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Spinal Prostaglandins Facilitate Exaggerated A- and C-fiber–mediated Reflex Responses and Are Critical to the Development of Allodynia Early after L5–L6 Spinal Nerve Ligation

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Background: **Spinal prostaglandins are important in the early pathogenesis of spinal nerve ligation (SNL)–induced allodynia. This study examined the effect of SNL on the expression of cyclooxygenase (COX)-1, COX-2, and prostaglandin E_2 receptors in the rat lumbar spinal cord, and the temporal and pharmacologic relation of these changes to the exaggerated A- and C-fiber–mediated reflex responses and allodynia, 24 h after injury.**

Methods: **Male Sprague-Dawley rats, fitted with intrathecal catheters, underwent SNL or sham surgery. Paw withdrawal threshold, electromyographic analysis of the biceps femoris flexor reflex, and immunoblotting of the spinal cord were used.**

Results: **Both allodynia (paw withdrawal threshold of ≤ 4 g) and exaggerated A- and C-fiber–mediated reflex responses (*i.e.*, decrease in activation threshold, increase in evoked activity, including windup; $P < 0.05$) were evident 24 h after SNL but not sham surgery. Allodynic animals exhibited significant increases in prostaglandin E_2 receptor (subtypes 1–3) and COX-1 (but not COX-2) expression in the ipsilateral lumbar dorsal horn. The corresponding ventral horns and contralateral dorsal horn were unchanged from sham controls. Exaggerated A- and C-fiber–mediated reflex responses were significantly attenuated by intrathecal SC-560 or SC-51322, but not SC-236, given 24 h after SNL.**

Conclusion: **These results provide further evidence that spinal prostaglandins, derived primarily from COX-1, are critical in the exaggeration of A- and C-fiber input and allodynia, 24 h after SNL.**

THE mechanisms underlying neuropathic pain (including allodynia) are known to be complex, multifactorial, and subject to plasticity.[1–3] Understanding the time course and pharmacology of the mechanisms comprising the cascade leading to allodynia can enable the identification of interceptive measures that prevent acute nerve injury from becoming a chronic neuropathic state. In view of the complex and time-dependent changes triggered by nerve injury, targeting vital signaling events early after injury would be an obvious strategy. The early contribution of spinal prostaglandins in the development of experimental allodynia,[4–9] and the clinical availability of drugs with which to disrupt their effects, make prostanoids an interesting potential target.

A role for spinal prostaglandins in allodynia is consistent with their direct excitatory effects on dorsal horn neurons, as well as their ability to exaggerate cellular responses to afferent sensory input,[10–14] and the early induction of molecular changes (*e.g.*, increases in *N*-methyl-D-aspartic acid receptor phosphorylation,[15] $\alpha_2\delta_1$ calcium channel subunit expression,[16] and the induction of phospho-ERK[17]) coupled to spinal cyclooxygenase (COX)-1 and COX-2 expression.[4,8] These changes are temporally correlated with the onset of allodynia[4,8,15–17] and support the hypothesis of an early pathogenic role of spinal prostaglandins.

Previous pharmacologic studies with the L5–L6 spinal nerve ligation (SNL) model of allodynia revealed a preferential sensitivity to COX-1 inhibitors given 2–8 h after injury.[18] Intrathecal SC-560 (a highly selective COX-1 inhibitor) completely prevented the neurochemical, pharmacologic, and behavioral features of SNL-induced allodynia for at least 20 days. This result was comparable to that observed with intrathecal *S*(+)-ibuprofen.[18] In contrast, *R*(−)-ibuprofen and SC-236 (a highly selective COX-2 inhibitor) were without effect.[18] These data suggested that COX-1 is primarily responsible for the generation of spinal prostaglandins up to 24 h after SNL, which contribute to the development of allodynia in this model. However, the expression profile and time course of the COX isoforms affecting allodynia, and their relevance (or not) to changes in central sensitivity early after SNL, have not been characterized.

Treatment with a nonselective prostaglandin E_2 (EP) receptor antagonist (SC-51322), beginning 2 days after SNL, significantly reversed tactile allodynia.[9] These results indicate that spinal EP receptors mediate the proallodynic effects of prostaglandins in the spinal cord. EP receptor subtypes are known to be coupled to discrete signaling pathways, each of which could contribute to central sensitivity and allodynia. Increased EP receptor immunoreactivity has been reported in the dorsal root ganglia after partial sciatic nerve ligation.[19] However, the effect of experimental nerve injury, including SNL, on the expression of EP receptors in the spinal cord has not been investigated.

To further study the early contribution of spinal prostaglandins in experimental allodynia, we investigated: (1) the effect of SNL on the expression of COX-1, COX-2,

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and EP receptors in the rat lumbar dorsal horn; and (2) the temporal and pharmacologic relation of these changes to exaggerated A- and C-fiber–mediated reflex responses (AFRR and CFRR, respectively) and allodynia, 24 h after SNL or sham surgery.

Materials and Methods

All studies were approved by, and experiments were conducted in accordance with, the guidelines of the Institutional Animal Care Committee of Memorial University (St. John's, Newfoundland, Canada).

Animals

Male Sprague-Dawley rats (130–150 g) were obtained from the Vivarium of Memorial University of Newfoundland and housed in standard cages with wood-chip bedding. Animals had free access to food and water. A 12-h light–dark cycle (lights on at 07:00 h) was used throughout.

Intrathecal Catheterization

Intrathecal catheters (6.5-cm length terminating near the lumbar enlargement) were implanted according to the method of Yaksh and Rudy,[20] as modified by Hefferan *et al.*[9] The catheter was sterilized with 70% alcohol and filled with sterile saline. During halothane anesthesia, the catheter was inserted through an incision in the atlanto-occipital membrane of the cisterna magna. The catheter was externalized behind the head and sealed with a piece of stainless steel wire. Intrathecal catheters (ID, 240 μm; OD, 290 μm) were constructed from triple-lumen PE-5 tubing (Spectranetics, Colorado Springs, CO) using the modified method of Marsala *et al.*[21] Rats with normal motor, grooming, and feeding behavior were housed separately and allowed to recover for 24 h before SNL or sham surgery.

Neuropathy

Neuropathy was induced using the method of Kim and Chung,[22] as previously described.[9,18] Briefly, rats were anesthetized with halothane, and the left L4 and L5 spinal nerves were isolated and separated. The L5 and L6 spinal nerves were tightly ligated with 6-0 silk thread. In sham controls, the L5 and L6 spinal nerves were isolated but not ligated. All animals were allowed to recover for 24 h before experimentation. Allodynia, defined as a paw withdrawal threshold of 4 g or less, was confirmed using von Frey filaments before experimentation.

Electrophysiologic Recordings

Rats were anesthetized with halothane, and cannulae were placed in the trachea, left carotid artery, and right external jugular vein. Halothane anesthesia was then replaced by sodium thiobutabarbitone (1 mg/kg intravenous; Inactin; Sigma-Aldrich, Oakville, Ontario, Canada). The depth of anesthesia was assessed by testing for hind limb withdrawal and corneal reflexes, which had to be absent. Blood pressure was continuously monitored *via* a left carotid artery catheter, and the mean arterial pressure was maintained between 100 and 130 mmHg by additional anesthetic as required. Systolic blood pressure did not fall below 100 mmHg throughout the experiment. In the event that the blood pressure dropped and consistently remained below 100 mmHg, the experiment was stopped and the animal was euthanized. Core temperature was maintained close to 37°C using a homeothermic blanket system. The animal preparation was allowed to stabilize for at least 30 min before data collection.

Spinal flexor reflexes were evoked by subcutaneous electrical stimulation applied to the first toe of the hind paw. Needle location was based on the innervation pattern of the sural nerve[23] and square-wave pulses (0.2, 0.6, 1.0 Hz) of 1-ms duration were used. Stimulation at each frequency was repeated three times to ensure stability and extracellular electromyographic responses were recorded from the biceps femoris muscle using a pair of tungsten needle electrodes. Intervals of 3–5 min were introduced between successive stimulus trains to prevent a conditioning effect by the preceding stimulus. A low-intensity suprathreshold stimulus refers to a voltage twice the AFRR activation threshold (5 pulses at 0.2 Hz). A high-intensity suprathreshold stimulus refers to a voltage twice the CFRR activation threshold (20 pulses at 1.0 Hz).

The AFRR and CFRR were distinguished on the basis of activation threshold and response latency. Electrical stimulation sufficient to activate the CFRR resulted in two distinct components, an early and a late phase, separated by a quiescent period of variable duration. The AFRR and CFRR were classified as those occurring less than 100 ms after the stimulus artifact and greater than 100 ms up to a maximum of 600 ms, respectively. These criteria were confirmed by examining the effect of intrathecal morphine (100 μg) and naloxone (100 μg) on the AFRR and CFRR of the biceps femoris reflex in naive animals. Based on the measured length of the sural nerve in rats weighing 130–150 g (*i.e.*, 12 cm), afferent fibers mediating the late phase ($>$ 100–600 ms) had an average conduction velocity of 1.2 m/s, a value within the accepted range for C-fibers.[24] The AFRR and CFRR intervals used in the present experiments are in agreement with previous reports on the rat biceps femoris reflex.[24–26]

To determine the AFRR activation threshold, stimulus trains of 5 pulses (0.2–1.0 Hz) were applied at increasing voltages until a positive response was obtained. A positive response was deemed to have occurred when at least 3 of the 5 stimulus pulses produced a spike (*i.e.*, amplitude $>$ 20 μV). To determine the CFRR activation threshold, stimulus trains of 20 pulses (0.2–1.0 Hz) were

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applied at increasing voltages until a positive response was obtained. A positive response was deemed to have occurred when at least 10 of the 20 stimulus pulses produced a spike (*i.e.*, amplitude > 20 μV).

Data were collected, stored, and analyzed using a PowerLab data acquisition system (ADInstruments, Inc., Colorado Springs, CO). Off-line data analysis was initially performed using the data acquisition software provided by the manufacturer (Chart 5.0). The data were further analyzed using a customized computer program developed in collaboration with the Department of Computer Science, Memorial University of Newfoundland.

Western Blotting

Animals were deeply anesthetized with urethane (1.2 mg/kg intraperitoneal) and perfused intracardially with ice cold saline (0.9% NaCl). The spinal cord was extracted hydraulically,[27] immediately frozen in 2-methylbutane (Sigma Chemical, Oakville, Ontario, Canada), and stored at −80°C. The lumbar region (*i.e.*, L2–L6) of the spinal cord was isolated, removed, and subsequently divided into the left and right, ventral and dorsal quadrants. The L2–L6 region was needed to provide sufficient tissue for Western analysis in each quadrant. Spinal cord was homogenized in ice-cold lysis buffer (1% Nonidet-P40, 10% glycerol in Tris buffer plus a protease inhibitor cocktail tablet; Roche Diagnostics, Laval, Quebec, Canada; 1 mM sodium vanadate, 1 mM sodium fluoride, and 0.025% sodium dodecyl sulfate); and centrifuged at 10,000 rpm for 5 min (4°C). Samples, diluted to achieve equal protein concentrations (30 μg), were separated by electrophoresis on 8% sodium dodecyl sulfate–polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and incubated for 1.5 h in Tris buffer (25 mM Tris, 192 mM glycine, 200 ml methanol, pH 8). Prestained protein markers were used for molecular weight determination. Blots were initially stained with Ponceau red, and later probed with an antibody directed against the cytosolic protein, actin (1:1,000; Sigma Chemical) to assess the equivalency of protein loading. Blots were subsequently probed with the following primary antibodies: polyclonal rabbit COX-1 antibody (1:250), polyclonal rabbit COX-2 antibody (1:1,000), polyclonal rabbit EP_1 receptor antibody (1:1,000), polyclonal rabbit EP_2 receptor antibody (1:2,000), and polyclonal EP_3 receptor antibody (1:1,000). These primary antibodies were purchased from Cayman Chemical (Ann Arbor, MI). Protein standards for COX-1, COX-2, and EP receptors were probed with antibodies that were preabsorbed with their corresponding blocking peptide (Cayman Chemical) to determine the antibody specificity. Western blots were corrected for nonspecific binding by subtracting the optical density in the presence of the corresponding blocking peptide from that of each test sample. The membranes were incubated overnight at 4°C with the primary antibodies and diluted in Tris buffer (containing 3% milk powder and 0.05% Tween-20). Protein bands were treated with a goat antirabbit horseradish peroxidase–conjugated secondary antibody (1:5,000; Chemicon International Inc., Temecula, CA) for 1 h at room temperature, washed for 30 min in Tris buffer, visualized using enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA), and exposed to x-ray film (Cronex MRF Clear base; Agfa Corp., Greenville, SC).

Drugs

SC-236, a selective COX-2 inhibitor, and SC-560, a selective COX-1 inhibitor, were generous gifts from Searle (Skokie, IL). SC-51322, a nonselective EP receptor antagonist, was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Drugs were dissolved in 100% dimethyl sulfoxide and diluted with normal saline at the time of injection to yield a final dimethyl sulfoxide concentration of 50%. All equipment was sterilized with 70% alcohol and thoroughly rinsed with 0.9% sterile saline before injection. Intrathecal drugs were injected into anesthetized rats using a handheld microsyringe. Drugs were delivered in a volume of 5 μl followed by 5 μl sterile saline. The intrathecal catheter was immediately resealed with a stainless steel plug. The position of the catheter tip was verified visually after death in randomly selected animals.

Data Analysis

Electromyographic recordings in sham-operated and nerve-ligated animals were compared using the mean responses elicited by three stimulus trains of 0.2, 0.6, and 1.0 Hz. Data were collected (4,000 samples/s), and high-pass digital filters (cutoff frequency 60 Hz) were used to remove noise and movement artifacts. Spontaneous activity was defined as the average spike count integrated over a 60-s period before experimentation. In all cases, the magnitude of the AFRR and CFRR was calculated by integrating the spike count over the stimulation period (*i.e.*, area-under-the-curve analysis). Spikes were integrated from 0.5 to 99.9 ms and 100 to 600 ms for the AFRR and CFRR, respectively. Windup was assessed by comparing the response evoked by the last stimulus (*e.g.*, 20th pulse) in each train of sham-operated and nerve-ligated animals. The area under the curve was calculated using trapezoidal integration. Western blots were analyzed by optical density using ImageQuant software (Amersham Biosciences Corp., Piscataway, NJ). Expression data are presented as nanograms of protein relative to COX (50 ng) or EP receptor (25 ng) standards, which were corrected for background optical density and normalized using the cytosolic protein, actin. Dose–response analysis was performed using methods from Tallarida and Murray.[28] Comparisons within each treatment group were performed using one-way, repeated-measures analysis of variance, followed by the Newman–Keuls test. Comparisons across all drug- and vehicle-

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treated groups at each time point were determined using one-way, completely randomized analysis of variance, followed by the Newman–Keuls test (SigmaStat 2.0; Systat Software Inc., San Jose, CA).

Results

After SNL, rats displayed a significant decrease in paw withdrawal threshold from 15 g or greater (baseline) to 4 g or less (data not shown). This change was apparent 24 h after SNL, was stable for at least 20 days, and remained confined to the plantar surface of the left hind paw (*i.e.*, ipsilateral to nerve ligation). Generally, the affected hind paw was kept in an elevated and cupped position to minimize contact with the cage floor. All nerve-ligated rats were otherwise healthy, showed normal feeding and grooming behavior and regular weight gain. Sham surgery had no effect on paw withdrawal threshold compared with presurgical values, as reported previously.

Western analysis revealed a 4.2-fold increase ($P < 0.05$) in the expression of COX-1 in the ipsilateral lumbar dorsal but not ventral horn of nerve ligated rats compared with sham controls 24 h after surgery (figs. 1A and B). In contrast, COX-2 was unchanged from sham controls ($P > 0.05$; fig. 1C). In addition, the expression of COX-1 or COX-2 in the contralateral lumbar cord was unchanged from their respective ipsilateral or contralateral sham controls ($P > 0.05$; figs. 1A and C). The increased expression of COX-1 (and of the EP receptors described below) in the lumbar segments was not an artifact of the intrathecal catheter. There were no differences in expression between nerve-ligated animals with or without intrathecal catheters 24 h after SNL (data not shown).

Electromyographic recordings of the AFRR and CFRR of the biceps femoris in naive animals are shown in figure 2A. Intrathecal morphine (100 μg) had no significant effect on the AFRR. In contrast, the CFRR was nearly eliminated (fig. 2B). There was no change in the activation threshold of the AFRR. However, the activation threshold of the CFRR (ipsilateral to SNL) was significantly reduced compared with sham controls (fig. 3A). The reduction in the CFRR ranged from 56–60% evoked at voltages triggering the AFRR in sham controls (figs. 3A–D). There were no significant differences between the contralateral hind limb of SNL animals and either side of sham controls for the AFRR or CFRR (data not shown).

The magnitude of both the AFRR and CFRR evoked by suprathreshold stimulation was significantly increased 24 h after SNL compared with sham controls (figs. 4A–D). This increase in evoked activity was greater in the AFRR (51.9%) than the CFRR (41.8%). The AFRR and CFRR evoked by the first suprathreshold stimulus were

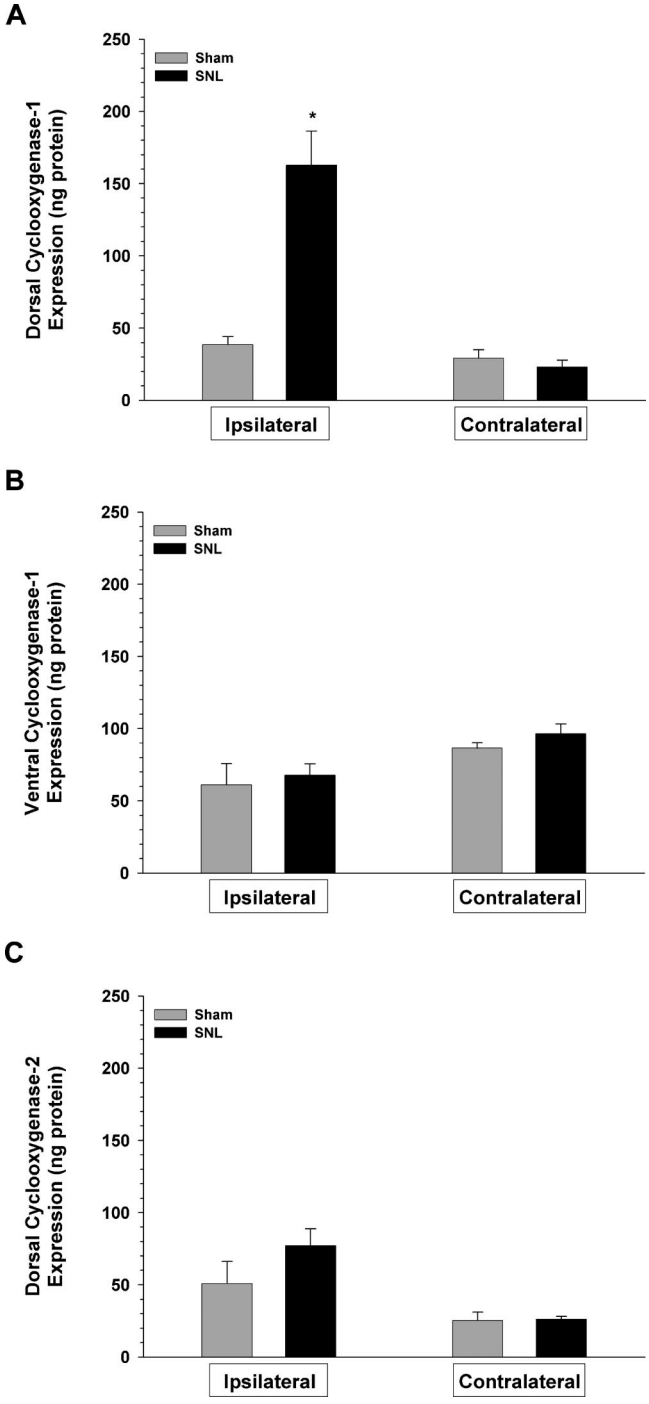

Fig. 1. The expression of cyclooxygenase-1 (*A* and *B*) and cyclooxygenase-2 (*C*) in the dorsal or ventral horns of the lumbar spinal cord 24 h after spinal nerve ligation (SNL) or sham surgery. Data were normalized using total protein content, corrected for nonspecific immunoreactivity, and expressed as nanograms of protein (see Materials and Methods). *Asterisk* indicates a significant difference from sham-operated animals: * $P < 0.05$. Each *bar* represents the mean ± SEM of three to five animals.

significantly increased in nerve-ligated compared with sham-operated animals (table 1). Repeated low- and high-intensity suprathreshold stimulation induced significant amplification of the AFRR (*i.e.*, a windup-like ef-

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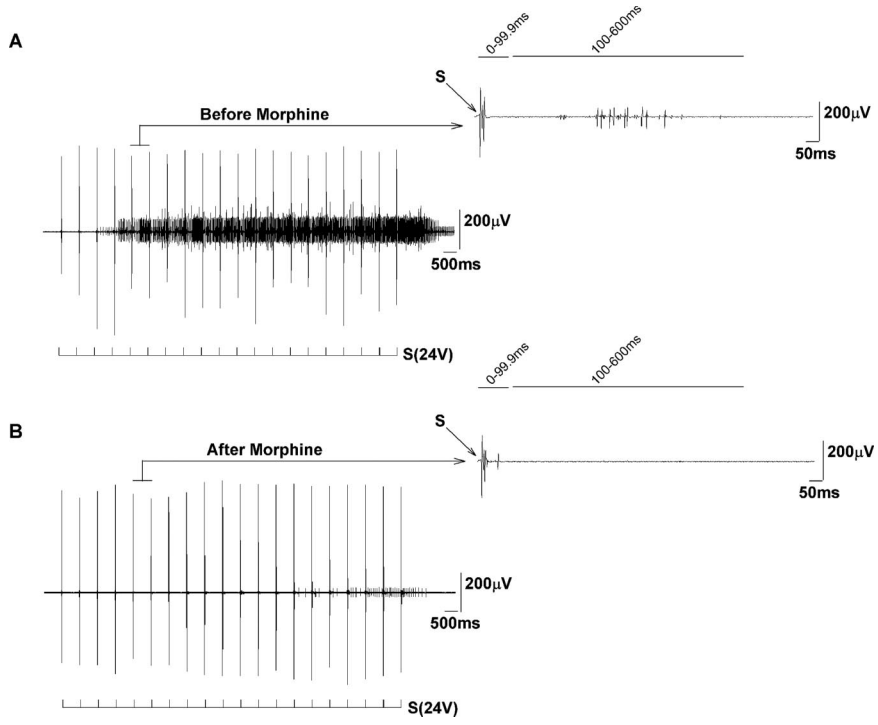

Fig. 2. Representative electromyographic tracings of the A- and C-fiber–mediated reflex responses from naive animals. An *indicator bar* (S) illustrating individual stimuli (1.5 Hz; 24 V) is included below each tracing. Stimuli were applied to the hind paw before (*A*) and after (*B*) intrathecal morphine (100 μg). *Inserts* represent the A- (0.5–99.9 ms) and C-fiber–mediated reflex responses (100–600 ms) evoked by a single stimulus. Note the virtual absence of evoked activity between 100 and 600 ms after intrathecal morphine; the 0.5- to 99.9-ms interval remained unchained (*B*).

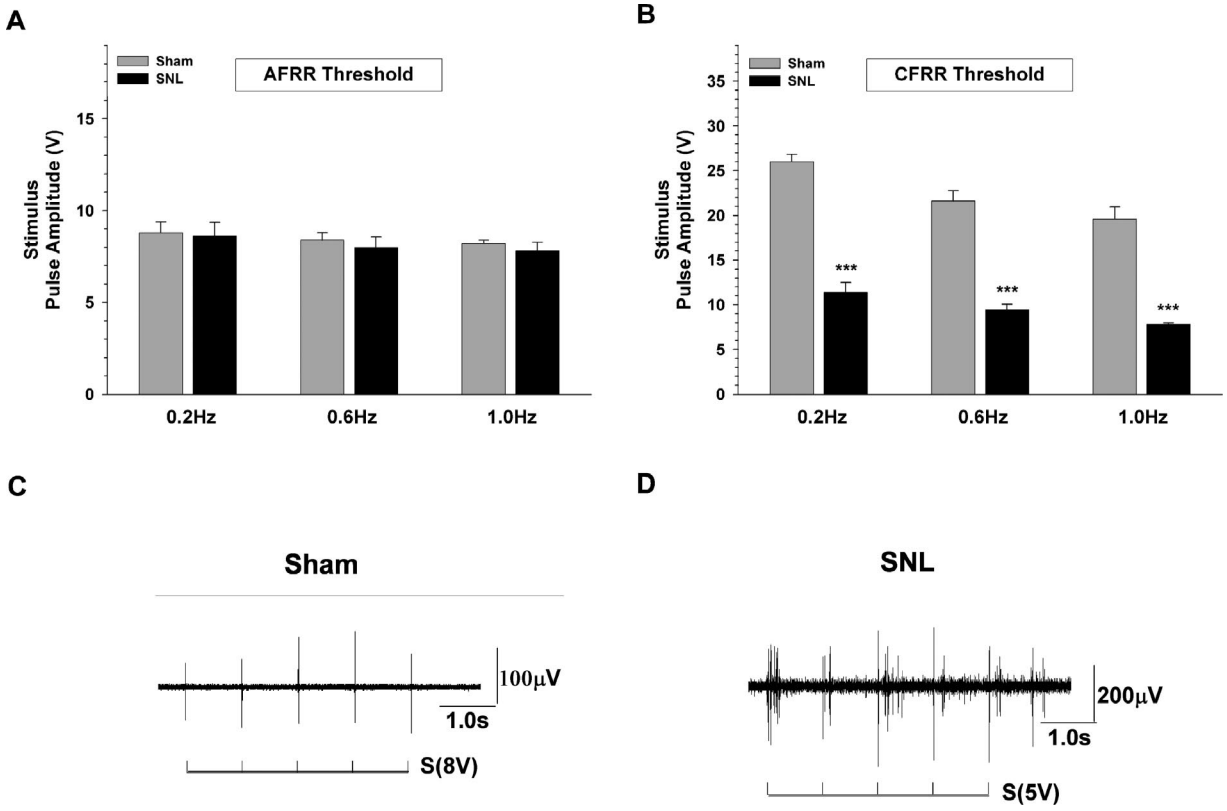

Fig. 3. Activation threshold (V) eliciting the A- (AFRR; *A*) and C-fiber–mediated reflex responses (CFRR; *B*) in the ipsilateral biceps femoris 24 h after spinal nerve ligation (SNL) or sham surgery. Each *bar* represents the mean ± SEM of five to seven animals. *Asterisks* indicate a significant difference from the respective sham control: *** $P < 0.001$. Note the differences in activation threshold (V) for the AFRR and CFRR. Representative electromyographic tracings of the AFRR and CFRR after sham (*C*) and SNL surgery (*D*). Note the differences in both the stimulus (S) and response voltages between nerve-ligated and sham-operated rats, and the presence of the CFRR evoked by threshold stimulation of the AFRR after SNL.

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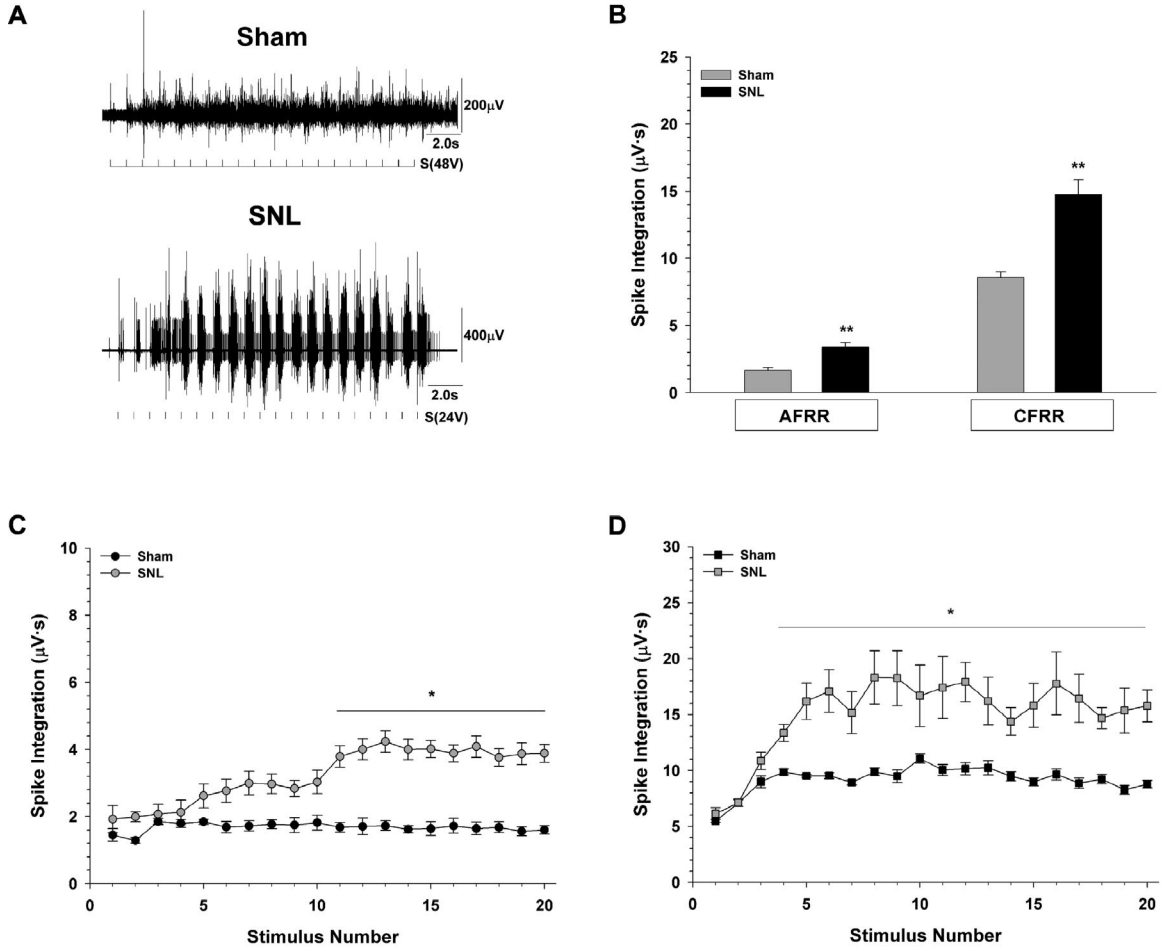

Fig. 4. Individual electromyographic tracings (*A*), spike integration of the A- (AFRR) and C-fiber–mediated reflex responses (CFRR) in the ipsilateral biceps femoris (*B*), and representative windup *versus* stimulus number curves for the AFRR (*C*) and CFRR (*D*). Electromyographic responses were evoked by high-intensity suprathreshold stimulation (1.0 Hz) 24 h after spinal nerve ligation (SNL) or sham surgery. A pulse stimulus *indicator bar* is shown under each tracing. Data are presented as the mean ± SEM of four to six animals. *Asterisks* indicate a significant difference from sham controls: * $P < 0.05$, **$P < 0.01$. Note the differences in the stimulation (S) and response voltages between SNL (24 V and 400 μV) and sham controls (48 V and 200 μV) in *A*. Also note the differences in the spike integration scale for the AFRR (*C*) and CFRR (*D*).

fect) as evidenced by the response to the last (*i.e.*, 20th) stimulus in nerve-ligated as compared with sham-operated animals (table 1). A comparable effect was observed with the CFRR (table 1). Spontaneous activity was also increased in nerve-ligated rats compared with sham controls but did not reach statistical significance ($P > 0.05$; data not shown). There was no increase in spontaneous activity on the contralateral hind limb (data not shown). At suprathreshold activation, the AFRR had a pooled average latency of 12.3 ± 0.4 ms, whereas the CFRR had a pooled average latency of 173.2 ± 9.9 ms. These latencies were unaffected by SNL irrespective of the stimulus intensity (data not shown). In addition, there were no significant differences in the latencies of the

Table 1. Spike Integration of Reflex Responses Recorded from the Ipsilateral Biceps Femoris 24 h after SNL or Sham Surgery

		A-fiber–mediated Reflex Response, μV · s		C-fiber–mediated Reflex Response, μV · s	
Stimulus Number	Stimulus Intensity	Sham	SNL	Sham	SNL
1st	Low	1.01 ± 0.25	2.70 ± 0.60*	5.30 ± 0.25	5.45 ± 0.41
	High	1.45 ± 0.29	2.96 ± 0.56*	6.60 ± 0.79	6.14 ± 0.74
20th	Low	1.07 ± 0.15	2.02 ± 0.22‡	5.21 ± 0.14	7.44 ± 0.58†
	High	1.60 ± 0.12	3.23 ± 0.26*	8.62 ± 0.48	13.62 ± 2.07*

Reflex responses were evoked by suprathreshold stimulation (see Materials and Methods).

Significant difference from sham-operated animals: * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

SNL = spinal nerve ligation.

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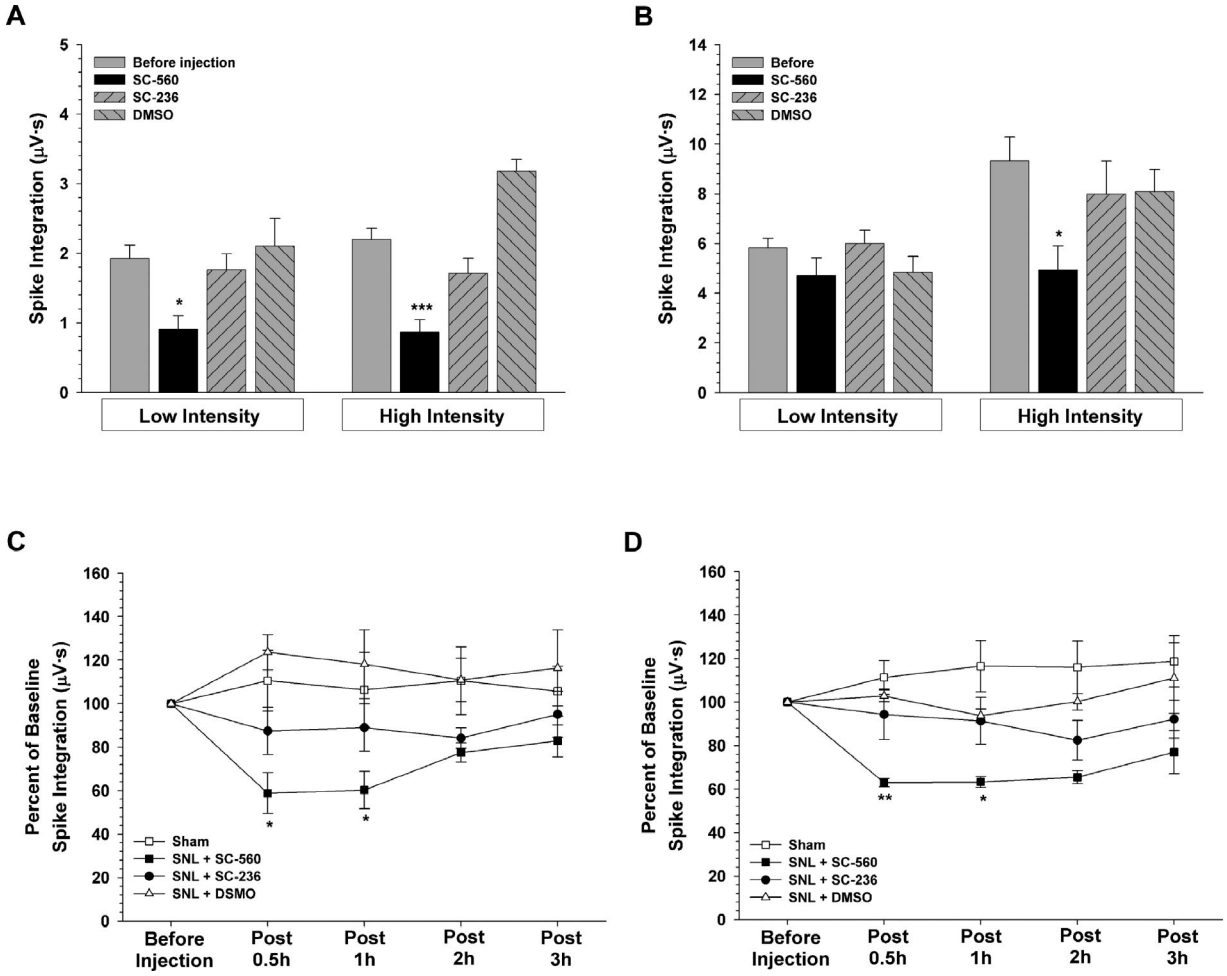

Fig. 5. The effect of intrathecal SC-560 and SC-236 on spinal nerve ligation (SNL)–induced exaggeration of the A- (*A* and *C*) and C-fiber–mediated reflex responses (*B* and *D*). Electromyographic responses were recorded from the ipsilateral biceps femoris, 24 h after surgery. All data are presented as the mean ± SEM of four to six animals. *A* and *B* represent the peak effect (*i.e.*, 30 min after injection) of SC-560 and SC-236, respectively. *Asterisks* indicate a significant difference from before injection: * $P < 0.05$, *** $P < 0.001$. Note the differences in scale in *A* and *B*. The time courses of drug effects on the A- and C-fiber–mediated reflex responses evoked by high-intensity suprathreshold stimulation are shown in *C* and *D*, respectively. *Asterisks* indicate a significant difference from sham controls: * $P < 0.05$, ** $P < 0.01$. DMSO = dimethyl sulfoxide.

AFRR or CFRR evoked by suprathreshold stimulation recorded from the contralateral hind limb of SNL animals (data not shown).

Treatment with intrathecal SC-560 (100 μg) 30 min before low- or high-intensity suprathreshold stimulation significantly reduced the AFRR in nerve-ligated rats, 24 h after SNL (fig. 5A). Whereas SC-560 also significantly inhibited the CFRR evoked by high-intensity suprathreshold stimulation, no such effect was observed using low-intensity suprathreshold stimulation (fig. 5B). SC-236 had no effect on A- or C-fiber activity using either paradigm (figs. 5A and B). The duration of action of SC-560 was 2 h (figs. 5C and D). The AFRR evoked by the first stimulus was inhibited for 1 h by SC-560 but not SC-236 (table 2). Neither SC-560 nor SC-236 had any effect ($P > 0.05$) on the CFRR evoked by the first stimulus. SC-560 but not SC-236 significantly reduced the AFRR and CFRR evoked by the last stimulus (*i.e.*, wind-up-like response) for up to 2 h (table 2). Neither SC-560 nor SC-236 had a significant effect on spontaneous activity, response latency, or duration after SNL or sham surgery (data not shown). Dimethyl sulfoxide had no significant effect ($P > 0.05$) on any evoked responses except for the exaggeration of the AFRR at high-intensity suprathreshold stimulation (figs. 5A and B). The inhibitory effect of SC-560 on the AFRR and CFRR, determined 30 min after intrathecal injection, was dose dependent (figs. 6A and B). SC-560 had no effect in sham controls ($P > 0.05$; data not shown). There was a lack of a dose–response effect of SC-236 on the AFRR and CFRR, determined after intrathecal injection, respectively (data not shown). SC-560 and SC-236 had no effect in sham controls ($P > 0.05$).

Treatment with intrathecal SC-51322 (100 μg) 30 min before low- and high-intensity suprathreshold stimulation significantly reduced the AFRR in nerve-ligated rats, 24 h after SNL (fig. 7A). SC-51322 also significantly inhibited the CFRR evoked by high-intensity but not low-

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Table 2. Effect of Intrathecal SC-560 (100 μg) on Reflex Responses Recorded Ipsilaterally 24 h after SNL or Sham Surgery

		A-fiber–mediated Reflex Response, % Baseline		C-fiber–mediated Reflex Response, % Baseline	
Stimulus Number	Hours after Injection	Sham	SNL	Sham	SNL
1st	0.5	120.15	60.48*	128.94	59.85*
	1.0	116.52	62.53*	123.43	59.58*
	2.0	126.23	80.91*	131.18	59.19*
	3.0	115.77	93.47	110.40	68.30
20th	0.5	109.29	42.72*	118.81	85.68
	1.0	110.29	46.68*	106.77	82.25
	2.0	110.80	64.06	110.69	93.00
	3.0	111.73	70.57	102.33	101.30

Reflex responses were evoked by suprathreshold stimulation (see Materials and Methods).
Significant difference from before intrathecal drug injection (*i.e.*, baseline): * $P < 0.05$.
SNL = spinal nerve ligation.

intensity suprathreshold stimulation (fig. 7B). The duration of action of SC-51322 on the AFRR was 2 h, whereas the inhibitory effect on the CFRR was 1 h (figs. 7C and D). SC-51322 had no effect on the latency or duration of the AFRR and the CFRR evoked by the first stimulus in either nerve-ligated animals or sham controls (data not shown). SC-51322 exhibited a similar 2-h time course for the AFRR and CFRR evoked by the last stimulus (data not shown). SC-51322 had no effect on the AFRR or CFRR in sham controls ($P > 0.05$; data not shown). Spontaneous activity was unaffected with intrathecal SC-51322. Dimethyl sulfoxide had no significant effect ($P > 0.05$) on any evoked responses except for the exaggeration of the AFRR at high-intensity suprathreshold stimulation (figs. 7A and B).

Western analysis revealed a significant increase in the expression of EP_1 (2.0-fold), EP_2 (2.2-fold), and EP_3 (2.5-fold) receptors in nerve-ligated animals compared with sham controls in the ipsilateral lumbar dorsal horn 24 h after SNL (fig. 8), an effect confined to the dorsal horn (data not shown). The expression of all EP receptor subtypes in the contralateral lumbar cord was unchanged from their respective ipsilateral or contralateral sham controls ($P > 0.05$; figs. 8A–C).

Discussion

Changes in the spinal expression of COX isoforms and EP receptor subtypes, and their relation to changes in the excitability of spinal neurons, were investigated 24 h after SNL. Western analysis revealed a significant increase in the expression of COX-1 but not COX-2 in nerve-ligated rats compared with sham controls. This effect was confined to the ipsilateral lumbar dorsal horn—the contralateral dorsal horn and ventral horns were unchanged after SNL—and was accompanied by a significant increase in the hyperexcitability of spinal neurons. Both the AFRR and CFRR exhibited a reduced activation threshold and an exaggerated response to single (*i.e.*, activity before spatial and temporal summation) and repetitive stimulation. These changes, characteristic of peripheral and central sensitization, trigger the neural generation of prostanoids[29–30] and paralleled the

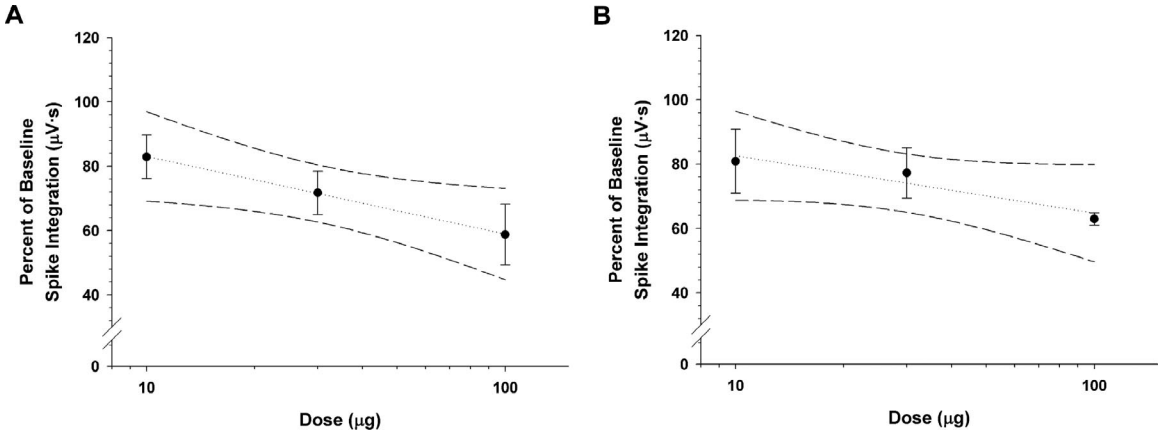

Fig. 6. The dose–response effect of intrathecal SC-560 on the A- (*A*) and C-fiber–mediated reflex responses (*B*) of the biceps femoris. Electromyographic responses to high-intensity suprathreshold stimulation were recorded ipsilaterally, 24 h after spinal nerve ligation. Data are expressed as the mean ± SEM of four to six animals and represent the peak effect of SC-560 (30 min after injection). *Dashed lines* indicate the 95% confidence intervals around the regression (*dotted*) line.

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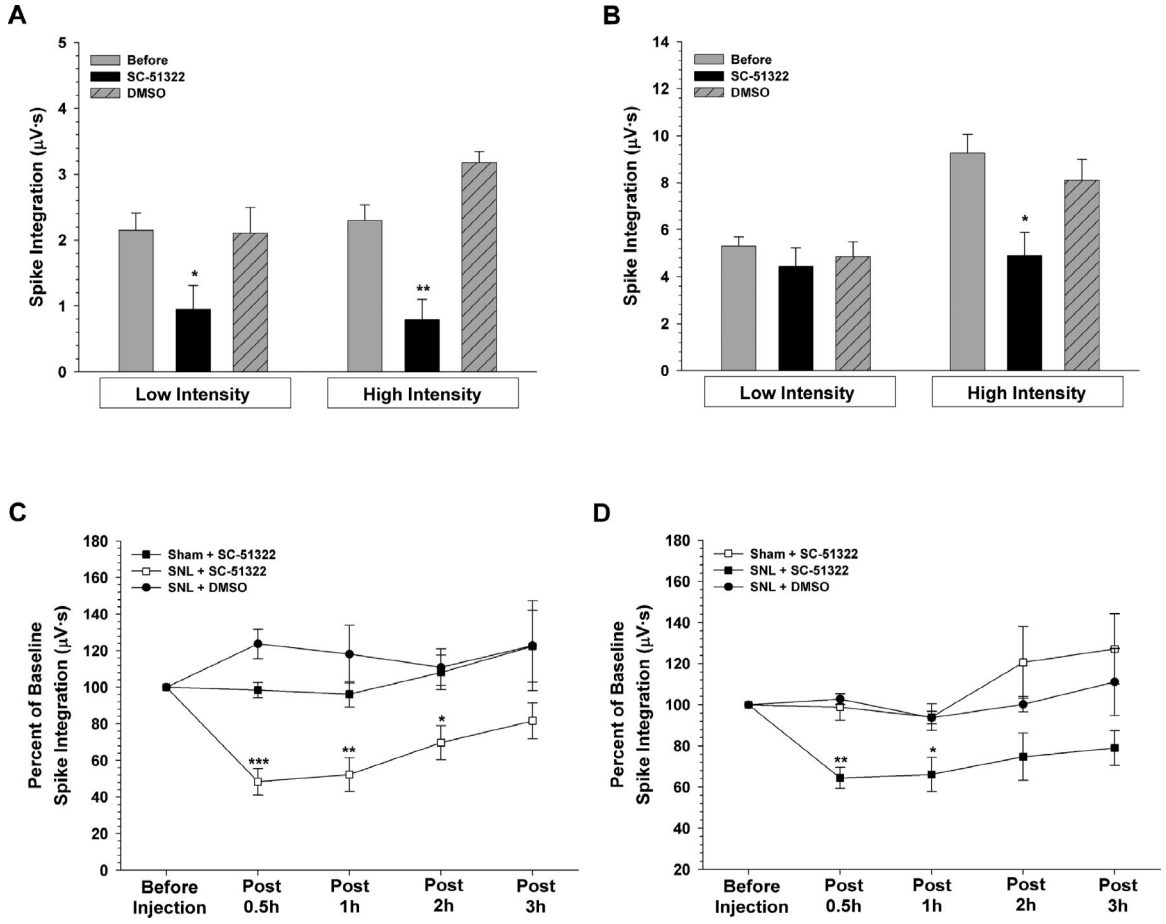

Fig. 7. The effect of intrathecal SC-51322 on the A- (*A* and *C*) and C-fiber–mediated reflex responses (*B* and *D*) of the biceps femoris. Electromyographic responses to suprathreshold stimulation were recorded ipsilaterally, 24 h after spinal nerve ligation (SNL). All data are presented as the mean ± SEM of four to six animals. In *A* and *B*, *asterisks* indicate a significant difference from before injection: * $P < 0.05$, ** $P < 0.01$. Note the differences in scale in *A* and *B*. The time courses of SC-51322 on the A- and C-fiber–mediated reflex responses evoked by high-intensity suprathreshold stimulation are shown in *C* and *D*, respectively. *Asterisks* indicate a significant difference from sham controls: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. DMSO = dimethyl sulfoxide.

development of mechanical allodynia (*i.e.*, paw withdrawal threshold ≤ 4 g). The effect on the AFRR is especially interesting given that SNL-induced mechanical allodynia is elicited by impulses carried along surviving Aβ-afferents.[22] To our knowledge, this is the first report of exaggerated AFRR (including windup) after SNL.

The AFRR and CFRR were investigated using accepted activation and conduction criteria[31] and were further validated by their differential sensitivity to the inhibitory effects of intrathecal morphine. Responses were highly reproducible within the treatment groups at low- and high-intensity suprathreshold stimulation. No attempt was made to separate the A-fiber subtypes primarily because of altered conductivity to injured and uninjured A-fibers.[32] The absence of detectable changes in the latencies of the AFRR and CFRR was not surprising considering that synaptic reorganization in the spinal cord is delayed, normally taking weeks to months after nerve injury.[33–34] The results of the current study illustrate the influence of SNL on the excitability of spinal neurons to both single and repetitive stimulation 24 h after SNL, effects temporally related to the increased expression of COX-1 but not COX-2 in the ipsilateral lumbar dorsal horn.

Comparable increases in spinal excitability have been described in other nerve injury models, including chronic constriction injury,[34–36] complete sciatic nerve transection,[33–34] partial sciatic nerve ligation,[36] spared nerve injury,[34] and spinal cord transection.[37] Interestingly, Chapman *et al.* [38] reported no change in the excitability of Aβ- and C-fibers, 7-17 days after SNL. Although this result may be due, in part, to methodologic differences from the current study (*e.g.*, degree of constriction injury), there is evidence that the timing after SNL is important. For example, a transition from spinal prostaglandin-dependent to prostaglandin-independent allodynia was reported 7–10 days after SNL,[18] and early results in our laboratory indicate a similar transition and time course for the AFRR and CFRR (data not shown). Moreover, the exaggerated reflex responses described in the current study were significantly attenuated by intrathecal SC-51322 or SC-560, but not SC-236.

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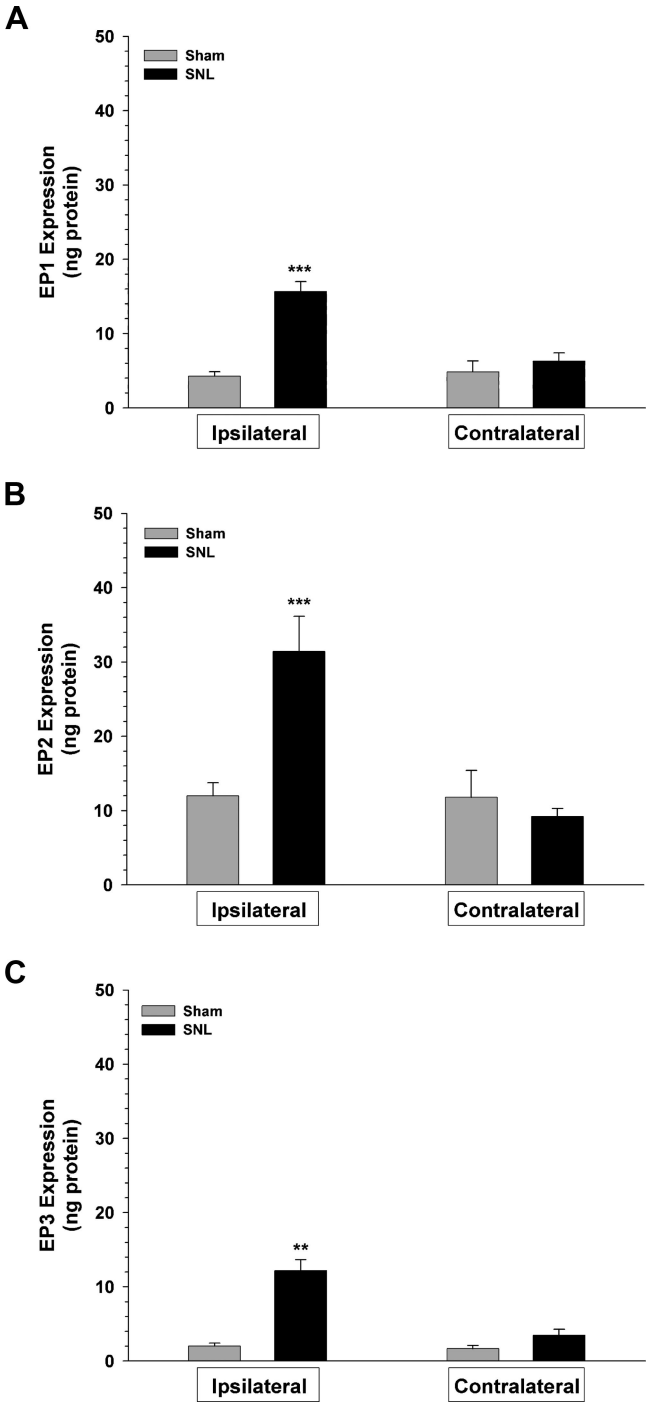

Fig. 8. The expression of EP_1 (*A*), EP_2 (*B*), and EP_3 (*C*) receptors in the ipsilateral and contralateral lumbar dorsal horn 24 h after spinal nerve ligation (SNL) or sham surgery. Each *bar* represents the mean ± SEM of three to five animals. *Asterisks* indicate a significant difference from the corresponding sham control: ** $P < 0.01$, *** $P < 0.001$.

The increased excitability of spinal neurons recorded 24 h after SNL was not only spinal prostaglandin dependent and EP receptor mediated, but also consistent with the concurrent, preferential expression of COX-1 in the ipsilateral lumbar dorsal horn. They are also consistent with previous studies demonstrating the stereoselective, dose-dependent inhibition of mechanical[18] and brush-evoked allodynia[4] by intrathecal ibuprofen in conscious, unrestrained rats.

Western analysis also revealed a significant increase in the expression of EP_1, EP_2, and EP_3 receptors in the ipsilateral dorsal but not ventral horns, 24 h after SNL. Reliable and selective staining of EP_4 receptors could not be achieved with the commercially available antibodies precluding their further investigation in this study. EP_1 receptors, located on primary afferent terminals, facilitate neurotransmitter release[30] (*e.g.*, glutamate) and likely mediate, at least in part, the central sensitizing effect of spinal prostaglandins. Postsynaptic EP_2 receptors in the dorsal horn[10] attenuate glycine-mediated inhibitory postsynaptic currents.[14] That the resulting disinhibition is conducive to allodynia is indicated by the robust and selective allodynia after the intrathecal injection of low-dose strychnine in conscious rats.[39] The EP_2-mediated disinhibitory state would also be exaggerated by the enhanced production/release of and sensitivity to prostaglandin E_2, abnormalities known to occur in the affected spinal cord early after SNL.[4,9,14] The contribution of the EP_3 receptor subtype to central sensitization is more complex, reflecting its multiple variants and signaling pathways. Some EP_3 receptors in the dorsal horn are positively coupled to nitric oxide synthase, with the resulting nitric oxide[40] amplifying prostaglandin synthesis and prostaglandin-dependent allodynia.[41–43] The up-regulation of EP_3 variants which are negatively coupled to adenylyl cyclase explains the previously reported increase in glutamate release from slices of nerve-ligated rats pretreated with SC-51322 and evoked by high concentrations of prostaglandin E_2.[4]

In summary, SNL triggered the increased expression of COX-1 and three EP receptor subtypes (EP_1–EP_3) in the ipsilateral lumbar dorsal horn, and an exaggeration of the AFRR and CFRR 24 h after injury. These changes paralleled the development of mechanical allodynia, were evident only in animals exhibiting allodynia, and were significantly attenuated by pharmacologic agents disrupting COX-1 and EP receptors given 24 h after SNL. The results demonstrate for the first time the influence of spinal prostaglandins on SNL-induced neuronal hyperexcitability. Although an effect on motor neurons cannot be completely excluded at this time, the absence of altered expression in either ventral horn and the lack of detectable motor effects in the SNL model suggest that afferent sensory input is preferentially, if not selectively, affected.

The results also implicate COX-1 as the dominant isoform catalyzing the synthesis of spinal prostaglandins which contribute to the spinal hyperexcitability evident 24 h after nerve injury. This is not to suggest that COX-2 is unaffected by SNL. In fact, the latter exhibits significant up-regulation at 72 h.[4] The results of the current study strongly support the hypothesis that spinal prostaglandins play a critical early role in the development of SNL-induced allodynia[4–9,18,19] and indicate a time-depen-

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dent shift in the isoform primarily responsible for nerve injury–induced spinal prostaglandin synthesis.

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