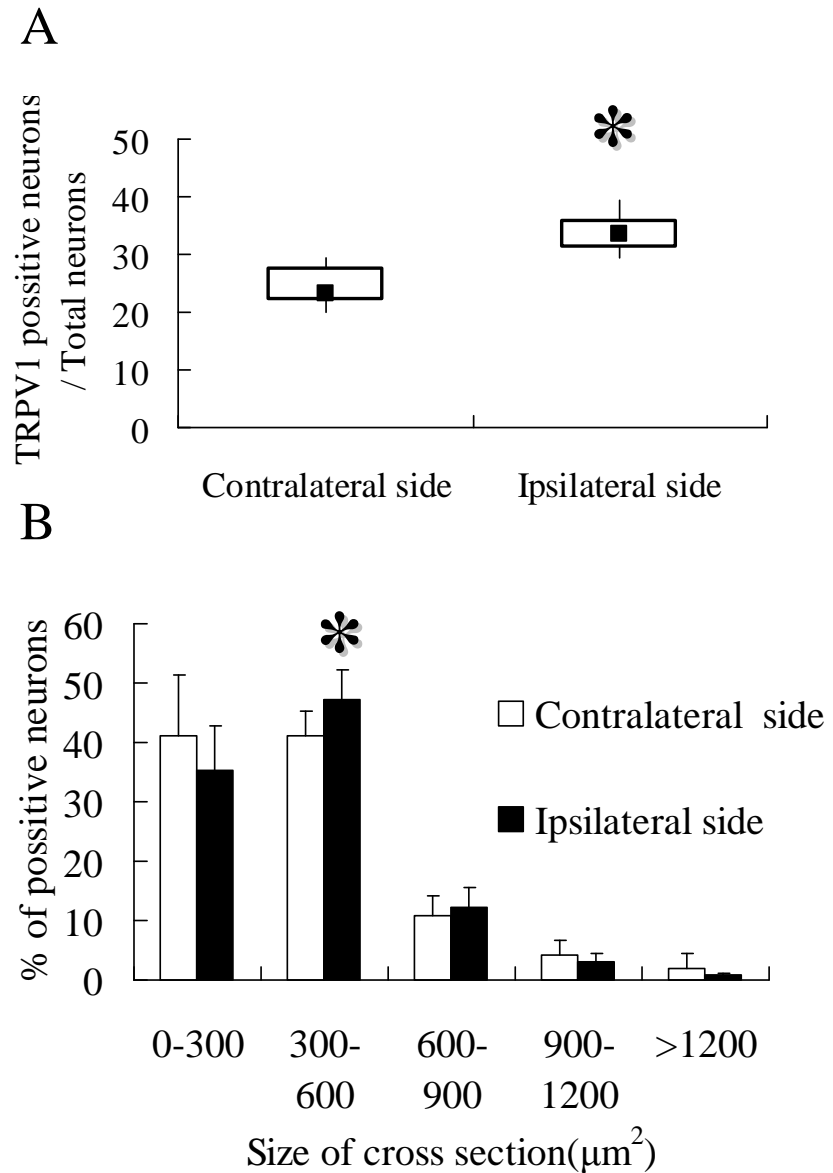
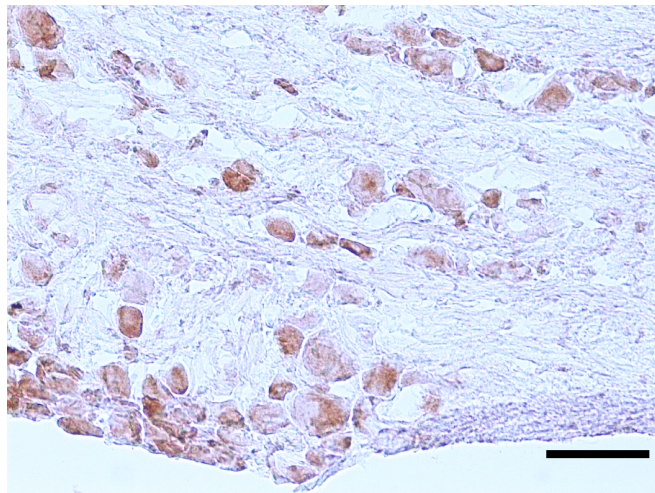


Fig.1. Significant differences in the number of TRPV1-IR DRG neurons to total neurons were found between ipsilateral and contralateral sides (A) The TRPV1 IR cell size on the ipsilateral side significantly increased in 300-600 μm² compared to contralateral side (B). *p<0.05.



Contralateral side



Ipsilateral side

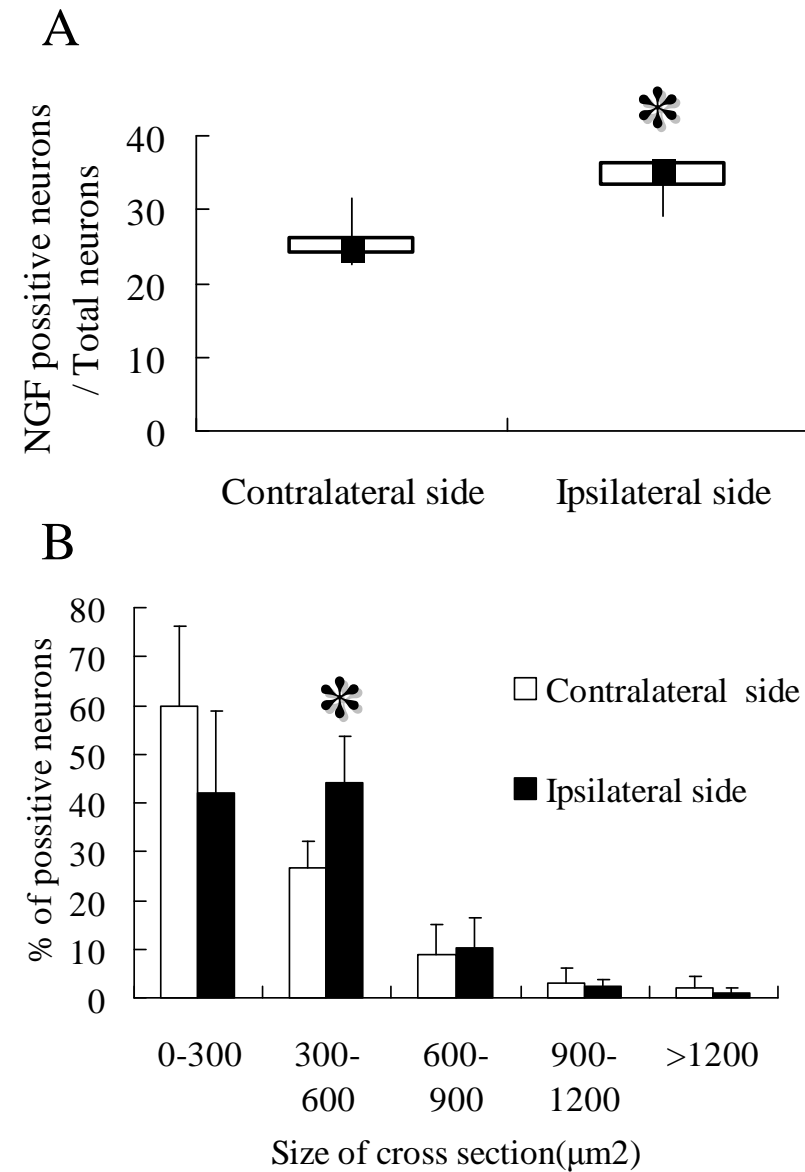


Fig.2. Significant differences in the number of NGF-IR DRG neurons to total neuron were found between ipsilateral and contralateral sides (A). The NGF-IR cell size on the ipsilateral side significantly increased in 300-600 μm^2 compared to contralateral side (B). * $p < 0.05$.