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Time Course of Substance P Expression in Dorsal Root Ganglia Following Complete Spinal Nerve Transection

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Abstract

Recent evidence suggests that substance P (SP) is upregulated in primary sensory neurons following axotomy, and that this change occurs in larger neurons that do not usually produce SP. If so, this upregulation may allow normally neighboring, uninjured, and non-nociceptive dorsal root ganglion (DRG) neurons to become effective in activating pain pathways. Using immunohistochemistry, we performed a unilateral L5 spinal nerve transection upon male Wistar rats, and measured SP expression in ipsilateral L4 and L5 DRGs and contralateral L5 DRGs, at 1 to 14 days postoperatively (dpo), and in control and sham operated rats. In normal and sham operated DRGs, SP was detectable almost exclusively in small neurons ($\leq 800 \mu\text{m}^2$). Following surgery, the mean size of SP-positive neurons from the axotomized L5 ganglia was greater at 2, 4, 7 and 14 dpo. Among large neurons ($> 800 \mu\text{m}^2$) from the axotomized L5, the percentage of SP-positive neurons increased at 2, 4, 7, and 14 dpo. Among small neurons from the axotomized L5, the percentage of SP-positive neurons was increased at 1 and 3 dpo, but was decreased at 7 and 14 dpo. Thus, SP expression is affected by axonal damage, and the time course of the expression is different between large and small DRG neurons. These data support a role of SP-producing, large DRG neurons in persistent sensory changes due to nerve injury.

INTRODUCTION

Nerve injuries that partially denervate an extremity often lead to persistent spontaneous pain and sensory hypersensitivities (Mantyh, 1991; Woolf and Mannion, 1999). The mechanisms underlying these changes remain unclear. Substance P (SP) has been implicated as an important pain-signaling peptide at the first central synapse in the spinal cord and in the brainstem (reviewed by DeVane, 2001). In normal animals SP is expressed almost exclusively in smaller dorsal root ganglion (DRG) neurons, but typically not in larger neurons (Lee et al., 1985; Szucs et al., 1999). Substance P is released in the dorsal horn following noxious peripheral stimulation (Abbadie et al., 1996; Allen et al., 1999; Schicho et al., 2005), and activates neurokinin-1 (NK-1) receptors on nociceptive spinal neurons,

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thereby transmitting nociceptive signals. Furthermore, spinal neurons bearing the SP receptor are important for triggering and maintaining central sensitization (Khasabov et al., 2002), and hence they may contribute to the sensory hypersensitivities that occur in partially denervated areas, as well as surrounding areas (Nichols et al., 1999).

There is considerable evidence implicating the activity of A β fibers, which are from large DRG neurons, in the type of tactile hypersensitivity termed allodynia (reviewed by Gracely, 1999), and that large DRG cellular SP may play a role. Tactile hypersensitivity in rodents occurs as early as 1 day after nerve injury (Sorkin and Doom, 2000; Chacur et al., 2001; Wallas et al., 2003), the same time that substantial ectopic firing begins in A β fibers (Sun et al., 2005). Large DRG neurons express preprotachykinin, the precursor of SP, after nerve injury and inflammation (Noguchi et al., 1994; Marchand et al., 1994; Kawakami et al., 1994; Noguchi et al., 1995; Neumann et al., 1996; Ma and Bisby, 1998b). While exogenous SP has little effect upon large DRG neurons from normal rats, it is excitatory to large DRG neurons following axotomy (Abdulla et al., 2001). Also, following sciatic nerve transection, stimulation of low threshold A β afferents axons can cause SP release in the spinal cord (Malcangio et al., 2000; Meyer-Tuve et al., 2001). These data support a mechanism for the involvement of larger DRG neurons in tactile hypersensitivity following nerve injury.

Although there are numerous reports that chronicle sensory changes following nerve injury, there are no studies addressing the modulation of SP in DRG neurons in the first few days following nerve injury. In this study, we have used immunocytochemical labeling to evaluate the time course of SP expression in the DRG, before and following a spinal nerve transection in rats. Here we report that the percentage of DRG neurons expressing SP is increased in the first few days following a spinal nerve lesion. We also report that for large neurons this increase is maintained, while for smaller neurons the percentage of SP-positive neurons decreases at 1 and 2 weeks following the nerve injury.

MATERIALS AND METHODS

Animals

Experiments were carried out using 42 adult male Wistar rats weighing 250-300g. The protocol was approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center and was in accordance with the guidelines of the International Association for the Study of Pain.

Surgery

Anesthesia was induced using 2.5-3% isoflurane in oxygen, and maintained with 1.5 - 2% isoflurane in oxygen. Tight ligation and transection of the left fifth lumbar spinal nerve (L5) was performed, using technical measures aimed at minimizing tissue damage. Using strict aseptic technique, the skin over the lower lumbar transverse processes was cut over L3 - L6, and the paraspinal muscles were retracted following natural fascial planes. A specially designed retractor was made with shaped blades, and was used to maintain a small window that revealed the left L6 transverse process. The superior portion of this process was removed to reveal the ventral ramus of the left L5 spinal nerve. This nerve was freed from the surrounding tissue, tightly ligated using 7-0 prolene ~4 mm distal to the DRG, and transected just distal to the ligature. The procedure unavoidably destroyed the L5 dorsal ramus; thus all sensory, motor, and sympathetic axons from this level were cut. The incision was closed in layers using 4-0 prolene. After surgery, the animals were randomly assigned to one of the survival time groups of 1, 2, 3, 4, 7, or 14 days postoperative (dpo; 4 rats per group). Four rats that did not undergo any procedures were used as controls. Twelve rats underwent sham surgery, which included all procedures except for the nerve ligation, to

control for possible effects of the surgical procedure. These animals were randomly assigned to survival times of 1, 4, and 7 dpo (4 per group).

Immunohistochemistry

Animals were anesthetized with an overdose of sodium pentobarbital (200 mg/kg, i.p.), perfused transcardially with heparinized 0.1 M phosphate buffered saline (pH 7.4), and fixed with 300 ml of a 4% paraformaldehyde, 4% sucrose solution over 15 minutes. The axotomized L5, ipsilateral unoperated L4, and the contralateral L5 DRGs were removed from all animals and placed in the same fixative for 1 hour. They were then transferred to 30% sucrose in 0.1 M phosphate buffered saline overnight for cryoprotection. Ganglia were mounted for sectioning upon their ventral surface, as they appeared during tissue harvest; thus, frontal sections were made. Eight μm sections were cut using a cryostat and sequentially thaw-mounted on glass slides. Sections were washed in Tris buffer (pH 7.6), blocked in 4% normal goat serum with buffer (60 minutes) and incubated in anti-SP primary antibody (ImmunoStar, USA; PN 20064; 1:3000 dilution) overnight at 4°C. This polyclonal antibody was raised in rabbit against SP coupled to a carbodiimide/keyhole limpet hemocyanin conjugate. The manufacturer reports that preadsorption with SP abolished staining, and that preadsorption with neurokinin A, neurokinin B, somatostatin, and neuropeptide K did not affect staining. We performed control studies using the same antibody dilution after preadsorption with 100 μM substance P (Fisher, USA, catalog #AP70-1-10) at 37°C for 2 hours. We controlled for secondary antiserum specificity through omission of the primary antibody.

Sections were washed and reacted with biotin-conjugated goat anti-rabbit IgG (Jackson Labs; 1:300) for 90 minutes. For product visualization we used the ABC Elite Kit (Vector Labs, USA) in Tris buffer for 90 minutes, followed by DAB/H₂O₂/buffer mixed according to manufacturer recommendations (Vector Labs), for 30 minutes. Finally, sections were rinsed in distilled water for 10 minutes, counterstained with cresyl violet, dehydrated with ascending concentrations of alcohol, defatted with xylene, and coverslipped with DPX (Biochemika, Switzerland).

Data Collection

All slides were relabeled to blind the investigator as to the identity of the sections being analyzed. At least 2 randomly selected sections were analyzed. Reasons for rejection of a section for analysis included processing artifacts such as folding of the section, lack of adhesion to the slide, or irregular counterstaining. Each second section selected was at least 32 μm away (4 sections) from the first.

Using a digital camera (SPOT, USA), the entire section was photographed piecemeal at 200X and images were combined to form a montage. The same section was observed microscopically at 400X and 600X to identify neurons containing a visible nucleolus and to judge whether such neurons had SP-like immunoreactivity. The montage was used for record keeping; to minimize bias, all neurons with visible nucleoli from each chosen section, whether SP-positive or SP-negative, were marked. Because many neurons were elliptical, measurements of the long and short axes (AL, AS) of each nucleolated cell were made (Farel, 2002) using the SPOT software, after calibration with a precision reticle. Areas were estimated from these long and short axes (see below). The images were resized and adjusted for appropriate resolution using Adobe Photoshop, assembled using Adobe Illustrator, but not otherwise modified (e.g., contrast or color).

Our *a priori* target was to count a minimum of 100 neurons per ganglion, in at least 2 representative sections per ganglion. If this number was not reached in the 2 sections

chosen, a 3rd section was counted, also in its entirety. However, because identification and counting continued even after the minimum 100 neurons were obtained, this method inevitably led to the counting of many more than 100 neurons per DRG (mean = 230 ± 55 neurons/DRG).

Statistical Analysis

Cell measurements were expressed as cross-sectional areas calculated using the long and short axes (AL and AS, respectively) and the formula $\text{Area} = \pi \text{AL AS} / 4$. The data were compared using ANOVA for each of SP-positive neurons and SP-negative neurons, using Fisher's LSD, post-hoc. P-values of 0.05 or less were considered significant differences.

Proportions of SP-positive neurons were expressed as Log odds, LN(SP-positive/SP-negative), fit by a Generalized Linear Model (McCullagh and Nelder, 1989) with dispersed binomial variance, and with model effects of ganglion (ipsilateral L5 and L4, and contralateral L5), treatment (unoperated; operated 1, 2, 3, 4, 7 and 14 dpo; sham 1, 4, and 7 dpo), and the interaction of ganglion X treatment. Parameters and their standard errors were estimated by maximum likelihood fitting. The differences of interest were unoperated control vs postoperative day, and postoperative day vs sham control (i.e.; data from lesion studies were compared to unoperated control data, and also to sham control data from matching days). Differences were deemed significant when calculated t-values on N - p degrees of freedom (119 - 29 = 90 df) yielded p-values 0.05 or less. In the results, values were expressed as group means with their standard errors.

RESULTS

Animals

Surgery was performed upon 42 animals. Of these, 2 became moribund following surgery (2 and 3 days), and were euthanized. Necropsy showed no gross abnormality except for, in both rats, a highly distended and hyperemic bladder. The 40 other rats recovered without complication until euthanized. Most rats limped following the surgery, and the posture of the foot on the operated side was usually inverted and extended until sacrifice. Such posture is consistent with denervation of the muscles that cause ankle eversion and flexion and, potentially, the presence of pain in the limb. In all rats, the level and adequacy of the nerve lesion was visually confirmed during tissue harvest.

Substance P Expression

We evaluated 27,620 neurons from 119 DRGs for cross-sectional area and the presence of SP-like immunoreactivity. SP-like immunoreactivity was prominent in small neurons ($\leq 800 \mu\text{m}^2$; see below) in all ganglia (Fig. 1). In the axotomized L5 ganglia, SP was also observed in large neurons ($>800 \mu\text{m}^2$; Fig. 1 C-D). SP-like immunoreactivity was only very rarely observed in large neurons in any ganglia from control or sham animals (Fig. 1A-B), or from the contralateral L5 or ipsilateral L4 DRGs (data not shown). There was no specific staining in control ganglia, to which primary antibody was not applied, or in sections reacted with antibody preadsorbed to 100 μM SP.

The mean areas of SP-positive neurons in axotomized ganglia were significantly higher at 2, 4, 7, and 14 dpo ($437 \pm 64 \mu\text{m}^2$, $p = 0.04$; $505 \pm 100 \mu\text{m}^2$, $p < 0.001$; $515 \pm 99 \mu\text{m}^2$, $p = 0.001$; $526 \pm 45 \mu\text{m}^2$, $p < 0.001$, respectively) when compared to control ($320 \pm 7 \mu\text{m}^2$) and shams from matching days (4 dpo: $325 \pm 50 \mu\text{m}^2$, $p = 0.003$; 7 dpo: $348 \pm 49 \mu\text{m}^2$, $p = 0.004$; Fig 2A). There were no other significant differences between the other groups of SP-positive neurons (from the ipsilateral L4 or the contralateral L5 ganglia, Fig. 2 B-C). There

were no significant size differences between SP-negative neurons from any ganglion at any time point (data not shown).

The choice of where to divide DRG neurons into large and small neurons was based upon the cell sizes taken from 10,729 neurons from the contralateral L5 ganglia ($n = 40$; there were no significant differences among the cell characteristics between any of these ganglia). The size distribution was highly skewed, but when plotted as a frequency histogram with 0.1 log area binning a clear bimodality appeared (Fig 3). The two peaks supported the presence of 2 overlapping populations by size (Lawson, 1979). The SP-positive neurons seemed to be associated with the first peak, as has been previously reported (Lawson, 1992). Fitting the 2 size distributions by maximum likelihood placed the intersection at approximately $800 \mu\text{m}^2$. This number was consistent with previous reports (Lawson, 1992) and was used as justification to separate the neurons from other ganglia into two subsets, small ($\leq 800 \mu\text{m}^2$) and large ($>800 \mu\text{m}^2$) for further analysis.

Frequency histograms of the axotomized, control, and sham L5 neurons were plotted (Fig 4). After axotomy, the SP-positive neurons expressed a rightward shift in frequency, towards larger neurons. The rightward shift could also be appreciated in the SP-negative neurons. Combined, these findings suggest a relative loss of smaller neurons following surgery, though our methods did not allow a quantitative confirmation of this possibility. Similar histograms made using the ipsilateral L4 and contralateral L5 data did not reveal such differences (data not shown).

Percentages of SP-positive neurons by day were calculated after separation into two groups, small neurons ($\leq 800 \mu\text{m}^2$) and large neurons ($>800 \mu\text{m}^2$), and statistically and graphically analyzed (Fig 5). Data from each postoperative day were compared to their controls, and also to day-matched shams as appropriate.

Large cell analysis—Compared to large neurons in control ipsilateral L5 ganglia, the percentages of SP-positive neurons from axotomized L5 ganglia were increased at 2, 4, 7, and 14 dpo [$1.1\% \pm 2.1$ (control) vs $10.3\% \pm 3.1$ (2 dpo), $p = 0.004$; vs $14\% \pm 3.2$ (4 dpo), $p = 0.001$; vs $7.4\% \pm 2.1$ (7 dpo), $p = 0.017$; and vs $7.5\% \pm 2.6$ (14 dpo), $p < 0.001$]. At 4 dpo and 7 dpo the percentages of large neurons were higher compared to day-matched sham ganglia [4 dpo: $10.3\% \pm 3.1$ vs $3.2\% \pm 2$ (sham), $p = 0.001$; 7 dpo: $14\% \pm 3.2$ vs. $1\% \pm 2.3$ (sham), $p = 0.01$]. There were no significant differences among ipsilateral L4 or contralateral L5 large neurons.

Small cell analysis—Compared to small neurons in control ipsilateral L5 ganglia, the percentages of SP-positive neurons from axotomized L5 ganglia were increased at 1 and 3 dpo [$21.4\% \pm 2.6$ (control) vs $31.3\% \pm 3$, $p = 0.014$ (1 dpo) and vs $31.4\% \pm 3.2$, $p = 0.017$ (3 dpo)]. At 1 dpo the percentage of small neurons was increased compared to neurons from day-matched sham ganglia ($31.3\% \pm 3$, $p = 0.014$ vs $18.4\% \pm 2.8$ (sham), $p = 0.003$). However, at 7 and 14 dpo, the percentages of SP-positive neurons were significantly less than control [$21.4\% \pm 2.6$ (control) vs $13.3\% \pm 2.5$ (7 dpo), $p = 0.035$ and vs $12.5\% \pm 3.1$ (14 dpo), $p = 0.048$]. At 7 dpo, the percentages of SP-positive small neurons were also less than in day-matched sham ganglia ($13.3\% \pm 2.5$ vs $25.6\% \pm 2.9$ (sham), $p = 0.004$). The only differences between the ipsilateral L4 and contralateral L5 ganglia were for 1 dpo versus their matched shams (1 dpo ipsilateral L4 vs sham: $28\% \pm 3.2$ vs $16\% \pm 2.8$, $p = 0.03$; 1 dpo ipsilateral L5 vs sham: $31.3\% \pm 3$ vs $18.8\% \pm 2.8$, $p = 0.005$).

DISCUSSION

We have shown that during the first few days following spinal nerve transection close to the DRG, the size spectrum of SP-positive neurons and the percentage of SP-positive neurons increase. These changes occurred in both small and large neurons, but with different time courses. The smaller neurons responded to the axotomy sooner after injury (1 dpo), but this increased SP expression was not maintained, and in fact decreased at 7 and 14 dpo. The expression of SP in larger neurons was not observed until 2 dpo, but remained significantly increased compared to unoperated and sham ganglia until 14 dpo.

Two possible explanations for the size increase of SP-positive neurons following axonal transection were considered: cell swelling due to injury, and *de novo* synthesis of SP, by neurons that normally do not produce SP, including larger neurons. Cell swelling could account for the appearance of large SP-positive neurons. However, cell shrinkage rather than swelling is the typical response of DRG neurons to axotomy (Vestergaard et al., 1997; Degn et al., 1999). Moreover, blinded measurements from our own material failed to reveal any statistical indication of axotomy-induced swelling, as reflected in cell size histograms of neurons from all ganglia, considering both SP-positive and SP-negative neurons (data not shown). As expected, however, some neurons showed chromatolytic changes, including eccentric nuclei and cytoplasmic pallor, especially at longer survival times. It is therefore more likely that the increased size spectrum and percentage of positive neurons were the result of *de novo* synthesis of SP by larger neurons.

Following axotomy of primary sensory neurons there are profound and persistent changes in the content of neuropeptides (for review see Hokfelt et al., 1994). Substance P is a neuromodulator present in 6 - 20% of the DRG neuronal population, usually limited to small sensory neurons (our findings and Otsuka and Yoshioka, 1993). Peptide content and gene expression products in DRG neurons, axons, and projections into the dorsal horn are generally down-regulated when assayed a week or more after peripheral nerve transection or tight ligation (Ahmed et al., 1995; Zhang et al., 1995a; Zhang et al., 1995b; Ji et al., 1996; Rydh-Rinder et al., 1996; Sterne et al., 1998; Ma and Bisby, 1998a; Mohiuddin et al., 1999; Antunes Bras et al., 1999; Sondell et al., 1999; Honore et al., 2000; Siri et al., 2001; Xiao et al., 2002; White and Kocsis, 2002; Wang et al., 2002; Partata et al., 2003; Hofmann et al., 2003; Valder et al., 2003; Sanderson et al., 2004; Swamydas et al., 2004); also see Hokfelt et al., 1994) for earlier studies). Nerve compression also modulates the content of SP and gene expression in primary sensory neurons (Bisby and Keen, 1986; Cameron et al., 1997; Sondell et al., 1999; Lee et al., 2001; Wong and Tan, 2002; Kobayashi et al., 2004; Swamydas et al., 2004). However, there are also reports of no changes in SP metabolism following nerve injury (Murphy et al., 1999; Macdonald et al., 2001).

In contrast, a number of studies of primary somatic afferent neurons examined shortly (up to 10 days) after nerve injury have shown increases in SP or products of gene expression for SP (Noguchi et al., 1994; Marchand et al., 1994; Noguchi et al., 1995; Neumann et al., 1996; Ma and Bisby, 1998b). Following nerve section and using in situ hybridization to detect PPT mRNA, a dramatic *de novo* expression of PPT mRNA was found in large and moderate sized DRG neurons but not in small DRG neurons at 7 dpo (Noguchi et al., 1994; Noguchi et al., 1995). Masseter nerve section also resulted in a upregulation of PPT mRNA in the mesencephalic trigeminal nucleus at 7 dpo (Umemoto et al., 1994).

Data regarding changes in SP at time points earlier than 1 week dpo are more limited. An apparent phenotypic switch to producing SP was also observed 2 days following the peripheral injection of turpentine to produce an inflammatory response (Neumann et al., 1996). In this study the upregulation of SP was not exclusive to large primary sensory

neurons; increases also were observed in small diameter A δ and C fibers, and enhanced SP-excitation of deep dorsal horn neurons elicited by the activation of A δ and C fibers was demonstrated (Neumann et al., 1996). In the chronic constriction injury model of neuropathic pain, upregulation of PPT mRNA was observed in all cell size classes at 2, 5, and 10 dpo (Marchand et al., 1994). Similar patterns of up-regulation of PPT mRNA were observed following partial nerve injury and chronic constriction injury (Ma and Bisby, 1998a). The present results are consistent with these studies, as we observed an increased number of SP-positive neurons in all size classes of DRG neurons. Our study extends the observational resolution in the first week, showing that smaller neurons appear to be affected as early as 1 dpo and large neurons affected as early as 2 dpo. Of significant note is that the present methods involved confirmed axotomy of every axon of the operated spinal nerve, whereas other models involved variable levels of axotomy. This could account for some differences in the various reports.

It is uncertain what factors are responsible for the increase in SP expression we and others have observed in the first week following nerve injury, but such factors are likely to be at the injury site, and related to the inflammatory response. While there are little supportive data for this concept, local inflammation of the rear paw causes the PPT gene to be induced in A β fibers (Neumann et al., 1996), and SP is expressed de novo in large diameter neurons innervating the lung that have been exposed to an allergen (Myers et al., 2002; Chuaychoo et al., 2005). Nerve growth factor (NGF) may also be involved. Axotomy such as performed in our experiments reduces the transport of NGF from peripheral tissue (Hendry et al., 1974; Campenot and MacInnis, 2004). Nerve growth factor is important for long term maintenance of SP expression following peripheral nerve injury (Verge et al., 1995; Csillik et al., 2003); thus the relative reduction of SP-positive neurons after 1 week may follow reduced NGF. Also, transgenic mice that over express NGF develop ectopic fibers that express SP (Ma et al., 1995; Ribeiro-da-Silva et al., 2000) and these mice show allodynia and hyperalgesia (McLeod et al., 1999).

Alterations in the amount of SP and the type of neurons expressing it following nerve injury may alter the way the organism processes nociception in response to somatosensory stimuli. In the spinal cord, SP depolarizes and activates dorsal horn neurons bearing NK-1 receptor (Radhakrishnan et al., 1998), and may trigger and maintain spinal central sensitization (McLeod et al., 1999; Cahill andCoderre, 2002; Khasabov et al., 2002). Furthermore, dorsal horn wide dynamic range neurons in mice devoid of the gene for the NK-1 receptor do not develop “wind up” as do normal wide dynamic range neurons (Weng et al., 2001), although PPT-A knockout mice do not show sensitization of wide dynamic range neurons subsequent to the peripheral application of mustard oil (Martin et al., 2004). When sensory axons are injured, spontaneous activity ensues in axons of all sizes, though there is no consensus regarding the time course of the ongoing activity (Tal et al., 1999; Lee et al., 1999; Michaelis et al., 2000; Boucher et al., 2000; Gorodetskaya et al., 2003); for review see Abdulla et al., 2003). Such activity releases SP from primary afferent neurons in the spinal cord (Schaible et al., 1990; Duggan et al., 1995). We have confirmed that SP, usually produced only in small DRG neurons, is produced in larger DRG neurons following axotomy, and in fewer smaller neurons at later time points. Therefore, it is reasonable to presume that spinal cord SP levels will increase following axotomy due to increased SP release from spontaneously active primary afferent neurons. At earlier time points DRG neurons of all sizes may participate, but because at later time points there are fewer smaller SP-positive neurons, it is more likely that larger neurons are more critically involved.

It is possible that the increase in SP in larger neurons may increase the area of spinal cord exposed to SP, which may affect secondary nociceptive and somatosensory spinal cord neurons. Through this mechanism, increased levels of SP could be expected to affect spinal

cord levels beyond the lesioned segment. Upon entering the spinal cord, primary afferent axons branch rostrally and caudally to terminate at many levels of the dorsal horn (Wall and Werman, 1976; Bullitt, 1991; Ling et al., 2003; Woodbury and Koerber, 2003). This arborization was reflected by increased SP immunoreactivity in the dorsal horn 3 segments rostrally and 1 segment caudally following nerve transection (Abbadie et al., 1996). Thus, SP released from axotomized neurons, especially those with A β axons, which have more elaborate ramifications, could have widespread effects on NK1-receptor bearing nociceptive neurons over spinal segments that continue to receive normal input from their respective segmental primary afferent neurons. Sensitized wide dynamic range neurons that receive inputs from both nociceptive and non-nociceptive neurons are the logical sites for cross-modality sensory confusion (i.e., touch versus pain). Once sensitized, these neurons could be driven by normal function of intact low threshold mechanoreceptors, potentially resulting in activation of nociceptive pathways via normally non-noxious stimuli.

We have shown that more DRG neurons begin to express SP as soon as one day after total spinal nerve transection, and that large neurons but not small neurons maintain this expression 1 and 2 weeks following the lesion. The pattern of increased SP expression seems to parallel numerous reports of increased mechanical sensitivity of the hindpaw following similar nerve injuries (e.g., Kim and Chung, 1992; Ringkamp et al., 1999; Hammond et al., 2004; Sun et al., 2005). Augmented spinal release of SP from spontaneously firing, damaged primary afferent neurons, including larger DRG neurons, may trigger central sensitization and thus facilitate pain mediated by normal activity in neighboring, intact receptors.

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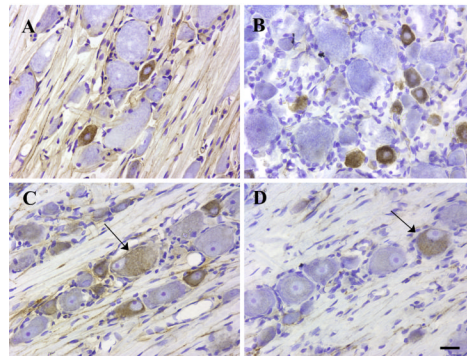
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**Figure 1.**

Substance P (SP) expression in intact and axotomized dorsal root ganglia. A. Sections from unoperated animals showed SP staining (brown) limited to smaller neurons. B. Sham surgery led to staining similar to unoperated sections. Labeling in sections from 4 (C) and 7 (D) days postoperatively demonstrated SP in larger neurons (arrows). Scale bar = 25 μ m.

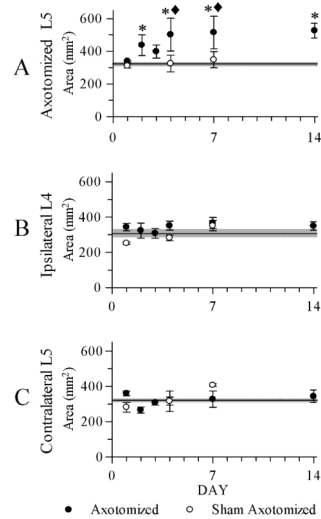


Figure 2.

A plot of mean areas of SP - positive dorsal root ganglion neurons showing changes following axotomy. Unoperated control means are indicated in all graphs by a horizontal line, with gray shaded zones indicating the SEM of these means. A. Neuron size was significantly increased in axotomized L5 ganglia at 2, 4, 7, and 14 days postoperatively (dpo), compared to control (*) and to day-matched 4 dpo and 7 dpo sham ganglia (◆). B, C. There were no significant differences with similar comparisons of the ipsilateral L4 or contralateral L5 ganglia. N = 4 in all groups except n = 3 for ipsilateral L4 sham, 1 dpo. Error bars = SEM.

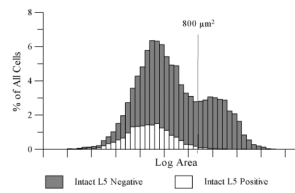


Figure 3.

Size distributions of intact L5 dorsal root ganglion neurons separated by SP content. All counted neurons ($n = 10,729$) from the intact L5 ganglia ($n = 40$) were separated by presence (open bars) or absence (shaded bars) of SP and binned by area in 0.1 natural log increments. The 2 distributions were found to intersect at $800 \mu\text{m}^2$ (grey line).

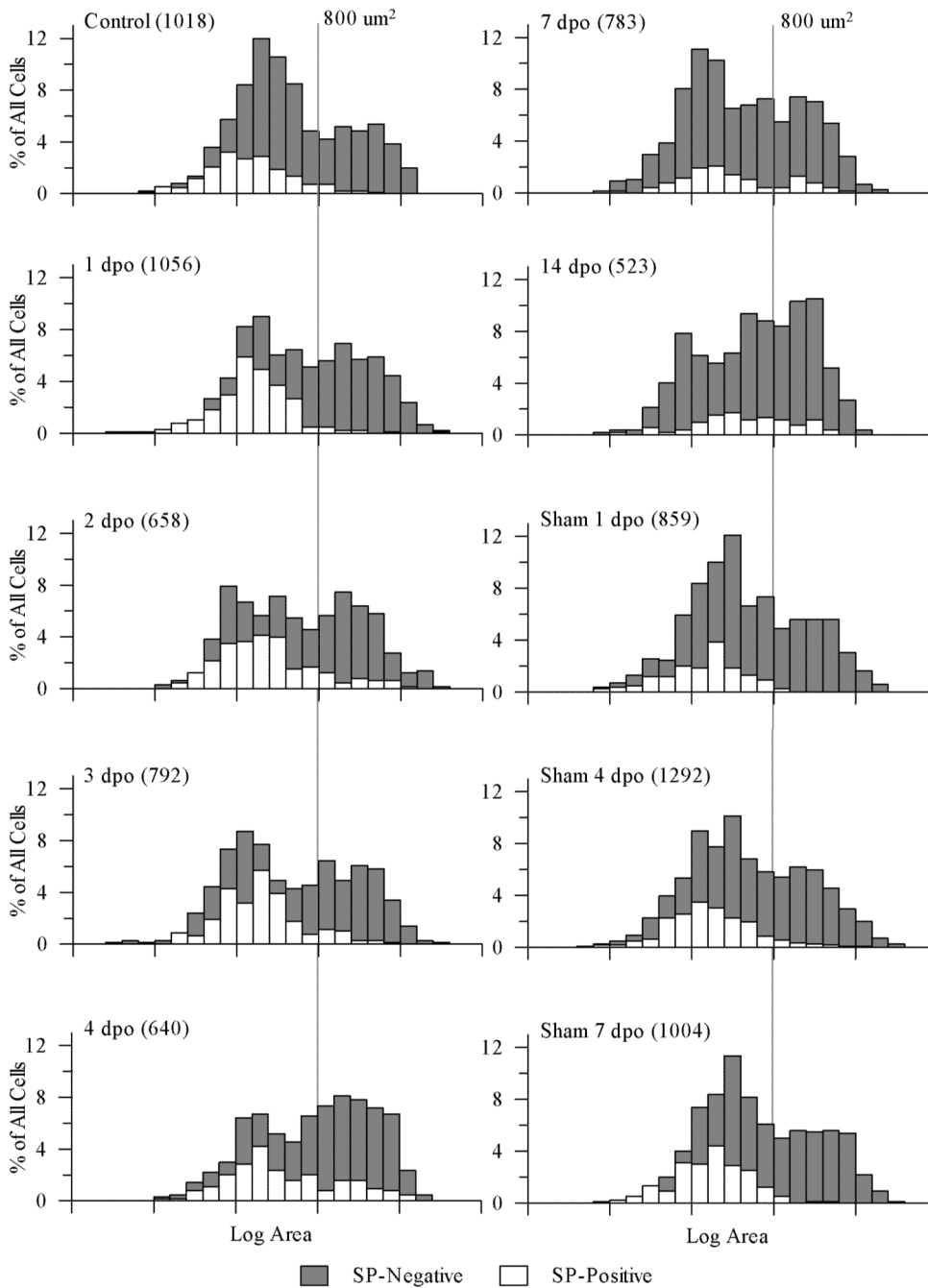


Figure 4.

Size distributions of axotomized L5 dorsal root ganglion neurons separated by SP content, over time. Neurons from control and axotomized L5 ganglia were separated by presence (open bars) or absence (shaded bars) of SP and binned by area in 0.2 natural log increments. A greater number of larger neurons positive for SP was present starting at 2 dpo. A smaller number of SP-positive neurons was seen at 7 and 14 dpo. The number of neurons used for the histograms is in parentheses.

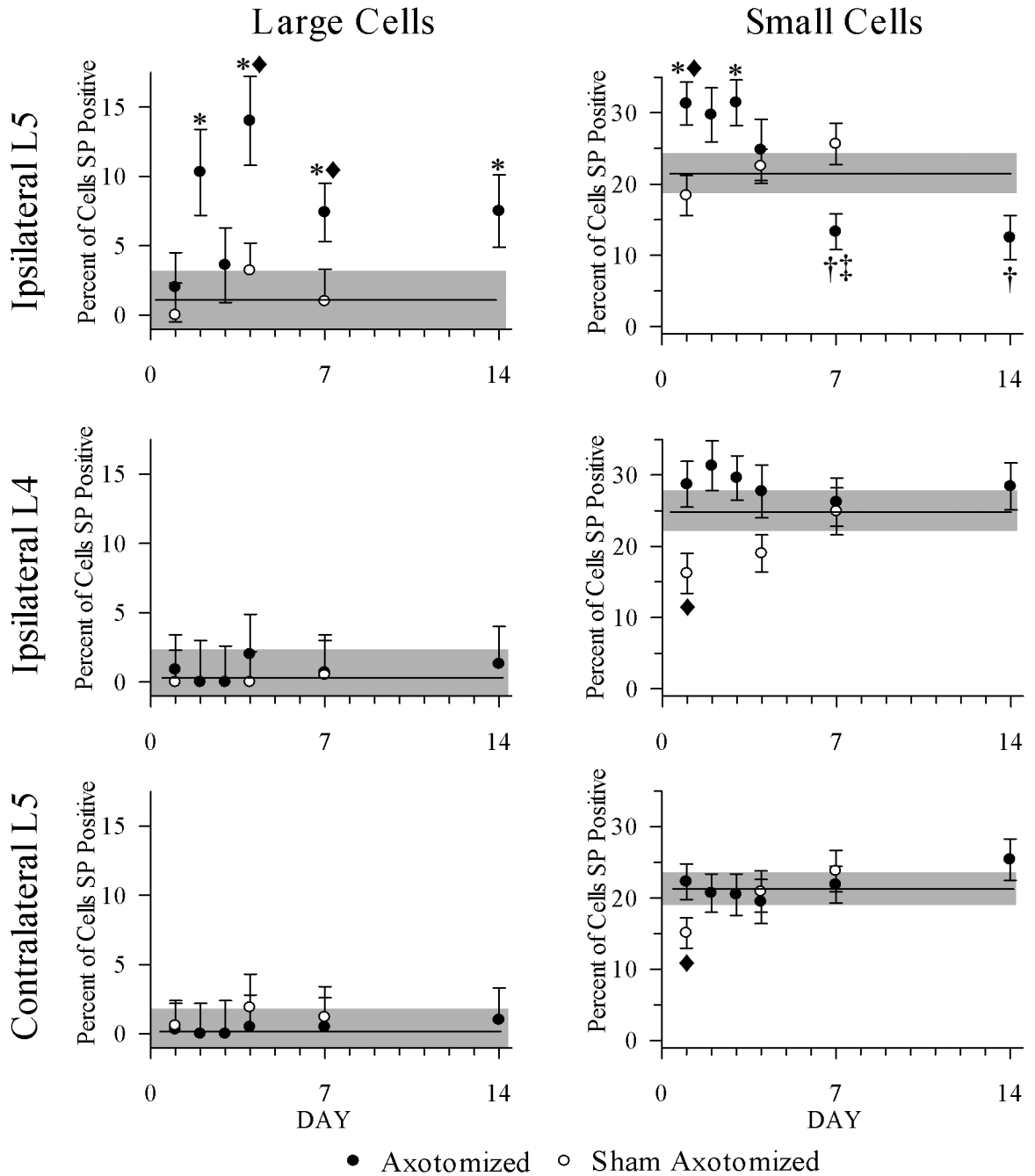


Figure 5.

Percentages of SP-positive neurons $>800 \mu\text{m}^2$ (left column) and $\leq 800 \mu\text{m}^2$ (right column) graphed by day. Unoperated control means are indicated in all graphs by horizontal line, with gray shaded zones indicating the SEM of these means. Left column: for large neurons from L5, there were higher percentages of SP positive neurons $>800 \mu\text{m}^2$ compared to control L5 ganglia at 2, 4, 7, and 14 dpo, (* = $p < 0.05$), and compared to sham ganglia at days 4 and 7 (◆ = $p < 0.05$). There were no changes in L4 or contralateral L5. Right column: for small neurons from L5, there were higher percentages of SP positive neurons $\leq 800 \mu\text{m}^2$ compared to intact ganglia at 1 and 3 dpo (* = $p < 0.05$) and compared to sham ganglia at day 1 (◆ = $p < 0.05$). At 7 and 14 dpo, the percentages of SP positive neurons

were less than control (†) and at 7 dpo was less than the day matched sham ganglia (‡). Proportions from intact L4 and L5 ganglia were different from sham ganglia at 1 dpo (◆ = $p < 0.05$). Error bars = SEM.