Published in final edited form as:

J Pain. 2010 April ; 11(4): 388–398. doi:10.1016/j.jpain.2009.09.008.

A NOVEL METHOD TO QUANTIFY HISTOCHEMICAL CHANGES THROUGHOUT THE MEDIOLATERAL AXIS OF THE SUBSTANTIA GELATINOSA AFTER SPARED NERVE INJURY: CHARACTERIZATION WITH TRPV1 AND SUBSTANCE P

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Abstract

Nerve injury dramatically increases or decreases protein expression in the spinal cord dorsal horn. Whether the spatial distribution of these changes is restricted to the central innervation territories of injured nerves or could spread to adjacent territories in the dorsal horn is not understood. To address this question, we developed a simple computer software-assisted method to precisely distinguish and efficiently quantify immunohistochemical staining patterns across the mediolateral axis of the dorsal horn 2 wk after transection of either the tibial and common peroneal nerves (thus sparing the sural branch, spared nerve injury, SNI), the tibial nerve, or the common peroneal and sural nerves. Using thiamine monophosphatase (TMP) histochemistry, we determined that central terminals of the tibial, common peroneal, sural, and posterior cutaneous nerves occupy the medial 35%, medial-central 20%, central-lateral 20%, and lateral 25% of the substantia gelatinosa, respectively. We then used these calculations to show that SNI reduced the expression of SP and TRPV1 immunoreactivity within the tibial and peroneal innervation territories in the L4 dorsal horn, without changing expression in the uninjured, sural sector. We conclude that SNI-induced loss of SP and TRPV1 in central terminals of dorsal horn is restricted to injured fibers. Our new method enables direct comparison of injured and uninjured terminals in the dorsal horn so as to better understand their relative contributions to mechanisms of chronic pain.

INTRODUCTION

Numerous neurotransmitters, neuromodulators, and receptors of primary afferent neurons relay pain signals from the periphery to the central nervous system. In many cases, their expression is dramatically altered by peripheral nerve injury, as reflected by increases or decreases in immunoreactivity at the dorsal root ganglion (DRG) soma and their terminals in the dorsal

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horn of the spinal cord[12,35]. As these changes in protein expression often correlate with spinal neuronal excitability and behavioral hypersensitivity, many of them are believed to cause and maintain signs of abnormal sensory function, and thus contribute to the neural pathophysiology underlying chronic pain [22,34]. We do not, however, understand the *spatial distribution* of nerve injury-induced changes in spinal cord protein expression. Are they restricted to the central innervation territories of injured nerves, or do they spread to adjacent territories in the dorsal horn?

In an elegant stereological study, Beggs and Salter recognized the powerful potential of the spared nerve injury (SNI) model of neuropathic pain as a means to simultaneously assess, within the same transverse section, the immunohistochemical changes in dorsal horn that correspond to injured and uninjured afferents. They used staining of isolectin-B4 (IB4) to extensively map deficits in afferent innervation throughout the lumbar spinal cord. They found that SNI (transection of the tibial and common peroneal branches of the sciatic nerves, leaving the sural branch intact) induced the expression of immunoreactivity for the microglial marker Iba-1 not only in the central terminal fields of injured nerves, but also in adjacent regions [1]. These results indicate that nerve injury-induced glial proliferation extends beyond the zone of injured nerve terminals, and point to the unique potential of the SNI model to explore mechanisms of neuropathic pain. However, this particular approach does not readily yield a rapid and efficient solution for the *quantitative* analysis of somatotopically-determined immunohistochemical staining, and the authors did not attempt to quantify loss of IB4 or Iba-1 staining within subdivisions of the mediolateral extent of the substantia gelatinosa (SG). Of the studies that did quantify dorsal horn staining following sciatic branch transection, the mediolateral length of the SG was measured and then divided into equivalent sectors [26,32]. Because this arbitrary delineation did not accurately reflect the innervation zones of the tibial, common peroneal and sural nerves, our first goal was to develop an accurate somatotopic map for the quantification of immunohistochemical staining after SNI. To this end, we developed a computer-assisted method to precisely distinguish and quantify immunohistochemical staining patterns across the mediolateral extent of the dorsal horn. We produced three variations of the rat SNI surgery, evaluated deficits in thiamine monophosphatase (TMP) histochemistry in the L4 substantial gelatinosa, and then determined the relative width of the zones occupied by tibial (medial sector), peroneal (medial-central sector), and sural (central-lateral sector) nerve terminals (see Figure 1). We then used our algorithm to evaluate the effect of sciatic nerve branch transection on the expression of substance P, a key pronociceptive neurotransmitter released from the terminal endings of primary afferent C-fibers. We found that SNI clearly reduced the expression of SP immunoreactivity within the tibial and peroneal, but not sural sectors of the L4 dorsal horn.

Transient Receptor Potential Vanilloid 1 receptors (TRPV1) are sensory neuron-specific, non-selective cation channels on the peripheral terminals of primary afferent peptidergic and IB4-expressing C-fibers that relay noxious information to lamina I and II of the dorsal horn [4,5, 11,36,37]. In addition, mounting evidence suggests that TRPV1 receptors on the central terminals of C-fibers contribute to pain transmission in the dorsal horn [8,30]. Despite numerous studies indicating that TRPV1 contributes to behavioral signs of neuropathic pain, however, the effect of peripheral nerve injury on TRPV1 expression in the dorsal horn is poorly understood. We used our new methodology to re-evaluate this question for the first time in the SNI model.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles Rivers Laboratories, Inc), weighing 200–250g at time of delivery, were housed in individual cages in a temperature controlled room on a 12-hour light/

dark cycle (6am/6pm), and were given food and water *ad libitum*. All animal use protocols were approved by the Institutional Animal Care and Use Committees of both Tulane University and the University of Kentucky.

Spared Nerve Injury (SNI) surgery

Animal models of peripheral neuropathic pain following traumatic injury to the distal sciatic nerve or the L5 spinal nerve lead to pain hypersensitivity and mimic clinical signs of neuropathic pain such as hyperalgesia and allodynia [34]. The model used in the current study involves transection of the distal tibial and/or common peroneal branches of the sciatic nerve, leaving the sural nerve intact [9,17]. Frequently referred to as spared nerve injury (SNI), inherent advantages of this model include surgical simplicity, reproducible damage to specific peripheral axons, severe and prolonged mechanical and cold allodynia lasting at least 6 months, and reliability (hypersensitivity occurs in every animal).

Anesthesia was induced and maintained throughout surgery with 5% and ~2% isoflurane, respectively. The left hind-leg area was shaved and wiped clean with alcohol and betadine. An incision was made in the skin at the level of the trifurcation of the left sciatic nerve. The overlying muscles were retracted, exposing the common peroneal (CP), tibial (T), and sural (S) nerves. CP and T were ligated with 6.0 silk (Ethicon, Somerville, NJ) and then the knot and adjacent nerve (2 mm) were transected (T_xCP_x) as described previously [9]. Care was taken to avoid touching the S branch. Two additional models were tested in which the tibial nerve alone or the common peroneal and sural nerves were transected (T_x and CP_xS_x , respectively). The muscle was sutured with absorbable 5-0 sutures (Ethicon) and the wound was closed with 9 mm metal clips. Sham surgery was produced by skin incision at the level of the trifurcation, and by exposing but not touching the three sciatic branches. Behavioral tests and then perfusions were performed 2 wk after nerve injury.

Behavioral pain tests

Tactile threshold—Tactile threshold was assessed with an incremental series of 8 von Frey filaments of logarithmic stiffness (Stoelting, Inc.; approximately 0.7–11.8 g). The 50% withdrawal threshold was determined using the up-down method of Dixon, modified by Chaplan et al [6]. First, an intermediate von Frey filament (2.0 g) was applied perpendicular to the hind-paw surface with sufficient force to cause a slight bending of the filament. In case of a positive response (rapid withdrawal of the paw within 3 sec), the next smaller filament was tested. In case of a negative response, the next larger filament was tested.

Response to cool temperature—Using a syringe connected to PE-90 tubing, flared at the tip to a diameter of 3½ mm, we applied a drop of acetone to the plantar paw. Surface tension maintained the volume of the drop to 10–12 µl. The length of time the animal lifted or shook its paw was recorded. The duration of paw response was recorded for 30 sec. Three observations were averaged for analysis.

Noxious response to noxious mechanical stimulus—We gently applied the tip of a diaper pin to the footpad, avoiding damage to the skin. The duration of paw withdrawal was recorded for 30 sec. Three observations were averaged for analysis.

Perfusion

Substance P—Animals were deeply anesthetized with an overdose of ketamine/xylazine (Vedco, St. Joseph, MO, Henry Schein, Melville, NY, respectively) by intramuscular injection (1 ml/kg of 88.9 mg/ml ketamine/11.1 mg/ml xylazine). Animals were transcardially perfused with ~200 ml chilled 2% sodium nitrate followed by ~400 ml 4% paraformaldehyde with acrolein (2.5 ml/100 ml paraformaldehyde). The spinal cords were removed and post-fixed in

4% paraformaldehyde at 4° for 3 hrs and transferred to 0.05 M PBS overnight. Spinal cord sections from the L4 region were cut at 50 µm on a vibratome (Lancer Series 3000, St. Louis, MO).

TRPV1—Animals were deeply anesthetized with pentobarbital (60 mg/kg, i.p., Sigma, St. Louis, Missouri) and spinal cords were perfused with 150–250 ml of heparinized PBS followed by 250–500 ml of 10% buffered formalin. Spinal cords were removed and postfixed for 48h in 30% sucrose. Forty micron sections were cut on a microtome (Microm International HM 450, Walldorf, Germany) with attached freezing stage at approximately –20°C.

Dorsal Horn Immunohistochemistry

Tissue sections were first washed in 0.1 M phosphate buffered saline (PBS), blocked in 1% H₂O₂, 0.02% Triton X, and 1% normal goat serum (NGS), and then blocked in 0.4% Triton X and 2% NGS. Next, they were incubated with a guinea pig anti-Substance P (1:20,000, BGP 450-01, Accurate Chemical, Westbury, NY) or anti-TRPV1 antibody (1:10,000, Neuromics, Edina, MN) at 4°C for 24–72 h. After further washes in PBS and block in 0.2% Triton X and 1% NGS, sections were incubated in biotinylated goat anti-guinea pig secondary antibody for 2 h at room temperature. After further washes in PBS, signal was enhanced with the ABC method according to the manufacturer's instructions (Vectastain Elite ABC kit, PK-6100, Vector Labs) for 1 h at room temperature. After further washes in PBS, tissues were incubated with diaminobenzidine (DAB) for approximately 5 min. After final washes in 0.01M PB, sections were mounted on Superfrost/Plus glass slides (Fisherbrand, Houston, TX), allowed to dry, dehydrated in an alcohol series, and then cover-slipped using Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI) or Permount (Fisherbrand, Houston, TX).

Dorsal Horn Thiamine Monophosphatase Histochemistry

We used the TMP staining protocol of Shields et al. [26], which is a modified version of the Knyihar-Csillik protocol [16]. The sections were first washed 2 × 5 min in 0.04 M Tris maleate (pH 5.6), and then incubated for 90 min at 37° in 6 mM TMP/Pb(NO₃)₂ buffer. The sections were rinsed in 0.04 M Tris maleate (pH 5.6) for 10 min and then incubated in 1% sodium sulfide for 5–10 sec. The sections were rinsed 2 × 5 min in dH₂O, mounted on Superfrost/Plus glass slides (Fisherbrand, Houston, TX), allowed to dry, dehydrated in an alcohol series, and then cover-slipped with Permount.

Dorsal Horn Image Acquisition

Digitized images of immunostained sections taken from lumbar segment L4 and the rostral part of L5 were captured on a Nikon TE2000-E microscope with a 4X objective on black and white Roper Scientific camera at 12-bit resolution (4096 intensity levels), using Metamorph Imaging software (Version 6.1r4, Universal Imaging Corp.). To standardize image acquisition across staining sessions, we first used the “Shading Correction” tool to normalize light intensity across the field of view. More importantly, we captured images only after adjusting exposure time with the “Image Gamma” tool. This resulted in essentially identical contrast from picture to picture. All procedures were performed blind to treatment.

Densitometric Analysis of SP and TRPV1 Staining within Mediolateral Sub-regions of the Substantia Gelatinosa

We faced a unique challenge in the design of an automated, computer-assisted method to quantify staining intensity within the T, CP, and S sub-regions of the dorsal horn: the width of the SG is highly variable between sections. Figure 2 illustrates our normalization procedure using an L4 tissue section from an SNI animal that was stained for Substance P. The image of Fig 2A was digitized with Metamorph imaging software as shown in Fig 2B. As previously

described [29], the upper and lower threshold optical densities were adjusted to encompass and match the immunoreactivity, providing an image with immunoreactive material appearing red. The area and density of pixels within the threshold values representing immunoreactivity were calculated and the integrated density was the product of the area and density.

Second, as illustrated in Fig 2C, we created a 900 μm in width template consisting of 224 narrow adjacent boxes, 260 μm in height and 4 μm in width, and manually placed it over the left or right digitized image of the dorsal horn such that the right-most or left-most box overlaid the most medial edge of contralateral or ipsilateral SG, respectively (boxes placed over the ipsilateral side in Fig 2). Even if nerve injury abolished medial staining (as is often the case following transection of the tibial nerve), the experimenter was easily able to distinguish between the labeled gray matter of the SG from the white matter of the dorsal columns.

Third, as illustrated in Fig 2D, all boxes that extended beyond the lateral edge of the gray matter were deleted with a computerized algorithm. The lateral edge of the gray matter was always strongly stained following tibial, common peroneal, and/or sural nerve transection, because these surgeries do not target the posterior cutaneous nerve (which innervates the most lateral aspect of the L4 SG). By contrast, TMP histochemistry and SP or TRPV1 immunohistochemistry never yielded much reaction product within the white matter. Therefore, boxes whose measured intensity value was less than 30% of the overall average intensity were reliably and accurately removed in an automated, computer-assisted fashion.

Finally, using the boundaries calculated with TMP histochemistry and tibial/common peroneal/sural nerve transection studies (see Results), the remaining boxes were divided into tibial (most medial 30.5% of boxes, here-on referred to as the medial subdivision), common peroneal (the next medial 18.8% of boxes) or sural (the next lateral 24.6% of boxes) subregions of the SG. We refer to these regions as the “medial”, “medial-central”, and “central-lateral” subdivisions, respectively, which correspond to the tibial, common peroneal, and sural innervation territories of the hindpaw (Figure 1).

Using standard Metamorph procedures, integrated density was calculated for each sub-region, and then imported into a Microsoft Excel spread sheet. We evaluated staining on both sides of the dorsal horn, ipsilateral and contralateral to treatment. We averaged the results of 4–6 sections per rat, and studied 4–6 rats per surgery. Data were reported as ipsilateral/contralateral ratio.

TRPV1 Dorsal Root Ganglion Immunohistochemistry

Following dissection, DRG were placed in fixative for 1–2 hours, cryoprotected in 30% sucrose, frozen in O.C.T. and sectioned at 14 μm with a cryostat. Slide-mounted sections were then washed three times for 10 min in 1% Normal Goat Serum in PBS with Triton (NGST), slides were blocked for 60 min with 5% NGST, washed again, and then incubated for 72 hrs with primary antibody (guinea pig anti-TRPV1, 1:1000, Neuromics, Edina, MN) They were then rinsed 4 $\times$ 10 min in 1% NGST, re-blocked for 30 min with 5% NGST and incubated overnight with a fluorescent secondary antibody (in 1% NGST). The slides were then rinsed 3 $\times$ 10 min in 0.1 PB, and cover-slipped with Prolong Gold Anti-Fade Reagent (Invitrogen, Eugene, OR). Unless otherwise mentioned, all steps were performed at 4°.

Image Acquisition and Quantification of TRPV1 Staining in DRG

Digital photomicrographs (100 $\times$: 10 $\times$ ocular, 10 $\times$ objective) of at least 5 randomly selected sections were taken from both L4 and L5 DRG. To standardize exposure time, we used the ‘autoexpose’ function on 3 stained photomicrographs; the average exposure time was applied to all further image captures. Only cells with a visible nucleus were analyzed. Background

luminosity was determined by averaging the maximum luminosity of five clearly unlabeled cells. Only cells with luminosity at least twice the background luminosity were counted as labeled cells. To ensure that cells were not counted more than once, sections that were analyzed were at least 144 μm apart. Cells with a diameter $\geq 50 \mu\text{m}$ were designated as “large” cells, and all others were designated as “small/medium”. Percentages of labeled cells within a given size range were calculated for each DRG and averaged for each group.

Statistical analyses

Behavior—To analyze behavioral data, we used GraphPad Prism 5 software to conduct one-way ANOVA using Surgery (SHAM, T_xCP_x , T_x and CP_xS_x) as the between groups factor. If the F-value was significant ($P < 0.05$), then Bonferroni’s post hoc test was used to determine differences compared to SHAM.

Immunohistochemistry—We conducted a two-way analysis of variance, with Surgery and Region (medial, medial-central and central-lateral) as the between-subjects factors. When F values were significant ($p < 0.05$), Bonferroni post hoc tests were used to determine differences between groups. All data are presented as mean $\pm$ SEM.

RESULTS

Tibial but not sural nerve transection elicits allodynia in the rat

To determine the behavioral signs of neuropathic pain following SNI and variants of SNI, we evaluated responses to mechanical and cool stimuli at the central and/or lateral aspects of the plantar hindpaw 14 d after T_xCP_x , T_x , CP_xS_x , or sham surgery. Figure 3A illustrates robust mechanical allodynia when applying von Frey hairs to the ventrolateral aspect of the hindpaw after T_xCP_x [$P < 0.001$] and T_x alone [$P < 0.001$], but not after CP_xS_x [$P > 0.05$]. Fig 3B illustrates that, compared to SHAM, the coolness associated with acetone application/evaporation produced robust increases in paw withdrawal duration after T_xCP_x [$P < 0.001$] and T_x alone [$P < 0.001$]. CP_xS_x produced a smaller, statistically insignificant change ($P > 0.05$). Similarly, Fig 3C illustrates robust mechanical hyperalgesia when applying a noxious stimulus to the ventrolateral hindpaw after T_xCP_x [$P < 0.001$] and T_x alone [$P < 0.001$], but not after CP_xS_x [$P > 0.05$]. When we applied mechanical stimuli to the central region of the ventral hindpaw, we observed no effect of any combination of nerve transections when compared to SHAM [$p > 0.05$, data not shown].

Topographic determination of mediolateral sub-regions within the dorsal horn

We developed a reliable method to *quantify* immunohistochemical staining within precise mediolateral borders defined by the spinal innervation territory of each sciatic nerve branch. Extending the strategy of Swett and Woolf[33] and Shields et al.[26], we used thiamine monophosphatase (TMP) histochemistry to estimate the topographical boundaries of the tibial, common peroneal and sural innervation regions of the dorsal horn. TMP is expressed in cell bodies and central terminals of a subpopulation of small diameter dorsal root ganglion neurons that express the lectin IB4 and are predominantly non-peptidergic C-fibers [28]. Our method is based on the assumption that the distribution of injured primary afferent terminals (tibial, peroneal, sural, and posterior cutaneous nerves innervating the medial, medial-central, medial-lateral, and lateral L4 dorsal horn) is reflected by transganglionic degenerative atrophy, indicated by loss of TMP staining [16]. This assumption was used by Shields et al to roughly (but not precisely) estimate the central innervation territories of sciatic nerve branches across the medial-lateral extent of lamina II in the mouse [1,26].

As illustrated in Fig 4A, TMP spans the mediolateral extent of the dorsal horn in SHAM rats. To estimate the mediolateral extent of innervation of T, CP, and S within the dorsal horn, we

evaluated TMP staining after T_x , T_xCP_x , and CP_xS_x [26]. In accordance with the somatotopic maps of Swett and Woolf [33], T_x abolished TMP staining in the medial region (Fig 4B). For each of 10 sections, we divided the width of this staining deficit (T_d), by the maximum width of the SG, yielding the % of TMP staining due to tibial innervation (**T%**). When averaged across all T_x tissue samples, we calculated that the tibial nerve innervates the most medial 34.5% of the dorsal horn.

Fig 4C illustrates loss of TMP staining in the medial and medial-central regions of the SG after T_xCP_x . Because our three surgical techniques did not disrupt staining in the most lateral territory (innervated by the non-sciatic, post-cutaneous nerve), analysis of T_xCP_x staining could not reveal the territory innervated by the sural nerve. However, using subtraction, we were able to determine the medial-lateral extent of TMP staining within the spinal innervation territory of the common peroneal nerve branch. To do this, we measured the width of the staining deficit (T_d,CP_d), divided by SG, and then subtracted T% from the average T_d/CP_d % ($T_d,CP_d/SG \times 100$) using the formula:

$$\% \text{ of TMP due to CP Innervation (CP\%)} = (T_d, CP_d / SG \times 100) - T\%$$

The average of 10 sections yielded an estimate that the common peroneal nerve innervates approximately 18.9% of gray matter, localized immediately lateral to the tibial territory.

Fig 4D illustrates loss of TMP staining in the medial-central and central-lateral regions of the SG after CP_xS_x . To determine the medial-lateral extent of TMP staining within the spinal innervation territory of the sural nerve branch, we measured the width of the staining deficit (CP_d,S_d), divided this value by SG, averaged these percentages, and then subtracted CP% using the formula:

$$\% \text{ of TMP due to S Innervation (S\%)} = CP_d, S_d / SG \times 100 - CP\%$$

The average of 10 sections yielded the following estimate: the sural nerve innervates 26.1% of gray matter, localized immediately lateral to the common peroneal territory.

The sum of T% + CP% + S% equals 79.5%. The remaining TMP staining, spanning the far lateral region of the SG, predominantly arises from non-sciatic innervation of the posterior cutaneous nerve (pC), with a small contribution from the tibial nerve (Fig 1) [26,33]. Thus, pC % is approximately 20.5% of SG.

In addition to Fig 4, Fig 2D also summarizes the distribution of SP staining within the dorsal horn regions innervated by T, CP, and S.

Peripheral nerve injury dramatically decreased SP-LI, specifically in the innervation territories of severed afferents

As described previously, SP-ir appeared as a discrete band in laminae I-II of control animals (Fig 5, top panels). Two-way ANOVA revealed that nerve injury decreased SP-ir [$F(3,60)=44$, $P<0.0001$], and this decrease was dependent upon the sub-region analyzed [$F(2,60)=14$, $P<0.0001$]. As quantified in Figure 5 (bottom), subsequent Bonferroni tests revealed that T_xCP_x (SNI) decreased staining in the medial and medial-central sub-regions by 73% and 38%, respectively. By contrast, T_x significantly decreased staining only in the medial region of tibial innervation (by 76%), while CP_xS_x significantly decreased staining in the medial-central and central-lateral region of common peroneal and sural nerve innervation by 27% and 29%, respectively. Analysis of total staining across all sub-regions also yielded significant drops in

SP-ir after each nerve injury. The magnitude of these changes were much less than observed in the specific innervation territories of the injured nerve branches, illustrating the power of our new quantification technique.

TRPV1 DH Staining

As described previously [7,10,36], TRPV1-ir was concentrated in lamina I and the inner part of lamina II (Fig 6). Two-way ANOVA revealed that nerve injury decreased TRPV1-ir [$F(3,57) = 9.8$, $P < 0.0001$]. As quantified in Figure 6 (bottom), subsequent Bonferroni tests revealed that SNI decreased staining in the medial (by 61%) and medial-central sub-regions. T_x significantly decreased staining only in the medial region of tibial innervation (by 54%), while CP_xS_x significantly decreased staining in the medial-central and central-lateral region of common peroneal and sural nerve innervation, respectively.

TRPV1 DRG Expression and Neuronal Size Distribution

To compare the effect of nerve injury on CNS terminal TRPV1 expression with cell body immunostaining, we evaluated TRPV1 in the L4 and L5 dorsal root ganglion (DRG). The frequency of TRPV1-positive profiles relative to total cell count was approximately 25%, largely restricted to small and medium size neurons (mean cross-sectional area of $480 \mu m^2$). As previously reported [11,20], Fig 7 illustrates that the most intense staining was observed in smaller diameter cells (perhaps unmyelinated C-fibers), while less intense staining was noted in medium diameter cells (perhaps thinly myelinated A- δ fibers). Immunoreactivity within larger profiles ($1,100$ to $1,400 \mu m^2$) was rare.

Fig 7 illustrates that each of the nerve injury surgeries decreased TRPV1-ir as compared to contralateral or SHAM levels ($P < 0.001$), without changing mean neuron size (Sham ipsi/contra = $395/384 \mu m^2$; T_x ipsi/contra = $436/435 \mu m^2$; SNI ipsi/contra = $346/333 \mu m^2$; CP_xS_x ipsi/contra = $355/334 \mu m^2$, all $p > 0.05$).

DISCUSSION

Tracing studies in the rat demonstrate that the central projections of hind limb sensory nerves are highly organized, and occupy well-delineated compartments within the medial 2/3 to 3/4 of the spinal L4 segment of the SG. These medial, medial-central, and central-lateral zones represent the innervations territories of the T, CP, and S nerves [21,27,33,38]. Consistent with these tracing studies and with mouse TMP studies [26], we report that transection of specific sciatic nerve branches results in topographically-coordinated losses in TMP and SP staining. Furthermore, assuming that deficits in TMP staining adequately represent the somatotopic boundaries of small primary afferent terminal fields in lamina II of the L4 segment, we calculated that the tibial, peroneal and sural nerves innervate approximately 35%, 20%, and 25% of the medial, medial-central, and central-lateral regions of the rat SG, respectively. Although inter-individual differences are present [21], these percentages are certainly more accurate than those used in previous studies, which arbitrarily divided the medial-lateral axis of the SG into equivalent sectors [26,31]. Second, because our computer-assisted analysis corrects for variations in width of the SG, we are able to quickly analyze staining intensity within the three innervation zones.

SNI decreases SP in medial dorsal horn

The precision and power of our new methodology is best-illustrated with the SP data showing that we can accurately quantify changes in dorsal horn neurochemistry following various combinations of sciatic nerve branch injury. Numerous studies in other models of peripheral neuropathic pain have shown that nerve injury alters immunoreactivity in the dorsal horn; however, it has been difficult to determine the physiological relevance of such changes in terms

of pain transmission. For example, both partial sciatic nerve injury (pSCI) and chronic constriction injury to the sciatic nerve (CCI) arbitrarily damage central terminals of primary afferents throughout the width of lamina II [2] [25]. And while spinal nerve ligation (SNL) produces a discreet loss of SP-ir within the L5 segment, technical difficulties in assessing rostral-caudal boundaries in transverse sections hampers the analysis [15]. By contrast, with the simple demonstration that SP deficits do not extend beyond somatotopic medial-lateral boundaries (e.g. into the central innervation territory of the sural nerve after SNI), we can conclude that nerve injury-induced loss of spinal SP does not directly control sural nerve hypersensitivity. For example, T_x decreased SP-ir only in the medial region corresponding to the injured tibial nerve, while CP_xS_x decreased SP-ir only in the central-lateral region corresponding to the injured sural nerve. Thus, deficits in SP are largely if not exclusively imprinted into the dorsal horn regions that are innervated by damaged neurons. Furthermore, because SP-ir did not increase in adjacent lateral territories of uninjured neurons, our results suggest that any elevations in SP of spared DRG neurons [18] are not coincident with increases in their terminal staining.

SNI decreases TRPV1 in medial dorsal horn and DRG

We found that common peroneal and/or tibial nerve transection decreased TRPV1-ir within the medial sector of the L4 dorsal horn, and did not change TRPV1 within the central-lateral, spared sector occupied by sural nerve terminals. These results do *not* support the suggestion that nerve injury increases TRPV1 in the central terminals of undamaged neurons [14]. We speculate that this discrepancy could be due in part to differences in surgical model (CCI vs SNI), quantification assay (western-blot vs IHC), and TRPV1 antibody (Trans-Genic vs Neuromics). Indeed, the Trans-Genic antibody used by Kanai et al was associated with relatively robust staining of the most superficial territory of lamina I, which likely represents immunoreactive astrocytes [10].

The loss of spinal TRPV1 was accompanied by down-regulation of protein expression in DRG neurons. Similar results were observed in L5 DRG after sciatic nerve axotomy [20], L5 spinal nerve ligation [19], chronic constriction injury [23] or partial sciatic nerve ligation (but only in damaged DRG [13]). Our results in dorsal horn suggest that TRPV1 decreases in injured DRG neurons. If TRPV1 increased in the cell bodies of uninjured neurons as suggested by the partial sciatic nerve ligation studies of Hudson and colleagues [13], our results would have to reflect relatively greater decreases in TRPV1 expression in injured DRG.

Tibial nerve injury elicits more allodynia than peroneal or sural nerve injury

Transection of the tibial nerve, either alone or in combination with CP, significantly increased mechanical and cold sensitivity. By contrast, transection of CP and S did not increase sensitivity in the territory of the spared tibial nerve (data not shown). These results are consistent with rat studies showing that transection of the tibial nerve (with or without CP or S) yielded more mechanical and cold allodynia than surgeries involving CP and/or S [17]. Our results are also consistent with those in the mouse, which also showed that transection of the tibial nerve (with or without CP), but not the CP and S nerve together, increased mechanical sensitivity [3]. The tibial nerve is the largest branch of the rat sciatic nerve [24], and this could explain the relatively strong behavioral sensitivity seen after T_x and T_xCP_x , but not CP_xS_x . Bourquin et al. proposed that a minimum amount of fibers must be damaged in order to trigger allodynia, and therefore the tibial nerve must be transected in order to reach this threshold [3].

Summary

We developed a strategy to precisely quantify the regional loss of immunohistochemical staining across the medial-lateral extent of the dorsal horn. Our method of spinal cord immunohistochemistry provides a powerful approach to simultaneously *quantify*, within the

same transverse section, the immunohistochemical changes in L4 dorsal horn that correspond to injured and uninjured afferents. This refined method of immunohistochemical analysis is uniquely suited for studies in spared nerve injury models of neuropathic pain, and will allow more detailed exploration of the mechanisms underlying neuropathic pain.

PERSPECTIVE

A simple computer software-assisted algorithm was developed to precisely distinguish and efficiently quantify immunohistochemical staining patterns across the mediolateral axis of the dorsal horn after distal sciatic branch transection. This method will facilitate a better understanding of the relative contribution of injured and uninjured terminals to mechanisms of chronic pain.

Acknowledgments

Supported by grants R01NS45954, R21DA18732, and K02DA19656 to BKT and the VA and ONR to JEZ. Portions of this study were presented in abstract form at the Society for Neuroscience meeting in New Orleans, Program No. 178.5, 2003. Contents do not represent the views of the VA or the US Government. We thank R. Denis Sognier for his assistance with DRG removal and cell counting methodology, Yonatan Carl for his assistance with formulation of the automated Metamorph analysis procedures, Shannon Shields for a critical review of the manuscript, and Jerone Farley for final touch-up of Figure 1.

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Figure 1. Diagram of the tibial, common peroneal, and sural branches of the sciatic nerve, their dorsal root origins, and the topography of their distal and central termination sites

The three primary distal branches of the sciatic nerve innervate the lateral (sural, blue color), central plantar (tibial, red color), and central dorsal (common peroneal, green color, dorsum not shown) sensory territories of the rat hindpaw (adapted from Decosterd and Woolf, 2000). At the level of L4 spinal cord, these branches terminate within the medial (tibial, red color), medial-central (common peroneal, green color) and central-lateral (sural, blue color) zones of the dorsal horn.

Figure 2. Densitometric Analysis of immunostaining within medial-lateral sub-regions of the dorsal horn

Panel A is a representative transverse section through the L4 spinal cord depicting SP immunoreactivity in the dorsal horn of an SNI rat. Panel B illustrates the image after digitization using Metamorph software. Panel C illustrates placement of a 224-box template such that the left-most box overlaid the most medial edge of the ipsilateral (right) substantia gelatinosa. Panel D illustrates the results of an algorithm-assisted deletion of boxes that extended beyond the lateral edge of the gray matter. The remaining boxes were divided into tibial (T), common peroneal (CP), sural (S), or posterior cutaneous (wide boxes) sub-regions of the SG. Measure bar = 200 μ m.

Figure 3. Tibial nerve transection increases sensitivity at the distal innervation territory of the sural nerve

Transection of the common peroneal (CP) and/or tibial (T) nerves decreased von Frey threshold (Panel A), increased duration of hindpaw withdrawal responses to the plantar application of acetone (cold response, Panel B), and increased duration of hindpaw withdrawal responses to plantar application of a noxious mechanical stimulus (pinprick response, Panel C). $n = 4-8$. Values represent mean $\pm$ SEM. * $P < 0.001$ as compared to SHAM.

Figure 4. Topographic loss of TMP staining

Representative photomicrographs from the L4 spinal segment of rats euthanized 2 wk after sham surgery (A), unilateral tibial and common peroneal nerve transection (B), unilateral tibial nerve transection (C), or unilateral common peroneal and sural nerve transection (D) surgery. Note that TMP staining is abolished in a surgery-specific manner. Arrows highlight areas of decreased immunoreactivity. Measure bar = 200 μ m.

Figure 5. Substance P immunohistochemistry in dorsal horn after transection of select primary branches of the sciatic nerve

SP levels are abolished in a surgery-specific manner in the dorsal horn. The top 4 panels illustrate representative photomicrographs of SP staining at 2 weeks after SHAM, T_xCP_x, T_x, or CP_xS_x surgery. The histogram at the bottom illustrates that tibial, common peroneal, and sural nerve transection decreased the staining intensity of SP at the medial, medial-central, and central-lateral zones of the dorsal horn, respectively. This is topographically-consistent with the innervation territories of each sciatic nerve branch. Values represent mean ± SEM.

*P<0.0001 as compared to SHAM. n = 4–7. Measure bar = 200 μm.

Figure 6. TRPV1 immunohistochemistry in dorsal horn after transection of select primary branches of the sciatic nerve

When compared to SHAM controls (top panel) or to the contralateral side (left), TRPV1 staining decreased on the ipsilateral side (right) after T_xCP_x, T_x, or CP_xS_x. The histogram at the bottom illustrates that T_xCP_x, T_x and CP_xS_x significantly decreases TRPV1 in a manner that is consistent with their innervation territories along the mediolateral axis of the dorsal horn. *P<0.05 as compared to SHAM. Values represent mean ± SEM. n = 4–10. Measure bar = 200 μm

Figure 7. TRPV1 immunohistochemistry in DRG after transection of select primary branches of the sciatic nerve

When compared to Sham controls (top panel) or to the contralateral side (SNI contra), TRPV1 staining decreased on the ipsilateral side after T_xCP_x (SNI ipsi). The histogram at the bottom shows that the percentage of TRPV1+ neurons was smaller in the ipsilateral as compared to the contralateral L4/L5 DRG two weeks after each of the peripheral nerve injuries. *n* = 3–5. Values represent mean ± SEM. **P* < 0.05 as compared to contralateral side. Measure bar = 50 μm.