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Inhibition of Hyperalgesia by Ablation of Lamina I Spinal Neurons Expressing the Substance P Receptor

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Substance P is released in the spinal cord in response to painful stimuli, but its role in nociceptive signaling remains unclear. When a conjugate of substance P and the ribosome-inactivating protein saporin was infused into the spinal cord, it was internalized and cytotoxic to lamina I spinal cord neurons that express the substance P receptor. This treatment left responses to mild noxious stimuli unchanged, but markedly attenuated responses to highly noxious stimuli and mechanical and thermal hyperalgesia. Thus, lamina I spinal cord neurons that express the substance P receptor play a pivotal role in the transmission of highly noxious stimuli and the maintenance of hyperalgesia.

A subpopulation of dorsal root ganglion neurons synthesize (*1*) and transport (*2*) substance P (SP) to the spinal cord, where it is released upon noxious stimulation of the innervated peripheral tissue (*3*). Although SP excites spinal cord nociresponsive neurons (*4*), the role that SP and the substance P receptor (SPR) play in signaling nociceptive information remains unclear. In the normal animal, SP, upon release from primary afferents, diffuses to and interacts primarily with SPR-expressing neurons located in lamina I of the spinal cord (*5–7*). A high proportion of spinothalamic and spinobrachial neurons located in lamina I express SPR (*8*), suggesting that these SPR-expressing neurons play a role in the ascending conduction of nociceptive information.

To investigate the functions of lamina I SPR-expressing neurons in nociceptive signaling, we selectively ablated these neurons by infusing a cytotoxin conjugated to SP into the intrathecal space of the spinal cord in rats. When SP binds to spinal cord neurons expressing the SPR, both SP and SPR are rapidly internalized (*5–7*). Using SP-induced internalization of SPR as a specific portal of entry into SPR-expressing spinal cord neurons, we conjugated SP to the ribosome-inactivating protein saporin (SAP). This substance P–saporin conjugate (SP-SAP), like other saporin conjugates, must be internalized to exert its toxicity as it inactivates and ultimately kills cells by blocking protein synthesis (*9*). We performed a series of correlative in vitro and in vivo studies to determine the specificity and toxicity of SP-SAP, as well as functional changes in somatosensory processing.

Competition binding studies with ^{125}I-SP binding to membranes of the adult rat spinal cord demonstrated that SP-SAP [median inhibitory concentration (IC_{50}) = 2.2 nM] was nearly equipotent with SP (IC_{50} = 2.0 nM) in blocking the binding of ^{125}I-SP to the SPR (*10*), whereas neurokinin A (IC_{50} = 5 nM) was less potent, and SAP alone (1 μM) or the unrelated peptide endothelin-1 (1 μM) were totally inactive.

SP-SAP internalization and cytotoxicity were examined in primary cultures of neonatal rat spinal cord neurons (*11*), in which ~15% of the neurons express the SPR (*12*). Both SP (10^{-7} M) and SP-SAP (10^{-7} M) induced a rapid and similar extent of SPR

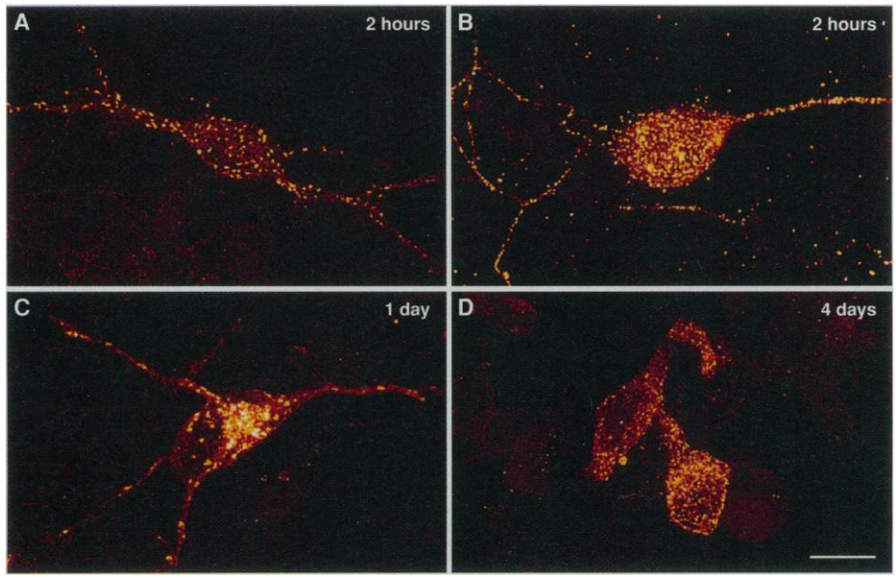

Fig. 1. Internalization and cytotoxicity of SP-SAP in primary cultures of neonatal spinal cord neurons (*11*). Confocal image of neurons where the SPR immunofluorescence (A, C, D) appears red, areas of concentrated SPR immunofluorescence appear yellow. (**A, C,** and **D**) SPR immunofluorescence in neurons 2 hours, 1 day, and 4 days, respectively, after treatment with SP-SAP. (**B**) Confocal image showing SAP immunofluorescence (yellow) 2 hours after SP-SAP treatment. These images were projected from 14 optical sections acquired at 0.8-μm intervals with a 60× lens. Bar, 25 μm.

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internalization that was blocked by 5×10^{-6} M of the nonpeptide SPR antagonist RP67580. Two hours after treatment with 10^{-7} M SP-SAP, but not 10^{-7} M SAP, SPR internalization was visualized with an antibody that recognized SPR (Fig. 1A), and intracellular accumulation of SAP was visualized with an antibody that recognized SAP (Fig. 1B).

One day after SP-SAP treatment, there was no significant loss of cultured SPR-immunoreactive neurons, although in most of these neurons the SPR-immunoreactivity was localized within intracellular endosomes (Fig. 1C). In contrast, 1 day after treatment with SP alone, most of the SPR neurons had recycled to the plasma membrane. Thus, within 24 hours after SP-SAP internalization, these neurons could no longer efficiently recycle SPR back to the plasma membrane. Four days after SP-SAP treatment, there was an 82% decrease in the number of SPR-immunoreactive neurons, at 7 days a 95% reduction, and at 10 days there were no SPR-immunoreactive neurons remaining in culture. At 4 and 7 days after treatment, the surviving SPR-immunoreactive neurons showed shrunken cell bodies, diffuse SPR immunoreactivity throughout the cytoplasm (Fig. 1D), and shortened dendritic processes. In contrast, nearby neurons that did not express SPR immunoreactivity, but did express the neuronal marker microtubule-associated protein–2 (MAP-2), appeared morphologically normal. Treatment of cultured spinal cord neurons with saline, SP, or SAP alone resulted in no significant morphological or cytotoxic changes in either the SPR-expressing neurons or the non-SPR, MAP-2–immunofluorescent neurons.

To estimate the placement of the intrathecal catheter and the potential spread of the SP-SAP, we injected 10 μl of the dye Fast Green with the end of the intrathecal catheter placed at L4; 1 hour later, the dye had intensely labeled the spinal cord from spinal segments L2 to L5 (*13*). One hour after injection of 10 μl of 5.0×10^{-6} M SP-SAP, SPR internalization was observed in SPR-immunoreactive dendrites and cell bodies in lamina I of the spinal cord at spinal segments L2 to L5 (Fig. 2). Internalization of SPR presumably reflected the sites where SP-SAP had bound to SPR and induced the internalization of both SPR and SP-SAP (*5–7*). After injection of SP-SAP, a significant loss of SPR immunoreactivity was first detected at 7 days after treatment. This loss of SPR immunoreactivity was confined to lamina I in spinal segments L2 to L5, and the loss of SPR immunofluorescence in lamina I was observed through 28 days after treatment (Fig. 3B), which was the last time point examined. In contrast, injection of saline, SP, or SAP alone produced no change in SPR immunoreactivity in lamina I in spinal segments L2 to L5 at any of the time points examined (Fig. 3A).

Twenty-eight days after injection of saline, SP, SAP, or SP-SAP, spinal cords and dorsal root ganglia (L4) were histologically examined (*12*) to determine which cell populations had been affected by these treatments (Table 1). Measurements were made of neuronal cell populations expressing SPR (labels lamina I, III to V, and the preganglionic sympathetic neurons at spinal segment T10), calbindin (labels a subset of lamina I and II neurons), ChAT (labels motor neurons), SP (labels cell bodies in the L4 dorsal root ganglia), as well as immunofluorescence for SP in lamina I (labels SP primary afferent inputs), MAP-2 (labels all neurons), glial fibrillary acidic protein (GFAP; labels astrocytes), and OX-42 (labels microglia).

Examination of neuronal markers and immunofluorescence intensity values showed that the only significant changes observed at 28 days after treatment with saline, SP, SAP, or SP-SAP was that SP-SAP treatment reduced the number of lamina I SPR–immunoreactive neurons in lamina I and in the levels of SPR immunofluorescence in lamina I (Fig. 3 and Table 1). Infusion of SP-SAP produced an 85% reduction in the number of SPR immunofluorescent neurons in lamina I at spinal cord segment L4. The surviving 15% of the lamina I immunoreactive neurons showed shrunken cell bodies, shortened cell processes, and SPR immunoreactivity that was diffusely distributed throughout the cytoplasm with little SPR present on the plasma membrane. In contrast, there was not a significant reduction in the total number, or evidence of cytotoxicity, in SPR-immunoreactive neurons located in lamina III to V or X at the L4 spinal segment or in preganglionic sympathetic neurons at spinal segment T10 (Table 1).

Examination of the spinal cords treated with saline, SP, or SAP alone showed that these treatments did not produce a signifi-

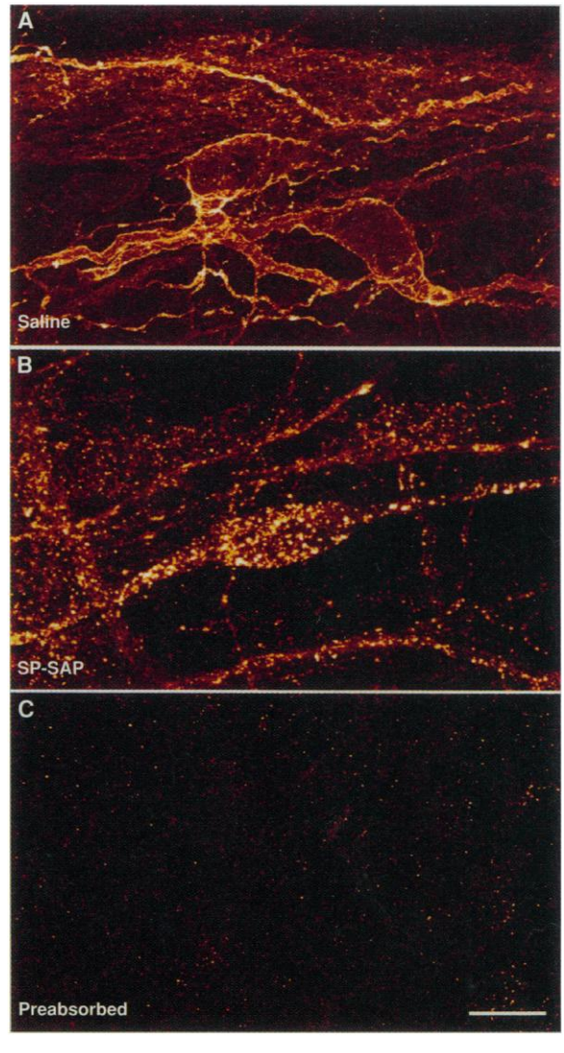

Fig. 2. SPR immunofluorescence in lamina I neurons of the spinal cord after SP-SAP treatment (*13*). SPR immunofluorescence in lamina I neurons in sagittal sections of the L4 spinal segment of the spinal cord at 1 hour after infusion of saline (**A**), 1 hour after infusion of SP-SAP (**B**), and an absorption control for SPR immunofluorescence in a saline-infused animal (**C**). In these confocal images the SPR immunofluorescence appears red and areas of intense SPR immunofluorescence appear yellow. Infusion of SP-SAP induced a marked translocation of the SPR from the plasma membrane (A) into intracellular endosomes (B). These images were projected from 18 optical sections acquired at 0.7-μm intervals with a 60× lens. Bar, 25 μm.

cant change in cell number, morphology, or fluorescence level of any of the cell markers examined (Table 1). Thus, the cytotoxicity of intrathecally infused SP-SAP was limited to the SPR-expressing lamina I neurons in spinal segments L2 to L5.

Intrathecal infusion of saline, SP, SAP, or SP-SAP produced no detectable changes in body weight, food intake, alertness, locomotion, or grooming behavior for 28 days after injection. Behavioral testing indicated that all animals had normal withdrawal latencies to heat applied to the plantar surface of the hindpaw before treatment with capsaicin (*14*). In untreated rats, intraplantar injection of 10 μg of capsaicin produced nocifensive behavior for a duration of ~3 min and produced about a 50% decrease in withdrawal latency to heat and a 40 to 60% increase in the frequency of withdrawal from the mechanical stimuli (*15*). Animals treated with SP-SAP exhibited a significant attenuation of mechanical (85% decrease at day 28) and heat (60% decrease at day 28) hyperalgesia produced by intraplantar injec-

Table 1. Cytotoxicity of intrathecally infused saline, SP, SAP, and SP-SAP in the L4 segment of the spinal cord at 28 days after treatment (*12*). Cell numbers and immunofluorescence levels were determined by confocal microscopy. In all instances the saline, SP, and SAP animals were not significantly different from normal untreated control animals, and thus only the values for the saline-, SAP-, and SP-SAP–infused animals are shown. The only significant difference in the SP-, SAP-, or SP-SAP–treated animals as compared with saline-treated controls was the loss of lamina I SPR-immunoreactive neurons and the loss of SPR immunoreactivity in lamina I of the spinal cord in the SP-SAP–treated animals. Data points are expressed as the mean ± SEM (n = 6), and significant differences were calculated by a one-way ANOVA and Bonferroni comparisons (*P < 0.01).

Treatment (neuronal cell population)	Percent immunoreactive positive cells (saline = 100)		
	Saline	SAP	SP-SAP
SPR (laminae I and II)	100 ± 27	80 ± 16	**15 ± 13***
SPR (laminae III to V)	100 ± 12	79 ± 13	86 ± 13
SP (DRG)	100 ± 23	91 ± 11	103 ± 13
Calbindin (laminae I and II)	100 ± 10	96 ± 7	93 ± 9
ChAT (motor neurons)	100 ± 16	116 ± 24	107 ± 11
	Percent immunofluorescence level (saline = 100)		
SPR (laminae I and II)	100 ± 9	95 ± 3	**65 ± 8***
SPR (preganglionic sympathetics)	100 ± 14	100 ± 9	92 ± 7
SP (laminae I and II)	100 ± 24	96 ± 3	97 ± 13
GFAP (lamina I)	100 ± 11	90 ± 13	116 ± 7
MAP-2 (lamina I)	100 ± 12	100 ± 14	100 ± 17

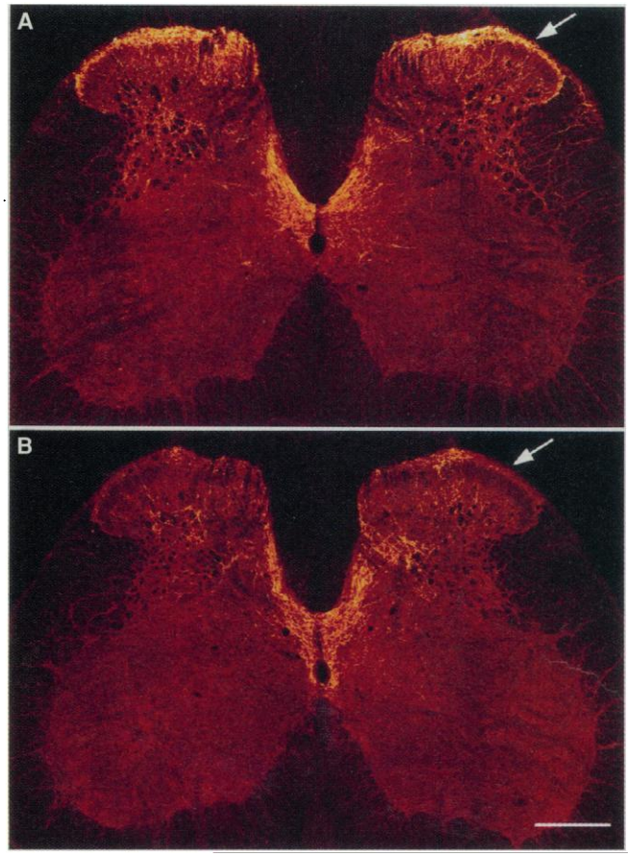

Fig. 3. Cytotoxicity after intrathecal infusion of SP-SAP in the spinal cord (*13*). Confocal images of SPR immunofluorescence in the spinal cord 28 days after infusion of saline (**A**) or SP-SAP (**B**), where the SPR immunofluorescence appears yellow. The only difference between saline- and SP-SAP–treated animals is the marked reduction in SPR immunofluorescence in lamina I (arrows) of the SP-SAP–treated animals. These images are 60-μm-thick tissue sections acquired with a 10× lens. Bar, 400 μm.

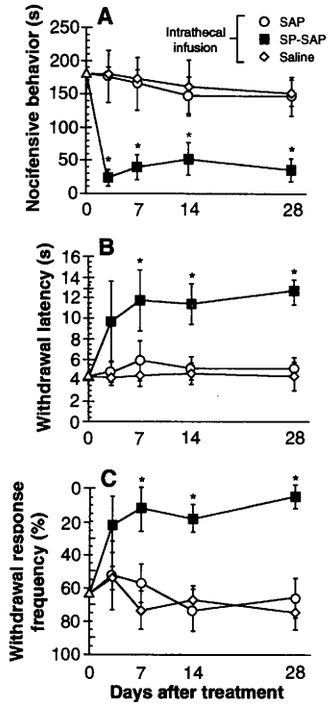

Fig. 4. Three behavioral parameters after intrathecal infusion of saline, SAP, or SP-SAP at day 0 (*14*). There was no significant difference between normal untreated animals, SP-infused animals, or saline-infused animals at any of the time points examined, and thus only the values for the saline-, SAP-, and SP-SAP–infused animals are shown. All animals (normal, saline-, SP-, SAP-, and SP-SAP–treated) had normal withdrawal latencies to heat applied to the plantar surface of the hindpaw before treatment with capsaicin (*14*). (**A**) Nocifensive behavior during the first 5 min after unilateral injection of capsaicin. (**B** and **C**) Thermal and mechanical hyperalgesia at 5 min after intraplantar injection of capsaicin. The triangles at time 0 are the baseline measurements for the saline-treated animals after injection of capsaicin. Data for each experimental group and at each time point were obtained from separate animals (n = 6 for each group) and represent withdrawal responses of the paw injected with capsaicin. All data points are expressed as the mean ± SEM, and significant differences were calculated by a one-way analysis of variance (ANOVA) and Bonferroni comparisons (*P < 0.01).

tion of capsaicin (Fig. 4, B and C). Additionally, there was a marked reduction (75% decrease at day 28) in the nocifensive behavior induced by unilateral injection of capsaicin into the hindpaw at days 7, 14, and 28 after intrathecal treatment (Fig. 4A). In contrast, infusion of saline, SP, or SAP produced no significant change in mechanical or thermal hyperalgesia, or in nocifensive behavior produced by capsaicin as compared with normal untreated animals in any corresponding time point examined (Fig. 4).

Although we ablated only the SPR-expressing neurons, which constitute less than 10% of all lamina I neurons (*6*, *7*), capsaicin-induced nocifensive behavior and mechanical and thermal hyperalgesia were depressed by 60 to 90% (Fig. 4). Our assumption is that intrathecal infusion of SP-SAP is more cytotoxic to SPR-expressing lamina I cells than to SPR-expressing cells in laminae III to V because further spread of bioactive SP-SAP into deeper laminae was prevented by degradation of the SP moiety by characterized proteases. One reason that ablation of such a small percentage of lamina I neurons could produce such a large change in behavioral nociceptive responses may be that, because most lamina I spinothalamic and spinoparabrachial neurons express SPR (*8*) and internalize SP-SAP, SP-SAP treatment is ablating a major part of the system for the ascending conduction of nociceptive information.

Hyperalgesia produced by capsaicin is mediated in part by sensitization of spinothalamic neurons, and SP is involved in the excitation and sensitization of spinothalamic neurons (*16*) and the development of hyperalgesia (*17*). However, it has been surprisingly difficult to block noxious stimulus-evoked pain behavior with either SP antagonists (*18*) or "knockout" of SPR in mice (*19*). In this study we did not block or inactivate only the SPR, but rather we killed a specific population of SPR-expressing cells that also express a variety of other neurotransmitter receptors (*20*). These data suggest that while this small population of SPR-expressing neurons is pivotal in the maintenance of hyperalgesia, a variety of other non-SPR receptors expressed by these neurons are also involved in nociceptive signaling. Understanding the repertoire of receptors that are expressed by this small population of SPR-immunoreactive lamina I neurons and how these receptors interact to generate persistent pain states should provide valuable information on how chronic pain states are generated, maintained, and potentially managed.

Whether receptor internalization can serve as a specific portal for introducing other therapeutic compounds into other receptor-bearing cells remains to be determined. This approach is, however, promising as a substantial number of other receptors have been shown to undergo ligand-induced receptor internalization (*21*). Specific targeting of spinal neurons involved in transmitting chronic as opposed to acute pain may have substantial therapeutic potential because most analgesics block both acute and chronic pain (*22*), and tolerance and dependence are major problems in long-term treatment with narcotics (*23*). Because the present findings suggest that the conduction of mild pain can be dissociated from highly noxious and hyperalgesic pain, SP-SAP treatment may be therapeutically useful in the treatment of persistent pain. However, before such therapies can be considered, the long-term consequences of removal of the superficial SPR-immunoreactive neurons must be defined.

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10. SP and endothelin-1 were obtained from Bachem (Torrence, CA), RP67580 was from RBI (Natick, MA), and SAP and SP-SAP were provided by Advanced Targeting Systems (San Diego, CA). SAP and SP-SAP were in liquid form and sterile-filtered, and in all other instances the compounds were weighed, dissolved in sterile saline, pipetted into microfuge tubes, and frozen at −70°C until use. The IC_{50} of these compounds was determined through ^{125}I-SP (Amersham, Oak Park, IL) binding to plasma membranes obtained from adult rat (7 to 10 weeks, 250 g) spinal cord as described [G. Nicol, D. K. Klingberg, M. R. Vasko, *J. Neurosci.* **12**, 1917 (1992)].
11. All procedures were approved by the Animal Care Committee at the VA Medical Center and the University of Minnesota. Neurons were cultured from the spinal cords of 18-day embryonic Holtzman rats (Harlan Sprague-Dawley, Madison, WI). The whole spinal cord was dissected out of the rat and placed in 4°C Puck's saline supplemented with 40 mM glucose, 50 mM sucrose, and 10 mM Hepes (DISG media, pH 7.4). The spinal cords were then dissociated by trituration (15 to 20 times) through a small-bore serological pipette. The resulting cell suspension was centrifuged at 4°C for 5 min at 1500 rpm in a Sorvall RC-3B centrifuge (DuPont, Newtown, CT). The supernatant was removed and the pellet resuspended in Dulbecco's modified Eagle's medium (DMEM; Sigma) plus 5% equine serum and 5% calf serum (v/v) (Hyclone, Logan, UT). The cell suspension was plated on poly-L-ornithine coated two-well chamber slides at a density of 100,000 cells per milliliter and incubated at 37°C with 9% CO_2. After 4 days the mitotic inhibitors, 5-fluoro-2′-deoxyuridine and uridine were added. At 7 days the media was replaced with DMEM plus 10% equine serum (v/v). The cells were incubated until 14 days after culture with media changes every fourth day. At day 14 after culture, either saline, SP, SP-SAP conjugate, or SAP was added to a final concentration of 10^{-7} M. The cells were incubated for 12 hours with the SP-SAP or SAP compounds at which time the compounds were removed from the culture and fresh media was added. The cultures were then allowed to continue until the desired time points (2 hours or 1, 4, 7, and 10 days) at which time the experiment was terminated and the cells were processed for immunohistochemistry by fixing for 20 min at 22°C with 4% formalin in phosphate-buffered saline (PBS) as described (*5*–*7*).
12. Immunohistochemistry and fluorescent confocal microscopy were done as described (*5*–*7*) with an MRC-1024 Confocal Imaging System (Bio-Rad, Boston, MA) and an Olympus BH-2 microscope equipped for epifluorescence (Lake Success, NY). For cell counts the spinal cords were cut in the sagittal plane, whereas for immunofluorescence measurements the spinal cords were cut in either the sagittal or coronal plane. Both of the microscopes were set up as described [T. C. Brelje, D. W. Scharp, R. L. Sorenson, *Diabetes* **38**, 808 (1989); P. W. Mantyh *et al.*, *J. Neurosci.* **15**, 152 (1995)]. SPR was detected by polyclonal rabbit antibody to SPR (anti-SPR) (1:5000) raised against a 15–amino acid peptide sequence ($SPR_{393-407}$) at the COOH-terminus of the rat SPR. SP was detected by polyclonal guinea pig anti-SP (1:1000). Calbindin was detected by monoclonal mouse anti-calbindin (Sigma, 1:300). Microtubule-associated protein (MAP-2) was detected by monoclonal mouse anti–MAP-2 (Sigma, 1:750). Choline acetyltransferase (ChAT) was detected by monoclonal mouse anti-ChAT (Chemicon, 1:500). Glial fibrillary acidic protein was detected by polyclonal rabbit anti-GFAP (DAKO, 1:450). OX-42 was detected by a monoclonal mouse antibody (Chemicon, 1:2000). Saporin was detected by polyclonal goat anti-Saporin (Advanced Targeting Systems, 1:350). Secondary antibodies conjugated to fluorescent markers Cy3 (used with SPR, Calbindin, ChAT, GFAP, OX-42, and Saporin) and fluorescein isothiocyanate (used with SP and MAP-2) (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a dilution of 1:600 and 1:150, respectively. All primary (overnight, 22°C) and secondary (3 hours, 22°C) antibodies were applied in cocktails with 1% goat serum and 0.3% Triton X-100 in PBS solution. Finally, the tissue sections were washed for 20 min in PBS (pH 7.4, 22°C), mounted onto gelatin-coated slides, and applied onto cover slips with PBS-glycerine containing 1.0% *p*-phenylenediamine to reduce photobleaching. To determine the number of immunofluorescent cell bodies (Table 1), we viewed the slides through a 1.0-cm^2 eyepiece grid, which was divided into 100 1 mm by 1 mm units, and counted the total number of immunofluorescent cell bodies per unit area. To calculate SPR immunofluorescence intensity, we obtained images of the 60-μm-thick tissue sections with the Bio-Rad MRC1024 laser-scanning confocal fluorescent-imaging system and analyzed them using NIH Image 1.7 software.
13. Animals were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and acepromazine (1 mg/kg) and placed in a stereotaxic frame. An 8.5-

cm length of PE-10 tubing (inner diameter, 0.28 mm; outer diameter, 0.61 mm), serving as an inflow cannula, was inserted into the subarachnoid space through an incision in the atlanto-occipital membrane. The cannula terminated in the caudal region of the lumbar enlargement (approximately L4). The spinal cord was superfused with 10 μl of either saline or 5.0×10^{-6} M SP, SAP, or SP-SAP followed by a 5 μl saline flush with a 25-μl Hamilton syringe. Five minutes after spinal superfusion, the cannula was carefully withdrawn and the wound closed with 3-0 silk sutures.

14. Measurements of nocifensive behavior and hyperalgesia produced by intraplantar injection of capsaicin were obtained as described [H. D. Gilchrist, B. L. Allard, D. A. Simone, *Pain* **67**, 179 (1996)]. We and most other pain researchers define hyperalgesia as an increase in withdrawal responses. The capsaicin model of hyperalgesia used in the present experiments was chosen as it has been well characterized, it produces hyperalgesia in humans, and it is the only model of hyperalgesia that can be used in parallel animal and human studies. Capsaicin (Sigma) was dissolved in a vehicle of 7.5% polyxyethylene sorbitan monooleate (Tween-80) and saline, and given into the plantar surface of one hindpaw. Each animal received one injection of 10 μg in a volume of 10 μl. The duration of nocifensive behavior, defined as lifting and guarding the injected paw, was measured for the first 5 min after injection. Withdrawal responses to heat were determined by means of a previously described procedure [K. Hargreaves, R. Dubner, F. Brown, C. Flores, J. A. Joris, *Pain* **32**, 77 (1988)]. Rats were placed under a nonbinding cage on a 3-mm-thick glass plate that was elevated to allow maneuvering of a radiant heat source from below. Controlled radiant heat stimuli were applied to the plantar surface of the hindpaw by means of a 50-W light bulb placed in a custom-built case. The start of each trial activated a timer, and withdrawal latencies to the nearest 0.1 s were measured automatically by a photocell that terminated each trial and stopped the timer upon withdrawal of the paw. Four stimuli, spaced at least 1 min apart, were applied to each hindpaw. Withdrawal latency for each paw was defined as the mean latency of the last three trials. The intensity of the heat was adjusted and maintained to produce withdrawal latencies of about 12 s under normal conditions. Heat hyperalgesia was defined functionally as a decrease in the withdrawal latency. To measure withdrawal responses to mechanical stimuli, we placed rats under a clear plastic cage on an elevated plastic mesh floor (1-cm^2 perforations). A von Frey monofilament with a bending force of 95.0 mN was applied to the plantar surface from below the floor. The stimulus was applied 10 times, each for a duration of 1 to 2 s, at random locations on the plantar surface. The frequency was determined for each hindpaw. Mechanical hyperalgesia was defined as an increase in withdrawal response frequency.
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Preserved Acute Pain and Reduced Neuropathic Pain in Mice Lacking PKCγ

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In normal animals, peripheral nerve injury produces a persistent, neuropathic pain state in which pain is exaggerated and can be produced by nonpainful stimuli. Here, mice that lack protein kinase C gamma (PKCγ) displayed normal responses to acute pain stimuli, but they almost completely failed to develop a neuropathic pain syndrome after partial sciatic nerve section, and the neurochemical changes that occurred in the spinal cord after nerve injury were blunted. Also, PKCγ was shown to be restricted to a small subset of dorsal horn neurons, thus identifying a potential biochemical target for the prevention and therapy of persistent pain.

Neuropathic pain is a devastating consequence of nerve injury that is characterized by spontaneous, often burning, pain, an exaggerated response to painful stimuli (hyperalgesia), and pain in response to normally innocuous, for example touch, stimuli (allodynia). Neuropathic pain syndromes are among the most difficult to manage. Although the pain produced by tissue injury can usually be controlled by anti-inflammatory drugs and opioids, neuropathic pains such as postherpetic neuralgia, reflex sympathetic dystrophy, and phantom limb pain are often refractory to these treatments.

Some studies suggest that nerve injury leads to neuropathic pain because it triggers an *N*-methyl-D-aspartate (NMDA) receptor–mediated hyperexcitability of dorsal horn neurons in the spinal cord. Events downstream of the NMDA receptor, including activation of various protein kinases, have also been implicated; these are presumed to underlie the persistence of the pain (*1*). Conclusions from these studies, however, are very limited. For example, although there is evidence for a contribution of protein kinase C (PKC), those studies not only used inhibitors that are not specific for PKC but they also provided no information about the contribution of specific isoforms of PKC, at least 10 of which have been identified (*2*). In the present study we examined nerve injury–induced neuropathic pain in mice with a deletion of the gene that encodes for the neuronal-specific (gamma) isoform of PKC.

The deletion (knock-out) of PKCγ produces viable mice with normal appearance. The mice have a slight ataxia, modest impairments in tests of learning and memory (*3*), and some motor incoordination (*4*) that may be related to a defect in elimination of multiple climbing fiber innervation of Purkinje cells (*5*). Although synaptic transmission appears normal, long-term potentiation is impaired (*3*).

In the absence of nerve injury, we found no difference in paw withdrawal responses to thermal or mechanical stimulation in mutant and wild-type mice (Fig. 1). Thus, transmission of acute "pain" messages was intact in the mutant mice. To study pain behavior produced by nerve injury, we tightly ligated one-third to one-half of the diameter of the sciatic nerve; this partial nerve injury produces a neuropathic pain syndrome characterized by a marked and long-lasting reduction in the paw withdrawal threshold to both thermal and mechanical stimulation on the injured side (*6*). In the wild-type mice thermal response laten-

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