Neuropathy-Induced Spinal GAP-43 Expression Is Not a Main Player in the Onset of Mechanical Pain Hypersensitivity

Robby J. Jaken,^{1,3} Sebastiaan van Gorp,^{1,3} Elbert A. Joosten,^{1,3} Mario Losen,^{2,3} Pilar Martínez-Martínez,^{2,3} Marc De Baets,^{2,3} Marco A. Marcus,^{1,3} and Ronald Deumens^{1,3,4}

Abstract

Structural plasticity within the spinal nociceptive network may be fundamental to the chronic nature of neuropathic pain. In the present study, the spatiotemporal expression of growth-associated protein-43 (GAP-43), a protein which has been traditionally implicated in nerve fiber growth and sprouting, was investigated in relation to mechanical pain hypersensitivity. An L5 spinal nerve transection model was validated by the presence of mechanical pain hypersensitivity and an increase in the early neuronal activation marker cFos within the superficial spinal dorsal horn upon innocuous hindpaw stimulation. Spinal GAP-43 was found to be upregulated in the superficial L5 dorsal horn from 5 up to 10 days after injury. GAP-43 was co-localized with calcitonin-gene related peptide (CGRP), but not vesicular glutamate transporter-1 (VGLUT-1), IB4, or protein kinase- γ (PKC- γ), suggesting the regulation of GAP-43 in peptidergic nociceptive afferents. These GAP-43/CGRP fibers may be indicative of sprouting peptidergic fibers. Fiber sprouting largely depends on growth factors, which are typically associated with neuro-inflammatory processes. The putative role of neuropathy-induced GAP-43 expression in the development of mechanical pain hypersensitivity was investigated using the immune modulator propentofylline. Propentofylline treatment strongly attenuated the development of mechanical pain hypersensitivity and glial responses to nerve injury as measured by microglial and astroglial markers, but did not affect neuropathy-induced levels of spinal GAP-43 or GAP-43 regulation in CGRP fibers. We conclude that nerve injury induces structural plasticity in fibers expressing CGRP, which is regarded as a main player in central sensitization. Our data do not, however, support a major role of these structural changes in the onset of mechanical pain hypersensitivity.

Key words: glial cell response to injury; neuroplasticity; peripheral nerve injury

Introduction

NEUROPATHIC PAIN is a common cause of chronic pain, for which there is no effective treatment. It is thought that more effective and specific treatment approaches may be based on neuropathic pain mechanisms (Baron et al., 2010). Therapeutic targets have been identified by the progress made in elucidating cellular and/or molecular mechanisms contributing to neuropathy-induced pain hypersensitivity (Basbaum et al., 2009; Berger et al., 2011; Ren and Dubner, 2010). After a focus on the mechanisms involved in the onset of pain hypersensitivity following nerve injury, more and more attention is being given to describing the mechanisms that are responsible for pain chronicity (Eijkelkamp et al.,

2010; Ji et al., 2006; Marchand et al., 2005). It is likely that the mechanisms of pain chronicity develop in the early stages after nerve injury. Indeed, structural plasticity in the nociceptive network, which is considered an important contributor to chronicity of neuropathic pain symptoms such as mechanical pain hypersensitivity, occurs within days after nerve injury (Doubell et al., 1997; Shortland et al., 1997; Woolf et al., 1992,1995).

Peripheral neuropathy has been reported to induce expression of growth-associated protein-43 (GAP-43) in two vital locations of the nociceptive network: the dorsal root ganglion and the superficial dorsal horn of the spinal cord (Coggeshall et al., 1991; Woolf et al., 1990). GAP-43 is traditionally regarded as a marker of sprouting fibers, but it can

¹Pain Management and Research Center, Department of Anesthesiology, and ²Department of Neuroscience, School for Mental Health and Neuroscience, Maastricht University Medical Center, Maastricht, The Netherlands.

³European Graduate School of Neuroscience (EURON), Maastricht, The Netherlands.

⁴Dutch Polymer Institute (DPI), Eindhoven, The Netherlands.

also be indicative of regeneration or neuronal plasticity (Oestreicher et al., 1997). Thus increased GAP-43 expression may be an early indicator of structural changes within the nociceptive network, which alters the processing of noxious and innocuous information following nerve injury. Although this pathological process has been claimed to be particularly relevant to mechanical pain hypersensitivity (Hughes et al., 2003; Latremoliere and Woolf, 2009; Woolf et al., 1990), a limited understanding of the regulators of GAP-43 expression explains why it is still unknown whether such structural changes play a role in neuropathy-induced mechanical pain hypersensitivity.

Spinal GAP-43 expression after nerve injury is considered to reflect a sprouting response of primary afferent fibers, including unmyelinated fibers that have not been further identified (Coggeshall et al., 1991). Fiber sprouting depends on the presence of growth factors, which are largely produced by immune-competent cells such as glial cells which respond to nerve injury (McMahon and Malcangio, 2009). However, it is not known whether glial modulators affect spinal GAP-43 expression following nerve injury.

In the present study, the rat L5 spinal nerve transection (SNT) model for neuropathic pain was used to investigate the spatiotemporal expression of spinal GAP-43 and to identify the fibers regulating GAP-43 expression. The SNT model was validated by several means, including the development of mechanical pain hypersensitivity and an increase in early neuronal activation within the superficial lumbar dorsal horn upon light mechanical stroking of the hindpaw. The glial modulator propentofylline (PPF) was used to study (1) the effect on neuropathy-induced GAP-43 expression, and (2) the putative relationship between spinal GAP-43 expression and pain hypersensitivity following SNT.

Methods

Animals and surgery

All experiments were approved by the Animal Experiments Committee of the Maastricht University (DEC #2009-068), and were performed in accordance with the recommendations of the European Commission (European Communities Council Directive of 24 November 1986; 86/609/EEC). Sixty-eight adult female Sprague-Dawley rats (Charles River, Utrecht, The Netherlands) weighing about 200–250 g were used for these experiments. L5 SNT was performed according to the method described by Chung and co-workers (Chung et al., 2004), with a slight modification. Briefly, under isoflurane anesthesia and aseptic conditions, the L6 transverse vertebral process was exposed and removed, thereby exposing the L5 spinal nerve. The L5 spinal nerve was freed from surrounding connective tissue, then gently lifted and transected. A 5-mm piece of L5 spinal nerve was resected to prevent spontaneous nerve regeneration. The wound was then closed with muscle sutures (4/0 resorbable catgut) and skin sutures (3/0 nylon). The animals were allowed to survive for 3 ($n=6$), 5 ($n=6$), 7 ($n=6$), 10 ($n=10$), or 21 ($n=5$) days post-operation (DPO). Animals receiving identical surgery but without nerve injury were used as sham controls ($n=4$). Another cohort of rats received an intrathecal (IT) polyethylene catheter 5 days prior to SNT. Under isoflurane anesthesia, a sterile pre-measured IT catheter was introduced through a slit in the atlanto-occipital membrane and threaded down to the lumbar enlargement.

The location of the catheter was verified by observation of paralysis of both hindpaws upon IT injection of lidocaine (10 μ L of 2% lidocaine followed by 10 μ L of saline flush) 2 days following placement of the catheter. Moreover, the location of the catheter tip was verified after the animal was sacrificed. Three animals were excluded from the study due to behavioral signs of paralysis following surgery. One hour prior to SNT, the animals received either a bolus injection of PPF (10 μ g PPF in 20 μ L saline, followed by a 10- μ L saline flush, $n=12$; Sigma-Aldrich, St. Louis, MO) or vehicle (30 μ L saline, $n=10$). The animals were treated daily until day 5 post-operation. The experimenter recording the behavioral data was blinded to treatment.

Behavioral testing

The animals were habituated to the testing environment for 3–5 days before baseline testing. For testing mechanical sensitivity, the animals were placed in transparent plastic cages on an elevated wire mesh floor. Before testing, acclimatization was allowed for approximately 15 min, until exploration and major grooming stopped. A dynamic plantar aesthesiometer (electronic von Frey device; Ugo Basile, Comerio, Italy) was used to measure the withdrawal threshold to mechanical stimulation of the mid-plantar surface of the hindpaws (stimulus intensity was increased from 0–50 g in 10 sec). Importantly, only withdrawal responses associated with aversive behavior to the stimulus (e.g., paw licking, postural changes, and/or attacking the metal probe at paw stimulation) were considered. Withdrawal thresholds were measured three times per animal per time point and averaged to render the paw withdrawal threshold (PWT) of the animal.

Non-nociceptive stimulation of L5 SNT animals

Five days following SNT, another cohort of animals ($n=6$) received non-nociceptive stimulation as previously described (Zhang et al., 2007). The animals were habituated to the experimenter. Then the animal was gently held by the investigator, and the rat's plantar hindpaw was rubbed using the flat portion of the investigator's thumb. Strokes were made towards the distal footpad over a period of 2 sec. This procedure was done for both hindpaws and for a total duration of 10 min. As such, each hindpaw was stimulated about 150 times within 10 min. The animals were sacrificed 2 h after the 10-min stimulation period. The animals ($n=6$) in which the hindpaws were not stimulated and that were sacrificed at 5 days after SNT served as controls.

Tissue processing

The rats were anesthetized with an overdose of pentobarbital (150 mg/kg body weight) via IP injection and subsequently transcardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by a 4% paraformaldehyde/15% picric acid solution (PF-P). The lumbar spinal cord were isolated and post-fixed in PF-P overnight at 4°C. Cryoprotection was performed by overnight incubation in 10% sucrose solution (in 0.1 M phosphate buffer, pH 7.6 at 4°C), followed by at least 72 h incubation in 30% sucrose solution (in 0.1 M phosphate buffer, pH 7.6 at 4°C). The spinal cord segments were frozen using dry ice powder and stored at -80°C until further processing. For cryosectioning, the spinal cords

were mounted in Tissue-Tek Optimal Cutting Temperature solution (O.C.T.; Sakura FineTek, Alphen aan den Rijn, The Netherlands). Then 30- μ m serial sections were mounted on gelatin-coated glass slides and stored at -20°C until immunohistochemical processing.

Immunohistochemistry

Glass slides were thawed for about 2 h at room temperature followed by three washing steps of 10 min each with PBS. Next, the spinal sections were incubated with the following primary antibodies: rabbit anti-PKC- γ (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-GAP-43 (1:16,000; Chemicon, Temecula, CA), rabbit anti-CGRP (1:1000; Biotrend, Wangen, Switzerland), rabbit anti-VGLUT-1 (1:1000; Synaptic Systems, Goettingen, Germany), and rabbit anti-cFos (1:2000; Santa Cruz Biotechnology). In addition, biotinylated IB4 (1:1000; Sigma-Aldrich, St. Louis, MO) was used for histological staining. All primary antibodies were diluted in PBS-T (PBS with 0.3% Triton X-100) and incubated overnight at room temperature. The following day, the sections were rinsed three times for 10 min each with PBS. Next, the sections were incubated with the following secondary antibodies: Alexa 594-conjugated donkey anti-rabbit (for PKC- γ , CGRP, and VGLUT-1), Alexa 488-conjugated donkey anti-mouse (for GAP-43 and cFos), and Alexa 594-conjugated streptavidin (for IB4). All secondary antibodies were diluted in PBS-T (1:100; Invitrogen, Carlsbad, CA) and incubated for 2 h at room temperature. Spinal sections were washed again three times for 10 min each (PBS) and embedded in 80% glycerol/PBS. For double-labeling experiments, spinal sections were incubated with a mixture of the two respective primary antibodies in PBS-T overnight at room temperature. The secondary antibodies were also mixed and sections were incubated with this mixture for 2 h at room temperature. Glial fibrillary acidic protein (GFAP) was stained to visualize astrocytes. Here, rabbit anti-GFAP (1:1000; Dako North America, Inc., Carpinteria, CA) was used as the primary antibody, and Alexa 488-conjugated donkey anti-rabbit (1:100) as the secondary antibody. In contrast to previous staining, Tris-buffered saline (TBS) and TBS containing 1% Triton X-100 (TBS-T) were used for the washing steps; the antibodies were dissolved in TBS-T. Ionized calcium binding adaptor-1 protein (Iba-1) was used to stain microglia. Here, spinal sections were incubated with blocking serum (TBS-T containing 5% normal donkey serum [NDS]) for 1 h at 4°C . Next, the sections were incubated with rabbit anti-Iba-1 (1:1000 in TBS-T containing 1% NDS; Wako Chemicals USA, Inc., Richmond, VA) overnight at 4°C . The following day, the sections were rinsed three times for 10 min each with TBS. Then the sections were incubated with the Alexa 488-conjugated donkey anti-rabbit antibody (1:100 in TBS-T) and incubated for 1 h at 4°C . The spinal sections were washed again three times for 10 min each (TBS) and embedded in 80% glycerol/TBS. Immunostainings that were performed identically, but with omission of the primary antibody, served as negative controls.

Image analysis

GAP-43-immunostained sections were examined under a fluorescence microscope. Photomicrographs of the ipsilateral and contralateral dorsal horn were taken of immunostained sections with a $10\times$ objective (and a $12.5\times$ condenser) using a

grayscale F-view camera. Analysis was performed using Cell Profiler software (Cell^P; Olympus, Zoeterwoude, The Netherlands). The superficial dorsal horn was defined as the area of the dorsal horn from the most dorsal part to the lower border of the spinal PKC- γ -immunoreactive layer. As such, the PKC- γ immunostaining was used to delineate the superficial dorsal horn in each section. GAP-43 expression (grey value of immunoreactivity) was determined separately for the superficial dorsal horns in the L3, L4, and L5 spinal cord. For each animal a total of 12 sections were analyzed. No changes were observed for GAP-43 expression over various time points within the contralateral dorsal horn. Therefore, the contralateral dorsal horn was used as an internal control. Values at various time points were then expressed in relation to those of sham-operated animals. To investigate the double-labeling of GAP-43 (green) and the different primary afferent markers (red), individual photomicrographs were taken using a fluorescence microscope, and two photomicrographs were merged together using Cell^P software. To quantify the GFAP and Iba-1 immunoreactivity signal, photomicrographs of the ipsilateral and contralateral dorsal horn were taken of the immunostained sections with a $4\times$ objective using a grayscale F-view camera. Analysis was performed using Cell^P. Regions of interest (ROIs) were drawn of the ipsilateral and contralateral dorsal horn, and the mean gray value within each ROI was measured. Results are expressed as the percent difference in gray value between the ipsilateral and contralateral dorsal horn.

Stereology-based cell counting

Stereology-based cell counting was performed with a stereology workstation consisting of a modified light microscope and stereology software (StereoInvestigator; MBF Bioscience, Williston, VT). Total numbers of cFos-positive cells were evaluated with an optical fractionator (West et al., 1991). Consecutive sections were used to count cFos-positive cells in the superficial dorsal horn of the L4 and L5 spinal dorsal horn. Again the PKC- γ layer was used to delineate the superficial dorsal horn. Using the optical fractionator, cFos-positive cells were counted at $20\times$ magnification in a counting frame of $22,500\ \mu\text{m}^2$ and with an optical disector height of $16\ \mu\text{m}$. Fixed distances (guard zones) of $2\ \mu\text{m}$ were set to prevent counting the same cell twice. All neurons for which the nucleus top came into focus within unbiased virtual counting spaces distributed in a systematic-random fashion throughout the delineated regions were counted (Schmitz and Hof, 2005). Then the total neuron numbers were calculated from the density of counted neurons and the corresponding corrected counting volume.

Confocal microscopy

Confocal images of GAP-43 and CGRP double-immunostained areas were analyzed with a Confocal Spinning Disk (SI-SD) system (MBF Bioscience), consisting of a BX51 microscope (Olympus), a customized spinning disk unit (DSU; Olympus), and controlling software (StereoInvestigator). A digital RGB image stack showing the same microscopic field in 47 consecutive focal planes with a distance of $0.5\ \mu\text{m}$ between the focal planes were generated with a $100\times$ objective. From these image stacks, three-dimensional reconstructions (maximum intensity projections) were

produced with Imaris 4.0 software (Bitplane Scientific Software, Zurich, Switzerland).

Statistical analysis

All data were analyzed with the statistical program SPSS 15.0 (SPSS Inc., Chicago, IL). Statistical comparisons between baseline and post-injury paw withdrawal thresholds were performed with an unpaired Student's *t*-test. GAP-43 expression in SNT animals was compared to sham-operated animals using one-way analysis of variance (ANOVA) and Dunnett's *post-hoc* test (sham-operated animals were used as controls). Unpaired Student's *t*-tests were used to compare the total number of cFos-positive cells between stimulated and non-stimulated animals, and the GFAP and Iba-1 expression between PPF-treated and vehicle-treated animals. All data are expressed as mean $\pm$ standard error of the mean (SEM). A *p* value of 0.05 was regarded as statistically significant.

Results

Validation of the SNT model: Presence of mechanical allodynia and neuronal activation in the superficial dorsal horn following innocuous paw stimulation

Peripheral neuropathy is known to render hypersensitivity to low-threshold (innocuous) stimuli. Indeed, withdrawal thresholds to mechanical stimulation of the hindpaw ipsilateral to SNT were found to be clearly reduced up to at least 3 weeks (Fig. 1A). Pain hypersensitivity is thought to be due to altered processing of signals in the spinal nociceptive network. This is evidenced by altered spinal neuronal activity

upon innocuous peripheral stimulation. Tactile stimulation of the hindpaws for a period of 10 min increased the expression of cFos, an immediate early gene and a marker of early neuronal activation (Fig. 1B–E). Indeed, the total number of cFos-immunoreactive cells was doubled in the superficial dorsal horn ipsilateral to SNT in animals that received innocuous hindpaw stimulation (1080 ± 107), compared to the superficial dorsal horn ipsilateral to SNT in non-stimulated animals (518 ± 67 ; $p < 0.01$; Fig. 1F). Innocuous stimulation of the contralateral hindpaw of SNT animals induced only a slight increase in cFos-immunoreactive cell numbers in the superficial dorsal horn contralateral to SNT (743 ± 74 in stimulated animals versus 500 ± 118 in non-stimulated animals), but this increase did not reach statistical significance (Fig. 1F).

SNT increases spinal GAP-43 expression in a clear spatiotemporal manner

Transection of the L5 spinal nerve (SNT) was performed to study spinal GAP-43 expression in the superficial dorsal horn of the L5, L4, and L3 spinal cord at 3, 5, 7, 10, and 21 days after nerve injury. The innermost part of lamina II was identified by PKC- γ immunostaining (Fig. 2B–D, red), and used to delineate the superficial dorsal horn. SNT induced a substantial increase in GAP-43 expression (Fig. 2A), which was restricted to the superficial dorsal horn (Fig. 2B–D). The most prominent regulation of GAP-43 expression was found to occur at the L5 spinal cord (ANOVA, $F_{(5,34)} = 2.6$, $p < 0.05$; Fig. 2E). GAP-43 expression was upregulated in the superficial L5 dorsal horn ipsilateral to SNT, up to levels around 30% over those found in sham-operated animals at 5, 7, and 10 days after nerve

FIG. 1. Validation of the spinal nerve transection (SNT) model for neuropathic pain hypersensitivity. (A) Paw withdrawal thresholds (PWT) of ipsilateral hindpaws are reduced by about 60–70% by transection of the L5 spinal nerve ($n=5$); the contralateral PWT remained unaffected. (B–E) Expression of the immediate early marker cFos in neurons of the ipsilateral (B and D) and contralateral (C and E) L5 superficial dorsal horn at 5 days after SNT. (B and C) An animal without 10 min of innocuous hindpaw stimulation. (D and E) An animal with 10 min of innocuous hindpaw stimulation. (F) Graph showing that 10 min of innocuous hindpaw stimulation strongly increased the number of cFos-positive neurons in the superficial spinal dorsal horn ipsilateral, but not contralateral, to SNT ($n=6$ animals in both the non-stimulated and stimulated groups; PWT, paw withdrawal threshold in grams; time in days after SNT; $t=0$ days corresponds to presurgical baseline values; * $p < 0.05$; ** $p < 0.01$).

FIG. 2. Temporal upregulation of growth-associated protein-43 (GAP-43) ipsilateral to spinal nerve transection (SNT). (A) GAP-43 immunostaining of the L5 spinal cord at 5 days after SNT (SNT is on the left side). (B–D) GAP-43 expression in the superficial ipsilateral L5 spinal cord of a sham-operated rat (B), at 5 days after SNT (C), and at 21 days after SNT (D). Protein kinase- γ (PKC- γ) immunoreactivity demarcates the superficial dorsal horn; insets show spinal GAP-43 expression at higher resolution. (E) GAP-43 expression in the superficial dorsal horn of the L3, L4, and L5 spinal cord (expression is shown in relation to values of sham-operated animals; sham averages $\pm$ standard error of the mean is indicated by dotted lines and grey shading; $n=4$ for sham animals; $n=5$ for 21 days after SNT; $n=6$ each for 3, 5, and 7 days after SNT; $n=10$ for 10 days after SNT; $*p<0.05$).

injury ($p<0.05$). This increase was no longer observed at 21 days after nerve injury. In the L4 spinal cord, GAP-43 expression was also increased at 5 and 7 days after SNT, although this increase did not reach statistical significance compared to levels in sham-operated animals. GAP-43 expression was unaffected in the L3 spinal cord.

In order to characterize GAP-43 expression in the superficial dorsal horn (boxes in Fig. 3A and B indicate the location studied for co-localization), double-fluorescence immunostaining was performed for GAP-43 and a range of marker proteins. A clear co-localization was found for GAP-43 and CGRP, a marker for peptidergic primary afferent fibers (Fig. 3F). GAP-43/CGRP co-localization was clearly observed in fiber-like structures (Fig. 3G and H), and was confirmed using confocal microscopy (Fig. 3I). On the contrary, GAP-43 expression did not co-localize with PKC- γ (Fig. 3C), VGLUT-1 (Fig. 3D), a marker for A fibers, or IB4 (Fig. 3E), a marker for non-peptidergic primary afferents. Moreover, GAP-43 expression was not observed in astrocytes as evidenced by non-overlapping staining of GAP-43 and GFAP (Fig. 3J and K).

PPF attenuates nerve injury-induced microglial responses and hypersensitivity, but not spinal GAP-43 expression

Daily intrathecal bolus injections of PPF for 5 consecutive days following SNT effectively attenuated mechanical hypersensitivity (Fig. 4A). At DPO 5, SNT animals treated with PPF displayed a less pronounced decrease in PWT in comparison to

saline-treated SNT animals (decrease of 26.6 ± 5.7 versus 57.3 ± 3.0 , respectively; $p<0.01$). Moreover, PPF treatment resulted in a marked decrease in the expression of the microglial marker Iba-1, but not of the astroglial marker GFAP. Saline-treated SNT animals displayed robust expression of Iba-1 in the ipsilateral dorsal horn (Fig. 4B, D, and E). PPF treatment significantly reduced Iba-1 expression in the dorsal horn of SNT animals (Fig. 4B, F, and G). Quantitative analysis showed that Iba-1 expression was significantly reduced in PPF-treated animals ($123.4 \pm 2.3\%$ compared to control levels) compared to saline-treated animals ($131.6 \pm 1.1\%$ compared to control levels; $p<0.05$). SNT was not found to change expression of the astroglial marker GFAP at this time point (5 days post-SNT; Fig. 4C, H, and I). Consequently, PPF treatment did not affect GFAP expression in the dorsal horn (Fig. 4C, J, and K).

Despite its effect on mechanical hypersensitivity and Iba-1 expression following SNT, PPF did not affect spinal GAP-43 expression (Fig. 5D). At DPO 5, spinal GAP-43 expression in the L5 superficial dorsal horn was at the same level in PPF-treated (Fig. 5C) and saline-treated animals (Fig. 5B), and both were significantly increased compared to sham-operated animals (Fig. 5A). Double staining for GAP-43 and CGRP showed that the GAP-43 was still highly regulated in peptidergic primary afferents (Fig. 5E).

Discussion

In this study, we investigated the spatiotemporal expression of GAP-43 in the superficial dorsal horn following

FIG. 3. Identification of neuronal growth-associated protein-43 (GAP-43) immunoreactivity. In order to investigate the cellular source of GAP-43 immunoreactivity following spinal nerve transection (SNT), several double immunostainings were performed. (A and B) Protein kinase- γ (PKC- γ)-immunostained sections were used to delineate the superficial dorsal horn. Insets in boxes A and B show the location in which double-stained sections were investigated. Combined immunohistochemical staining for GAP-43 with PKC- γ (C) and the primary afferent markers vesicular glutamate transporter-1 (VGLUT-1; D), isolectin B4 (IB4; E), and calcitonin-gene related peptide (CGRP; F) (all in red) at 5 days following SNT show a clear co-localization for CGRP/GAP-43 (yellow; F). High magnification of GAP-43 and CGRP shows an overlap in staining for fiber-like structures (G and H; indicated by white arrows). (I) Confocal analysis revealed an unambiguous co-localization of both GAP-43 and CGRP. (J and K) Double-staining of glial fibrillary acidic protein (GFAP) and GAP-43 shows no overlap in staining.

FIG. 4. Propentofylline (PPF) attenuates mechanical hypersensitivity and microglial ionized calcium binding adaptor 1 protein (Iba-1) expression following spinal nerve transection (SNT). (**A**) SNT induced a nearly 60% reduction of mechanical hypersensitivity of the ipsilateral hindpaw compared to the contralateral hindpaw in saline-treated animals, while PPF-treated animals only showed a reduction of about 30%, which is similar to previous observations (Raghavendra, 2003, #1268; $n=10$ for saline-treated SNT animals, and $n=12$ for PPF-treated SNT animals). (**B, D–G**) At 5 days after SNT, expression of Iba-1 was strongly upregulated in the ipsilateral spinal cord of SNT animals receiving daily intrathecal injections with saline (**D** and **E**). This was evidenced by a more than 30% increase of Iba-1 immunoreactivity compared to control animals (**B**). Daily intrathecal delivery of PPF for 5 days following SNT attenuated the expression of Iba-1 significantly ($p < 0.05$; **B, F** and **G**). In contrast to Iba-1 immunoreactivity, glial fibrillary acidic protein (GFAP) immunoreactivity was not affected by SNT (**C, H** and **I**). As a consequence, PPF did not affect GFAP expression in the dorsal horn of the lumbar spinal cord (**C, J** and **K**; $n=5$ for saline-treated SNT animals, and $n=5$ for PPF-treated SNT animals; $**p < 0.01$; $*p < 0.05$; Ipsi, ipsilateral; contra, contralateral).

transection of the L5 spinal nerve. GAP-43 expression was found to be clearly upregulated in the L5 superficial dorsal horn from 5 up to 10 days following nerve injury, while a trend toward an increase in GAP-43 expression was detected at 5 and 7 days in the ipsilateral L4 superficial dorsal horn. GAP-43 expression largely co-localized with CGRP, indicating its regulation in peptidergic primary afferent fibers. Treatment with the glial modulator PPF was effective in attenuating neuropathy-induced microglial Iba-1 upregulation and mechanical pain hypersensitivity, but neither affected spinal GAP-43 expression or its regulation in CGRP fibers.

Chronicity of pain hypersensitivity following nerve injury is thought to originate at least partly from neuropathy-induced processes of structural plasticity. Original work on changes in primary afferent innervations of the superficial dorsal horn led to the claim that upon peripheral nerve injury, large A fibers sprout into the upper substantia gelatinosa or even into lamina I (Woolf et al., 1992). As such, it was thought that low-threshold A fibers directly innervate nociception-specific neurons in lamina I, resulting in the gating of non-noxious stimuli to pain pathways. Although the evidence for A fiber sprouting has been shown to be subject to methodological bias by tracing procedures (Bao et al., 2002; Tong et al., 1999), neuropathy-induced increases in spinal GAP-43 expression still suggest that fiber sprouting occurs within the superficial dorsal horn. Spinal GAP-43 expression was previously reported to be regulated in unmyelinated fibers following nerve injury (Coggeshall et al., 1991). Moreover, it has

been suggested that nociceptive primary afferents from the L4 spinal cord re-innervate the superficial L5 dorsal horn after L5 SNT (Hu et al., 2004). We found that GAP-43 is regulated in peptidergic primary afferent fibers, based on a strong co-localization of GAP-43 and the neuropeptide CGRP. Although CGRP expression can be induced in large A fibers after nerve injury (Malcangio et al., 2000; Michael et al., 1999; Miki et al., 1998; Noguchi et al., 1995), GAP-43/CGRP fibers are most likely small fibers. Indeed, increased spinal GAP-43 expression was not observed in the deeper part of the dorsal horn, typically innervated by large A fibers (Lorenzo et al., 2008). Moreover, no co-localization between GAP-43 and the large A fiber marker VGLUT-1 (Todd et al., 2003) was observed in the present investigation. As an alternative to neuronal expression, GAP-43 expression may be regulated in glial cells. Schwann cells have been reported to upregulate GAP-43 following peripheral neuropathy (Plantinga et al., 1993a, 1993b), and astroglial GAP-43 expression has been found *in vitro* (Vitkovic et al., 1988). Importantly, we did not obtain any evidence for neuropathy-induced GAP-43 regulation in astroglial cells within the superficial dorsal horn.

The finding that GAP-43 is regulated predominantly in CGRP fibers is of particular interest to the functioning of the spinal nociceptive network, as CGRP has been strongly associated with both central sensitization and pain hypersensitivity in models of peripheral and central neuropathies (Christensen and Hulsebosch, 1997a, 1997b; Cridland and Henry, 1988; Lee and Kim, 2007; Neugebauer et al., 1996).

CGRP has been reported to increase the release of neurotransmitters from primary afferent fibers, and to regulate the expression of post-synaptic receptors, including the substance P receptor in spinal neurons (Oku et al., 1987; Seybold, 2009; Seybold et al., 2003). Hence, structural alterations in the wiring of the spinal nociceptive network, particularly in relation to CGRP-expressing fibers, may have a strong impact on the processing of noxious and innocuous information. However, our data do not support a major role for neuropathy-induced spinal GAP-43 expression in the onset of mechanical pain hypersensitivity. Treatment with the glial modulator PPF largely blunted mechanical pain hypersensitivity, while the increase in spinal GAP-43 expression induced by SNT remained unaffected, and GAP-43 was still regulated in CGRP fibers. On the basis of these data, we conclude that these structural changes are not a main player in the onset of mechanical pain hypersensitivity following nerve injury. However, it needs to be stressed that our study focused exclusively on mechanical hypersensitivity, which is one of the most debilitating features of neuropathic pain (Berger et al., 2011). Therefore we cannot exclude that the structural changes we observed in the superficial spinal dorsal horn may to some extent be related to other modalities such as cold and heat hypersensitivity.

GAP-43 regulation is most likely an early indicator of structural changes in the nociceptive network, and a return of GAP-43 expression to normal levels may point to establishment of synaptic contacts in the superficial dorsal horn, as developmental studies have shown a downregulation of GAP-43 expression after fibers reach their target (Fitzgerald et al., 1991). Consequently such structural changes may form the basis for a persistent mechanism of sensitization. The SNT model is a validated and common model to study mechanisms underlying neuropathy-induced pain sensitivity. In line with previous work by Zhang and colleagues, we found that stroking the hindpaw ipsilateral to spinal nerve injury resulted in an increased number of neurons expressing the early neuronal activation marker cFos (Zhang et al., 2007). This increase was observed in the superficial dorsal horn of the lumbar spinal cord. We propose that the innocuous stimuli are transmitted to a sensitized lumbar spinal cord via the intact L4 nerve, which is known to innervate multiple levels of the spinal cord (L2–L5 levels; Shehab et al., 2008). Although it is tempting to link structural plasticity, particularly that related to CGRP, a well-known sensitizing molecule, to neuropathy-induced pain hypersensitivity, we still lack

FIG. 5. Propentofylline (PPF) does not affect spinal growth-associated protein-43 (GAP-43) expression following spinal nerve transection (SNT). (A) Spinal GAP-43 expression in sham-operated animals sacrificed at 5 days after sham surgery. (B and C) Spinal GAP-43 expression at 5 days after SNT in a vehicle-treated (B) and a PPF-treated (C) animal. (D) PPF does not affect spinal GAP-43 expression in the superficial dorsal horn of the L5 spinal cord after SNT (GAP-43 expression is shown in relation to values of sham-operated animals; $n=4$ for sham-operated animals, $n=6$ each for SNT animals with saline or PPF treatment). (E) Representative example of GAP-43/CGRP double staining in the superficial dorsal horn of a PPF-treated animal at 5 days after SNT (* $p < 0.05$; CGRP, calcitonin gene-related peptide).

extensive knowledge about this scientific niche. Better understanding of such a link requires insights into the molecular and/or cellular cues that affect GAP-43 expression. Although the temporal character of spinal GAP-43 expression (between 5 and 10 days after SNT) coincides with glial responses to SNT (Romero-Sandoval et al., 2008), our data show that glial inhibition does not affect GAP-43 levels in the superficial dorsal horn after SNT. This observation was surprising, knowing that glial cells produce nerve growth factor (NGF; Krenz and Weaver, 2000), which affects cells expressing the receptor *trkC*, such as peptidergic primary afferent fibers (Hunt and Mantyh, 2001). However, we cannot exclude that NGF is still involved in regulation of spinal GAP-43 expression, as growth factor expression is not restricted to immune cells, but can also occur neuronally (Thoenen, 1995). Indeed, interference with NGF using an anti-NGF antibody has previously been reported to prevent the increased density of CGRP fibers in the superficial dorsal horn following experimental spinal cord injury (Christensen and Hulsebosch, 1997b). Importantly, structural changes in CGRP fibers following spinal cord injury have previously been linked to pain hypersensitivity (Christensen and Hulsebosch, 1997a; Hofstetter et al., 2005). Alternative approaches to investigate the relationship between spinal GAP-43 expression and neuropathy-induced pain hypersensitivity include strategies using antisense oligonucleotides against GAP-43 or GAP-43 antibodies, but such strategies have thus far only been exploited *in vitro* (Aigner and Caroni, 1995; Shea, 1994; Shea et al., 1991).

Conclusion

This study has identified the CGRP nature of GAP-43-expressing fibers in the spinal cord following transection of the rat L5 spinal nerve. Spinal GAP-43 expression was upregulated within the superficial dorsal horn of the L5 spinal cord from 5 up to 10 days after injury. This structural plasticity may be at least partly involved in increased early neuronal activation in these same areas following innocuous mechanical stimulation of the hindpaws. Our data do not, however, support a major role of these structural changes in the development of pain hypersensitivity. Increased understanding of the cellular and/or molecular processes involved in fiber sprouting is needed to study the direct relationship between fiber sprouting and pain hypersensitivity. This understanding may provide novel and so far largely unexploited insights that aid in the development of therapies to more effectively treat neuropathic pain.

Acknowledgments

The authors wish to thank Ms. Marijke Lemmens for her assistance in the use of the stereology-based cell counting method and the confocal SI-SD system. Thanks also to Ms. Liesbeth Knaepen for critically reading the manuscript. This work was financially supported by a grant from the Dutch Government (SENTERNOVEM grant no. IS 041064 to E.A.J. and M.A.M.), a grant from the Dutch Polymer Institute (DPI grant no. 608 to E.A.J., M.A.M., and R.D.), and two Kootstra fellowships of the Maastricht University (to R.J.J. and R.D.). The SI-SD system was funded by grant no. 911-06-003 from the Medical Section of the Dutch Scientific Organization (NWO).

Author Disclosure Statement

No competing financial interests exist.

References

- Aigner, L., and Caroni, P. (1995). Absence of persistent spreading, branching, and adhesion in GAP-43-depleted growth cones. *J. Cell Biol.* 128, 647–660.
- Bao, L., Wang, H.F., Cai, H.J., Tong, Y.G., Jin, S.X., Lu, Y.J., Grant, G., Hokfelt, T., and Zhang, X. (2002). Peripheral axotomy induces only very limited sprouting of coarse myelinated afferents into inner lamina II of rat spinal cord. *Eur. J. Neurosci.* 16, 175–185.
- Baron, R., Binder, A., and Wasner, G. (2010). Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment. *Lancet Neurol.* 9, 807–819.
- Basbaum, A.I., Bautista, D.M., Scherrer, G., and Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell* 139, 267–284.
- Berger, J.V., Knaepen, L., Janssen, S.P., Jaken, R.J., Marcus, M.A., Joosten, E.A., and Deumens, R. (2011). Cellular and molecular insights into neuropathy-induced pain hypersensitivity for mechanism-based treatment approaches. *Brain Res. Rev.*
- Christensen, M.D., and Hulsebosch, C.E. (1997a). Chronic central pain after spinal cord injury. *J. Neurotrauma* 14, 517–537.
- Christensen, M.D., and Hulsebosch, C.E. (1997b). Spinal cord injury and anti-NGF treatment results in changes in CGRP density and distribution in the dorsal horn in the rat. *Exp. Neurol.* 147, 463–475.
- Chung, J.M., Kim, H.K., and Chung, K. (2004). Segmental spinal nerve ligation model of neuropathic pain. *Methods Mol. Med.* 99, 35–45.
- Coggeshall, R.E., Reynolds, M.L., and Woolf, C.J. (1991). Distribution of the growth associated protein GAP-43 in the central processes of axotomized primary afferents in the adult rat spinal cord; presence of growth cone-like structures. *Neurosci. Lett.* 131, 37–41.
- Cridland, R.A., and Henry, J.L. (1988). Effects of intrathecal administration of neuropeptides on a spinal nociceptive reflex in the rat: VIP, galanin, CGRP, TRH, somatostatin and angiotensin II. *Neuropeptides* 11, 23–32.
- Doubell, T.P., Mannion, R.J., and Woolf, C.J. (1997). Intact sciatic myelinated primary afferent terminals laterally sprout in the adult rat dorsal horn following section of a neighbouring peripheral nerve. *J. Comp. Neurol.* 380, 95–104.
- Eijkelkamp, N., Heijnen, C.J., Willemsen, H.L., Deumens, R., Joosten, E.A., Kleibeuker, W., den Hartog, I.J., van Velthoven, C.T., Nijboer, C., Nassar, M.A., Dorn, G.W., 2nd., Wood, J.N., and Kavelaars, A. (2010). GRK2: a novel cell-specific regulator of severity and duration of inflammatory pain. *J. Neurosci.* 30, 2138–2149.
- Fitzgerald, M., Reynolds, M.L., and Benowitz, L.I. (1991). GAP-43 expression in the developing rat lumbar spinal cord. *Neuroscience* 41, 187–199.
- Hofstetter, C.P., Holmstrom, N.A., Lilja, J.A., Schweinhardt, P., Hao, J., Spenger, C., Wiesenfeld-Hallin, Z., Kurpad, S.N., Frisen, J., and Olson, L. (2005). Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. *Nat. Neurosci.* 8, 346–353.
- Hughes, D.I., Scott, D.T., Todd, A.J., and Riddell, J.S. (2003). Lack of evidence for sprouting of Abeta afferents into the superficial laminae of the spinal cord dorsal horn after nerve section. *J. Neurosci.* 23, 9491–9499.
- Hu, J., Mata, M., Hao, S., Zhang, G., and Fink, D.J. (2004). Central sprouting of uninjured small fiber afferents in the

- adult rat spinal cord following spinal nerve ligation. *Eur. J. Neurosci.* 20, 1705–1712.
- Hunt, S.P., and Mantyh, P.W. (2001). The molecular dynamics of pain control. *Nat. Rev. Neurosci.* 2, 83–91.
- Ji, R.R., Kawasaki, Y., Zhuang, Z.Y., Wen, Y.R., and Decosterd, I. (2006). Possible role of spinal astrocytes in maintaining chronic pain sensitization: review of current evidence with focus on bFGF/JNK pathway. *Neuron Glia Biol.* 2, 259–269.
- Krenz, N.R., and Weaver, L.C. (2000). Nerve growth factor in glia and inflammatory cells of the injured rat spinal cord. *J. Neurochem.* 74, 730–739.
- Latremoliere, A., and Woolf, C.J. (2009). Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J. Pain* 10, 895–926.
- Lee, S.E., and Kim, J.H. (2007). Involvement of substance P and calcitonin gene-related peptide in development and maintenance of neuropathic pain from spinal nerve injury model of rat. *Neurosci. Res.* 58, 245–249.
- Lorenzo, L.E., Ramien, M., St. Louis, M., De Koninck, Y., and Ribeiro-da-Silva, A. (2008). Postnatal changes in the Rexed lamination and markers of nociceptive afferents in the superficial dorsal horn of the rat. *J. Comp. Neurol.* 508, 592–604.
- Malcangio, M., Ramer, M.S., Jones, M.G., and McMahon, S.B. (2000). Abnormal substance P release from the spinal cord following injury to primary sensory neurons. *Eur. J. Neurosci.* 12, 397–399.
- Marchand, F., Perretti, M., and McMahon, S.B. (2005). Role of the immune system in chronic pain. *Nat. Rev. Neurosci.* 6, 521–532.
- McMahon, S.B., and Malcangio, M. (2009). Current challenges in glia-pain biology. *Neuron* 64, 46–54.
- Michael, G.J., Averill, S., Shortland, P.J., Yan, Q., and Priestley, J.V. (1999). Axotomy results in major changes in BDNF expression by dorsal root ganglion cells: BDNF expression in large trkB and trkC cells, in pericellular baskets, and in projections to deep dorsal horn and dorsal column nuclei. *Eur. J. Neurosci.* 11, 3539–3551.
- Miki, K., Fukuoka, T., Tokunaga, A., and Noguchi, K. (1998). 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* 82, 1243–1252.
- Neugebauer, V., Rumenapp, P., and Schaible, H.G. (1996). Calcitonin gene-related peptide is involved in the spinal processing of mechanosensory input from the rat's knee joint and in the generation and maintenance of hyperexcitability of dorsal horn-neurons during development of acute inflammation. *Neuroscience* 71, 1095–1109.
- Noguchi, K., Kawai, Y., Fukuoka, T., Senba, E., and Miki, K. (1995). Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. *J. Neurosci.* 15, 7633–7643.
- Oestreicher, A.B., De Graan, P.N., Gispen, W.H., Verhaagen, J., and Schrama, L.H. (1997). B-50, the growth associated protein-43: modulation of cell morphology and communication in the nervous system. *Prog. Neurobiol.* 53, 627–686.
- Oku, R., Satoh, M., Fujii, N., Otake, A., Yajima, H., and Takagi, H. (1987). Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats. *Brain Res.* 403, 350–354.
- Plantinga, L.C., Verhaagen, J., Edwards, P.M., Hol, E.M., Bar, P.R., and Gispen, W.H. (1993a). The expression of B-50/GAP-43 in Schwann cells is upregulated in degenerating peripheral nerve stumps following nerve injury. *Brain Res.* 602, 69–76.
- Plantinga, L.C., Verhaagen, J., and Gispen, W.H. (1993b). The expression of B-50/GAP-43 in Schwann cells. *Ann. NY Acad. Sci.* 679, 412–417.
- Raghavendra, V., Tanga, F., Rutkowski, M.D., and De Leo, J.A. (2003). Anti-hyperalgesic and morphine-sparing actions of propentofylline following peripheral nerve injury in rats: mechanistic implications of spinal glia and proinflammatory cytokines. *Pain* 104, 655–664.
- Ren, K., and Dubner, R. (2010). Interactions between the immune and nervous systems in pain. *Nat. Med.* 16, 1267–1276.
- Romero-Sandoval, A., Chai, N., Natile-McMenemy, N., and Deleo, J.A. (2008). A comparison of spinal Iba1 and GFAP expression in rodent models of acute and chronic pain. *Brain Res.* 1219, 116–126.
- Schmitz, C., and Hof, P.R. (2005). Design-based stereology in neuroscience. *Neuroscience* 130, 813–831.
- Seybold, V.S., McCarron, K.E., Mermelstein, P.G., Groth, R.D., and Abrahams, L.G. (2003). Calcitonin gene-related peptide regulates expression of neurokinin1 receptors by rat spinal neurons. *J. Neurosci.* 23, 1816–1824.
- Seybold, V.S. (2009). The role of peptides in central sensitization. *Handb. Exp. Pharmacol.* 194, 451–491.
- Shea, T.B. (1994). Delivery of anti-GAP-43 antibodies into neuroblastoma cells reduces growth cone size. *Biochem. Biophys. Res. Commun.* 203, 459–464.
- Shea, T.B., Perrone-Bizzozero, N.I., Beermann, M.L., and Benowitz, L.I. (1991). Phospholipid-mediated delivery of anti-GAP-43 antibodies into neuroblastoma cells prevents neurogenesis. *J. Neurosci.* 11, 1685–1690.
- Shehab, S.A., Al-Marashda, K., Al-Zahmi, A., Abdul-Kareem, A., and Al-Sultan, M.A. (2008). Unmyelinated primary afferents from adjacent spinal nerves intermingle in the spinal dorsal horn: a possible mechanism contributing to neuropathic pain. *Brain Res.* 1208, 111–119.
- Shortland, P., Kinman, E., and Molander, C. (1997). Sprouting of A-fibre primary afferents into lamina II in two rat models of neuropathic pain. *Eur. J. Pain* 1, 215–227.
- Thoenen, H. (1995). Neurotrophins and neuronal plasticity. *Science* 270, 593–598.
- Todd, A.J., Hughes, D.I., Polgar, E., Nagy, G.G., Mackie, M., Ottersen, O.P., and Maxwell, D.J. (2003). The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. *Eur. J. Neurosci.* 17, 13–27.
- Tong, Y.G., Wang, H.F., Ju, G., Grant, G., Hokfelt, T., and Zhang, X. (1999). Increased uptake and transport of cholera toxin B-subunit in dorsal root ganglion neurons after peripheral axotomy: possible implications for sensory sprouting. *J. Comp. Neurol.* 404, 143–158.
- Vitkovic, L., Steisslinger, H.W., Aloyo, V.J., and Mersel, M. (1988). The 43-kDa neuronal growth-associated protein (GAP-43) is present in plasma membranes of rat astrocytes. *Proc. Natl. Acad. Sci. USA* 85, 8296–8300.
- West, M.J., Slomianka, L., and Gundersen, H.J. (1991). Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat. Rec.* 231, 482–497.
- Woolf, C.J., Reynolds, M.L., Molander, C., O'Brien, C., Lindsay, R.M., and Benowitz, L.I. (1990). The growth-associated protein GAP-43 appears in dorsal root ganglion cells and in the dorsal horn of the rat spinal cord following peripheral nerve injury. *Neuroscience* 34, 465–478.

- Woolf, C.J., Shortland, P., and Coggeshall, R.E. (1992). Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* 355, 75–78.
- Woolf, C.J., Shortland, P., Reynolds, M., Ridings, J., Doubell, T., and Coggeshall, R.E. (1995). Reorganization of central terminals of myelinated primary afferents in the rat dorsal horn following peripheral axotomy. *J. Comp. Neurol.* 360, 121–134.
- Zhang, E.T., Ossipov, M.H., Zhang, D.Q., Lai, J., and Porreca, F. (2007). Nerve injury-induced tactile allodynia is present in the absence of FOS labeling in retrogradely

labeled post-synaptic dorsal column neurons. *Pain* 129, 143–154.

Address correspondence to:

Ronald Deumens, M.D.

Department of Anesthesiology

Maastricht University Medical Center

P. Debyelaan 25, P.O. Box 5800

6202 AZ, Maastricht, The Netherlands

E-mail: r.deumens@maastrichtuniversity.nl