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Characterization of primary sensory neurons mediating static and dynamic allodynia in rat chronic constriction injury model

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Abstract

Mechanical allodynia, such as static and dynamic allodynia, is a prominent feature of neuropathic pain syndromes. The aim of this study is to characterize primary sensory neurons mediating the mechanical allodynia in a rat chronic constriction injury (CCI) model with a combination of pharmacological and histological investigations. *N*-(4-Tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2*H*)-carbox-amide (BCTC), a selective and competitive antagonist of the vanilloid receptor 1 (TRPV1), and resiniferatoxin, which causes desensitization of TRPV1-expressing fibres, suppressed static allodynia but not dynamic allodynia in CCI rats. Immunohistochemical studies of TRPV1 and NF200, an A-fibre marker 200 kDa neurofilament, in dorsal root ganglion neurons demonstrated that each 48% of the positive-stained neurons were immunoreactive only for TRPV1 or NF200. The other 4% of stained neurons were double-positive for TRPV1 and NF200. Of the TRPV1-positive neurons, more than 99% were small- (diameter <25 μm) and medium- (25–45 μm) sized. In contrast, 97% of NF200 single-labelled neurons were medium- and large- (>45 μm) sized. These findings suggest that two types of mechanical allodynia are transmitted by different primary sensory neurons: static allodynia is mediated by TRPV1 positive small- and medium-sized neurons and dynamic allodynia might be signalled by TRPV1-negative medium- and large-sized neurons.

Introduction

Cutaneous stimuli of various modalities activate different classes of afferent fibres associated with various receptor mechanisms (Burgess & Perl 1973). Mechanical allodynia is a prominent feature of neuropathic pain syndromes. It has been reported that two distinct types of mechanical allodynia, static and dynamic allodynia, can be detected in patients suffering from neuropathic pain (Ochoa & Yarnitsky 1993) and in a rat neuropathic pain model (Field et al 1999).

The vanilloids, such as capsaicin and its ultrapotent analogue resiniferatoxin (RTX), which chemically ablate vanilloid receptor 1 (TRPV1)-expressing fibres (capsaicin-sensitive fibres), have been used as selective tools to study the action of capsaicin-sensitive fibres in mediating mechanical allodynia. Field et al (1999) reported that multiple capsaicin treatment blocked the maintenance of static allodynia, but not dynamic allodynia in rats with chronic constriction injury (CCI), suggesting that static and dynamic allodynia are mediated by distinct primary sensory neurons: capsaicin-sensitive fibres and capsaicin-insensitive fibres, respectively. On the other hand, Hao et al (1996) and Ossipov et al (1999) reported that RTX treatment did not prevent static allodynia for more than 4 weeks after the treatment in rat neuropathic pain models, suggesting that capsaicin-insensitive fibres mediate static allodynia. Methodological differences among these studies, such as reagents and routes of administration, might cause the discrepant results.

Recently, *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2*H*)-carbox-amide (BCTC) is reported as a selective and competitive TRPV1 antagonist in-vitro (Valenzano et al 2003) and in-vivo (Pomonis et al 2003; Kanai et al 2005). Therefore, compared with capsaicin and RTX used in previous studies, BCTC would be a better tool to examine the involvement of capsaicin-sensitive fibres in mediating mechanical allodynia.

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Furthermore, histological investigations would show a detailed classification of primary sensory neurons that transmit static and dynamic allodynia.

The aim of this study is to characterize primary sensory neurons mediating static and dynamic allodynia in CCI rats with a combination of pharmacological and histological investigations. As well as RTX, BCTC was used to examine the role of capsaicin-sensitive fibres in maintenance of static and dynamic allodynia. Furthermore, dorsal root ganglion (DRG) neurons expressing TRPV1 or an A-fibre marker 200 kDa neurofilament (NF200), or both, in CCI rats were categorized as small- (<25 μm), medium- (25–45 μm) and large- (>45 μm) sized according to their diameter.

Materials and methods

Animals

Male Sprague–Dawley rats (7 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). The rats were acclimatized to the laboratory conditions during experiments. The experiments were carried out according to a protocol approved by the animal ethics committee at the Nagoya Laboratories of Pfizer Global Research and Development.

CCI model

The CCI was made according to the method of Bennett & Xie (1988). Briefly, rats, 251–293 g, were anaesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.). The left common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through biceps femoris. Proximal to the sciatic's trifurcation, about 7 mm of nerve was freed of adhering tissue and 4 ligatures were loosely tied around it by using 4-0 silk thread (Ethicon Inc., Brussels, Belgium) with about 1 mm space. The length of nerve thus affected was 4–5 mm long. The incision was closed in layers. Sham operation was performed in the same manner except for the sciatic nerve ligation.

Measurement of static and dynamic allodynia

The effect of drugs on static and dynamic allodynia was measured 2 weeks after the surgery as described previously (Field et al 1999). The rats were habituated to grid bottom cages before the start of the experiment.

Static allodynia was evaluated by the application of von Frey hairs (VFHs; Semmes-Weinstein Monofilaments, North Coast Medical Inc., San Jose, CA) in ascending order of force (0.16, 0.4, 0.6, 1, 1.4, 2, 4, 6, 8, 10, 15 and 26 g) to the plantar surface of the hind paw. Each VFH was applied to the paw for 6 s, or until a withdrawal response occurred. Once a withdrawal response was observed, the paw was re-tested, starting with the next descending VFH until no response was noted. The lowest amount of force required to elicit a response was recorded as the withdrawal threshold (PWT) in g. Static allodynia was defined as being present if the rats responded to the 2 g VFH or below, which was innocuous to normal or sham-operated rats.

Dynamic allodynia was assessed by lightly stroking the plantar surface of the hind paw with a cotton bud. Care was taken to perform this procedure in fully habituated rats that were not active to avoid recording general motor activity. Latency to paw withdrawal (PWL) was noted. If no reaction was exhibited within 15 s the procedure was terminated and rats were assigned this withdrawal time. Thus, 15 s effectively represented no withdrawal. Dynamic allodynia was considered to be present if rats responded to the cotton stimulus after less than 8 s of stroking.

Immunohistochemistry and quantitative analysis

For immunohistochemistry, three rats that received CCI surgery 2 weeks before were used. These rats were deeply anaesthetized with sodium pentobarbital (70–80 mg kg^{-1}, i.p.) and perfused transcardially with 200 mL of 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 300 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The L4–L5 DRG was dissected out and post-fixed overnight in the same fixative at 4°C, and cryoprotected in graded sucrose solutions of 10%, 15% and 20% in PBS. The tissue was then embedded in Tissue-Tek OCT compound, quickly frozen to –50°C, sectioned on a cryostat at an 8 μm thickness and mounted onto glass slides.

The sections were blocked in CAS BLOCK (ZYMED Laboratories, South San Francisco, CA) containing 10% normal donkey serum and 0.3% Triton X-100 for 2 h, then incubated overnight at room temperature with the mixture of primary antibodies diluted in blocking solution. Subsequently, sections were washed in PBS and incubated with the secondary antibody mixture for 2 h at room temperature. Primary antibodies were used at the following dilution: goat anti-TRPV1 N-terminus (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA); mouse anti-NF200 (1:3000; Sigma, St Louis, MO). Secondary antibodies were used at the following dilutions: rhodamine red-X conjugated donkey anti-goat immunoglobulin G (3 μg mL^{-1}; Jackson ImmunoResearch Laboratories Inc., West Grove, PA); Alexa Fluor 488 conjugated donkey anti-mouse immunoglobulin G (3 μg mL^{-1}; Molecular Probes Inc., Eugene, OR). Finally, sections were rinsed, coverslipped and examined on a laser confocal microscope (LSM5 PASCAL; Carl Zeiss, Oberkochen, Germany).

For each ganglion, two sections were randomly selected to measure and calculate the size frequencies and percentages of the different cell populations. Only cell bodies with a clearly visible nucleus were counted. To analyse TRPV1-positive neuronal profiles, we categorized the DRG neurons as small- (<25 μm), medium- (25–45 μm) and large- (>45 μm) sized according to their diameter.

Reagents

BCTC was synthesized at Pfizer Global Research and Development Nagoya Laboratories according to the method described in patent application WO 02/08221, filed by Neurogen Corporation. The compound was suspended in vehicle (0.5% methylcellulose solution–Tween80, 95:5 v/v) and administered orally at a volume of 5 mL kg^{-1}. RTX (Sigma,

St Louis, MO) was dissolved in vehicle (ethanol–Tween80–saline, 10:10:80 v/v/v). Following baseline behavioural evaluation, rats were subcutaneously injected with either 0.3 mg kg^{-1} RTX or vehicle. The rats were then tested on days 1, 4 and 7 after treatment. Some rats were orally administered with BCTC (10 and 100 mg kg^{-1}) or vehicle, and tested at 1, 2 and 4 h after administration.

Data analysis

Static allodynia data was expressed as the median force (g) with vertical bars in the 1st and 3rd quartiles. The dynamic allodynia data were expressed as the mean PWL (s) ± s.e.m. The data from the static allodynia studies were subjected to a Kruskal–Wallis test followed by an individual Dunn's test (or just an individual Mann–Whitney *U*-test where there were only two groups). The data from the dynamic allodynia studies were subjected to a one-way analysis of variance followed by Dunnett's *t*-test (or just a *t*-test where there were only two groups). $P < 0.05$ was regarded as significant. All calculations were performed by EXSAS ver 7.10 (SAS Institute Inc., NC).

Results

Effect of BCTC and RTX on CCI-induced static and dynamic allodynia

A total of 90 rats underwent CCI surgery. Two weeks after the surgery, approximately 60 rats showed both static and dynamic allodynia. CCI rats displayed static allodynia as shown by the reduction in PWT to 2 g or lower force compared with PWT of 10–26 g in sham-operated rats. CCI rats also displayed dynamic allodynia as shown by a PWL of 8 s or less compared with 10–15 s in sham rats. These rats were divided into subgroups for drug evaluation. Each subgroup consisted of 6–8 rats.

The oral administration of BCTC partially suppressed the maintenance of CCI-induced static allodynia in a dose-dependent manner, and significant anti-allodynic effects were observed at 10 mg kg^{-1} after 1 h ($P = 0.023$) and at 100 mg kg^{-1} after 1 h ($P = 0.003$) and 2 h ($P = 0.0026$) compared with those of vehicle treatment (Figure 1A). In contrast, BCTC treatment did not block the maintenance of CCI-induced dynamic allodynia even at the dose of 100 mg kg^{-1} (Figure 1B). No

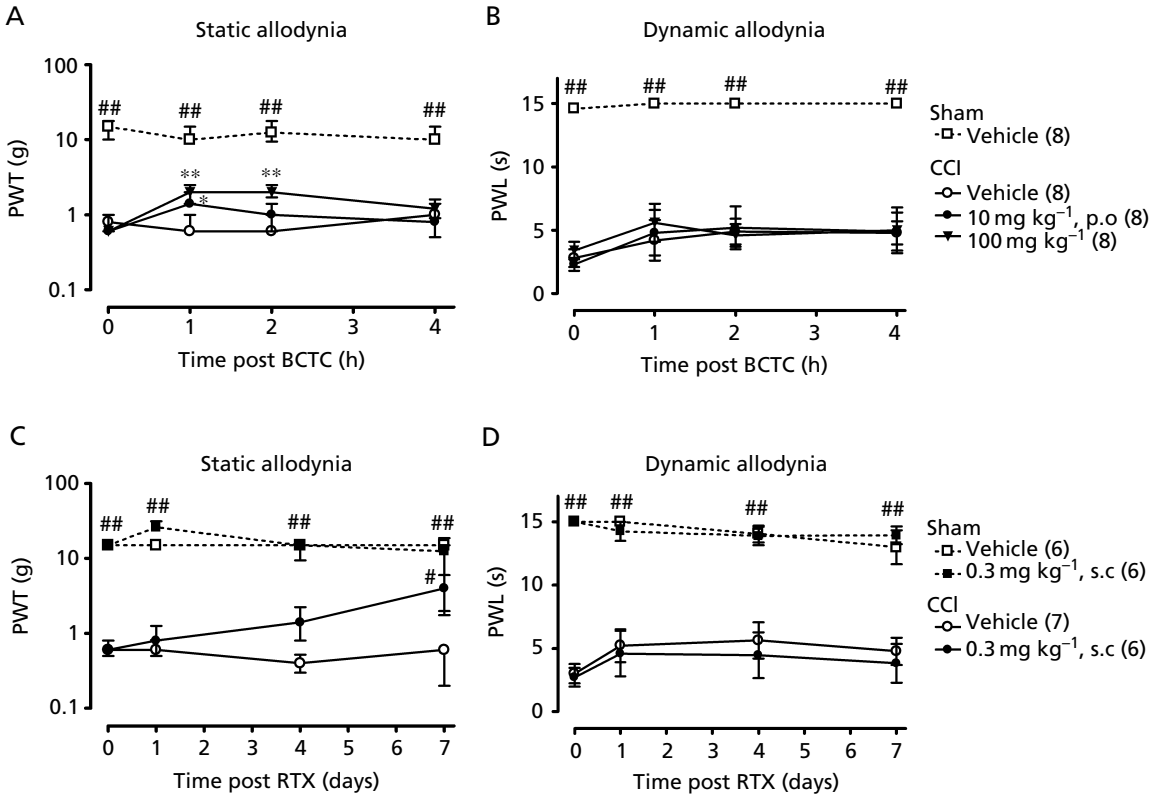

Figure 1 Effect of BCTC (A, B) and RTX (C, D) on CCI-induced static and dynamic allodynia in rats. Baseline paw withdrawal threshold (PWT) to von Frey hairs and paw withdrawal latencies (PWL) to a cotton bud stimulus were determined before drug administration and were reassessed at each time point. Results for static allodynia are expressed as the median force (g) required to induce paw withdrawal in 6–8 rats per group (vertical bars represent 1st and 3rd quartiles). Results for dynamic allodynia are expressed as the mean PWL (s) in 6–8 rats per group (vertical bars represent ± s.e.m.). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the CCI/vehicle group (Mann–Whitney test for A and C, *t*-test for B and D). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the CCI/vehicle group (Kruskal–Wallis test followed by Dunn's test). Parentheses indicates the number of rats used.

behavioural abnormalities were observed at any dose throughout the entire 4 h observation period.

The subcutaneous administration of RTX did not block the maintenance of static allodynia in CCI rats on day 1 after the treatment, although the anti-allodynic effect was markedly developed by day 7 ($P = 0.02$) (Figure 1C). On the other hand, RTX treatment failed to have any effect on dynamic allodynia throughout the experimental period (Figure 1D). RTX treatment did not affect either PWT or PWL in sham rats. To examine the desensitization of capsaicin-sensitive fibres, changes in paw withdrawal latencies to radiant heat were evaluated in sham and CCI rats at day 7 after RTX treatment. RTX significantly prolonged PWL of both sham and CCI rats (data not shown), suggesting that substantial desensitization of capsaicin-sensitive afferents was caused at this point in time.

Immunohistochemical examination of TRPV1 and NF200 in CCI rats

Immunohistochemical examination of TRPV1 and NF200 in the ipsilateral L4–L5 DRG sections was performed in CCI rats (Figure 2, Table 1). Among a total of 859 positive neurons in 11 DRG sections, 412 (48%) and 409 (48%) neurons were immunoreactive for only TRPV1 and only NF200, respectively.

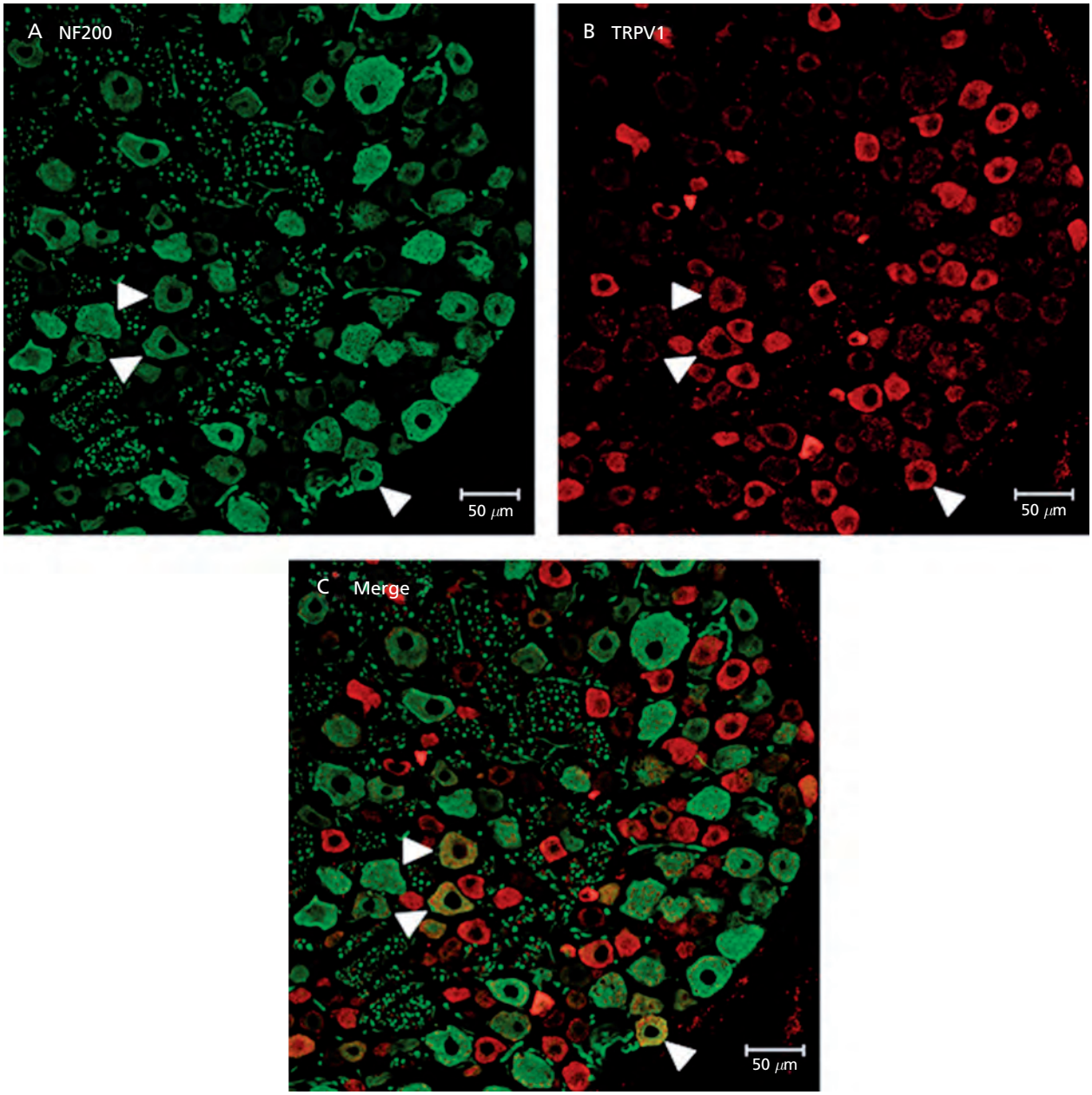

Figure 2 Double-labeling immunohistochemistry of TRPV1 and NF200 in L4–L5 DRG neurons from CCI rats. (A) NF200-immunoreactive neurons (green). (B) TRPV1-immunoreactive neurons (red). (C) Merged image: double-labeled neurons (arrowheads) appear as yellow staining. Scale bar: 50 μm.

Table 1 The number of DRG neurons that express TRPV1 or NF200, or both, in CCI rats

	TRPV1+[a]	TRPV1+/NF200+[b]	NF200+[c]
Small (<25 μm)	130	9	13
Medium (25–45 μm)	282	27	241
Large (>45 μm)	0	2	155

[a]Positive for TRPV1 only; [b]TRPV1 and NF200 double-labelled; [c]positive for NF200 only.

The other 38 (4%) neurons expressed both TRPV1 and NF200. In TRPV1-positive neurons, 99% (448/450 neurons) were small- (diameter < 25 μm) and medium- (25–45 μm) sized. In contrast, 97% of NF200 single-labelled neurons (396/409 neurons) were medium- and large- (>45 μm) sized.

Discussion

The aim of this study was to characterize primary sensory neurons, which mediate static and dynamic allodynia in CCI rats, using a combination of pharmacological and histological investigations. Although so far capsaicin and RTX have often been used as pharmacological tools to study the role of capsaicin-sensitive fibres on mechanical allodynia, there are discrepant reports regarding the involvement of capsaicin-sensitive primary afferents in mediating static allodynia in rat neuropathic pain models (Hao et al 1996; Field et al 1999; Ossipov et al 1999). The exact reason for this discrepancy is unclear, but it may be due to methodological differences. The difference in the site of nerve injury might be one of the reasons. Furthermore, the level of desensitization of capsaicin-sensitive fibres would vary depending on reagent type, dose and route of administration. Functional impairment of TRPV1 was not examined by capsaicin binding or Ca^{2+} influx assay, but thermal hypoalgesia was confirmed as a measure of desensitization in previous studies. Recently, BCTC has been reported as a selective and competitive TRPV1 antagonist (Pomonis et al 2003; Valenzano et al 2003; Kanai et al 2005). Therefore, BCTC could be a better tool to investigate the role of capsaicin-sensitive fibres in mediating the two types of mechanical allodynia. In this study, the effect of BCTC on static and dynamic allodynia was evaluated and compared with that of RTX. Both compounds suppressed static allodynia but not dynamic allodynia in CCI rats. This result is consistent with the finding of Field et al (1999) using capsaicin as a TRPV1-desensitizing agent. These data suggest that static and dynamic components of mechanical allodynia in the CCI model are transmitted by different primary sensory neurons and TRPV1-expressing fibres contribute to static allodynia but not dynamic allodynia.

For further examination of the primary sensory neurons mediating mechanical allodynia, DRG neurons expressing TRPV1 or NF200, or both, were histologically divided into 3 groups as small, medium and large according to their diameters. As several authors reported previously (Caterina et al 1997; Tominaga et al 1998; Michael & Priestley 1999; Ma 2002), our data shows that most of all TRPV1-positive neurons were classified as small- and medium-sized. On the other hand, most of the NF200-positive neurons were categorized into medium- and large-sized. Furthermore, only a few neurons were both TRPV1 and NF200 positive. Based on these results, static allodynia is mediated by TRPV1-positive small- and medium-sized neurons and dynamic allodynia might be signalled by TRPV1-negative medium- and large-sized neurons.

Field et al (1999) and preliminary experiments conducted in our laboratory revealed that pregabalin, the gold standard for the treatment of neuropathic pain, inhibited dynamic allodynia as well as static allodynia in the rat CCI model. Furthermore, it was reported that the analgesic actions of pregabalin are mediated through the alpha2-delta-1 subunit of voltage-gated calcium channels (Field et al 2006). It would be interesting to examine the DRG neurons expressing alpha2-delta-1 subunit and compare the size distributions of TRPV1-expressing neurons for further characterization of the primary sensory neurons mediating dynamic allodynia.

TRPV1 is a ligand-gated non-selective cation channel (Caterina et al 1997) and is recognized as a polymodal nociceptor, which integrates multiple pain stimuli (e.g., noxious heat, protons and vanilloids) (Tominaga et al 1998). As described earlier, BCTC effectively reversed hypersensitivity to mechanical stimuli (von Frey hair) after CCI. Detailed mechanisms of how TRPV1 participates in the pain transmission evoked by mechanical stimuli are unclear, since TRPV1 normally responds to heat, protons and vanilloids. However, TRPV1-immunoreactivity was increased not only in C-fibres, but also in myelinated A-fibres after nerve ligation (Hudson et al 2001), which could explain the ability of TRPV1 to participate in mechanical hypersensitivity after nerve injury. It is hypothesized that the TRPV1 receptor may contribute to the pain transmission evoked by mechanical stimuli via the release of glutamate and neuropeptides (calcitonin gene-related peptide and substance P) in the central terminal of primary afferents. This possibility is supported by recent studies (Hwang et al 2004; Kanai et al 2005; Roh et al 2007). Based on the present results, TRPV1 antagonists may have therapeutic potential in treating neuropathic pain elicited by some, but not all, modalities of stimulation.

Conclusions

We have provided evidence, based on the results from pharmacological and histological examinations, that two types of mechanical allodynia are transmitted by different primary sensory neurons. Static allodynia is mediated by TRPV1-positive small- and medium-sized neurons, and dynamic allodynia would be transmitted by TRPV1-negative medium- and large-sized neurons.

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