

MOUSE STRAINS THAT LACK SPINAL DYNORPHIN UPREGULATION AFTER PERIPHERAL NERVE INJURY DO NOT DEVELOP NEUROPATHIC PAIN

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Abstract—Several experimental models of peripheral neuropathy show that a significant upregulation of spinal dynorphin A and its precursor peptide, prodynorphin, is a common consequence of nerve injury. A genetically modified mouse strain lacking prodynorphin does not exhibit sustained neuropathic pain after nerve injury, supporting a pronociceptive role of elevated levels of spinal dynorphin. A null mutation of the γ isoform of protein kinase C (PKC γ KO [knockout]), as well as an inbred mouse strain, 129S6, also does not manifest behavioral signs of neuropathic pain following peripheral nerve injury. The objective of this study was to extend our observations to these genetic models to test the hypothesis that elevated levels of spinal dynorphin are essential for the maintenance of abnormal pain. In PKC γ wild-type mice and the outbred mouse strain ICR, ligation of the L5 and L6 spinal nerves (SNL) elicited both tactile hypersensitivity and thermal hyperalgesia. Both strains showed a significant elevation in dynorphin in the lumbar spinal dorsal horn following SNL. Spinal administration of an anti-dynorphin A antiserum blocked the thermal and tactile hypersensitivity in both strains of mice. However, the PKC γ KO mice and the 129S6 mice (which express PKC γ) did not show abnormal pain after SNL; neither strain showed elevated levels of spinal dynorphin. The multiple phenotypic deficits in PKC γ KO mice confound the interpretation of the proposed role of PKC γ -expressing spinal neurons in neuropathic pain states. Additionally, the data show that the regulation of spinal dynorphin expression is a common critical feature of expression of neuropathic pain. © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: dynorphin, PKC, spinal nerve injury, transgenic mice, neuropeptide, opioid.

Experimental models of peripheral neuropathy elicit abnormal pain behavior that is characterized by a persistent

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Abbreviations: CGRP, calcitonin gene-related peptide; DLF, dorsal lateral funiculus; i.th., intrathecal; PKC, protein kinase C; PKC γ , protein kinase C γ isoform; SDS, sodium dodecyl sulfate; SNL, spinal nerve ligation; WT, wild type.

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doi:10.1016/j.neuroscience.2003.08.021

hypersensitivity to normally innocuous tactile stimuli as well as to noxious stimuli. Such sensory hypersensitivity may be sustained, at least in part, by the spontaneous firing of the injured nerves (Kajander et al., 1990; Liu et al., 2000; for review, see Gold, 2000) and by a sensitization of the CNS to sensory input (for review, see Woolf and Salter, 2000). The latter appears to be mediated by both spinal and supraspinal mechanisms that collectively confer a lower threshold to neuronal excitation (for reviews, see Urban and Gebhart, 1999; Ossipov et al., 2000; Porreca et al., 2002). One potential mediator of neuropathic pain is the endogenous opioid dynorphin, which is consistently upregulated in the spinal cord upon chronic inflammation (Iadarola et al., 1988; Draisci et al., 1991; Pohl et al., 1997), nerve injury (Cho and Basbaum, 1988; Kajander et al., 1990; Malan et al., 2000), and chronic opioid treatment (which paradoxically induces abnormal pain; Vanderah et al., 2000). Importantly, spinal administration of an anti-dynorphin antiserum can effectively block nerve injury-induced pain (Bian et al., 1999; Malan et al., 2000), as well as opioid induced pain and antinociceptive tolerance (Vanderah et al., 2000). A transgenic model that consists of a deletion mutation of dynorphin's precursor peptide, prodynorphin, does not exhibit persistent abnormal pain behavior after nerve injury (Wang et al., 2001), further supporting a role of spinal dynorphin in the maintenance of neuropathic pain (for review, see Lai et al., 2001).

Pharmacological application of non-opioid fragments of dynorphin produces an enhancement in the evoked release of calcitonin gene-related peptide (CGRP), an excitatory transmitter found in the primary afferent (Gardell et al., 2002b). It is well established that non-opioid actions of dynorphin are pronociceptive and possibly excitotoxic (Skilling et al., 1992; Vanderah et al., 1996), and that these effects of dynorphin are consistent with excitatory effects on neurons (Hauser et al., 1999; Tang et al., 2000). A recent study in our laboratory shows that elevated levels of spinal dynorphin, and presumably its enhanced release, in morphine "tolerant rats" enhanced the evoked release of CGRP from primary afferents (Gardell et al., 2002b). This enhancing effect on excitatory neurotransmission by spinal dynorphin may underlie some aspects of opioid-induced abnormal pain and the expression of antinociceptive tolerance to morphine. While the mechanisms that mediate the action of dynorphin is at present unknown, many excitatory signal transduction processes that may contribute to the manifestation of abnormal pain have been proposed; among these, the activation of the *N*-methyl-D-aspartate (NMDA) receptors and protein kinase C (PKC) has been

proposed to be key elements that drive central sensitization (Woolf and Thompson, 1991; Chen and Huang, 1992; Mayer et al., 1999). Blockade of NMDA receptor activity by MK-801 (Woolf and Thompson, 1991; Bian et al., 1999), or inhibition of PKC activation (Mao et al., 1992), reduces some aspects of neuropathic pain. Other data support the hypothesis that NMDA receptors may mediate the activation/translocation of PKC resulting in neuropathic pain (Mao et al., 1995).

Transgenic mice with a null mutation of the gene encoding the γ isoform of PKC (PKC γ KO [knockout]) do not exhibit abnormal pain following partial sciatic nerve section (Malmberg et al., 1997). These mice also do not develop inflammation-induced hypersensitivity of the spinal cord (Martin et al., 2001). PKC γ is expressed exclusively in the CNS (Nishizuka, 1988); in the spinal cord, it is localized to the inner region of lamina II of the dorsal horn. This isoenzyme thus potentially defines a discrete population of spinal neurons that mediate chronic pain. Extending the findings of dynorphin to this transgenic strain may provide clues to the excitatory mechanism of dynorphin. As a parallel approach, an inbred mouse strain, the 129S6 (formerly known as 129/SvEv; Festing et al., 1999), was also selected for this study. This mouse strain is noted for its inability to develop opioid antinociceptive tolerance after chronic opioid treatment (Kolesnikov et al., 1998); based on the potential mechanistic similarity between opioid tolerance and abnormal pain as noted above, we hypothesized that this mouse also might not develop abnormal pain after nerve injury. The genetic basis of this phenotype is not known.

The present study determined whether PKC γ KO and/or 129S6 mice develop the behavioral manifestations of abnormal pain after spinal nerve ligation (SNL) injury and if such pain is associated with an upregulation of spinal dynorphin; wild-type (WT) mice and an outbred strain, ICR, were also analyzed in parallel experiments for comparison. Our goal is to extend our observations to these genetic models to test the hypothesis that elevated levels of spinal dynorphin are essential for the maintenance of abnormal pain, and to evaluate if PKC γ mediates the pronociceptive actions of dynorphin.

EXPERIMENTAL PROCEDURES

Animals

Male ICR (Harlan Industries, Cleveland, OH, USA), 129S6 (Taconic Farms, Germantown, NY, USA) and PKC γ KO and the corresponding WT, B6129F2/J mice (Jackson Laboratory, Bar Harbor, ME, USA) were used in these experiments. The mice weighed between 20 and 30 g; animals were maintained on a 12-h light/dark cycle and provided food and water *ad libitum* before the experimental procedures. All efforts were made to minimize the number of animals used for the experiments. All animal experiments were performed under an approved protocol in strict accordance with institutional guidelines and in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health (NIH publications no. 80-23, revised 1996).

SNL surgery

Nerve injury was induced in the mice surgically under anesthesia based on the technique described by Kim and Chung (1992). Anesthesia was induced with 2% halothane in O₂ at 2 l/min and maintained with 0.5% halothane in O₂. After surgical preparation of the mice and exposure of the dorsal vertebral column from L4 to S2, the L5 and L6 spinal nerves were exposed and tightly ligated distal to the dorsal root ganglion with 6-0 braided silk suture (Surgical Specialties, Reading, PA, USA). The incisions were closed and the animals were allowed to recover for 7 days prior to any further experimentation. Mice that exhibited motor deficiency (such as paw-dragging or dropping) were excluded from subsequent testing (less than 10% of the animals were not used). Sham control mice underwent the same operation and handling as the experimental animals, but without nerve ligation.

Intrathecal drug administration

Intrathecal (i.th.) injections were made into the subarachnoid space (L5–L7) in unanesthetized mice by the method of Hylden and Wilcox (1980) as modified by Porreca and Burks (1983). Antiserum to dynorphin A(1–13) (Bachem/Peninsula Laboratories, Belmont, CA, USA; Cat. No. T-4278.0500) or control, non-immune rabbit serum was administered in a dose of 200 μ g (5 μ l). This antiserum exhibits high affinity for dynorphin A(1–17), dynorphin A(1–13), and dynorphin A(1–12), and has little or no cross-reactivity with shorter fragments of dynorphin A, and does not react with dynorphin B, α -neoendorphin, β -endorphin, [Leu⁵]- or [Met⁵]-enkephalin. Dynorphin A(1–17) was given at a single dose of 10 nmol in 5 μ l saline.

Assessment of tactile hypersensitivity

Tactile sensitivity was determined by measuring the paw withdrawal in response to probing with a series of finely calibrated von Frey filaments according to that described by Chaplan et al. (1994). The paw withdrawal threshold was estimated by the Dixon non-parametric test (Dixon, 1980). The data were expressed as mean withdrawal threshold \pm S.E.M. Paw withdrawal thresholds were determined to the nearest 0.1 g before surgery (baseline) and on the morning and afternoon of day 7 after SNL.

Assessment of thermal hyperalgesia

The method of Hargreaves et al. (1988) was used to assess thermal hyperalgesia. A maximal cutoff of 30 s was used to prevent tissue damage. Paw withdrawal latencies were thus determined to the nearest 0.1 s before SNL (baseline) and on the morning and afternoon of day 7 after SNL.

Immunoassay of dynorphin in spinal cord extracts

Sham-operated and SNL animals were tested for tactile hypersensitivity and thermal hyperalgesia on day 7 after surgery. Following behavioral testing, mice were deeply anesthetized with ether and killed by decapitation. The spinal column was cut through the S1/S2 level. A 22-gauge needle attached to a syringe was inserted into the sacral vertebral canal and the spinal cord was extruded through the cervical opening with ice-cold saline. The ipsilateral dorsal lumbar quadrant was rapidly dissected and immediately frozen on dry ice and stored at -70 °C until use. Tissue extraction and immunoassay of dynorphin A(1–17) were carried out as previously described (Malan et al., 2000). Briefly, spinal cord tissues were homogenized and extracted with acetic acid (1.0 M) at 95 °C for 20 min followed by centrifugation at 10,000 \times g for 20 min at 4 °C. Protein concentration of the supernatant was determined by the bicinchoninic acid method. Immunoassay for dynorphin A(1–17) was performed using a commercial enzyme immunoassay kit (Bachem/Peninsula Laboratories,

Belmont, CA, USA; Cat. No. S-1203.0001). The antiserum is highly specific for dynorphin A(1–17) and exhibits no cross-reactivity with dynorphin A(1–13) or other shorter fragments of dynorphin A. It also has no affinity for dynorphin B, α -neoendorphin, β -endorphin, [Leu⁵]- or [Met⁵]-enkephalin, or orphanin FQ (Gardell et al., 2002a). Dynorphin content in the tissue extracts was calculated from a standard curve generated by non-linear least squares analysis using known concentrations of dynorphin A (1–17) (GraphPad Prism, La Jolla, CA, USA).

Western transblot analysis of spinal tissues

Lumbar sections of the spinal cords were homogenized and extracted with an ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 5 mM EDTA in PBS, pH 7.4) in the presence of protease inhibitors (0.3 mg/ml antipain dihydrochloride, 0.05 mg/ml bestatin, 0.1 mg/ml chymostatin, 0.3 mg/ml E-64, 0.05 mg/ml leupeptin, 0.05 mg/ml pepstatin, 0.3 mg/ml phosphoramidon, 2.0 mg/ml pefabloc s.c., 0.05 mg/ml aprotinin, 0.1 mg/ml phenylmethylsulfonyl fluoride). This preparation was incubated on a rotator at 4 °C for 1 h, and centrifuged at 35,000 \times g for 45 min at 4 °C. The protein content of the supernatant was determined by the Coomassie Plus protein assay (Pierce, Rockford, IL, USA). Spinal cord extracts were separated by 8% SDS-PAGE, and transblotted onto nitrocellulose membranes. The membranes were pre-blocked with 5% non-fat milk in TTBS (20 mM Tris-buffer saline, pH 7.6, 0.1% Tween 20) for 2 h, then incubated with a rabbit polyclonal antibody against the carboxyl terminus of the mouse PKC γ (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. The membranes were washed three times with TTBS, then incubated with a horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:2000) for 1 h followed by three washes with TTBS. Immunolabeled proteins on the membranes were detected by the ECL method (Amersham, Piscataway, NJ, USA). All incubations were carried out at room temperature.

Double fluorescent immunostaining for prodynorphin and PKC γ

The perfusion-fixation of spinal cords from mice and the immunostaining procedure were carried out according to that previously described (Wang et al., 2001). Frozen frontal sections (20–30 μ m) of the lumbar spinal cords were processed for labeling as floating sections. They were blocked with TBST (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) containing 0.1% BSA at room temperature for 1 h, then incubated with the primary antibody for prodynorphin (a gift from Dr. Robert Elde, University of Minnesota) raised in guinea-pig (1:20,000) and that for PKC γ (1:10,000; Santa Cruz Biotechnology) in TBST/3% BSA overnight at 4 °C. For control experiments, the primary antibodies were omitted. After 3 \times 15 min washes with TBST/0.1% BSA, sections were incubated with secondary antibodies at room temperature for at least 2 h. For prodynorphin staining, the secondary antibody was a Texas Red-labeled anti-guinea-pig IgG (Vector Laboratories, Burlington, CA, USA) diluted to 1:2000. The secondary antibody for PKC γ staining was a biotinylated goat anti-rabbit IgG (1:1000) followed by fluorescein-labeled streptavidin (1:2000; Vector Laboratories, Burlington, CA, USA). The sections were then washed with TBST/0.1% BSA, mounted onto gelatin-coated slides with Vectashield (Vector Laboratories) and coverslipped. Images were acquired with a Nikon Eclipse E800 microscope outfitted with a Plan Apo 20X objective lens, filter cubes for fluorescein and Texas Red, and a Hamamatsu C5810 color CCD camera. Fluorescence images for Texas Red (prodynorphin) and fluorescein (PKC γ) were acquired sequentially from the same field, then superimposed using Metamorph software (Universal Imaging Corporation, West Chester, PA, USA). To aid direct comparison of the immunolabeling of prodynorphin and PKC γ across strains and treatment

groups, tissue sections from the different experimental groups shown in Fig. 5 were processed in parallel under identical conditions. All images presented were acquired under identical time and exposure settings.

RESULTS

Tactile hypersensitivity

The paw withdrawal thresholds to tactile stimulus were not significantly different across the four strains of mice (ICR, 129S6, PKC γ KO and WT; Fig. 1) before surgery was performed. Baseline paw withdrawal thresholds ranged from 1.7 \pm 0.13 g to 1.8 \pm 0.12 g (Fig. 1). After sham SNL surgery, such paw withdrawal thresholds remained similar to that prior to sham surgery and were not different among the four strains, and ranged from 1.5 \pm 0.19 g to 1.8 \pm 0.16 g (Fig. 1). However, SNL produced significant ($P\leq 0.05$) reductions in paw withdrawal thresholds in the ICR and WT mice after SNL. The mean paw withdrawal threshold of the ICR mice was reduced to 0.10 \pm 0.03 g 7 days after SNL compared with a baseline value of 1.8 \pm 0.13 g (Fig. 1). The mean paw withdrawal thresholds of the WT mice were reduced to 0.05 \pm 0.02 g 7 days after SNL compared with a baseline value of 1.8 \pm 0.12 g (Fig. 1). The spinal injection of an anti-dynorphin A antiserum 20 min prior to testing reversed the tactile hypersensitivity in the ICR and WT mice. The paw withdrawal thresholds of ICR mice and of WT mice upon the antiserum treatment were 1.6 \pm 0.27 g and 1.7 \pm 0.17 g, respectively (Fig. 1). Moreover, spinal administration of the dynorphin A antiserum did not alter the paw withdrawal thresholds of mice that underwent sham surgery.

Unlike the ICR or the WT mice, however, SNL did not elicit tactile hypersensitivity in either the 129S6 or the PKC γ KO mice (Fig. 1). The mean paw withdrawal thresholds of 129S6 mice before and after SNL were 1.7 \pm 0.11 g and 1.7 \pm 0.16 g, respectively (Fig. 1), and that of the PKC γ KO mice before and after SNL were 1.7 \pm 0.12 g and 1.6 \pm 0.18 g, respectively. The spinal administration of dynorphin A antiserum did not alter the paw withdrawal thresholds of either the 129S6 or PKC γ KO mice after SNL (Fig. 1). Control serum did not produce any changes in paw withdrawal thresholds in any group.

Thermal hyperalgesia

The mean paw withdrawal latencies to noxious radiant heat of the four strains of mice were not significantly different ($P>0.05$) prior to SNL (Fig. 2). The baseline paw withdrawal latencies ranged from 10 \pm 0.17 s to 11 \pm 0.15 s. Sham operations did not produce any significant changes ($P>0.05$) in paw withdrawal latencies in any of the four strains tested. The paw withdrawal latencies of sham-operated mice ranged from 10 \pm 0.28 s to 10.6 \pm 0.31 s (Fig. 2). However, the paw withdrawal latencies were significantly ($P\leq 0.05$) reduced in the ICR and WT mice after SNL, which were 5.3 \pm 0.30 s and 5.1 \pm 0.25s, respectively (Fig. 2). The spinal administration of antiserum to dynorphin A reversed thermal hyperalgesia in these mice to control levels, showing a

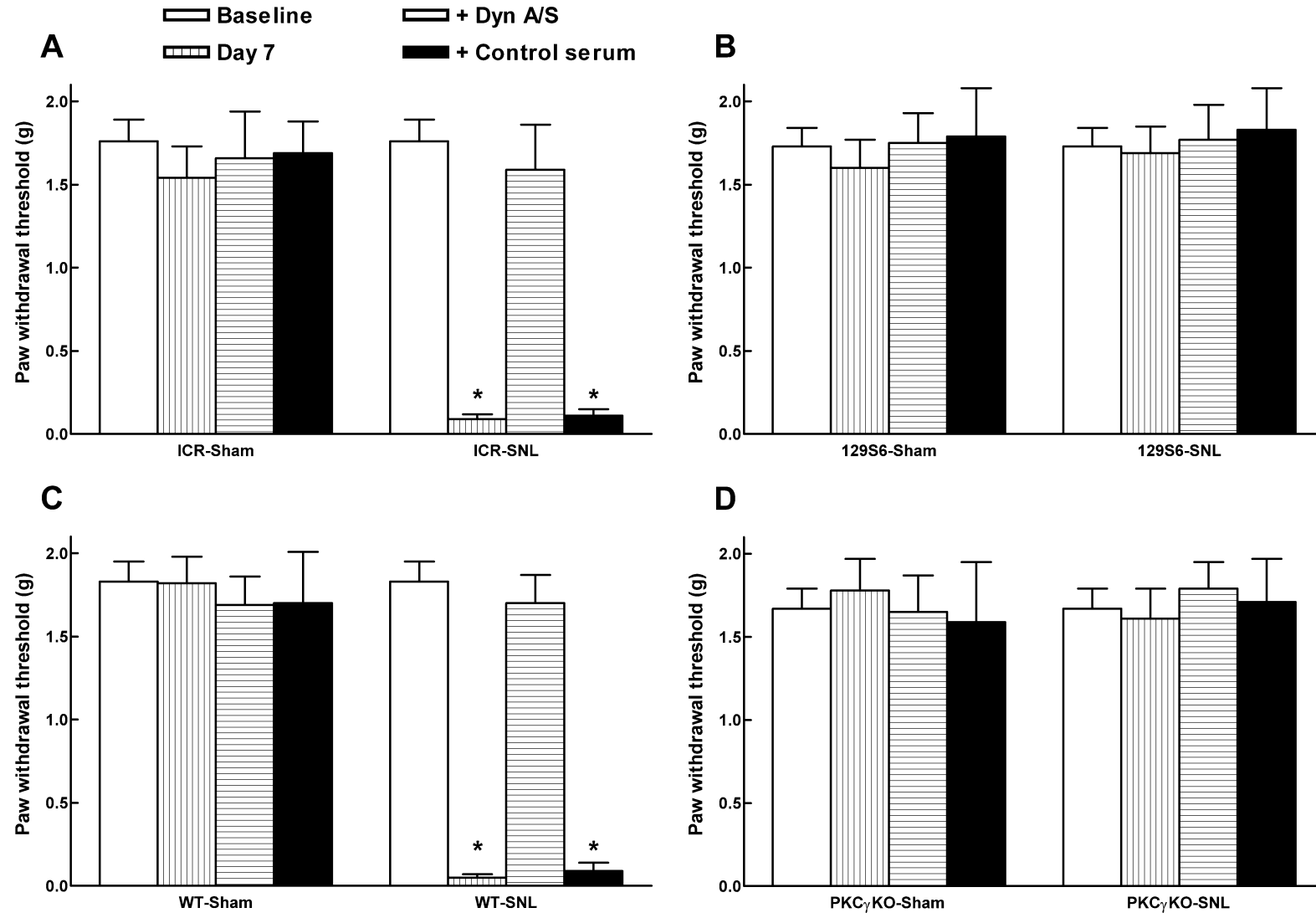


Fig. 1. Paw withdrawal thresholds for sham-operated and L5/L6 SNL mice are presented for the ICR (A), 129S6 (B), WT (C) and PKC γ KO (D). Each group consists of five to nine animals. Paw withdrawal thresholds were determined prior to surgery (open bars) and 7 days after either sham or SNL surgery (vertical stripes). The mice then received either an i.t. injection of 200 μ g of either an antiserum to dynorphin A(1–13) (horizontal stripes) or control serum (black bars) and were tested 20 min later. Only the ICR (A) and WT (C) mice demonstrated tactile hypersensitivity after SNL, demonstrated by a significant decrease in paw withdrawal threshold (* $P \leq 0.05$). No significant ($P > 0.05$) change in paw withdrawal threshold was observed after SNL in 129S6 (B) or PKC γ KO mice (D) after SNL. Antiserum to dynorphin A(1–13) reversed tactile hypersensitivity in the ICR and WT mice, as indicated by increased paw withdrawal thresholds to baseline values. Antiserum to dynorphin A(1–13) had no effect on paw withdrawal thresholds of 129S6 or PKC γ KO mice with SNL or any mice with sham surgery. Control serum did not produce any changes in paw withdrawal thresholds.

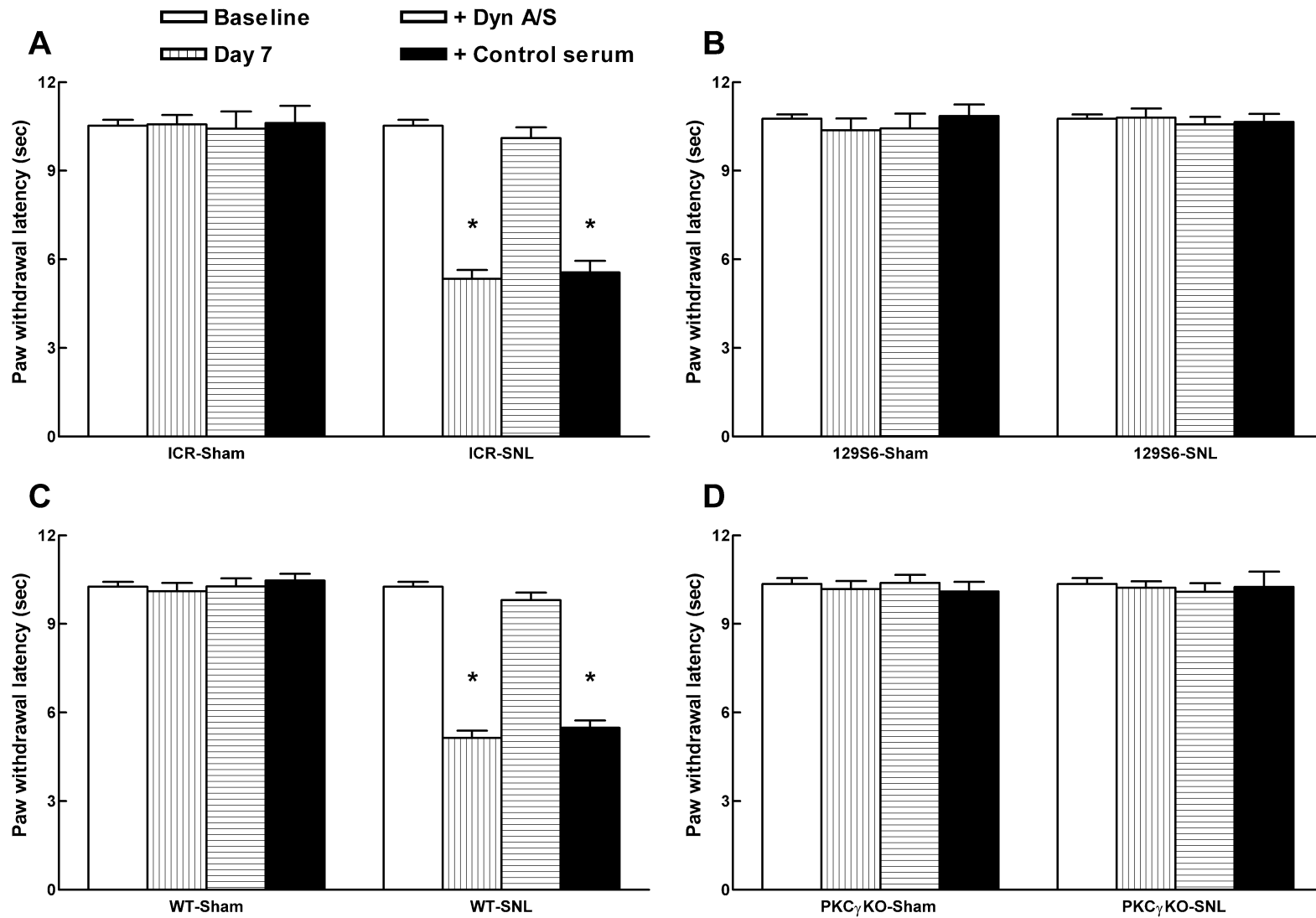


Fig. 2. Paw withdrawal latencies for sham-operated and L5/L6 SNL mice are presented for the ICR (A), 129S6 (B), WT (C) and PKC γ KO (D). Each group consists of five to nine animals. Paw withdrawal latencies were determined prior to surgery (open bars) and 7 days after either sham or SNL surgery (vertical stripes). The mice then received either an i.th. injection of 200 μ g of either an antiserum to dynorphin A(1–13) (horizontal stripes) or control serum (black bars) and were tested 20 min later. Only the ICR (A) or WT (C) mice demonstrated thermal hyperalgesia after SNL, demonstrated by a significant decrease in paw withdrawal latency (* $P \leq 0.05$). No significant ($P > 0.05$) changes in paw withdrawal latencies were observed after SNL in 129S6 (B) or PKC γ KO mice (D) after SNL. Antiserum to dynorphin A(1–13) reversed thermal hyperalgesia in the ICR and WT mice, as indicated by increased paw withdrawal latencies to baseline values. Antiserum to dynorphin A(1–13) had no effect on paw withdrawal latencies of 129S6 or PKC γ KO mice with SNL or any mice with sham surgery. Control serum did not produce any changes in paw withdrawal latencies.

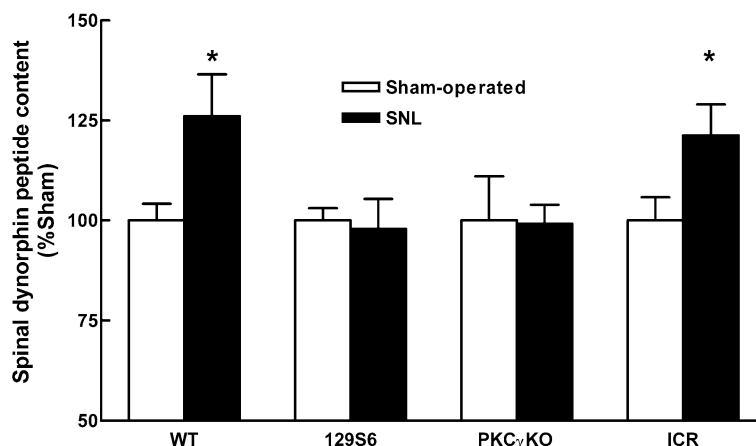


Fig. 3. Male WT, 129S6, PKC γ KO and ICR mice underwent either sham surgery or L5/L6 SNL. The animals were assessed for the presence of tactile hypersensitivity and thermal hyperalgesia on the seventh day after surgery. Spinal cords were then removed and the lumbar section isolated. Spinal dynorphin A(1–17) content was determined for each spinal cord separately by immunoassay. The dynorphin content (in pg/mg tissue) in each extract obtained from the same strain of mice (sham control and SNL) is expressed as a percent of the mean of the values from the sham control samples. Data ($n=7-10$ per treatment group) from the sham control group and the SNL group are then expressed as mean \pm S.E.M as shown. The WT and ICR mice demonstrated a significant increase in spinal dynorphin content (* $P \leq 0.05$), whereas the 129S6 and PKC γ KO groups showed no significant increase in spinal dynorphin A(1–17) content.

mean withdrawal latency of 10 ± 0.36 s in the ICR mice and 9.8 ± 0.25 s in the WT mice after dynorphin antiserum injection (Fig. 2). Moreover, the spinal injection of dynorphin A antiserum did not alter the paw flick latency of mice that underwent sham surgery.

Neither the 129S6 nor the PKC γ KO mice developed thermal hyperalgesia after SNL (Fig. 2). The mean baseline paw withdrawal latency of the 129S6 mice (11 ± 0.15 s) was not significantly different ($P > 0.05$) from the value of 11 ± 0.31 s observed 7 days after SNL (Fig. 2). Similarly, the baseline paw withdrawal threshold of PKC γ KO mice was 10 ± 0.19 s, which was not significantly different ($P > 0.05$) from the mean latency of 10 ± 0.22 s observed 7 days after SNL (Fig. 2). The administration of dynorphin A antiserum did not alter the paw withdrawal latency of either the 129S6 or the PKC γ KO mice after SNL. Control serum also did not produce any changes in paw withdrawal latencies in any group.

Dynorphin peptide content in the spinal cord

The average spinal dynorphin A content in the sham-operated WT and ICR mice was 432 ± 18.0 pg/mg protein and 441 ± 25.4 pg/mg protein, respectively. After SNL, spinal cord from the injured WT and ICR mice exhibited a significantly higher level of dynorphin A, with a mean of 544 ± 44.9 pg/mg protein and 535 ± 33.8 pg/mg protein, respectively (Fig. 3). On the contrary, SNL did not produce significant ($P > 0.05$) changes in the spinal dynorphin content in the PKC γ KO or the 129S6 mice. The mean spinal dynorphin A(1–17) content was 424 ± 19.9 pg/mg protein and 410 ± 31.6 pg/mg protein, respectively, compared with mean values of 428 ± 47.5 pg/mg protein and 419 ± 13.0 pg/mg protein, respectively, in the sham controls (Fig. 3).

Effect of i.th. dynorphin A(1–17) on the sensory thresholds of 129S6 mice

The 129S6 mice responded to intrathecally administered dynorphin A(1–17) as seen previously in rat (Vanderah et al., 1996) and in mice (Laughlin et al., 1997). A single dose of i.th. dynorphin A(1–17) induced a lasting reduction in the response thresholds to both von Frey probing and noxious radiant heat in the 129S6 mice (Fig. 4), suggesting that these mice are not deficient in the spinal mechanisms that mediate the pronociceptive actions of dynorphin.

Expression of PKC γ and prodynorphin in the spinal cord

As expected, PKC γ immunoreactivity was absent in the PKC γ KO mice, but was found in the WT, ICR, and 129S6 mice (Fig. 5). Lumbar spinal cords were also harvested from the four strains of mice on day 7 after sham or SNL surgery; cross-sections were prepared for double immunofluorescence labeling for prodynorphin and PKC γ . As shown in Fig. 6, immunoreactivity for PKC γ was discretely localized to cell bodies in the lamina II of the dorsal horn in all but the PKC γ KO mice (Fig. 5, center column). Nerve injury did not produce any significant alteration in the localization or the number of cells that expressed PKC γ in the WT, ICR or 129S6 mice when compared with the respective sham control tissues. Immunoreactivity for prodynorphin was detected in all four strains of mice (Fig. 6, left column). Under control conditions (after sham surgery), the immunolabeling for prodynorphin was similarly restricted to lamina I in all four strains of mice. After SNL injury in the WT and ICR mice, the immunoreactivity for prodynorphin was enhanced in the lamina I region when compared with the respective sham controls. In addition,

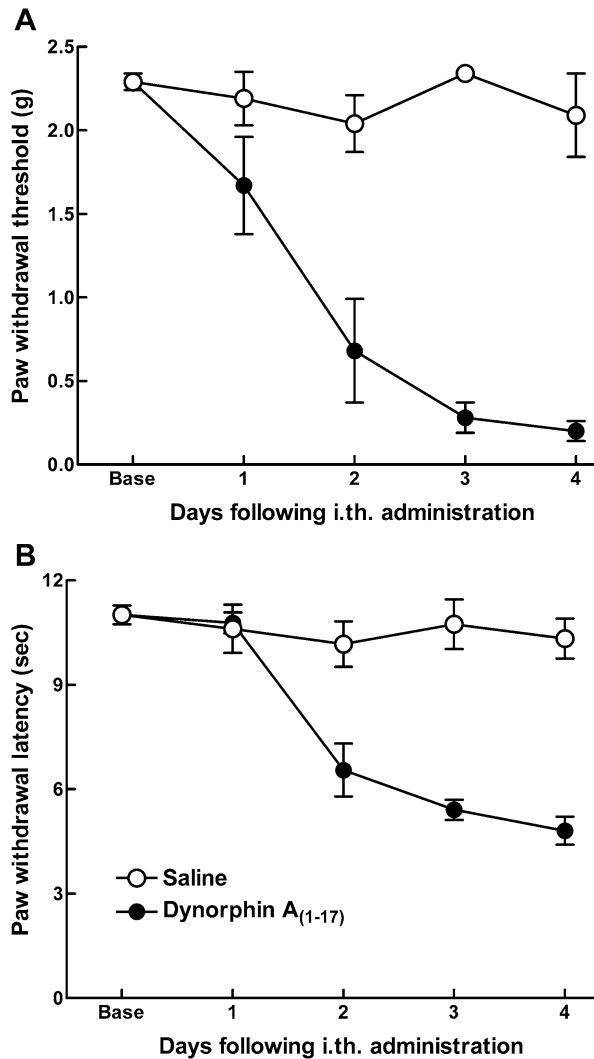


Fig. 4. Time course of (A) paw withdrawal response thresholds to non-noxious mechanical stimuli (von Frey filaments), or (B) paw withdrawal latencies to noxious thermal stimuli, in naive 129S6 mice across a 4-day period following a single i.th. injection of dynorphin A(1–17) (10 nmol; filled circles; $n=8$), or saline (open circles; $n=4$). I.th. dynorphin A(1–17) produced tactile hypersensitivity (A) and thermal hyperalgesia (B) by day 2 and persisted for at least 4 days. Saline-injected mice did not exhibit tactile hypersensitivity or thermal hyperalgesia.

discrete cell bodies that showed intense immunoreactivity for prodynorphin could be seen in the lamina V region in these two strains of mice after SNL. On the other hand, there was no significant change in either the distribution or the relative intensity of labeling for prodynorphin in the 129S6 or the PKC γ KO mice after SNL when compared with their respective sham controls. Furthermore, there was no overlap between the immunoreactivity for prodynorphin (left column) and that for PKC γ (center column) when the images were overlaid (right column), demonstrating that prodynorphin and PKC γ are expressed in distinct cell populations in the spinal dorsal horn.

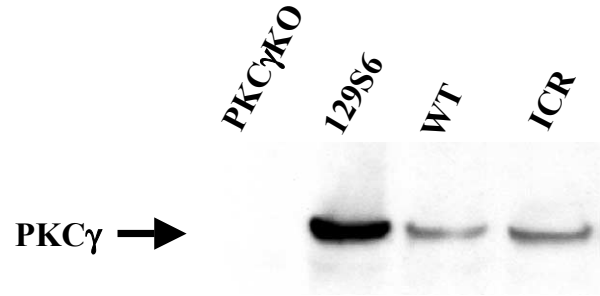


Fig. 5. Western blotting analysis of PKC γ expression was performed in lumbar spinal cord tissues obtained from PKC γ KO, 129S6, WT and ICR mice. Each lane contains 30 μ g of solubilized proteins separated on an 8% polyacrylamide SDS gel. Proteins were transblotted to nitrocellulose and incubated with an anti-PKC γ antibody (1:2000), and developed with the ECL method. A predominant band of approximately 80 kDa was detected in extracts from 129S6, WT and ICR mice but not from PKC γ KO mice. Data are representative of three independent determinations.

DISCUSSION

The four strains of mice that were examined in this study can be divided into two groups based on their behavioral response to nerve injury (Table 1): group I consists of the ICR and WT mice which display behavioral signs of neuropathic pain, and group II consists of the 129S6 and the PKC γ KO mice which do not exhibit abnormal pain. To these two groups we may also include the WT littermates of the prodynorphin KO mice to the former, and the prodynorphin KO mice to the latter based on previous observations (Wang et al., 2001). It is also evident from the data that the three strains in group I express elevated levels of spinal dynorphin as a consequence of nerve injury, whereas the three strains in group II do not (the third one, prodynorphin KO, does not produce dynorphin). Critically, administration of anti-dynorphin antiserum reverses both hyperalgesia and tactile hypersensitivity in all three strains of mice in group I. This causal relationship between the upregulation of spinal dynorphin and abnormal pain states thus exists in multiple mouse strains and is in agreement with previous observations in rats (Bian et al., 1999; Malan et al., 2000; Vanderah et al., 2000). This relationship is also supported by the characteristics of group II in which an absence of elevated spinal dynorphin after injury coin-

Table 1. Comparison of pain behavior, spinal dynorphin upregulation and PKC γ among six strains of mice

Strain	Signs of abnormal pain after SNL	Upregulation of spinal dynorphin after SNL	Expression of PKC γ
Group I			
ICR	+	+	+
PKC γ WT	+	+	+
Prodynorphin WT ^a	+	+	+
Group II			
129S6	-	-	+
PKC γ KO	-	-	-
Prodynorphin KO ^a	-	-	+

^aWang et al., 2001.

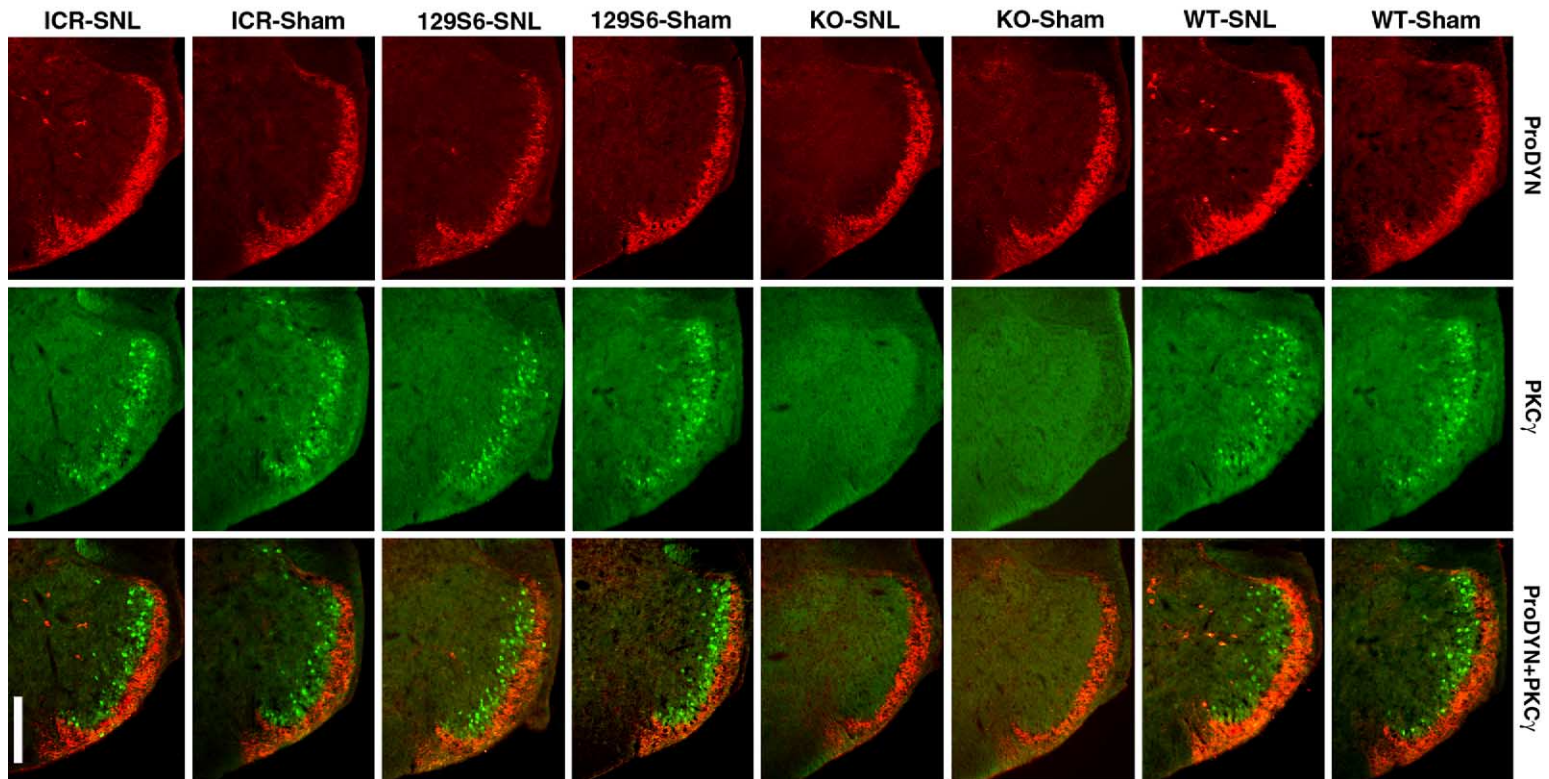


Fig. 6. Immunolocalization of prodynorphin (red; left column) and PKC γ (green; center column) double labeling (right column) in the ipsilateral lumbar spinal dorsal horn sections obtained from WT, PKC γ KO, 129S6, and ICR mice on day 7 after sham or SNL surgery. Representative sections show that prodynorphin is upregulated in laminae I and V after SNL in the WT and ICR mice, but not in the PKC γ KO or the 129S6 mice (left column). PKC γ immunoreactivity is located to discrete cell bodies in lamina II in all but the PKC γ KO mice (center column). No qualitative difference is apparent in the PKC γ labeling between tissues from the sham and SNL subjects. PKC γ and prodynorphin are localized to discrete cell populations in laminae I/II (right column). PKC γ immunoreactive cells lie in a deeper cell layer within laminae I/II compared with that where prodynorphin is normally expressed. The relative distribution correlates with a localization of PKC γ in lamina II inner region. There is no evidence for co-localization of these two molecules in sham or SNL tissues. Scale bar=200 μ m.

cides with an absence of abnormal pain. Another salient feature of the anti-dynorphin antiserum treatment is that it does not alter the baseline sensory thresholds in any of the strains of mice or rats tested to date, showing that dynorphin is unlikely to be an endogenous regulator of sensory thresholds under physiological conditions, but its enhanced expression acts to promote abnormal pain upon nerve injury.

The observed relationship between PKC γ and neuropathic pain is less clear based on the findings presented here. While the PKC γ KO mice clearly do not exhibit hyperalgesia or tactile hypersensitivity after SNL, the other two strains in group II, i.e. the 129S6 mice (Fig. 5) and the prodynorphin KO mice (Wang et al., 2001) are not deficient in PKC γ ; these two strains of mice, however, also do not exhibit abnormal pain after nerve injury. In fact, because the PKC γ KO mice do not show upregulation of prodynorphin or dynorphin after nerve injury, it is not possible to conclude that the failure of PKC γ KO mice to show behavioral expression of nerve injury-induced pain is due to the lack of expression of PKC γ , or the lack of spinal dynorphin upregulation. The role, if any, that PKC γ may have in regulating spinal dynorphin expression has not been explored; however, because dynorphin and PKC γ do not colocalize to the same spinal cord neurons (Fig. 6), it is unlikely that PKC γ is an intracellular regulator of dynorphin expression in the spinal cord (this does not preclude the possibility that prodynorphin expression may be regulated by other PKC isoforms).

Further complicating the interpretation of the mechanisms underlying the resistance of PKC γ KO mice to nerve-injury-induced pain is the genetic background of these mice. The PKC γ KO was derived from a background containing the 129S6 strain (Abeliovich et al., 1993). It is quite possible that the deficiency in the upregulation of spinal prodynorphin in the PKC γ KO mice is an inheritable trait from the 129S6 background, and is independent of the function of PKC γ . It should be noted that the WT mice used in this study were not littermates of the PKC γ KO mice (which were unavailable), but a related strain, B6129F2/J, such that the genetic background may not be identical to that of the PKC γ KO mice.

The underlying deficits in the 129S6 mice that confer a resistance to nerve-injury induced pain are deserving of further investigation as a possible genetic key to the fundamental mechanisms by which such pain is expressed. Previous findings suggest that 129S6 mice do not develop opioid antinociceptive tolerance (Kolesnikov et al., 1998), and the authors suggest that it may be due to a deficiency in the nitric oxide signaling pathway resulting from an inability of the NMDA receptors to activate nitric oxide synthase. Such a deficit may also impact on the expression of nerve-injury induced pain as seen here because NMDA receptor inhibition has been shown to prevent neuropathic pain produced by i.th. dynorphin in rats (Vanderah et al., 1996) and reverses nerve injury-induced abnormal pain in mice (Wang et al., 2001). Data from the 129S6 given i.th. dynorphin, however, appear to argue against a deficit in the downstream response to dynorphin because

these mice develop both thermal and tactile hypersensitivity upon i.th. dynorphin A administration (Fig. 4). The potential contribution of the 129S6 background to the genotype of the PKC γ KO mice calls for caution in the interpretation of data obtained from animals with specific gene deletions. Specifically, these data highlight the potential bias in the interpretation of cause and effect when using transgenic animal models, and reinforce the concept that multiple observations through multiple experimental approaches are needed to validate a potential mechanism.

The mechanisms that underlie the upregulation of spinal dynorphin A upon chronic opioid treatment or nerve injury are at present unknown. We have found that the upregulation of spinal dynorphin may be prevented by manipulations that block the development of neuropathic pain that normally arises after peripheral nerve injury. For example, the lesion of the dorsal lateral funiculus (DLF) prior to SNL injury also prevented the maintenance of neuropathic pain (Burgess et al., 2002). The spinal dynorphin content in these animals was not different from DLF lesion/sham-SNL, or sham-DLF lesion/sham-SNL controls (Gardell et al., 2003). In this regard, we may speculate that dynorphin upregulation may be promoted by enhanced primary afferent input upon nerve injury that is in part promoted by the descending pain facilitatory input via the DLF that develops after nerve injury. The lack of dynorphin A upregulation in the 129S6 mice could be due to deficits in these input pathways to the spinal cord; alternatively, it could be specifically associated with some characteristics in the synthesis or the processing of prodynorphin that is particular to this strain. In conclusion, the present data show that upregulation of spinal dynorphin A consistently correlates with the expression of nerve injury induced pain in multiple strains of mice, as previously observed in rats, and continue to support the hypothesis that the upregulation of spinal dynorphin A is a crucial factor in neuropathic pain.

Acknowledgements—This work was supported by NIH grants DA 12656 and DA 11823. The authors would like to thank Dr. Cheng-min Zhong for his technical assistance.

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