

# Distinct calcitonin gene-related peptide expression pattern in primary afferents contribute to different neuropathic symptoms following chronic constriction or crush injuries to the rat sciatic nerve

Yu Zou, PhD<sup>1,\*</sup>, Fangting Xu, PhD<sup>1,\*</sup>, Zhaohui Tang, MD<sup>1</sup>,  
Tao Zhong, PhD<sup>1</sup>, Jiawei Cao, MD<sup>1</sup>, Qulian Guo, PhD<sup>1</sup> and  
Changsheng Huang, MD<sup>1</sup>

## Abstract

Although calcitonin gene-related peptide is a recognized pain transducer, the expression of calcitonin gene-related peptide in primary afferents may be differentially affected following different types of nerve injury. Here, we examined whether different calcitonin gene-related peptide expression patterns in primary afferents contributes to distinct sensory disturbances in three animal models of sciatic nerve injury: chronic constriction injury, mild (100g force) or strong (1000g force) transient crush in rats. Assessments of withdrawal reflexes and spontaneous behavior indicated that chronic constriction injury and mild crush resulted in positive neuropathic symptoms (static/dynamic mechanical allodynia, heat hyperalgesia, cold allodynia, spontaneous pain). However, strong crush led to both positive (dynamic mechanical allodynia, cold allodynia, spontaneous pain) and negative symptoms (static mechanical hypoesthesia, heat hypoalgesia). Calcitonin gene-related peptide immunoreactivity in dorsal root ganglia and corresponding spinal cord segments, and calcitonin gene-related peptide mRNA levels in dorsal root ganglia, indicated that the primary afferent calcitonin gene-related peptide supply was markedly reduced only after strong crush. This reduction paralleled the development of negative symptoms (static mechanical hypoesthesia and heat hypoalgesia). Administration of exogenous calcitonin gene-related peptide intrathecally after strong crush did not alter heat hypoalgesia but ameliorated static mechanical hypoesthesia, an effect blocked by a calcitonin gene-related peptide receptor antagonist. Thus, reducing the primary afferent calcitonin gene-related peptide supply contributed to subsequent negative neuropathic symptoms, especially to static mechanical stimuli. Moreover, nerve injury caused a subcellular redistribution of calcitonin gene-related peptide from small- and medium-size dorsal root ganglia neurons to large-size dorsal root ganglia neurons, which paralleled the development of positive neuropathic symptoms. Intrathecal administration of the calcitonin gene-related peptide receptor antagonist ameliorated these positive symptoms, indicating that the expression of calcitonin gene-related peptide in large-size dorsal root ganglia neurons is important for the positive neuropathic symptoms in all three models. Taken together, these results suggest that distinct calcitonin gene-related peptide expression pattern in primary afferents contribute to different neuropathic symptoms following chronic constriction or crush injuries to the rat sciatic nerve.

## Keywords

Calcitonin gene-related peptide, animal model, neuropathic pain, allodynia, hyperalgesia, sciatic neuropathy

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<sup>1</sup>Department of Anesthesiology, Xiangya Hospital of Central South University, Changsha, China

\*Yu Zou and Fangting Xu contributed equally to this paper.

## Corresponding author:

Changsheng Huang, Department of Anesthesiology, Xiangya Hospital of Central South University, 87 Xiangya Road, Changsha, China.  
Email: docthuang2005@hotmail.com

## Introduction

Nerve injury leads to a variety of sensory disturbances, which can be categorized into positive and negative symptoms. The positive symptoms include spontaneous pain and increased sensitivity to innocuous stimuli



(allodynia) or painful stimuli (hyperalgesia).<sup>1,2</sup> The negative symptoms manifest as hypoesthesia or hypoalgesia.<sup>3–5</sup> Different combinations of these symptoms are frequently observed in patients, contributing to the complexity of chronic neuropathic sensory abnormalities.<sup>6</sup> Maier et al.<sup>7</sup> found that nearly half of 1236 patients with neuropathic pain had a mixture of positive and negative sensory symptoms, 26.1% had only negative symptoms, with fewer (19.7%) showing only positive symptoms. In order to translate the symptoms into mechanisms, a variety of animal models of nerve injury has been developed<sup>8–11</sup>; however, most address only positive symptoms. The mechanisms underlying the interplay between positive and negative symptoms are not well understood.<sup>12</sup>

Somatic injury is detected by somatosensory neurons located in the dorsal root ganglia (DRG), and this information is relayed to the postsynaptic neurons in the dorsal spinal cord.<sup>13,14</sup> Calcitonin gene-related peptide (CGRP) has been recognized as a pain transducer in the primary afferents.<sup>15–19</sup> Intrathecal injection of CGRP produces significant reductions in the withdrawal latency to thermal and mechanical stimulation, indicating increased pain sensitivity,<sup>20–22</sup> which could be normalized by CGRP antiserum.<sup>23,24</sup> CGRP knockout mice fail to demonstrate hyperalgesia in response to capsaicin<sup>25</sup> or after induction of knee joint inflammation.<sup>26</sup> Together, these studies demonstrate that CGRP in the afferents is necessary for the transmission and regulation of pain information.

CGRP belongs to a group of neuropeptides that can be synthesized peripherally and have a definite role in the transmission of pain.<sup>27,28</sup> The peripheral nerve-induced upregulation of many of these neuropeptides in primary neurons and their projections has been linked to pain behavior, including neuropeptide Y and vasoactive intestinal peptide as well as substance P, bradykinin, and others.<sup>29–32</sup> The expression pattern of CGRP in primary afferents is markedly different in different models of nerve injury. Most studies have reported that after CCI, CGRP immunoreactivity remains unchanged<sup>33–35</sup> or only slightly reduced<sup>36</sup> in the primary afferents, although significant positive symptoms were observed. However, in models associated with negative symptoms, that is, nerve transection or crush injury, CGRP is markedly reduced in the injured afferents.<sup>37,38</sup>

In this study, we hypothesized that different expression patterns of CGRP may contribute to distinct sensory disturbances following nerve injury. We induced sciatic nerve injury by either CCI or crush with a 100g or 1000g force. The subsequent positive or negative symptoms were evaluated, and the expression pattern of CGRP was examined in the primary afferents. Additionally, exogenous CGRP and a CGRP receptor antagonist were used to ascertain the effect of CGRP

on the generation of positive or negative symptoms following sciatic nerve injury.

## Material and methods

### Animals

Adult male Sprague-Dawley rats weighing 200 to 250 g were housed in a temperature-controlled environment ( $23 \pm 2^\circ\text{C}$ ) with a 12-h reverse light-dark cycle. Food and water were available ad libitum. All protocols were performed in accordance with the Animal Care and Use Committee of Central South University and followed the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 8023, revised 1978). Every possible effort was made to minimize animal numbers and suffering.

### Nerve crush and CCI

Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), and the left sciatic nerve was exposed at mid-thigh level by blunt dissection. An artery clamp or hemostat forceps were applied to induce a crush lesion. The artery clamp and hemostat forceps were instrumented with strain gauges and calibrated by a force-sensing resistor (FSR400, Interlink Electronics, CA, USA) linked to an Avometer. The applied forces were 100 g and 1000 g for the artery clamp and hemostat forceps, respectively. The artery clamp or the hemostat forceps were placed perpendicularly to compress the middle of sciatic nerve for 10 s. Compression was repeated 3 times with a 5-s interval, as described by Attal et al.<sup>39</sup> For CCI, 4 snug ligatures (4–0 chromic gut) were placed approximately 1 mm apart around the nerve proximal to the trifurcation, according to the method of Bennett and Xie.<sup>40</sup> In sham-operated rats, the left sciatic nerve was exposed without being compressed or ligated. All surgeries were performed by the same operator.

### Behavioral testing

The behavioral tests performed included an evaluation of spontaneous pain-related behavior, withdrawal reflexes to static/dynamic mechanical and heat stimuli, and motor function. The rat sciatic nerve has a relatively distinct innervating territory in the plantar skin of the rat paw.<sup>9,41</sup> The lateral and the central portions of the plantar skin are innervated by the sciatic nerve, whereas the medial portion is innervated by the saphenous nerve. Therefore, the sciatic and saphenous nerve innervation territories were measured separately in both hind paws when rats received static/dynamic mechanical and heat stimuli. In each innervated territory, the stimuli were

applied to three separate places, which included footpad and non-footpad areas,<sup>41</sup> and the results from these three locations were averaged (Figure 1(a)). Behavioral testing was performed 1 day before and 3, 10, 21, and 28 days post injury (dpi) or sham surgery. For each time point following the operation, six animals were tested in each nerve injury or sham surgery group. Animals were habituated to the testers, the environment, and the handling procedures before the commencement of testing. All behavioral measurements were performed by the same observers, who were blinded to the animal groups.

Spontaneous pain was assessed by evaluating autotomy behavior and examining the spontaneous pain-related score (SPS). Animals were excluded from the study if autotomy induced severe damage to the paw, and the SPS was used to quantify spontaneous pain-related behaviors. Each animal was placed on the floor of a glass cylinder and observed for up to 5 min. Different positions of the hind paw were rated according to a numerical scale adapted from Attal et al.<sup>42</sup> as follows: (1) the paw rested lightly on the floor and the toes were in a ventroflexed position; (2) only the internal edge of the paw was pressed to the floor; (3) only the heel was pressed to the floor and the hind paw was in an inverted position; (4) the entire paw was elevated; (5) the animal licked the paw. The SPS was calculated by multiplying the amount of time the rat spent in each category by the scales reported above, and dividing by the total observation time. This is expressed by the following formula:  $(t1 + (2 \times t2) + (3 \times t3) + (4 \times t4) + (5 \times t5))/300$  s, where  $t1$ ,  $t2$ ,  $t3$ ,  $t4$ , and  $t5$  represent the duration of time (in seconds) spent in categories 1, 2, 3, 4, or 5, respectively. Three values corresponding to three blocks of 300 s each were averaged to determine the baseline SPS for each rat.<sup>42</sup>

The Hargreaves test<sup>43</sup> was used to evaluate the response of the rats to an infrared heat stimulus by means of a plantar algesimeter (Tes7370, Ugo Basile, Comerio, Italy). Rats were placed in clear plastic cages on an elevated glass plate. A constant-intensity radiant heat source was focused underneath the glass and aimed at the ventral portion of the hind paw. A digital timer automatically recorded the duration between the start of the stimulus and paw withdrawal. The heat withdrawal latency (HWL) was measured to the nearest 0.1 s. Measurements were repeated three times at intervals of 5 min, and the mean value of the three measurements was calculated. A cutoff time of 35 s was established to avoid tissue damage.

Mechanical sense includes static and dynamic components, which are typically signaled by different afferents. While static mechanical sensation is mediated by nociceptive small-fiber/neurons,<sup>44,45</sup> dynamic mechanical sensation is mediated by large-fiber/neurons.<sup>46,47</sup>

Therefore, these two components were measured separately in rats.

Von Frey filaments were applied to determine the static mechanical withdrawal threshold (SMWT). Animals were tested with von Frey filaments (Stoelting, Wood Dale, Italy) corresponding to the following forces: 0.6, 1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g. Each filament was applied to the ventral portion of the hind paw until the filament just bent and then was maintained in this position for 6–8 s.<sup>48</sup> The smallest filament that elicited a foot withdrawal response was considered the SMWT stimulus.

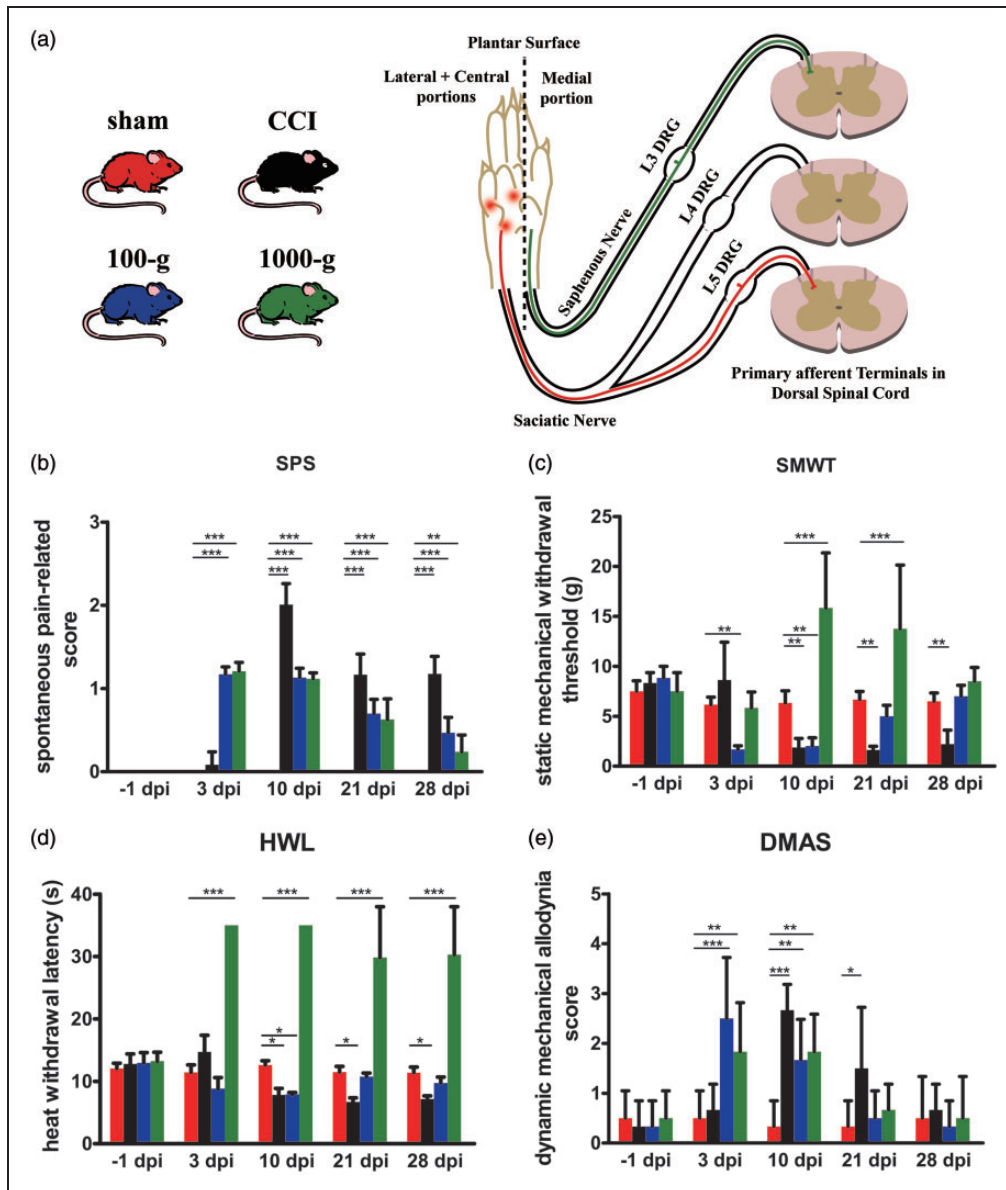
Hind paw withdrawal responses to a soft paintbrush were used to assess dynamic mechanical sensation.<sup>49</sup> The rat was placed in a cylinder with a wire mesh floor, and a soft paintbrush was used to stroke the plantar surface of the hind paw from heel to toes. The stimulus was applied five times with a 5-s interval. The dynamic mechanical allodynia score (DMAS) was defined as the total number of withdrawals (from 0 to 5).

### *Terms for stimulus-evoked positive and negative symptoms*

Increased and decreased sensitivity to stimuli were referred to as positive or negative symptoms, respectively. Probing with von Frey filaments and lightly stroking with a paintbrush are normally innocuous. Therefore, increased sensitivity to these stimuli was termed allodynia; reduced sensitivity was termed hypoesthesia. The Hargreaves test is painful for a non-injured animal. Therefore, an increase in sensitivity on this test was referred to as hyperalgesia, whereas a decrease was called hypoalgesia.

### *Immunohistochemistry and microscopy*

At 3, 10, 21, and 28 days following the operation, six rats in each nerve injury group and six rats in the sham-operated group on each day were deeply anesthetized and perfused transcardially with phosphate-buffered saline (PBS, 1 M, pH 7.4) followed by 4% paraformaldehyde. Because the rat sciatic nerve receives 98% of its sensory fibers from the fourth and fifth lumbar spinal nerves (L4 and L5)<sup>50</sup> (Figure 1(a)), the ipsilateral L4–L5 DRG and the related spinal cord segment were exposed and carefully harvested. Samples were postfixed in 4% paraformaldehyde at 4°C for 4 h (spinal cord segment) or 2 h (DRG), and cryoprotected in graded sucrose solutions (15% to 30%). Tissues were cut in 10- $\mu$ m transverse sections with a cryostat and collected onto gelatin-coated glass slides. Sections were blocked for 1 h with Animal Free Blocker (Vector, Burlingame, CA, USA) and then incubated with mouse monoclonal CGRP antibody (1:500, Abcam, Cambridge, UK) for 12 h at room



**Figure 1.** Neuropathic symptoms induced by different injuries. (a) Rats underwent chronic constriction injury (CCI), 100g crush injury, 1000g crush injury or a sham operation of the sciatic nerve. Neuropathic symptoms assessed included spontaneous pain in the affected hind paw and stimuli-evoked symptoms in the affected sciatic nerve-innervated plantar surface (lateral + central portions). Stimuli were applied to three separate regions (red areas) of each sciatic nerve-innervated plantar area, containing footpad and non-footpad regions, and the values were averaged. Symptoms were individually evaluated 1 day before injury, as well as 3, 10, 21, and 28 dpi, with  $n = 6$  in each of the four groups at each postoperative time point. (b) Spontaneous pain is observed after CCI, 100g crush injury, and 1000g crush injury, as demonstrated by increased spontaneous pain-related scores (SPS). (c) Static mechanical allodynia is induced by CCI and 100g crush injury, as demonstrated by a decrease in the static mechanical withdrawal threshold (SMWT), whereas static mechanical hypoesthesia is induced by 1000g crush injury, as demonstrated by an increase in the SMWT. (d) Heat hyperalgesia is induced by CCI and 100g crush injury, as demonstrated by decreased heat withdrawal latency (HWL), whereas heat hypoalgesia is induced by 1000g crush injury, as demonstrated by increased HWL. The cutoff latency was set at 35 s. (e) Dynamic mechanical allodynia is induced by CCI, 100g crush injury and 1000g crush injury, as demonstrated by decreased dynamic mechanical allodynia score (DMAS). Compared with sham-operated rats,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ .

temperature. Secondary detection was performed with a biotin-conjugated antibody and processed according to the instructions of the manufacturer using Vectastain Elite ABC Kit (Vector). After washes, sections were mounted and stored at 4°C.

For quantitative analysis, at least three sections from each spinal cord segment and three consecutive sections around the midline of the DRG, at intervals of 50 µm, were used. Images were captured with a light microscope (LEICA DM LB2; Leica Microsystems, Wetzlar, Germany), and CGRP-immunoreactivity (IR) was analyzed using Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). For spinal cord sections, the integrated density of CGRP-IR was measured within a region of interest (ROI, 200 × 150 µm) containing the central and medial part of the superficial dorsal horn corresponding to the area innervated by afferent projections from the sciatic nerve.<sup>51</sup> The integrated density of the CGRP-IR in the ipsilateral ROI was normalized to the corresponding area in the contralateral dorsal horn. Three sections from each spinal cord were averaged. For the DRG, the total number of cells with visible nuclei positive for CGRP-IR was counted, and three sections from each DRG were averaged. To distinguish cell size-specific changes, the cells with CGRP-IR in the DRG were characterized as small and medium (<1200 µm<sup>2</sup>) or large (>1200 µm<sup>2</sup>) based on their cross-sectional area.<sup>52</sup>

### Real-time quantitative polymerase chain reaction

Ten days after injury or sham surgery, rats were sacrificed under deep anesthesia ( $n = 8$  in each nerve injury or sham surgery group). The ipsilateral L4–L5 DRG were collected, snap-frozen in liquid nitrogen, and stored at –80°C until use. Total RNA was extracted from L4–L5 DRG tissues using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and subsequently subjected to reverse transcription using SuperScript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time quantitative polymerase chain reaction analysis was performed using an ABI PRISM 7900 sequence detector (Applied Biosystems, Foster City, CA, USA). The cDNA was amplified using the following primer pairs (forward and reverse): *calca*, GCATGGCCACT CTCAGTGAAG and CCTGACTTTCATCTGCATA TAGTTCTG; *calcb*, GCTTTGGAGAGCAGCCTAGA and CTGAGACCCTCAGTCTCTTG. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Thermal cycling was initiated with a 2 min incubation at 50°C, followed by a 10 min denaturation step at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Relative quantities of the candidate genes and GAPDH were calculated using the previously described comparative threshold cycle ( $\Delta\Delta Ct$ ) method.<sup>53</sup>

### Administration of CGRP or a CGRP antagonist and behavioral testing

Rats were subjected to CCI, 100g or 1000g crush injury, or sham surgery. Before surgery and 3 and 10 dpi, SMWT, HWL, DMAS, and SPS were measured as described above. CGRP (Tocris, Bristol, UK) with or without CGRP<sub>8–37</sub> (Sigma, St. Louis, MO, USA), a specific antagonist at the CGRP 1 receptor, were administered intrathecally via L4–L5 lumbar puncture.<sup>54</sup> Drugs were dissolved in artificial cerebrospinal fluid (ACSF) containing 150 mM Na<sup>+</sup>, 3.0 mM K<sup>+</sup>, 0.8 mM Mg<sup>+</sup>, 155 mM Cl<sup>+</sup>, 1.0 mM P<sup>+</sup>, and 3.87 mM glucose (Tocris). The rats in both the CCI and 100g force-injured groups were subdivided into two groups ( $n = 24$  for each subgroup). One subgroup received CGRP<sub>8–37</sub> (10 µl, 5 µM) and the other received ACSF (10 µl) as a control. Rats that underwent 1000g crush injury were divided into four subgroups ( $n = 12$  for each subgroup). The first subgroup received CGRP (10 µl, 0.5 µM), the second received CGRP (5 µl, 1 µM) plus CGRP<sub>8–37</sub> (5 µl, 10 µM), the third received CGRP<sub>8–37</sub> (10 µl, 5 µM), and the fourth received ACSF (10 µl) as a control. The doses and routes of administration were based on a previous report.<sup>22</sup> SMWT, HWL, DMAS, and SPS were examined 15, 30, 45, and 60 min after dosing. Because of the short time interval, SMWT, HWL, DMAS, and SPS could not all be evaluated for the same rat. Therefore, six rats in each group were evaluated for only a single measurement.

### Statistical analysis

Data are presented as the mean ± standard deviation (SD). Kruskal–Wallis followed by Dunn's test was used when comparing behavioral responses, CGRP-IR, and CGRP mRNA among different groups of rats with sciatic nerve injury. Two-way analysis of variance (ANOVA) with repeated measures, followed by Dunnett's test, was used when making comparisons among different groups of rats subjected to the intrathecal treatment. Statistical significance was set at  $p < 0.05$ .

## Results

### CCI and 100 g crush injury leads to positive neuropathic symptoms, whereas 1000g crush injury induces mixed positive and negative neuropathic symptoms

Spontaneous pain-related behaviors, such as excessive grooming and mild autotomy, were observed in all the three groups of rats with sciatic nerve injury. However, one rat that underwent CCI with a survival duration

of 28 days following surgery exhibited severe autotomy. One toe of the affected hind paw of this rat was bitten off; thus, this rat was excluded from the study. The SPS was used to quantify the spontaneous neuropathic symptoms. Before the operation, rats did not show spontaneous pain behavior. Therefore, the SPS was 0 in all groups. The SPS in sham-operated rats remained 0 during follow-up. By contrast, spontaneous pain-related behaviors were induced by the various injuries to the sciatic nerve, reflected by increases in the SPS of the affected hind paw. Compared with that in sham-operated rats, the SPS in rats with CCI peaked 10 dpi ( $2.01 \pm 0.25$ ,  $p < 0.001$ ) and remained elevated 21 dpi ( $1.17 \pm 0.25$ ,  $p < 0.001$ ) and 28 dpi ( $1.18 \pm 0.21$ ,  $p < 0.001$ ). In rats with 100g crush and 1000g crush, the SPS peaked 3 dpi (100g crush:  $1.17 \pm 0.09$ ,  $p < 0.001$ ; 1000g crush:  $1.21 \pm 0.11$ ,  $p < 0.001$ ) and remained significantly increased compared with that in sham rats 10 dpi (100g crush:  $1.13 \pm 0.11$ ,  $p < 0.001$ ; 1000g crush:  $1.12 \pm 0.07$ ,  $p < 0.001$ ), 21 dpi (100g crush:  $0.70 \pm 0.17$ ,  $p < 0.001$ ; 1000g crush:  $0.63 \pm 0.25$ ,  $p < 0.001$ ), and 28 dpi (100g crush:  $0.47 \pm 0.18$ ,  $p < 0.001$ ; 1000g crush:  $0.24 \pm 0.20$ ,  $p < 0.01$ ). These results indicated that CCI and 100g and 1000g crush to the sciatic nerve all evoked spontaneous pain-related behavior, although the time course and magnitude of the elevation in SPS varied with the injury type (Figure 1(b)).

The SMWT and HWL are the two most frequently used measurements for reflecting stimulation-evoked neuropathic symptoms. The SMWT, which was the response to normally innocuous pressure against the skin, remained relatively constant throughout the follow-up in sham-operated rats:  $7.50 \pm 1.05$  g before operation;  $6.17 \pm 0.75$  g at 3 dpi;  $6.33 \pm 1.21$  g at 10 dpi;  $6.67 \pm 0.82$  g at 21 dpi;  $6.50 \pm 0.84$  g at 28 dpi. Compared with that in sham-operated rats, in rats with CCI, the SMWT in the plantar territory innervated by the affected sciatic nerve was significantly decreased 10 dpi ( $1.88 \pm 0.89$  g,  $p < 0.01$ ), 21 dpi ( $1.63 \pm 0.36$  g,  $p < 0.01$ ) and 28 dpi ( $2.38 \pm 1.40$  g,  $p < 0.05$ ). Similarly, after 100g crush injury, a decrease in the SMWT was observed 3 dpi ( $1.68 \pm 0.37$  g,  $p < 0.01$ ) and 10 dpi ( $2.02 \pm 0.82$  g,  $p < 0.01$ ). In these 100g crush-injured animals, the SMWT returned to the basal level 21 dpi ( $5.00 \pm 1.10$  g) and remained at the basal level 28 dpi ( $7.00 \pm 1.10$  g). By contrast, compared with that in sham-operated rats, animals receiving 1000g crush injury displayed a marked increase in the SMWT 10 dpi ( $15.83 \pm 5.53$  g,  $p < 0.001$ ) and 21 dpi ( $13.75 \pm 6.39$  g,  $p < 0.001$ ). However, 28 dpi following 1000g crush injury, the SMWT returned to the basal level ( $8.50 \pm 1.38$  g) with no significant difference compared with that in the sham group. These results demonstrated that CCI induced chronic static mechanical allodynia and 100g crush injury induced transient static mechanical allodynia in the territory

innervated by the injured sciatic nerve within 28 days after injury. The 1000g crush injury initially led to static mechanical hypoesthesia, but this was ameliorated by the final observation (Figure 1(c)).

The HWL in the plantar surface innervated by the affected sciatic nerve in sham-operated rats was stable during the follow-up ( $12.04 \pm 0.87$  s before operation;  $11.44 \pm 1.19$  s at 3 dpi;  $12.60 \pm 0.70$  s at 10 dpi;  $11.49 \pm 0.91$  s at 21 dpi;  $11.39 \pm 0.90$  s at 28 dpi). Compared with that in sham-operated rats, after CCI, the HWL was significantly decreased 10 dpi ( $7.83 \pm 1.04$  s,  $p < 0.05$ ), 21 dpi ( $6.71 \pm 0.65$  s,  $p < 0.05$ ) and 28 dpi ( $7.17 \pm 0.49$  s,  $p < 0.05$ ). Rats with 100g crush injury displayed a similar reduction in HWL 10 dpi ( $7.91 \pm 0.29$  s,  $p < 0.05$ ), but the HWL returned to the basal level 21 dpi ( $10.74 \pm 0.58$  s) and remained at the basal level 28 dpi ( $9.74 \pm 0.96$  s). Rats with 1000g crush injury reached a HWL equivalent to the cutoff time, which was 35 s, at 3 dpi and 10 dpi ( $p < 0.001$ , versus sham-operated rats). In these 1000g crush-injured rats, the HWL remained significantly elevated 21 dpi ( $29.86 \pm 8.13$  s,  $p < 0.001$ ) and 28 dpi ( $30.31 \pm 7.67$  s,  $p < 0.001$ ) compared with that in sham-operated rats (Figure 1(b)). These results indicated that CCI induces chronic heat hyperalgesia and 100g crush injury leads to transient heat hyperalgesia in the plantar territory innervated by the injured sciatic nerve. By contrast, 1000g crush injury results in chronic heat hypoalgesia that lasts at least 28 days after the injury (Figure 1(d)).

The DMAS, which is a measure of an animal's response to a normally innocuous moving mechanical stimulus,<sup>55-58</sup> also reflects neuropathic symptoms. In sham-operated rats, the DMAS remained below 1 in the plantar surface innervated by the affected sciatic nerve during the follow-up ( $0.50 \pm 0.55$  before operation;  $0.50 \pm 0.55$  at 3 dpi;  $0.33 \pm 0.52$  at 10 dpi;  $0.33 \pm 0.52$  at 21 dpi;  $0.50 \pm 0.84$  at 28 dpi). Compared with that in sham-operated rats, after CCI, the DMAS was increased 10 dpi ( $2.67 \pm 0.52$ ,  $p < 0.001$ ) and 21 dpi ( $1.50 \pm 1.23$ ,  $p < 0.05$ ). Following 100g crush injury, the DMAS was elevated compared with that in sham-operated rats, peaking 3 dpi ( $2.50 \pm 1.23$ ,  $p < 0.001$ ) and remaining elevated 10 dpi ( $1.67 \pm 0.82$ ,  $p < 0.01$ ). Similarly, the DMAS in rats receiving 1000g crush injury was increased as early as 3 dpi ( $1.83 \pm 0.98$ ,  $p < 0.01$ ) compared with that in sham-operated rats, and the increase was maintained until 10 dpi ( $1.83 \pm 0.75$ ,  $p < 0.01$ ). These results indicated that CCI as well as 100g and 1000g crush injury to the sciatic nerve all lead to dynamic mechanical allodynia in the territory innervated by the injured sciatic nerve.

At 28 dpi, the DMAS in all three groups of rats that underwent nerve injury was reduced to the basal level (CCI:  $0.67 \pm 0.52$ ; 100g crush:  $0.33 \pm 0.52$ ; 1000g crush:  $0.50 \pm 0.84$ ), with no significant difference in

DMAS compared with that in sham-operated rats. These results are consistent with previous findings,<sup>44,59</sup> which showed that duration of dynamic mechanical allodynia was shorter than that of static mechanical allodynia and heat hyperalgesia (Figure 1(e)). In the saphenous nerve innervated territory of the affected hind paw and the plantar skin of the contralateral hind paw, changes in SMWT, HWL, and DMAS were not remarkable following sham operation, CCI, 100g crush injury, or 1000g crush injury (Supplemental Results).

In addition to the measurement of static/dynamic mechanical and thermal sensations, the withdrawal reflex to cold stimuli was examined in all groups of rats (Supplemental Results). The results demonstrate that CCI as well as 100g and 1000g crush injury to the sciatic nerve all induced cold allodynia in the affected hind paw. The development of cold allodynia was unrelated to the changes of CGRP in the afferents, consistent with previous findings that CGRP is not essential for cold sensitivity.<sup>60–62</sup>

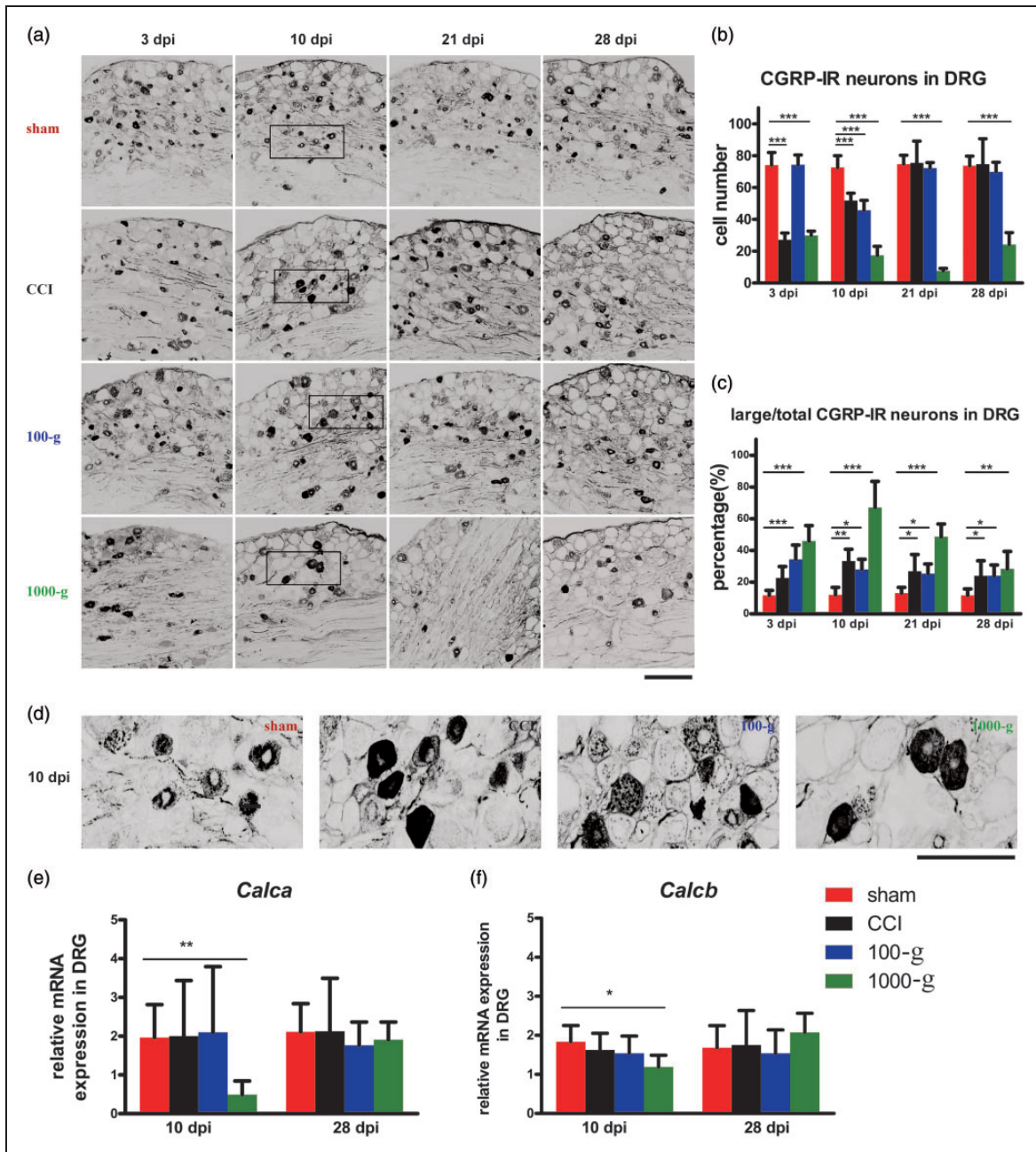
Sciatic nerve injury can also produce damage to motoneuron axons,<sup>63,64</sup> which was observed after CCI as well as 100g and 1000g crush injury in the present study (Supplemental Results). However, the protective flexor function of the withdrawal reflex was identified in all groups of rats. The reduced sensitivity to static mechanical and heat stimuli following 1000g crush injury was likely not due to an impairment of the flexor function or weakness of the paw as described previously<sup>65</sup> because the hyper-withdrawal reflex was observed when the same rats received dynamic mechanical and cold stimuli.

### **The supply of CGRP in the primary afferents is reduced after 1000g crush injury but remains unchanged following CCI or 100g crush injury**

CGRP is synthesized in DRG and transported anterogradely to the peripheral and central terminals of primary afferents.<sup>15,16,19</sup> In the present study, the dynamic expression of CGRP in L4–L5 DRG and dorsal spinal cord was determined using immunohistochemistry with a stereological technique. In sham-operated rats, the number of CGRP-IR neurons in the affected DRG remained stable at  $72.67 \pm 7.34$  to  $74.67 \pm 5.61$  from 3dpi to 28dpi. The normalized integrated density of CGRP staining within the region of the dorsal spinal cord where primary afferents of the sciatic nerve terminate also remained unchanged at  $100.50\% \pm 3.74\%$  to  $101.9\% \pm 9.13\%$  from 3dpi to 28dpi. However, CCI and 100g crush injury caused a transient reduction of CGRP-IR in the ipsilateral L4–L5 DRG. Compared with those in sham-operated rats, the number of

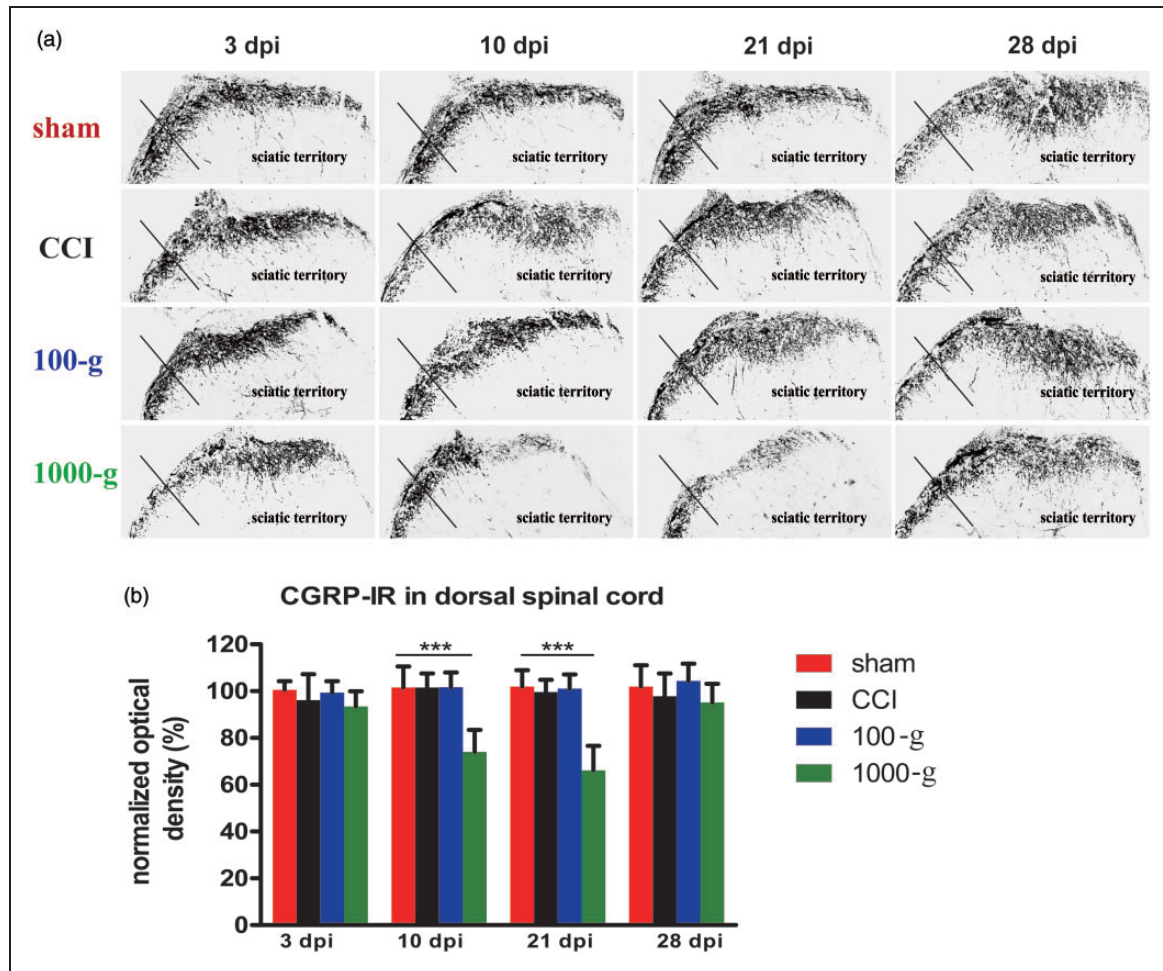
CGRP-IR neurons in rats with CCI was reduced to  $27.17 \pm 4.27$  ( $p < 0.001$ ) at 3dpi and  $51.83 \pm 4.62$  ( $p < 0.001$ ) at 10dpi, and in 100g crush injury to  $45.67 \pm 6.28$  ( $p < 0.001$ ) at 10dpi. At 21dpi and 28dpi in both CCI and 100g crush injury, the number of CGRP-IR neurons in DRG recovered to the same level as that in sham-operated rats (Figure 2(a) and (b)). By contrast, neither CCI nor 100g crush injury significantly altered the intensity of CGRP-IR in the sciatic nerve distributed region of the dorsal spinal cord (Figure 3(a) and (b)). The inconsistency of the CGRP-IR levels in the DRG and dorsal spinal cord might be because nociception enhanced the transportation and release of CGRP from DRG cell bodies to their central terminals.<sup>66,67</sup> Following 1000g crush injury, the number of CGRP-IR neurons in the affected L4–L5 DRG was markedly reduced from 3dpi until 28dpi ( $29.83 \pm 2.71$  at 3dpi,  $17.33 \pm 5.68$  at 10dpi,  $7.67 \pm 1.51$  at 21dpi, and  $24.17 \pm 7.52$  at 28dpi;  $p < 0.001$  for all days versus that in the respective sham-operated rats) (Figure 2(a) and (b)). Compared with that in sham-operated rats, 1000g crush injury also resulted in markedly reducing CGRP-IR expression in the sciatic nerve distribution region of the dorsal spinal cord at 10dpi ( $74.00\% \pm 9.42\%$ ,  $p < 0.001$ ) and 21dpi ( $66.17\% \pm 10.44\%$ ,  $p < 0.001$ ). However, the intensity level of the CGRP-IR in this region recovered at 28dpi, with no significant difference compared with that in sham-operated rats (Figure 3(a) and (b)).

We speculated that this simultaneous reduction of CGRP in both the DRG and spinal cord at 10 and 21 days post 1000g crush injury may be due to an insufficient supply of CGRP from DRG cell bodies to their central nerve endings. To verify this speculation, the expression levels of the two genes encoding CGRP, *calca* and *calcb*,<sup>68,69</sup> in L4–L5 DRG were examined at 10dpi and 28dpi following CCI as well as 100g and 1000g crush injury. The expression levels of *calca* and *calcb* remained unchanged 10 and 28 days after CCI or 100g crush injury. By contrast, the levels of these two genes were significantly decreased 10 days after 1000g crush injury (*calca*: 0.25 fold,  $p < 0.01$ ; *calcb*: 0.65 fold,  $p < 0.05$ ; versus sham-operated rats). However, 28 days after 1000g crush injury, both gene levels returned to those of sham-operated rats. These results indicated that 1000g crush injury but not CCI or 100g crush injury of the sciatic nerve reduced CGRP mRNA in primary afferents (Figure 2(e) and (f)). Thus, the transient reduction of CGRP in the DRG after CCI and 100g crush injury was more likely a CGRP redistribution in the primary afferents rather than a drop in CGRP production. By contrast, the supply of CGRP in the primary afferents was reduced following 1000g crush injury, although it increased again by the final observation.



**Figure 2.** CGRP expression in the affected L4-L5 dorsal root ganglia (DRG) following CCI, 100g crush injury, and 1000g crush injury. (a) and (b) Levels of CGRP immunoreactivity (IR) are transiently decreased in L4–L5 DRG at 3 and 10 dpi in rats with CCI and at 10 dpi in 100g crush-injured rats, whereas levels are decreased from 3 dpi to 28 dpi in rats with 1000g crush injury. Scale bar: 150  $\mu\text{m}$ . (c) Levels of CGRP-IR are increased in large-size DRG cells ( $>1200 \mu\text{m}^2$ ) following CCI, 100g crush injury, and 1000g crush injury. (d) Representative images obtained at 10 dpi show CGRP-IR in small- and medium-size cells in sham-operated rats and CGRP-IR in large-size cells in rats with CCI, 100g crush injury, and 1000g crush injury. Scale bar: 150  $\mu\text{m}$ . (e) and (f) The expression levels of two CGRP encoding genes, *calca* and *calcb*, are reduced in the affected L4–L5 DRG at 10 dpi in rats with 1000g crush injury but is not changed at any time in rats with CCI or 100g crush injury. For each of the four groups,  $n = 6$  at each postoperative time used to measure CGRP-IR. For each of the four groups,  $n = 8$  at each postoperative time used to measure the levels of the CGRP encoding genes. Compared with sham-operated rats,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ .





**Figure 3.** CGRP expression in the affected dorsal spinal cord following CCI, 100g crush injury, and 1,000g crush injury. (a) Representative images of CGRP-IR in the affected dorsal spinal cord. The territory receiving projections from the injured sciatic nerve is delineated in each image by the line. Scale bar: 200  $\mu$ m. (b) Normalized optical density of CGRP-IR in the affected dorsal spinal cord is reduced 10 and 21 dpi in rats with 1000g crush injury but is not changed at any time in rats after CCI or 100g crush injury. Compared with sham-operated rats, \*\*\* $p < 0.001$ .

### CGRP-IR is upregulated in the large-size DRG neurons following the three types of injury to the sciatic nerve

In addition to determining overall CGRP expression, we evaluated the subcellular distribution of CGRP-IR in the DRG. In the DRG of normal rodents, CGRP-IR is most often identified in small- and medium-size neurons.<sup>70,71</sup> Our results showed that the sub-cellular distribution of CGRP-IR in the ipsilateral L4–L5 DRG of sham-operated rats remained stable from 3 dpi to 28 dpi, with only 11.65%  $\pm$  3.19% to 13.18%  $\pm$  3.52% of the CGRP-IR neurons being large-size neurons, consistent with previous findings.<sup>72</sup> Compared with the sham-operated rats, CCI caused a significant increase in the percentage of CGRP-IR neurons with a large size from 10 dpi (33.34%  $\pm$  7.21%,  $p < 0.01$ ), and the increase was maintained at 21 dpi (26.93%  $\pm$  10.50%,  $p < 0.05$ ) and 28 dpi (24.09%  $\pm$  9.41%,  $p < 0.05$ ). Similarly, an increase in

CGRP-IR of the large-size neurons followed 100g crush injury, after which the percentage of large-size neurons with CGRP-IR compared with the total number of neurons with CGRP was increased 3 dpi (34.29%  $\pm$  9.13%,  $p < 0.001$ ), 10 dpi (28.02%  $\pm$  6.42%,  $p < 0.05$ ), 21 dpi (25.37%  $\pm$  6.10%,  $p < 0.05$ ), and 28 dpi (24.03%  $\pm$  6.80%,  $p < 0.05$ ). The 1000g crush injury resulted in a marked decrease in the total number of CGRP-IR neurons in the affected L4–L5 DRG. However, many of the remaining CGRP-IR neurons were large-size cells. The percentage of large-size CGRP-IR neurons compared with the total number of CGRP-IR neurons in rats with 1000g crush injury increased 3 dpi (45.90%  $\pm$  9.76%,  $p < 0.001$ ), 10 dpi (67.04%  $\pm$  16.51%,  $p < 0.001$ ), 21 dpi (48.66%  $\pm$  8.01%,  $p < 0.001$ ), and 28 dpi (28.33%  $\pm$  11.03%,  $p < 0.01$ ) (Figure 2(c) and (d)).

Nerve injury can lead to axonal degeneration distal to the focal lesion.<sup>73,74</sup> Although both large and small axons

can be affected by nerve injury, the small axons are more vulnerable than the large ones.<sup>75</sup> As demonstrated by the results of our histologic analysis (Supplemental Results), the three types of sciatic nerve injuries caused different degrees of axonal degeneration in the injured nerve. The most severe axonal degeneration was observed in rats with 1000g crush injury, in which most of the small axons were no longer apparent and most of the remaining axons were large. These results indicated that the primary afferents following 1000g crush injury are predominantly from large-size neurons. In contrast to axon degeneration distal to the lesion, sciatic nerve injury may not lead to a significant loss of DRG neurons within 4 weeks after damage.<sup>76</sup> Moreover, the proportions of small- to medium-size neurons and large-size neurons were also not changed following CCI, 100g crush injury or 1000g crush injury (Supplemental Results).

#### Negative neuropathic symptoms: Static mechanical hypoesthesia is alleviated by intrathecal treatment with CGRP

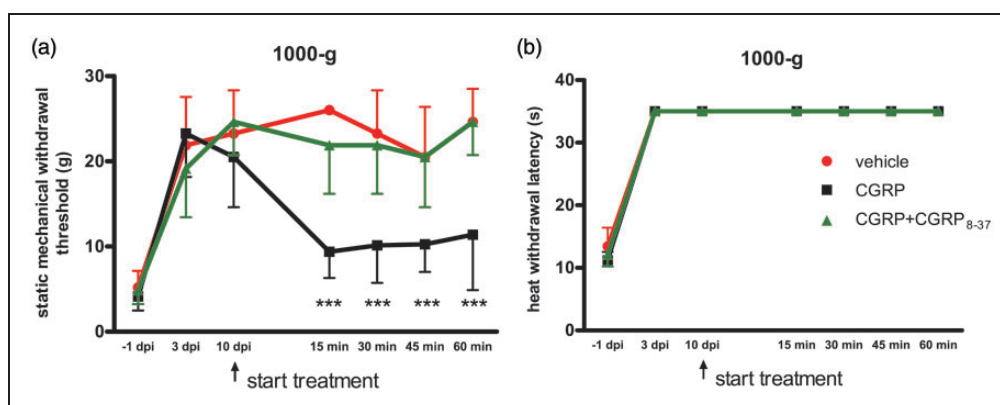
Our results demonstrated that the time course for the reduction in the overall level of CGRP paralleled the development of negative neuropathic symptoms, that is, static mechanical hypoesthesia and heat hypoalgesia, after 1000g crush injury. To determine whether the reduction of CGRP in the primary afferents contributed to the development of static mechanical hypoesthesia and heat hypoalgesia, 10 dpi after 1000g crush injury, the rats received an intrathecal injection of CGRP, CGRP plus the CGRP receptor antagonist (CGRP<sub>8-37</sub>), or vehicle. The dose of CGRP used in the present study has been shown sufficient to induce nociception in naive rats in a previous report.<sup>22</sup> Before treatment, prominent static mechanical hypoesthesia and heat hypoalgesia were observed, with a SMWT of

$20.50 \pm 2.08$  g and a HWL of  $35.00 \pm 0.00$  s for the affected hind paw. Rats receiving the vehicle injection did not show any significant changes in the SMWT after injection. By contrast, following an injection of CGRP, the SMWT was significantly decreased at 15 min ( $9.38 \pm 1.09$  g,  $p < 0.001$  vs. vehicle:  $26.00 \pm 0.00$  g) and remained downregulated until 60 min ( $11.38 \pm 2.29$  g,  $p < 0.001$  vs. vehicle:  $24.63 \pm 1.38$  g) after the injection. When both CGRP and CGRP<sub>8-37</sub> were given, the SMWT was not altered, suggesting that the effect of CGRP was mediated by CGRP receptors (Figure 4(a)). These results indicated that the reduction of CGRP in the primary afferents following 1000g crush injury contributes to the development of static mechanical hypoesthesia.

However, the injection of CGRP did not alter the HWL at any time after the injection (Figure 4(b)). In order to determine whether the dose of CGRP was sufficient, following 1000g crush, six rats were treated with a dose of CGRP 10 times larger than that used in the previous experiment. Once again, the HWL remained unchanged (Supplemental Results). Although ample evidence in previous studies has indicated that CGRP is essential for heat sensation transmission,<sup>61,77</sup> the heat hypoalgesia induced by 1000g crush injury may involve not only the downregulation of CGRP but also other factors, which will require further investigation.

#### Positive neuropathic symptoms: Static and dynamic components of mechanical allodynia, heat hyperalgesia, and spontaneous pain-related behavior are ameliorated by intrathecal treatment of a CGRP receptor antagonist

In the present study, static mechanical allodynia and heat hyperalgesia were induced by CCI and 100g crush



**Figure 4.** CGRP ameliorates static mechanical hypoesthesia but not heat hyperalgesia following 1000g crush injury. Rats received CGRP, CGRP plus the receptor antagonist CGRP<sub>8-37</sub>, or vehicle. Drugs were injected intrathecally at 10 dpi, and behavioral responses were evaluated 15, 30, 45, and 60 min later. (a) Static mechanical withdrawal threshold. (b) Heat withdrawal latency. For each of the three treatment groups,  $n = 8$  for each measurement. Compared with rats receiving vehicle, \*\*\* $p < 0.001$ .

injury. Spontaneous pain and dynamic mechanical allodynia were also induced by CCI and 100g crush injury as well as by 1000g crush injury. To determine whether CGRP-mediated signal transduction in the primary afferents is essential for the development of the positive neuropathic symptoms, including static and dynamic mechanical allodynia, heat hyperalgesia, and spontaneous pain, the CGRP receptor antagonist CGRP<sub>8-37</sub> was intrathecally injected 10 days after CCI, 100g crush, and 1000g crush injury. Before the injection, static mechanical allodynia and heat hyperalgesia had been developed in CCI and 100g crush injury rats, with a mean SMWT of  $1.62 \pm 0.15$  g in the CCI group and  $1.77 \pm 0.29$  g in the 100g crush group and a mean HWL of  $8.55 \pm 0.22$  s in the CCI group and  $8.29 \pm 0.30$  s in the 100g crush group. The SMWT and HWL were not altered following the injection of vehicle. By contrast, compared with that in vehicle-injected rats, 30 min after injection of CGRP<sub>8-37</sub>, the SMWT was significantly increased in CCI rats ( $7.50 \pm 0.96$  g,  $p < 0.01$  vs. vehicle:  $1.65 \pm 0.31$  g) and 100g crush injured rats ( $6.00 \pm 0.82$  g,  $p < 0.001$  vs. vehicle:  $1.68 \pm 0.30$  g). The SMWT remained elevated until 45 min after the antagonist injection in the CCI group ( $p < 0.05$ ) and 60 min after the injection in the 100g crush injury group ( $p < 0.01$ ). The HWL in CCI rats was also significantly increased 30 min after injection of CGRP<sub>8-37</sub> ( $13.70 \pm 1.97$  s,  $p < 0.05$  vs. vehicle:  $7.15 \pm 2.00$  s), and in the 100g crush group, the HWL was increased at 30 min ( $13.05 \pm 1.24$  s,  $p < 0.01$  vs. vehicle:  $9.07 \pm 0.94$  s) and 45 min ( $11.83 \pm 0.74$  s,  $p < 0.05$  vs. vehicle:  $8.43 \pm 0.22$  s) after treatment. Because the overall level of CGRP in the primary afferents was not changed after CCI or 100g crush injury, the results presented in this experiment indicated that CGRP-mediated signal transduction is important for developing of static mechanical allodynia and heat hyperalgesia following sciatic nerve injury (Figure 5(a) and (b)).

Before the treatment, spontaneous pain and dynamic mechanical allodynia had been developed in rats with CCI, 100g crush, and 1000g crush injury. The injured rats showed a mean SPS of  $2.01 \pm 0.31$  in the CCI group,  $1.16 \pm 0.11$  in the 100g crush group, and  $1.10 \pm 0.55$  in the 1000g crush group along with a mean DMAS of  $2.86 \pm 0.49$  in the CCI group,  $1.71 \pm 0.61$  in the 100g crush group, and  $2.11 \pm 0.40$  in the 1000g crush group. The SPS and DMAS were not altered following injection of vehicle, but intrathecal injection of the CGRP antagonist CGRP<sub>8-37</sub> caused a robust decrease in the SPS in each group compared with that for rats injected with vehicle. This was observed after the injection at 30 min ( $1.92 \pm 0.05$ ,  $p < 0.05$  vs. vehicle:  $2.14 \pm 0.06$ ) and 45 min ( $1.91 \pm 0.06$ ,  $p < 0.05$  vs. vehicle:  $2.12 \pm 0.05$ ) in CCI rats, at 60 min ( $1.06 \pm 0.01$ ,  $p < 0.05$  vs. vehicle:  $1.18 \pm 0.03$ ) in 100g crush rats, and at 30 min ( $1.05 \pm 0.01$ ,  $p < 0.01$  vs.

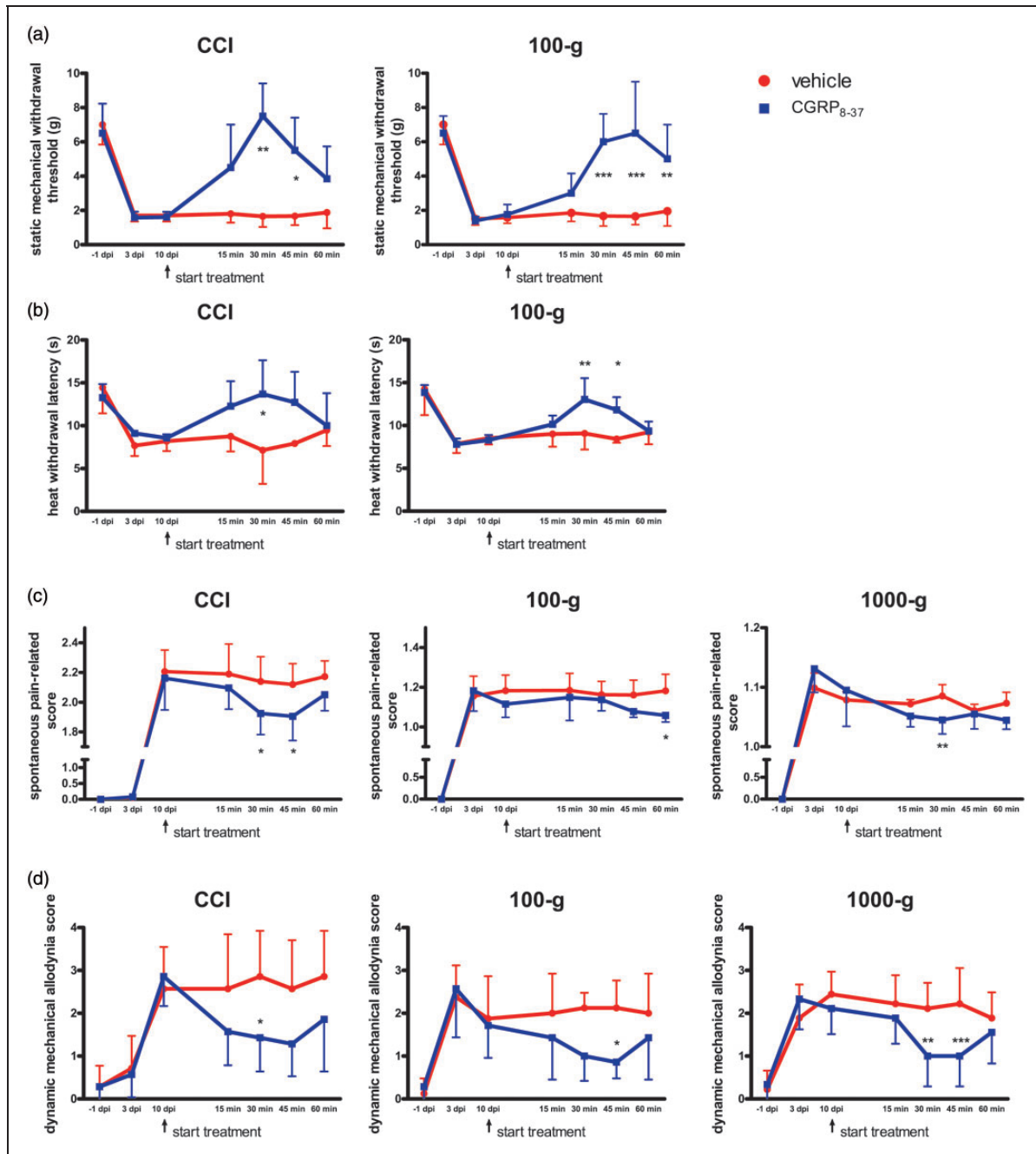
vehicle:  $1.09 \pm 0.01$ ) in 1000g crush rats. The injection of CGRP<sub>8-37</sub> also reduced the DMAS in all groups. Compared with rats injected with vehicle, a statistically significant difference was achieved at 30 min in CCI rats ( $1.43 \pm 0.30$ ,  $p < 0.05$  vs. vehicle:  $2.86 \pm 0.40$ ), at 45 min in 100g crush rats ( $0.86 \pm 0.14$ ,  $p < 0.05$  vs. vehicle:  $2.13 \pm 0.23$ ), and at 30 min ( $1.00 \pm 0.24$ ,  $p < 0.01$  vs. vehicle:  $2.11 \pm 0.20$ ), and 45 min ( $1.00 \pm 0.24$ ,  $p < 0.001$  vs. vehicle:  $2.22 \pm 0.28$ ) in 1000g crush rats. These results indicate that CGRP-mediated signal transduction contributes to the development of spontaneous pain and dynamic mechanical allodynia following sciatic nerve injury (Figure 5(c) and (d)).

## Discussion

In this study, rat peripheral nerve injury models were constructed by introducing CCI, 100g force crush injury with an artery clamp or 1000g force crush injury produced by hemostat forceps to the sciatic nerve. Neuropathic symptoms were determined by evaluating spontaneous pain behavior and the responses to static/dynamic mechanical and heat stimuli. The role of CGRP-mediated signal transduction in spontaneous and mechanical/heat stimuli-evoked pain transmission was investigated in these rat models of CCI or crush injuries to the sciatic nerve. These different models of rat sciatic nerve injury resulted in positive or negative neuropathic symptoms, which paralleled unchanged or reduced CGRP expression in the primary afferents, respectively.

The neuropathic symptoms resulting from the three different injuries to the sciatic nerve were characterized. Rats that underwent CCI demonstrated typical positive neuropathic symptoms in the ipsilateral hind paw that were consistent with those observed in previous studies.<sup>40,78</sup> Artery clamps and forceps have often been applied in nerve crush injury models. Similar to the results of this study, previous experiments have demonstrated differences between the neuropathic symptoms present following nerve crush injury with an artery clamp and those present following injury with forceps. After nerve crush injury with an artery clamp, animals have shown moderate and short-lasting positive sensory symptoms<sup>39</sup> in the area innervated by the injured nerve. By contrast, after crush injury produced by forceps, animals often demonstrate reduced sensitivity to heat or static mechanical stimuli.<sup>79-82</sup> The forceps typically produce a compression force much greater than that produced by an artery clamp. However, the exact compression forces produced by each tool have never been calculated and compared in a single study. This study, for the first time, calculated the forces produced by an artery clamp and forceps, which were 100 g and 1000 g, respectively.

CGRP in the primary afferents is produced in DRG neurons and transported to the dorsal spinal cord, where



**Figure 5.** CGRP receptor antagonist  $\text{CGRP}_{8-37}$  inhibits the positive neuropathic symptoms following CCI, 100g crush injury, and 1000g crush injury. Rats receiving artificial cerebrospinal fluid (vehicle) were used as control. Vehicle or  $\text{CGRP}_{8-37}$  was injected intrathecally at 10 dpi, and the behavioral responses were evaluated 15, 30, 45, and 60 min later: (a) Static mechanical withdrawal threshold (SMWT) in rats with CCI and 100g crush injury. (b) Heat withdrawal latency (HWL) in rats with CCI and 100g crush injury. (c) Spontaneous pain-related scores (SPS) in rats with CCI, 100g crush and 1000g crush injury. (d) Dynamic mechanical allodynia scores (DMAS) in rats with CCI, 100g crush, and 1000g crush injury. In each group,  $n = 8$  for each measurement. Compared with rats receiving vehicle, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

CGRP exerts its role as a pain transducer.<sup>15–19</sup> Evidence from dorsal rhizotomy surgeries has demonstrated that the synaptic endings containing CGRP in the dorsal spinal cord are on primary afferent fibers.<sup>16</sup> Therefore, we determined the overall level of CGRP in primary

afferents after sciatic nerve injury, which differs from previous studies that have investigated CGRP expression separately in either the DRG<sup>83,84</sup> or dorsal spinal cord.<sup>85,86</sup> Acute insult to the peripheral nerve injury may upregulate the uptake or degradation of CGRP in

the primary afferents,<sup>87</sup> which could explain our findings that CGRP-IR was reduced in the DRG within 10 days after CCI and 100g crush injury. However, noxious stimuli also increase the release of CGRP from primary neurons to their central nerve terminals.<sup>66,67</sup> Given the unchanged CGRP mRNA production, the level of CGRP in the dorsal spinal cord was maintained stable, whereas it was reduced in the DRG after CCI and 100g crush injury. By contrast, 1000g crush led to marked downregulation of the CGRP mRNA level 10 days after injury. Thus, the decrease of the CGRP-IR level in both the DRG and dorsal spinal cord may indicate a supply–demand imbalance of CGRP in the primary afferents after 1000g crush injury. With the return of CGRP mRNA levels 28 days after the 1000g crush injury, CGRP-IR levels in the dorsal spinal cord also recovered.

CGRP plays a critical role in the fiber function of small to medium primary neurons.<sup>61,70</sup> It has been demonstrated that loss of sensitivity of small to medium primary neurons evoked negative sensory symptoms.<sup>5,12,88</sup> In the present study, spontaneous and mechanical/heat stimuli-evoked positive sensory symptoms were developed in parallel with a stable supply of CGRP, whereas the negative symptoms, including static mechanical hypoesthesia and heat hypoalgesia, were accompanied by a shortage of CGRP in the primary afferents. Therefore, our results for the first time verified a previous speculation that CGRP availability rather than the increase of its expression is essential for the development of sensitization.<sup>77</sup> Moreover, static mechanical hypoesthesia was ameliorated either when exogenous CGRP was administered to the spinal cord or with the recovery of the CGRP supply, suggesting that the loss of CGRP accounts for the development of mechanical hypoesthesia after 1000g crush injury to the sciatic nerve. Many previous studies have demonstrated that CGRP in the primary afferents is essential for sensory transmission in response to a heat stimulus.<sup>61,77</sup> However, the heat hypoalgesia observed in rats with 1000g crush injury was not ameliorated by exogenous CGRP or with the recovery of the CGRP supply. Consistent with a previous finding,<sup>89</sup> our results indicate that the heat hypoalgesia demonstrated following 1000g crush injury to the sciatic nerve may be due not only to the reduction of CGRP but also to other factors that will need to be determined in future experiments.

In addition to changes in the overall expression of CGRP, peripheral nerve injury may cause a subcellular redistribution of CGRP in the primary afferents. Consistent with previous findings,<sup>90–92</sup> our study showed that the expression of CGRP was increased in the large-size DRG neurons after CCI, 100g crush injury, and 1000g crush injury to the sciatic nerve. Different from the small- to medium-size DRG neurons, which

are known to be nociceptors that respond to noxious heat and mechanical static stimuli,<sup>44,45,93</sup> large-size DRG neurons typically convey sensory information about innocuous stimuli.<sup>94–96</sup> In both clinical observations and experimental animals, the sensitization of large-size neurons and their fibers has been shown to contribute to spontaneous pain<sup>91,97</sup> and dynamic mechanical allodynia.<sup>56,98,99</sup> In the present study, the increased CGRP in the large-size DRG neurons may function as a pain-signaling neurotransmitter<sup>91,100</sup> and contribute to the development of spontaneous pain and dynamic mechanical allodynia following certain injuries to the sciatic nerve.

The effects of CGRP on nociceptive transmission are mediated by CGRP receptors.<sup>22,101</sup> The action of CGRP at CGRP receptors can be blocked by the competitive antagonist CGRP<sub>8–37</sub>.<sup>22</sup> Previous studies have shown that blockade of CGRP receptors by CGRP<sub>8–37</sub> inhibits pain-related responses to noxious mechanical and heat stimuli in rats.<sup>8,102</sup> Similarly, our results showed that intrathecal administration of CGRP<sub>8–37</sub> alleviated positive neuropathic symptoms, including spontaneous pain and static and dynamic mechanical allodynia, after CCI or 100g crush injury, as well as spontaneous pain and dynamic mechanical allodynia after 1000g crush injury. Moreover, the negative neuropathic symptom static mechanical hypoesthesia associated with 1000g crush was alleviated by an intrathecal administration of CGRP, and the effect of exogenous CGRP was neutralized by CGRP<sub>8–37</sub>. These results further demonstrate the important role of CGRP in the primary afferents in developing different neuropathic symptoms following various sciatic nerve injuries.

In conclusion, we found that different CGRP expression patterns contribute to distinct neuropathic symptoms following CCI, mild (100g force) or strong (1000g force) transient crush in rats. Moreover, we showed for the first time that a reduction of the CGRP supply in the primary afferents contributes to the development of negative neuropathic symptoms, especially the negative response to static mechanical stimuli. These findings may shed light on the mechanisms underlying the different neuropathic symptoms observed following peripheral nerve injury.

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### Authors' contributions

Yu Zou and Fangting Xu performed experiments, analyzed data, prepared figures, and drafted the manuscript. ZT performed experiments Zou et al. 13 and analyzed data. JC performed experiments. QG and CH designed, supervised the

experiments, and edited the manuscript. Yu Zou and Fangting Xu contributed equally to this work.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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### Supplemental material

All supplemental materials are available online at <http://mpx.sagepub.com/content/by/supplemental-data>.

### References

- Jensen TS and Finnerup NB. Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms. *Lancet Neurol* 2014; 13: 924–935.
- Baron R. Mechanisms of disease: neuropathic pain – a clinical perspective. *Nat Clin Pract Neurol* 2006; 2: 95–106.
- Baumgartner U, Magerl W, Klein T, et al. Neurogenic hyperalgesia versus painful hypoalgesia: two distinct mechanisms of neuropathic pain. *Pain* 2002; 96: 141–151.
- Huang C, Zou W, Lee K, et al. Different symptoms of neuropathic pain can be induced by different degrees of compressive force on the C7 dorsal root of rats. *Spine J* 2012; 12: 1154–1160.
- Matsushita Y, Araki K, Omotuyi O, et al. HDAC inhibitors restore C-fibre sensitivity in experimental neuropathic pain model. *Br J Pharmacol* 2013; 170: 991–998.
- Arning K and Baron R. Evaluation of symptom heterogeneity in neuropathic pain using assessments of sensory functions. *Neurotherapeutics* 2009; 6: 738–748.
- Maier C, Baron R, Tolle TR, et al. Quantitative sensory testing in the German research network on neuropathic pain (DFNS): somatosensory abnormalities in 1236 patients with different neuropathic pain syndromes. *Pain* 2010; 150: 439–450.
- Bennett AD, Chastain KM and Hulsebosch CE. Alleviation of mechanical and thermal allodynia by CGRP(8–37) in a rodent model of chronic central pain. *Pain* 2000; 86: 163–175.
- Decosterd I and Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* 2000; 87: 149–158.
- Seltzer Z, Dubner R and Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* 1990; 43: 205–218.
- Kim SH and Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 1992; 50: 355–363.
- Uchida H, Ma L and Ueda H. Epigenetic gene silencing underlies C-fiber dysfunctions in neuropathic pain. *J Neurosci* 2010; 30: 4806–4814.
- Woolf CJ and Ma Q. Nociceptors – noxious stimulus detectors. *Neuron* 2007; 55: 353–364.
- Basbaum AI, Bautista DM, Scherrer G, et al. Cellular and molecular mechanisms of pain. *Cell* 2009; 139: 267–284.
- Morton CR and Hutchison WD. Release of sensory neuropeptides in the spinal cord: studies with calcitonin gene-related peptide and galanin. *Neuroscience* 1989; 31: 807–815.
- Chung K, Lee WT and Carlton SM. The effects of dorsal rhizotomy and spinal cord isolation on calcitonin gene-related peptide-labeled terminals in the rat lumbar dorsal horn. *Neurosci Lett* 1988; 90: 27–32.
- Rosenfeld MG, Mermod JJ, Amara SG, et al. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* 1983; 304: 129–135.
- van Rossum D, Hanisch UK and Quirion R. Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. *Neurosci Biobehav Rev* 1997; 21: 649–678.
- Sun RQ, Lawand NB, Lin Q, et al. Role of calcitonin gene-related peptide in the sensitization of dorsal horn neurons to mechanical stimulation after intradermal injection of capsaicin. *J Neurophysiol* 2004; 92: 320–326.
- Oku R, Satoh M, Fujii N, et al. Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats. *Brain Res* 1987; 403: 350–354.
- Cridland RA and Henry JL. Intrathecal administration of CGRP in the rat attenuates a facilitation of the tail flick reflex induced by either substance P or noxious cutaneous stimulation. *Neurosci Lett* 1989; 102: 241–246.
- Sun RQ, Lawand NB and Willis WD. The role of calcitonin gene-related peptide (CGRP) in the generation and maintenance of mechanical allodynia and hyperalgesia in rats after intradermal injection of capsaicin. *Pain* 2003a; 104: 201–208.
- Kuraishi Y, Nanayama T, Ohno H, et al. Antinociception induced in rats by intrathecal administration of antiserum against calcitonin gene-related peptide. *Neurosci Lett* 1988; 92: 325–329.
- Kawamura M, Kuraishi Y, Minami M, et al. Antinociceptive effect of intrathecally administered antiserum against calcitonin gene-related peptide on thermal and mechanical noxious stimuli in experimental hyperalgesic rats. *Brain Res* 1989; 497: 199–203.
- Salmon AM, Damaj I, Sekine S, et al. Modulation of morphine analgesia in alphaCGRP mutant mice. *Neuroreport* 1999; 10: 849–854.
- Zhang L, Hoff AO, Wimalawansa SJ, et al. Arthritic calcitonin/alpha calcitonin gene-related peptide knockout mice have reduced nociceptive hypersensitivity. *Pain* 2001; 89: 265–273.
- Dickinson T and Fleetwood-Walker SM. Neuropeptides and nociception: recent advances and therapeutic implications. *Trends Pharmacol Sci* 1998; 19: 346–348.
- Saria A. The role of substance P and other neuropeptides in transmission of pain. *Acta Neurochir Suppl (Wien)* 1987; 38: 33–35.
- Ma W and Bisby MA. Partial and complete sciatic nerve injuries induce similar increases of neuropeptide Y and

- vasoactive intestinal peptide immunoreactivities in primary sensory neurons and their central projections. *Neuroscience* 1998b; 86: 1217–1234.
30. Noguchi K, Kawai Y, Fukuoka T, et al. Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. *J Neurosci* 1995; 15: 7633–7643.
  31. Chuang HH, Prescott ED, Kong H, et al. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P<sub>2</sub>-mediated inhibition. *Nature* 2001; 411: 957–962.
  32. Symons FJ, Wendelschafer-Crabb G, Kennedy W, et al. Degranulated mast cells in the skin of adults with self-injurious behavior and neurodevelopmental disorders. *Brain Behav Immun* 2009; 23: 365–370.
  33. Cameron AA, Cliffer KD, Dougherty PM, et al. Time course of degenerative and regenerative changes in the dorsal horn in a rat model of peripheral neuropathy. *J Comp Neurol* 1997; 379: 428–442.
  34. Bailey AL and Ribeiro-da-Silva A. Transient loss of terminals from non-peptidergic nociceptive fibers in the substantia gelatinosa of spinal cord following chronic constriction injury of the sciatic nerve. *Neuroscience* 2006; 138: 675–690.
  35. Ma W and Bisby MA. Increase of calcitonin gene-related peptide immunoreactivity in the axonal fibers of the gracile nuclei of adult and aged rats after complete and partial sciatic nerve injuries. *Exp Neurol* 1998a; 152: 137–149.
  36. Akkina SK, Patterson CL and Wright DE. GDNF rescues nonpeptidergic unmyelinated primary afferents in streptozotocin-treated diabetic mice. *Exp Neurol* 2001; 167: 173–182.
  37. Verge VM, Richardson PM, Wiesenfeld-Hallin Z, et al. Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons. *J Neurosci* 1995; 15(3 Pt 1): 2081–2096.
  38. Li XQ, Verge VM, Johnston JM, et al. CGRP peptide and regenerating sensory axons. *J Neuropathol Exp Neurol* 2004; 63: 1092–1103.
  39. Attal N, Filliatreau G, Perrot S, et al. Behavioural pain-related disorders and contribution of the saphenous nerve in crush and chronic constriction injury of the rat sciatic nerve. *Pain* 1994; 59: 301–312.
  40. Bennett GJ and Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988; 33: 87–107.
  41. Duraku LS, Hossaini M, Hoendervangers S, et al. Spatiotemporal dynamics of re-innervation and hyperinnervation patterns by uninjured CGRP fibers in the rat foot sole epidermis after nerve injury. *Mol Pain* 2012; 8: 61.
  42. Attal N, Jazat F, Kayser V, et al. Further evidence for ‘pain-related’ behaviours in a model of unilateral peripheral mononeuropathy. *Pain* 1990; 41: 235–251.
  43. Hargreaves K, Dubner R, Brown F, et al. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988; 32: 77–88.
  44. LaMotte RH, Shain CN, Simone DA, et al. Neurogenic hyperalgesia: psychophysical studies of underlying mechanisms. *J Neurophysiol* 1991; 66: 190–211.
  45. Magerl W, Fuchs PN, Meyer RA, et al. Roles of capsaicin-insensitive nociceptors in cutaneous pain and secondary hyperalgesia. *Brain* 2001; 124(Pt 9): 1754–1764.
  46. Torebjork HE, Lundberg LE and LaMotte RH. Central changes in processing of mechanoreceptive input in capsaicin-induced secondary hyperalgesia in humans. *J Physiol* 1992; 448: 765–780.
  47. Treede RD and Cole JD. Dissociated secondary hyperalgesia in a subject with a large-fibre sensory neuropathy. *Pain* 1993; 53: 169–174.
  48. Chaplan SR, Bach FW, Pogrel JW, et al. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994; 53: 55–63.
  49. Jain V, Pareek A, Bhardwaj YR, et al. Attenuating effect of standardized fruit extract of punica granatum L in rat model of tibial and sural nerve transection induced neuropathic pain. *BMC Complement Altern Med* 2013; 13: 274.
  50. Rigaud M, Gemes G, Barabas ME, et al. Species and strain differences in rodent sciatic nerve anatomy: implications for studies of neuropathic pain. *Pain* 2008; 136: 188–201.
  51. Swett JE and Woolf CJ. The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. *J Comp Neurol* 1985; 231: 66–77.
  52. Fukuoka T, Kondo E, Dai Y, et al. Brain-derived neurotrophic factor increases in the uninjured dorsal root ganglion neurons in selective spinal nerve ligation model. *J Neurosci* 2001; 21: 4891–4900.
  53. Zhu XY, Huang CS, Li Q, et al. p300 exerts an epigenetic role in chronic neuropathic pain through its acetyltransferase activity in rats following chronic constriction injury (CCI). *Mol Pain* 2012; 8: 84.
  54. Mothe AJ, Bozkurt G, Catapano J, et al. Intrathecal transplantation of stem cells by lumbar puncture for thoracic spinal cord injury in the rat. *Spinal Cord* 2011; 49: 967–973.
  55. Field MJ, Bramwell S, Hughes J, et al. Detection of static and dynamic components of mechanical allodynia in rat models of neuropathic pain: are they signalled by distinct primary sensory neurones? *Pain* 1999; 83: 303–311.
  56. Koltzenburg M, Lundberg LE and Torebjork HE. Dynamic and static components of mechanical hyperalgesia in human hairy skin. *Pain* 1992; 51: 207–219.
  57. Ochoa JL and Yarnitsky D. Mechanical hyperalgesias in neuropathic pain patients: dynamic and static subtypes. *Ann Neurol* 1993; 33: 465–472.
  58. Stiasny-Kolster K, Magerl W, Oertel WH, et al. Static mechanical hyperalgesia without dynamic tactile allodynia in patients with restless legs syndrome. *Brain* 2004; 127(Pt 4): 773–782.
  59. Ziegler EA, Magerl W, Meyer RA, et al. Secondary hyperalgesia to punctate mechanical stimuli. Central sensitization to A-fibre nociceptor input. *Brain* 1999; 122(Pt 12): 2245–2257.
  60. Caspani O, Zurborg S, Labuz D, et al. The contribution of TRPM8 and TRPA1 channels to cold allodynia and neuropathic pain. *PLoS One* 2009; 4: e7383.
  61. McCoy ES, Taylor-Blake B, Street SE, et al. Peptidergic CGRP $\alpha$  primary sensory neurons encode heat and itch and tonically suppress sensitivity to cold. *Neuron* 2013; 78: 138–151.

62. McCoy ES and Zylka MJ. Enhanced behavioral responses to cold stimuli following CGRP $\alpha$  sensory neuron ablation are dependent on TRPM8. *Mol Pain* 2014; 10: 69.
63. Lee BH, Yoon YW, Chung K, et al. Comparison of sympathetic sprouting in sensory ganglia in three animal models of neuropathic pain. *Exp Brain Res* 1998; 120: 432–438.
64. Datta S, Chatterjee K, Kline RHT, et al. Behavioral and anatomical characterization of the bilateral sciatic nerve chronic constriction (bCCI) injury: correlation of anatomic changes and responses to cold stimuli. *Mol Pain* 2010; 6: 7.
65. Cobianchi S, de Cruz J and Navarro X. Assessment of sensory thresholds and nociceptive fiber growth after sciatic nerve injury reveals the differential contribution of collateral reinnervation and nerve regeneration to neuropathic pain. *Exp Neurol* 2014; 255: 1–11.
66. Seybold VS, Galeazza MT, Garry MG, et al. Plasticity of calcitonin gene related peptide neurotransmission in the spinal cord during peripheral inflammation. *Can J Physiol Pharmacol* 1995; 73: 1007–1014.
67. Garry MG and Hargreaves KM. Enhanced release of immunoreactive CGRP and substance P from spinal dorsal horn slices occurs during carrageenan inflammation. *Brain Res* 1992; 582: 139–142.
68. Amara SG, Jonas V, Rosenfeld MG, et al. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* 1982; 298: 240–244.
69. Terrado J, Gerrikagoitia I, Dominguez L, et al. Expression of the genes for alpha-type and beta-type calcitonin gene-related peptide during rat embryogenesis. *Neuroscience* 1999; 92: 713–727.
70. Benarroch EE. CGRP: sensory neuropeptide with multiple neurologic implications. *Neurology* 2011; 77: 281–287.
71. Martinez-Gutierrez M and Castellanos JE. Morphological and biochemical characterisation of sensory neurons infected in vitro with rabies virus. *Acta Neuropathol* 2007; 114: 263–269.
72. McCarthy PW and Lawson SN. Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene-related peptide-like immunoreactivity. *Neuroscience* 1990; 34: 623–632.
73. Kanamori A, Catrinescu MM, Belisle JM, et al. Retrograde and Wallerian axonal degeneration occur synchronously after retinal ganglion cell axotomy. *Am J Pathol* 2012; 181: 62–73.
74. Chen P, Piao X and Bonaldo P. Role of macrophages in Wallerian degeneration and axonal regeneration after peripheral nerve injury. *Acta Neuropathol* 2015; 130: 605–618.
75. Guertin AD, Zhang DP, Mak KS, et al. Microanatomy of axon/glia signaling during Wallerian degeneration. *J Neurosci* 2005; 25: 3478–3487.
76. Tandrup T, Woolf CJ and Coggeshall RE. Delayed loss of small dorsal root ganglion cells after transection of the rat sciatic nerve. *J Comp Neurol* 2000; 422: 172–180.
77. Mogil JS, Miermeister F, Seifert F, et al. Variable sensitivity to noxious heat is mediated by differential expression of the CGRP gene. *Proc Natl Acad Sci USA* 2005; 102: 12938–12943.
78. Hu P, Bembrick AL, Keay KA, et al. Immune cell involvement in dorsal root ganglia and spinal cord after chronic constriction or transection of the rat sciatic nerve. *Brain Behav Immun* 2007; 21: 599–616.
79. Vogelaar CF, Vrinten DH, Hoekman MF, et al. Sciatic nerve regeneration in mice and rats: recovery of sensory innervation is followed by a slowly retreating neuropathic pain-like syndrome. *Brain Res* 2004; 1027: 67–72.
80. Casals-Diaz L, Vivo M and Navarro X. Nociceptive responses and spinal plastic changes of afferent C-fibers in three neuropathic pain models induced by sciatic nerve injury in the rat. *Exp Neurol* 2009; 217: 84–95.
81. Kerns JM, Braverman B, Mathew A, et al. A comparison of cryoprobe and crush lesions in the rat sciatic nerve. *Pain* 1991; 47: 31–39.
82. Bester H, Beggs S and Woolf CJ. Changes in tactile stimuli-induced behavior and c-Fos expression in the superficial dorsal horn and in parabrachial nuclei after sciatic nerve crush. *J Comp Neurol* 2000; 428: 45–61.
83. Shi TJ, Tandrup T, Bergman E, et al. Effect of peripheral nerve injury on dorsal root ganglion neurons in the C57 BL/6J mouse: marked changes both in cell numbers and neuropeptide expression. *Neuroscience* 2001; 105: 249–263.
84. Hirose K, Iwakura N, Orita S, et al. Evaluation of behavior and neuropeptide markers of pain in a simple, sciatic nerve-pinch pain model in rats. *Eur Spine J* 2010; 19: 1746–1752.
85. Sommer C and Myers RR. Neurotransmitters in the spinal cord dorsal horn in a model of painful neuropathy and in nerve crush. *Acta Neuropathol* 1995; 90: 478–485.
86. Na HS, Kim HJ, Sung B, et al. Decrease in spinal CGRP and substance P is not related to neuropathic pain in a rat model. *Neuroreport* 2001; 12: 175–178.
87. Lu CL, Pasricha PJ, Hsieh JC, et al. Changes of the neuropeptides content and gene expression in spinal cord and dorsal root ganglion after noxious colorectal distension. *Regul Pept* 2005; 131: 66–73.
88. Fields HL, Rowbotham M and Baron R. Postherpetic neuralgia: irritable nociceptors and deafferentation. *Neurobiol Dis* 1998; 5: 209–227.
89. Hsieh YL, Lin CL, Chiang H, et al. Role of peptidergic nerve terminals in the skin: reversal of thermal sensation by calcitonin gene-related peptide in TRPV1-depleted neuropathy. *PLoS One* 2012; 7: e50805.
90. Miki K, Fukuoka T, Tokunaga A, et al. Calcitonin gene-related peptide increase in the rat spinal dorsal horn and dorsal column nucleus following peripheral nerve injury: up-regulation in a subpopulation of primary afferent sensory neurons. *Neuroscience* 1998; 82: 1243–1252.
91. Nitzan-Luques A, Devor M and Tal M. Genotype-selective phenotypic switch in primary afferent neurons contributes to neuropathic pain. *Pain* 2011; 152: 2413–2426.
92. Nitzan-Luques A, Minert A, Devor M, et al. Dynamic genotype-selective “phenotypic switching” of CGRP expression contributes to differential neuropathic pain phenotype. *Exp Neurol* 2013; 250: 194–204.
93. Cho H, Yang YD, Lee J, et al. The calcium-activated chloride channel anoctamin 1 acts as a heat sensor in nociceptive neurons. *Nat Neurosci* 2012; 15: 1015–1021.



94. Castro-Lopes JM, Coimbra A, Grant G, et al. Ultrastructural changes of the central scalloped (C1) primary afferent endings of synaptic glomeruli in the substantia gelatinosa Rolandi of the rat after peripheral neurotomy. *J Neurocytol* 1990; 19: 329–337.
95. Woolf CJ, Shortland P and Coggeshall RE. Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* 1992; 355: 75–78.
96. Ueda H. Molecular mechanisms of neuropathic pain-phenotypic switch and initiation mechanisms. *Pharmacol Ther* 2006; 109: 57–77.
97. Djouhri L, Fang X, Koutsikou S, et al. Partial nerve injury induces electrophysiological changes in conducting (uninjured) nociceptive and nonnociceptive DRG neurons: possible relationships to aspects of peripheral neuropathic pain and paresthesias. *Pain* 2012; 153: 1824–1836.
98. Garrison SR, Kramer AA, Gerges NZ, et al. Sickie cell mice exhibit mechanical allodynia and enhanced responsiveness in light touch cutaneous mechanoreceptors. *Mol Pain* 2012; 8: 62.
99. Landerholm AH and Hansson PT. Mechanisms of dynamic mechanical allodynia and dysesthesia in patients with peripheral and central neuropathic pain. *Eur J Pain* 2011; 15: 498–503.
100. Weyer AD, O'Hara CL and Stucky CL. Amplified mechanically gated currents in distinct subsets of myelinated sensory neurons following in vivo inflammation of skin and muscle. *J Neurosci* 2015; 35: 9456–9462.
101. Sun W, Wang L, Zhang Z, et al. Intramuscular transfer of naked calcitonin gene-related peptide gene prevents autoimmune diabetes induced by multiple low-dose streptozotocin in C57BL mice. *Eur J Immunol* 2003b; 33: 233–242.
102. Yu LC, Hansson P and Lundberg T. The calcitonin gene-related peptide antagonist CGRP8-37 increases the latency to withdrawal responses in rats. *Brain Res* 1994; 653: 223–230.