Primary Afferent Fibers That Contribute to Increased Substance P Receptor Internalization in the Spinal Cord After Injury

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INTRODUCTION

Under normal circumstances, nociceptive information is transmitted to the CNS by unmyelinated (C) and thinly myelinated (Aδ) fibers (Willis 1985). C fibers are known to terminate almost exclusively in lamina I and II of the spinal cord, whereas Aδ fibers have been shown to terminate in laminae I, II, V, and VI (Nagy and Hunt 1983; Swett and Woolf 1985; Willis and Coggeshall 1991). It also should be noted that Aβ fibers terminate in laminae III–V of the dorsal horn, the exact pattern of termination depending on the receptor type (Brown and Fuchs 1981).

Several lines of evidence support the notion that the neuropeptides substance P (SP) and neurokinin A (NKA), which are synthesized and contained in 20–30% of dorsal root ganglion (DRG) neurons, are involved in the transmission of nociceptive information from primary afferent fibers to the spinal cord. First, SP/NKA are contained primarily in, and coreleased from, small-diameter primary afferent fibers on noxious stimulation (Boehmer et al. 1989; Dalsgaard et al. 1984; Duggan et al. 1990; Hokfelt et al. 1975; Hope et al. 1990; Levine et al. 1993). Second, nociceptive spinal neurons are excited by iontophoretic application of SP and NKA (Radhakrishnan and Henry 1991; Slater and Henry 1991). Third, destruction of SP-containing unmyelinated primary afferent fibers by the neurotoxin capsaicin attenuates withdrawal responses to noxious stimuli (Buck and Burks 1986; Buck et al. 1983). Fourth, opiate analgesics have been reported to inhibit the release of SP in the spinal cord (Aimone and Yaksh 1989; Jessell and Iversen 1977; Yaksh and Iversen 1977; Yaksh 1988). Although activation of nociceptive C and Aδ primary afferent fibers by electrical, chemical, or mechanical stimulation has been reported to release SP/NKA (Duggan and Hendry 1986, 1990; Garry and Hargreaves 1992; Hope et al. 1990; Lang and Hope 1994), the precise location of SP/NKA release in the spinal dorsal horn by Aδ or C fibers has not yet been definitively identified.

In the spinal cord, SP (and to a lesser extent NKA) primarily interacts with the substance P receptor (SPR), also known as the neurokinin-1 (NK-1) receptor. The SPR is a prototypical G-protein-coupled receptor with seven-transmembrane spanning domains that, when activated, induce inositol phospholipid hydrolysis and, in some cases, adenylyl cyclase (Garland et al. 1994; Reubi et al. 1990; Sjödin et al. 1980). It has been shown previously that a subpopulation of spinal cord neurons express SPR immunoreactivity and that this immunoreactivity is present along the majority of the plasma membrane, in both...
the cell body and dendrites (Liu et al. 1994). We have found that injection of SP into the striatum (Mantyh et al. 1995a) or intraplantar injection of the irritant capsaicin (Mantyh et al. 1995b), which causes release of SP, evoked massive endocytosis of SPRs in striatal and spinal cord neurons, respectively. Internalization of the SPR also may be attributed, in part, to release of neurokinin A (NKA) because the EC₅₀ value for NKA-evoked internalization of the SPR is 21.0 nM (Mantyh et al. 1995a). Thus internalization of the SPR can be used as a pharmacologically specific marker of SP/NKA release and peptide-receptor interaction.

One potential function of SP/NKA in nociceptive transmission is the sensitization of nociceptive dorsal horn neurons; this contributes to hyperalgesia and allodynia, characteristic symptoms of neuropathic and inflammatory pain. Sensitization of dorsal horn neurons in lamina I and in deeper laminae (III–VI) has been well documented after tissue injury and inflammation (for reviews, see Coderre et al. 1993; Dubner and Ruda 1992; Mense 1993; Simone 1992; Treede et al. 1992). Results from a variety of behavioral and electrophysiological studies suggest that SP/NKA is involved in the development of hyperalgesia. Intrathecal administration of SP produces itching and scratching behavior suggestive of nociception (Seybold et al. 1982), and administration of SP or NKA facilitates withdrawal responses in the tail flick and hot plate tests (Fleetwood-Walker et al. 1990; Yashpal et al. 1993). Intrathecal administration of NK-1 antagonists prevents the development of hyperalgesia produced by inflammation (Ma and Woolf 1995; Traub 1996). In electrophysiological studies, iontophoretic application of SP to the spinal cord produced long-lasting depolarization (Harvard rodent ventilator after being paralyzed with gallamine triethiodide (5 mg · kg⁻¹ · h⁻¹ iv). End-tidal CO₂ was monitored continuously and maintained at 3.5–4.5%.

A 3-cm incision was made ~0.5 cm below the pelvis. Using blunt dissectors, the biceps femoris and the gluteus superficialis muscles were separated until the sciatic nerve was isolated. Small sections of paraffilm (10 × 5 mm) were placed under the nerve and on the muscles surrounding the nerve to provide electrical insulation. The surgical field was filled with warm (37°C) mineral oil. The sciatic nerve was lifted carefully onto a silver bipolar electrode held by a micromanipulator. The electrode was adjusted so that the sciatic nerve was held firmly but not stretched. The electrode then was connected to the output of a constant current stimulus isolator (World Precision Instruments) for electrical stimulation.

To expose the sural nerve, a 1.5-cm incision was made over the ipsilateral calf. The flexor digitorum superficialis and the gastrocnemius muscles were separated by blunt dissection until the sural nerve was isolated. Once the nerve was free, a small pool was formed around the nerve by sewing the skin to a metal ring using silk sutures and filled with warm mineral oil. Using fine jewelers forceps and a pair of microscissors, the epineurium and perineurium sheaths were opened, exposing theafferent fascicles. The sural nerve was lifted carefully onto a platinum, bipolar recording electrode held by a micromanipulator. The compound action potential (CAP) recorded by the platinum recording electrode was amplified, and the output connected to an oscilloscope, audio monitor, and video tape recorder.

**METHODS**

**Subjects**

A total of 38 male, Sprague-Dawley, rats weighing 280–480 g were used. Animals were housed in pairs in plastic cages on a 12-h light-dark cycle and had access to food and water ad libitum. All procedures were approved by the Animal Care Committee of the University of Minnesota.

**Surgical preparation for recording the compound action potential**

Rats were deeply anesthetized with pentobarbital sodium (50 mg/kg ip). A feedback-controlled heating pad was used to maintain the rats core temperature close to 37°C. The right jugular vein was cannulated (0.28 mm ID, 0.61 mm, OD) for the administration of supplemental doses of pentobarbital sodium, which were given as needed. Depth of anesthesia was monitored by checking withdrawal responses to pinching the left hindpaw. A tracheotomy was performed, and a plastic Y tube inserted into the air way to allow the rat to be ventilated with a Harvard rodent ventilator after being paralyzed with gallamine triethiodide (5 mg · kg⁻¹ · h⁻¹ iv). End-tidal CO₂ was monitored continuously and maintained at 3.5–4.5%.
of activation of other fiber types as indicated by the CAP. Therefore in subsequent experiments, 90 μA was used to selectively activate Aβ fibers only.

To determine the electrical intensity that would activate Aβ and Aδ fibers maximally, we followed the same protocol described earlier for determining the electrical threshold and intensity that produced maximal activation of Aβ fibers only. It was determined that a stimulus intensity of 150 μA (0.2-ms duration) consistently recruited Aβ fibers (in addition to Aβ fibers) without activating C fibers.

To generate the maximum C-fiber component of the CAP, a stimulus intensity of 2 mA (0.5-ms duration) was used. This intensity reliably activated all three fiber types. In all experiments that followed, these standardized electrical stimuli were used to activate Aβ, Aδ, and C-fibers, and the CAP was not recorded from these animals. Figure 1 shows representative CAPs that illustrate activation of Aβ fibers only, Aβ and Aδ fibers, and all three primary afferent fiber types.

**Sciatic nerve transection**

Animals were anesthetized, and the right sciatic nerve isolated as described earlier. The right sciatic nerve was transected just proximal to the plexus using microscissors. The wound was closed with surgical staples, and an antibacterial ointment applied. Animals were allowed to recover for 14 days and then were anesthetized deeply for electrical stimulation. Day 14 after transection was chosen because it has been shown previously (Noguchi et al. 1995) that at this time after transection there is maximal increase in SP in DRG and dorsal roots.

**Hindpaw inflammation**

Animals were anesthetized as described above, and 100 μl of phosphate-buffered saline and complete Freund’s adjuvant (1:1 concentration) was injected subcutaneously into the plantar surface of the right hindpaw. Three days after injection, the time at which inflammation was developed fully, animals were anesthetized deeply and received electrical stimulation as described in the preceding text. Although quantitative measures of inflammation were not made, all animals exhibited robust edema of the injected paw.

**Preparation of the antibody**

The antibody used in the present study was raised in the rabbit against a 15-amino acid peptide sequence (SP393–407) at the carboxylterminus of the rat SPR (Vigna et al. 1994). The immunogen consisted of synthetic peptide conjugated to bovine thyroglobulin using glutaraldehyde. The antisera recognizes a protein band of 80–90 kDa on Western blots of membranes prepared from cells transfected with the rat SPR (Vigna et al. 1994). The antibody staining in the rat spinal cord was blocked by preabsorbing the antiserum with SPR 393–407. Light microscopy revealed an excellent correlation between the patterns of SPR immunoreactivity and of 125 I-SP binding sites in the CNS. In the stratum, SP-induced internalization of the SPR is dose dependent: EC50 = 0.93 nM for SP, EC50 = 21.0 nM for neurokinin A, and EC50 > 1.0 μM for neurokinin B (Mantyh et al. 1995a). These potencies correspond closely to the affinities of these peptides for the rat SPR (Mantyh et al. 1989). The SP-induced internalization of the SPR also appears to be due to interaction with the SPR agonist binding site because injection of RP-67,580, a nonpeptide antagonist, produced no significant internalization of the SPR by itself but potently blocked the SP-induced SPR internalization. The SP antibody was raised in guinea pig, allowing us to use double label sections with the ligand and receptor.

**Immunohistochemical localization of the SPR**

After electrical stimulation, rats were perfused via the ascending aorta with 500 ml of 0.1 M phosphate-buffered saline (PBS; pH 7.4, 22°C) followed by 750 ml PBS containing 12% paraformaldehyde and 11% picric acid (pH 6.9, 4°C). After perfusion, the spinal cord was removed, blocked in the transverse plane, postfixed in PBS containing 12% paraformaldehyde and 11% picric acid (pH 6.9, 4°C, 10 h), and placed in PBS containing 30% sucrose (pH 7.4, 4°C, 24 h). Lumber segments were cut into sagittal sections by inserting a catheter tip into the ventral region of the cord opposite the center of the dorsal root entry points in the spinal cord. Corridors processed coronally were cut between the dorsal root entry points. Sagittal sections were obtained from three animals in each group and processed for quantification of SPR + endosomes while coronal sections obtained from two animals in each group were processed for SPR + immunofluorescence. Spinal cords were sectioned at a thickness of 60 μm on a sliding, freezing microtome, and serial sections were collected in PBS.

Tissue sections were pretreated in PBS containing 0.1% saponin and 1.0% normal goat serum (pH 7.4, 22°C, 30 min) followed by a 12-h incubation in PBS containing 1.0% normal goat serum, 0.3% Triton X-100, and the anti-SP antibody (11884–5) (Vigna et al. 1994) at a concentration of 1:5,000 (pH 7.4, 22°C). After incubation with the primary antibody, the tissue sections were washed for 30 min at 22°C in PBS (pH 7.4) and then incubated in a second primary antibody containing guinea pig anti-SP antibody at 1:2,000 for 3 h at 22°C. Sections were once again washed for 30 min in PBS (pH 7.4) at 22°C. Afterward the sections were incubated in secondary antibody solution. This secondary antibody solution was identical to the primary antibody solution with the exception of cyanine (Cy-3)-conjugated donkey anti-rabbit IgG (711–165–152, Jackson Immmunoresearch Labs, West Grove, PA) present at a concentration of 1:600 and fluorescein isothiocyanate (FITC)-conjugated donkey anti-guinea pig IgG (706–095–148, Jackson ImmunoResearch Labs) at a concentration of 1:200 in place of either the anti-SP or anti-S antibodies. Finally, the tissue sections were washed overnight in PBS (pH 7.4, 22°C), mounted onto gelatin-coated slides and coverslipped with PBS glycerine containing 1.0% p-phenylenediamine to reduce photobleaching. Tissue sections from each group of animals were processed in parallel. To gauge the specificity of the antibody, the SPR was preabsorbed with the anti-SP at 1:1,000 in a control rat.

**SPR immunoreactivity**

To determine the intensity of SPR immunoreactivity, we used a confocal imaging system (BioRad MRC 1000) equipped with a Nikon Axiomat microscope. Images were collected using a Kalman averaging of 15 scans. A computer assigned each individual pixel a number ranging from 0 to 255 (0 is black and 255 is white), and the average intensity of SPR immunofluorescence was determined. The medial portion of the dorsal horn was chosen as the standard area to obtain fluorescent intensity. Fluorescent intensities were obtained from the L4 spinal segment of animals that were untreated or received sciatic nerve transection or inflammation (n = 5 measurements per group).

**Quantification SPR internalization in cell bodies**

The tissue sections that were processed for immunohistochemistry were analyzed by fluorescent and confocal microscopy to determine the spinal levels and laminae where significant SPR internalization occurred. To examine the sites of internalization within the cell, sections were viewed with an MRC-1024 Confocal Imaging System (Bio-Rad, Boston, MA) equipped with a ×60 oil immersion objective and an Olympus AX-70 microscope equipped for fluorescence (Lake Success, NY). The microscope was set up as previously described (Brelje et al. 1989; Mantyh et al. 1995a,b). To quantify internalization, sections were examined with an Olympus BX-60 microscope equipped for fluorescence. Endo-
somes were counted from sets of 50 neurons in laminae I, III, and IV in the fourth lumbar spinal segment in random sections from each group of animals. Tissue sections were sampled randomly from each animal. The experimenter performing the quantification was unaware of the treatment group from which the tissue was obtained. An SPR+ endosome was defined as an intense SPR-immunoreactive intracellular organelle between 0.1 and 0.7 μm in diameter that was clearly not part of the external plasma membrane. The SPR was considered to have undergone internalization if the cell body contained 20 or more SPR+ endosomes. The percentage of neurons exhibiting ≥20 SPR+ endosomes per cell body as well as the mean number of endosomes per cell body was determined.

**Experimental design**

Animals were divided randomly into groups of three to four each. In normal animals and in animals that received either transection of the sciatic nerve or hindpaw inflammation, the sciatic nerve was exposed and the stimulating electrode placed on the sciatic nerve as previously described. Electrical stimulation was performed 1 h later to allow the recycling of any SPRs that may have been internalized by the aforementioned nerve isolation procedure. In a separate group of sham-operated animals, the nerve was exposed and placed on the electrode but not stimulated to verify that the surgical procedure did not evoke internalization at the time of electrical stimulation. The electrode was placed on the sciatic nerve for 1 h and 5 min, and rats were perfused 8 min later. In additional groups of animals, the sciatic nerve was stimulated continuously for 5 min at intensities that activated either Aβ fibers alone, Aβ and Aδ fibers, and Aβ, Aδ, and C-fibers. A fibers were activated using a pulse duration of 0.2 ms, and a pulse duration of 0.5 ms was used to activate C fibers. All stimuli were delivered at a frequency of 10 Hz. Animals were perfused 8 min after completion of electrical stimulation, the time at which SPR internalization is maximal (Mantyh et al. 1995b).
Data analyses

A one-way ANOVA was used to determine differences in the intensity of SPR immunofluorescence between ipsilateral and contralateral dorsal horn in groups of animals that received sham surgery, nerve transection and inflammation. χ² tests with Bonferroni correction of the alpha level were used to determine differences between the groups in the proportion of cell bodies that exhibited >20 SPR+ endosomes after electrical stimulation. Two-way ANOVAs (and Fisher’s PLSD post hoc comparisons) were used to determine differences between groups in the mean number of endosomes per cell body in laminae I, III, and IV evoked by electrical stimulation. For each stimulus intensity, comparisons were made between cell bodies located in laminae I, III, and IV. A probability value <0.05 was considered significant.

RESULTS

SPR immunofluorescence after sciatic nerve transection and inflammation

In the spinal cord of normal, unstimulated rats, SPR-immunoreactive neurons comprise ~5–7% of the neurons within lamina I, and the SPR immunoreactivity is associated with the plasma membrane and decorates almost the entire somatic and dendritic surface. Within the substantia gelatinosa, SPR immunoreactivity is associated primarily with dorsally directed dendrites that originate from cell bodies in laminae III–V. SPR+ endosomes within cell bodies or dendrites rarely were observed, and when this did occur, the number of endosomes in the cell body was always less than five. A one-way ANOVA revealed significant differences in the intensity of immunoreactivity between untreated animals and those that received nerve transection and inflammation (P < 0.005). Transection of the sciatic nerve and hindpaw inflammation produced a mean ± SE increase in immunoreactivity of 33.7 ± 6.67% and 32.5 ± 4.93%, respectively, compared with untreated animals. The great majority of the SPR immunoreactivity was associated with the plasma membrane. In the absence of peripheral nerve stimulation, no differences were observed in the number of SPR+ endosomes in cell bodies and in dendrites or in the percentage of cells exhibiting >20 SPR+ endosomes between the control (normal, unstimulated, and sham-operated) and experimental (nerve transection and inflammation) groups. These results indicate that the surgical preparation itself did not evoke SPR internalization at the time tested and that nerve transection and inflammation produce an upregulation of SPRs without an increase in ongoing release of SP into the dorsal horn and are in agreement with a recent report demonstrating increased SPR+ immunoreactivity in the spinal cord dorsal horn after inflammation (Abbadie et al. 1996).

Electrical stimulation of primary afferent fibers and internalization of SPRs

NORMAL ANIMALS. Aβ fibers. Stimulation of Aβ primary afferent fibers, using a stimulus intensity of 90 μA, did not produce internalization of the SPR in laminae I–V in SPR+ cell bodies or distal dendrites. As illustrated in Fig. 1A, which provides an example of the recorded CAP and typical SPR+ cell body in lamina I of a normal animal, SPR immunoreactivity was found on the surface of the plasma membrane of cell bodies and dendrites with very few, if any, SPR+ endosomes present in the cytoplasm as in the unstimulated animal (0.60 ± 0.18 endosomes per cell body). In this and the other examples of CAPs and SPR internalization, the CAPs and representative confocal image of internalization in lamina I cell bodies were obtained from the same animal.

Aβ + Aδ fibers. Electrical stimulation of Aβ and Aδ afferent fibers with an intensity of 150 μA produced significant internalization of the SPR in cell bodies and dendrites in laminae I of the dorsal horn. A representative example of the CAP with Aβ and Aδ components, and a single lamina I cell body which shows characteristic internalization of SPRs following electrical stimulation of Aβ + Aδ primary afferent fibers in a normal animal is provided in Fig. 1B. Unlike the profile following stimulation of only Aβ fibers in which SPR immunoreactivity was located primarily on the plasma membrane, SPR+ endosomes were scattered throughout the cytoplasm of the cell body and dendrites. Additionally, the thin distal dendrites in laminae I and II exhibited a morphological reorganization by changing from a structure of rather uniform diameter to one characterized by large swollen varicosities that were packed with SPR+ endosomes and connected by thin fibers. It was found that 66% of lamina I SPR+ cell bodies in the lateral portion of the ipsilateral fourth lumbar segment of the normal rat spinal cord contained more than 20 SPR+ internalized endosomes, and the mean number of endosomes per cell body was 34.20 ± 3.05. Internalization of SPRs was not observed in cells or dendrites within laminae III–V.

Aβ + Aδ + C-fiber stimulation. Stimulation of C fibers (2 mA) produced the greatest amount of SPR internalization in the superficial dorsal horn, and a representative example is provided in Fig. 1C. Internalization of SPRs was found in cell bodies located in lamina I and in dendrites located in laminae I and II but not in deeper laminae. Many of the dendrites exhibiting SPR internalization originated from deep (laminae III–IV) neurons, which send dendritic arbors into the superficial dorsal horn. Interestingly, the pattern of SPR internalization differed from the pattern produced by stimulation of Aβ + Aδ fibers. Internalization of SPRs after Aδ fiber stimulation was restricted to the lateral portion of the superficial dorsal horn, whereas SPR+ endosomes were found throughout the lateral and medial superficial dorsal horn after stimulation of C fibers. Figure 2A shows that 90% of lamina I SPR+ cell bodies in the fourth lumbar segment exhibited >20 SPR+ endosomes. The mean number of endosomes per cell body was 43.7 ± 2.10 (Fig. 3A). Also thin distal dendrites in laminae I and II underwent morphological reorganization as a result of dense intracellular packing of SPR+ endosomes; however, this was not quantified.

SCIATIC NERVE TRANSECTION. Aβ fibers. Electrical stimulation of Aβ primary afferent fibers alone at 14 days after nerve transection did not produce significant internalization of the SPR in lamina I cell bodies or in laminae I and II dendrites (0.96 ± 0.32 endosomes per cell body). Additionally, no evidence for SPR internalization was found in SPR+ cell bodies or dendrites of the deeper laminae (III–V). Nearly all SPR immunoreactivity was associated with the plasma membrane of the cell bodies and the dendrites.

Aβ + Aδ fibers. Stimulation of Aβ + Aδ primary afferent fibers produced significant internalization of the SPR in laminae I cell bodies in the dorsal horn of the spinal cord. Eighty-
six percent of SPR+ lamina I cell bodies in the lateral portion only of the ipsilateral dorsal horn had >20 SPR+ endosomes per cell body and was significantly higher compared with control animals that received electrical stimulation of Aβ + Aδ fibers ($\chi^2 = 4.44, P < 0.035$).

A two-way ANOVA revealed significant differences in number of endosomes per cell bodies between the experimental groups ($P < 0.001$) and across intensities of stimulation ($P < 0.001$). The mean number of endosomes per cell body in lamina I evoked by Aδ stimulation was $42.3 \pm 2.38$ and was significantly increased after transection ($P < 0.024$). Although there was abundant internalization in lamina I, no internalization was observed in SPR+ cell bodies and dendrites of the deeper laminae (III–V).

$A\beta + A\delta + C$ fibers. Electrical stimulation that recruited C fibers produced significant SPR internalization in laminae I cell bodies and laminae I and II dendrites (Fig. 4A). As illustrated in Fig. 2B, 94% of SPR+ lamina I cell bodies had >20 SPR+ endosomes, and this proportion did not differ significantly from that produced by C-fiber stimulation in control animals. However, there was a significant increase in the proportion of lamina III cell bodies that exhibited SPR internalization compared with normal or sham-operated animals ($\chi^2 = 10.22, P < 0.001$). After nerve transection, 22% of lamina III cell bodies had >20 internalized SPR+ endosomes after stimulation of C fibers (Fig. 2B). For lamina III cell bodies that exhibited SPR internalization, typically the most superficial portion of their dorsally projecting dendrites (within laminae I–II) contained SPR+ endosomes. An example of SPR internalization in a lamina III neuron after nerve transection is provided in Fig. 5B. There was no evidence of SPR internalization in SPR+ cell bodies or dendrites located in laminae IV or in deeper laminae (Fig. 4C).

As illustrated in Fig. 3B, the mean number of endosomes per lamina I cell body was $46.8 \pm 1.32$ and did not differ from control values. However, a two-way ANOVA indicated differences between the groups in the mean number of endosomes per cell body in lamina III ($P < 0.001$). After transection, the mean number of endosomes per lamina III cell body evoked by C-fiber stimulation was $44.2 \pm 3.01 (P < 0.004)$.

HINDPAW INFLAMMATION. $A\beta$ fibers. At 3 days after intraplantar injection of CFA, inflammation was apparent and was characterized by edema of the hindpaw. In animals treated with CFA, there was no evidence that electrical stimulation at intensities that excite $A\beta$ primary afferent fibers evoked internalization of SPRs in SPR+ lamina I cell bodies or dendrites or in cell bodies and dendrites of deeper laminae (III–V).

$A\beta + A\delta$ fibers. Stimulation of $A\beta + A\delta$ fibers produced internalization of the SPR in lamina I cell bodies and dendrites in the lateral dorsal horn only, with many of the dendrites originating from deeper neurons. It was found that 90% of lamina I SPR+ cell bodies contained >20 SPR+ endosomes per cell body and this proportion was significantly higher than in control animals ($\chi^2 = 7.05, P < 0.008$). In addition, the mean number of endosomes per cell body increased significantly to $42.5 \pm 1.99 (P < 0.021)$. There was no evidence of internalization of the SPR in deeper laminae. Thus inflammation increased the proportion of lamina I neurons that exhibit SPR internalization evoked by stimulation of $A\delta$ primary afferent fibers as well as the number of internalized endosomes per cell body.

$A\beta + A\delta + C$-fiber stimulation. In rats with hindpaw inflammation, electrical stimulation at an intensity that recruited C fibers produced a novel pattern of SPR internalization in that SPR+ endosomes were found in cell bodies and dendrites throughout laminae I–IV of the ipsilateral dorsal horn (Figs. 4, B and D, and 5C). One hundred percent of lamina I SPR+ cell bodies contained >20 SPR+ endosomes, and this proportion did not differ from control values (Fig. 2C). However, the proportion of SPR+ neurons in the deep dorsal horn that exhibited internalization increased significantly compared with values obtained in control animals. In laminae III and IV, 32% ($\chi^2 = 16.74, P < 0.001$) and 36% ($\chi^2 = 19.58, P < 0.001$) of SPR+ cell bodies had >20 internalized endosomes (Fig. 2C).
Thus inflammation produced a spatial pattern of SPR internalization different from nerve transection in that cell bodies and dendrites in both lamina III and lamina IV exhibited internalization after stimulation of C fibers.

Similarly, significant differences occurred between the groups in the mean number of C-fiber-evoked internalized SPR+ endosomes per cell body in lamina III (<0.001) and lamina IV ($P < 0.001$). Figure 3C shows that the mean number of endosomes in lamina III and IV evoked by stimulation of C fibers increased to $43.6 \pm 2.85$ and $47.9 \pm 1.48$, respectively.

A schematic that summarizes the spatial extent of SPR internalization evoked by stimulation of A and C fibers in normal animals and after nerve transection and inflammation is provided in Fig. 6. In normal, untreated animals, internalization of the SPR is restricted to cell bodies and dendrites in the superficial dorsal horn. Internalization is observed in cell bodies and dendrites in lamina III after nerve transection and further ventral into lamina IV after inflammation.

**DISCUSSION**

Activation of the SPR, like several other G-protein-coupled receptors, is followed by receptor endocytosis (Caron and Lefkowitz 1993; Garland et al. 1996; Kobilka et al. 1992; Mantyh et al. 1995a; Senogles et al. 1990; Von Zastrow et al. 1993). Noxious stimulation, such as intraplantar injection of capsaicin (Mantyh et al. 1995b), produces internalization of SPRs in cell bodies and dendrites in the superficial dorsal horn of the spinal cord. The magnitude of SPR internalization, indicated by the proportion of neurons exhibiting internalization and by the number of endosomes per cell body, is dependent on the intensity of noxious stimulation. For example, we have shown that noxious heat and noxious cold stimuli evoke a graded internalization of the SPR that correlates with stimulus temperature (Allen et al. 1997a). Similarly, we have shown recently that the magnitude of SPR internalization produced by mechanical stimulation is also dependent on stimulus intensity (Allen et al. 1997b). Thus internalization of the SPR is an agonist-dependent process that provides a spatial image of the magnitude and localization of SP/NKA release.

In the present study, we investigated which primary afferent fibers contribute to SPR internalization after nerve injury and inflammation by measuring internalization after electrical stimulation of the sciatic nerve at varying intensities. Electrical stimulation provides a method to stimulate identified classes of primary afferent fibers (A$\beta$, A$\delta$, and C) because increasing stimulus intensity progressively recruits smaller caliber fibers. It was found that nerve injury and inflammation increased the proportion of neurons in the superficial and deep dorsal horn that exhibited SPR internalization and that A$\delta$ and C fibers contribute to the increase in SPR internalization. Stimulation of A$\beta$ fibers alone never evoked SPR internalization, and presumably SP/NKA release, in any of the pain models used. This is consistent with previous reports that electrical stimulation of A$\delta$ and C fibers, but not A$\beta$ fibers, evoked release of SP under normal conditions (Duggan and Hendry 1986; Go and Yaksh 1987; Hutchinson and Morton 1989; Klein et al. 1992) and parallels immunohistochemical studies of L4 DRG neurons that demonstrated SP was located in $\sim$50% of neurons with C fibers and in 20% of neurons with A$\delta$ fibers (McCarthy and Lawson 1989). SP was not found in neurons with fast-conducting A$a$ or A$\beta$ fibers under normal conditions.

Primary afferent fibers that evoke SPR internalization after nerve injury and inflammation

It has been reported that under pathological conditions, DRG neurons with A$\beta$ fibers may synthesize and release SP. After peripheral axotomy, mRNA and peptide levels of SP, calcitonin gene-related peptide, and somatostatin in DRG neurons are decreased (Baranowski et al. 1993; Henken et al. 1990; Jessell et al. 1979; Nielsch et al. 1987; Noguchi et al. 1989, 1990, 1993). Interestingly, Noguchi and colleagues (1995) subsequently demonstrated that preprotachykinin (PPT) mRNA and SP were expressed in some medium- and large-sized DRG neurons after nerve transection. Furthermore, they reported that

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**FIG. 3.** Mean ± SE number of SPR+ endosomes per cell body in laminae I, III, and IV evoked by C-fiber stimulation in normal (control) animals (A) and after sciatic nerve transection (B) and hindpaw inflammation (C). *Significant difference from control values.
unilateral sciatic nerve transection induced PPT mRNA and SP immunoreactivity in medium- to large-sized DRG neurons that project to the gracile nucleus and an increase in SP immunoreactivity in large myelinated dorsal root fibers and in the gracile nucleus at 2 wk after axotomy. An increase in SP immunoreactivity was not found in the spinal cord. In addition, fos-labeling in the gracile nucleus evoked by stimulation of the sciatic nerve at an intensity which excited only Aβ fibers increased after nerve injury, and this was attenuated by an NK-1 receptor antagonist. It was suggested that the newly acquired SP in large myelinated primary afferent fibers would enhance excitability of dorsal column-medial lemniscus pathway and thereby contribute to abnormal sensations after nerve injury.

It also has been suggested that inflammation produces novel expression of SP in Aβ mechanosensitive primary afferent fibers. Neumann et al. (1996) reported that inflammation of the hindpaw produced progressive tactile hypersensitivity (Ma and Woolf 1996) and an increase in the number of DRG neurons and large myelinated dorsal root fibers that expressed SP. Furthermore the proportion of dorsal horn neurons that exhibited afterdischarge produced by brief electrical stimulation of the sciatic nerve that excited only Aβ fibers increased after inflammation, and this was blocked by the NK-1 receptor antagonist RP67580. It was concluded that the phenotype of some Aβ fibers change and resemble C fibers in that they released SP and large myelinated dorsal root fibers that expressed SP. Although release of SP is frequency dependent, it is unlikely that higher frequencies of stimulus were needed because the stimulation frequency used (10 Hz) was in the range used previously (1–20 Hz) to produce NK-1-dependent afterdischarge in dorsal horn neurons after inflammation (Neumann et al. 1996). In addition, it could be argued that inflammation and transection changed electrical thresholds of afferent fibers resulting in activation of different populations of fibers by our standard electrical stimuli. We did not measure the compound action potential in animals that received sciatic nerve transection. However, in separate experiments, we found that the electrical threshold for evoking the Aβ, Aδ, and C-fiber components of the compound action potential after inflammation did not differ significantly from thresholds obtained in normal animals (unpublished observations). It is therefore unlikely that we were stimulating different classes of fibers in the experimental groups as compared with the control groups. It should be pointed out that in previous studies (Neumann et al. 1996), it was not determined whether an increase in SP-containing fibers or their distribution was present in the spinal cord. It is therefore unclear whether afterdischarge of wide dynamic range (WDR) neurons evoked by stimulation of Aβ fibers and attenuated by an NK-1 receptor antagonist was due to release of SP from primary afferent fibers or from indirect sources. Although results of the present study show that activation of Aβ fibers do not evoke SPR internalization, and presumably do not release SP/NKA after inflammation or nerve transection, approaches that measure directly the release of SP/NKA from Aβ afferent fibers, such as microdialysis and radioimmunoassay, are needed.

Potential mechanisms underlying increased internalization of SPRs after nerve transection and inflammation

Within the superficial and deep dorsal horn, the proportion of neurons that exhibited SPR internalization evoked by stimulation of Aδ or C fibers increased after nerve transection and inflammation. The mechanisms underlying enhanced internalization are unknown and there are several possibilities. One is that there is a greater amount of SP/NKA released from primary afferent fibers. This could be due to increased release of SP/NKA from those fibers that normally contain these peptides or from fibers in which their synthesis is increased, including small caliber fibers that normally do not contain SP/NKA. Another possibility that could account for increased SPR internalization is an increase in the affinity or sensitivity of the receptor (Stucky et al. 1993). It is also
possible that increased SPR internalization is due to upregulation of the SPR. Indeed, an increase in SPR-like immunoreactivity has been documented after inflammation (Abbadie et al. 1996) and was suggested to be due to an increase in the number of receptors for a given cell rather than an increase in the number of cells that expressed the SPR. In the present experiments, SPR immunoreactivity increased after nerve transection and inflammation, and it is therefore likely that upregulation of SPRs contributed to the increased SPR internalization.

Reorganization of SPR internalization after nerve transection and inflammation

Nerve transection and inflammation each produced reorganization of SPR internalization in cell bodies and dendrites located in lamina III and lamina IV. Many deep neurons that possess the SPR send dendrites dorsally into lamina I and II (Bleazard et al. 1994; Brown et al. 1995; Liu et al. 1994; Mantyh et al. 1995), and many get direct input from SP-containing primary afferent fibers (Naim et al. 1997). It is difficult to determine how much SPR internalization in deep neurons resulted from volume transmission or from direct synaptic transmission. If volume transmission accounted for much of the internalization, it is likely that there is a significant increase in the amount of SP/NKA released from primary afferent fibers. Volume transmission, however, is unlikely to account for all of the SPR internalization in deep layers of the dorsal horn. It recently has been shown that SP-immunoreactive fibers make direct synaptic
contact with cell bodies and their dendrites located in lamina III and IV (Naim et al. 1997), although the number of contacts made by SP-containing fibers is much greater in the dorsal portion of these dendrites within lamina I and II. It is unknown how synaptic contacts between SPRs and SP/NKA-containing fibers in deeper regions of the dorsal horn are modified by nerve transection and by inflammation. The same potential mechanisms that account for increased SPR internalization in the superficial dorsal horn are likely to underlie the novel internalization in the deep dorsal horn and include upregulation of the SPR, increased binding affinity of the SPR, or increased synthesis and/or release of SP/NKA from primary afferent fibers.

Our results are consistent with a previous report demonstrating that inflammation of the hindpaw increases the magnitude and localization of stimulus-evoked SPR internalization (Abbadie et al. 1997). In those studies, SPR internalization was observed in deep dorsal horn neurons after not only noxious heat and noxious mechanical stimuli but also nonnoxious mechanical (brush) stimulation. It could not be determined whether SPR internalization produced by innocuous stimulation was due to release of SP/NKA from sensitized nociceptors or from large caliber A-fiber mechanoreceptors that had undergone a change in phenotype to synthesize SP/NKA. Our studies suggest that brush-evoked SPR internalization after inflammation resulted from activity of sensitized Aδ and C nociceptors.

Conclusions

Reorganization of the spatial extent of SPR internalization produced by excitation of small caliber primary afferent fibers has important implications for the development of hyperalgesia. It is well known that after tissue injury and inflammation, hyperalgesia can occur at the injury site (primary hyperalgesia) and well as in a surrounding area of noninjured tissue (secondary hyperalgesia). The present studies suggest that the spread of hyperalgesia may involve not only enhanced responses of spinal neurons but also recruitment of activity from neurons that were not excited before the injury. The newly recruited neurons that come into play as a result of increased release of SP/NKA or upregulation of the SPR are likely to become sensitized and thereby contribute to the spatial and intensive characteristics of hyperalgesia. Understanding the mechanisms by which reorganization of SPR internalization occurs may have important implications for development of new therapeutic approaches for certain persistent painful conditions.

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