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Inhibition of Spinal Prostaglandin Synthesis Early after L5/L6 Nerve Ligation Prevents the Development of Prostaglandin-dependent and Prostaglandin-independent Allodynia in the Rat

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Background: **Prostaglandins, synthesized in the spinal cord in response to noxious stimuli, are known to facilitate nociceptive transmission, raising questions about their role in neuropathic pain. The current study tested the hypothesis that spinal nerve ligation–induced allodynia is composed of an early prostaglandin-dependent phase, the disruption of which prevents allodynia.**

Methods: **Male Sprague-Dawley rats, fitted with intrathecal drug delivery or microdialysis catheters, underwent left L5–L6 spinal nerve ligation or sham surgery. Paw withdrawal threshold, brush-evoked behavior, and the concentration of prostaglandin E_2 (PGE_2) in spinal cerebrospinal fluid ($[PGE_2]_{dialysate}$) were determined for up to 24 days. PGE_2-evoked glutamate release from spinal slices was also determined.**

Results: **Paw withdrawal threshold decreased from at least 15 g (control) to less than 4 g, beginning 1 day after ligation. Brushing the affected hind paw evoked nociceptive-like behavior and increased $[PGE_2]_{dialysate}$ (up to 257 ± 62% of baseline). There was no detectable change in basal $[PGE_2]_{dialysate}$ from preligation values. The EC_{50} of PGE_2-evoked glutamate release (2.4×10^{-11} M, control) was significantly decreased in affected spinal segments of allodynic rats (8.9×10^{-15} M). Treatment with intrathecal S(+)-ibuprofen or SC-560, beginning 2 h after ligation, prevented the decrease in paw withdrawal threshold, the brush-evoked increase in $[PGE_2]_{dialysate}$, and the change in EC_{50} of PGE_2-evoked glutamate release. R(−)-ibuprofen or SC-236 had no effect.**

Conclusions: **The results of this study provide solid evidence that spinal prostaglandins, synthesized by cyclooxygenase-1 in the first 4–8 h after ligation, are critical in the pathogenesis of prostaglandin-dependent and prostaglandin-independent allodynia and that their early pharmacologic disruption affords protection against this neuropathic state in the rat.**

PROSTAGLANDINS are synthesized in the spinal cord in response to repetitive C-fiber (noxious) input,[1–5] an effect independent of their role in pain and inflammation in the periphery. This finding is consistent with the localization of cyclooxygenase and prostaglandin binding sites in spinal laminae known to receive nociceptive afferent input[6–9] and the ability of intrathecal nonsteroidal antiinflammatory drugs to block nociceptive behaviors and the concurrent release of prostaglandins into spinal cerebrospinal fliud.[10]

The facilitatory effect of spinal prostaglandins on nociceptive transmission[11] suggests that prostaglandin synthesis early after nerve injury could be important in the development of allodynia, a neuropathic condition in which pain is evoked by a stimulus that does not normally evoke pain (*e.g.*, cold breeze or light touch[12]). Indeed, studies of the effects of spinal prostaglandins on normal nociceptive neurotransmission provide clues as to how this could occur. Prostaglandins directly stimulate wide dynamic range neurons in the rat dorsal horn,[13] sensitize these same neurons to noxious mechanical stimulation,[8] enhance glutamate and substance P release from primary afferent terminals in the spinal cord,[14,15] and reduce glycine-mediated inhibition in the spinal cord.[16]

Certainly, there is growing experimental evidence linking spinal prostaglandins to allodynia. Intrathecal prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) evoke robust allodynia in conscious mice and rats, an effect blocked by prostaglandin receptor antagonists.[14,17–19] Allodynia induced by intrathecal strychnine[20] features brush-evoked increases in the concentration of PGE_2 in spinal dialysate[21] ($[PGE_2]_{dialysate}$) and attenuation by intrathecal cyclooxygenase inhibitors.[22] Bicuculline-induced allodynia is also blocked by cyclooxygenase inhibitors given locally to the affected spinal segments.[23] In the spinal nerve ligation model,[24] significant upregulation of the cyclooxygenase-2 protein has been reported in the spinal cord as early as 1 day after ligation,[25] and allodynia can be temporarily reversed by an intrathecal cyclooxygenase inhibitor or prostaglandin subtype receptor antagonist.[26] Experimental allodynia is also sensitive to spinal N-methyl D-aspartate (NMDA) receptor blockade.[23,27–32] NMDA receptor activation, an essential trigger for central prostaglandin synthesis,[33] is not normally involved in low-threshold neurotransmission.[34] The emergence of NMDA and prostaglandin properties in allodynia suggests a convergence of cellular events by which low- and

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high-threshold input are processed. Thus, the enhancement of glutamate release, NMDA-mediated Ca^{2+} influx, activation of cyclooxygenase, and prostaglandin synthesis may be synaptic events beginning immediately after nerve injury that initiate the subsequent and more complex changes leading to permanent (prostaglandin-independent) allodynia.

In the current study, we used the rat spinal nerve ligation model to test the hypothesis that mechanical allodynia is composed of an early spinal prostaglandin-dependent phase, the disruption of which prevents the establishment of delayed (prostaglandin-independent) allodynia.

Materials and Methods

All studies were conducted in accordance with the guidelines of the Institutional Animal Care Committee of Memorial University of Newfoundland.

In Vivo *Experiments*

Animals. Male Sprague-Dawley rats were obtained from the Vivarium of Memorial University of Newfoundland and were housed in standard cages with wood chip bedding. Animals had free access to food and water and were housed singly after surgery. A 12- to 12-h light cycle (lights on at 0700 h) was used throughout.

Intrathecal Catheterization. Intrathecal microdialysis catheters were constructed using a method modified from Marsala *et al.*[35] and implanted in rats weighing 100–120 g, as previously described.[36] A 6.5-cm catheter was used for termination near the lumbar enlargement in these smaller animals. The catheter was sterilized with 70% alcohol and filled with sterile saline. Under halothane anesthesia, the catheter was inserted through an incision in the atlantooccipital membrane of the cisterna magna. The catheter was externalized behind the head and sealed with stainless steel wire. Drugs were delivered intrathecally through a separate channel of the microdialysis catheter. Rats with normal motor behavior were housed separately and allowed to recover for 3 days before spinal nerve ligation. A laminectomy was performed at the end of the experiments to visually confirm the position of the intrathecal catheter except for rats used in the *in vitro* experiments where this was not feasible. No animals were excluded from the results because of an improperly positioned catheter.

Neuropathy. Neuropathy was induced using the method of Kim and Chung,[24] as modified by Chaplan *et al.*[37] Rats were anesthetized with halothane, and a dorsal midline incision was made from L3–S2. Using blunt dissection, the left posterior interarticular process was located and resected to aid visualization of the L6 transverse process. This was partially removed to expose the L4 and L5 spinal nerves. Once separated, the L5 spinal root was tightly ligated with 6-0 silk thread. The L6 spinal root was then located medial and caudal to the sacroiliac junction and ligated in the same manner. The wound was closed with 4-0 silk sutures in two layers and cleaned with 70% alcohol, and a 5-ml bolus of lactated Ringer's solution was injected intraperitoneally. The animal was placed under a warming light and returned to the animal care facility after recovery from anesthesia.

Microdialysis. Intrathecal microdialysis was conducted in conscious, unrestrained animals 1 day before and on selected days after spinal nerve ligation. Briefly, the inflow channel of the microdialysis catheter was connected to a microsyringe pump. Artificial cerebrospinal fluid, sterilized by filtration through a 0.22-μm pore filter (Micron Separations, Inc., Edmonton, Canada), was perfused at a rate of 10 μl/min. This flow rate, selected on the basis of previous work,[10,26] produced an *in vitro* recovery rate of 31.7 $\pm$ 2.7% of the external PGE_2 concentration.[26] After a 30-min stabilization period, a control sample (100 μl, no paw stimulation) was collected. The plantar surface of the affected hind paw was then lightly brushed for 2 min with a cotton-tipped applicator followed by a 1-min rest period (no brushing). This stimulation protocol was repeated at regular intervals so that rats received a total of 6 min of brushing in a 10-min interval. Dialysate samples were collected on ice over 10 min and immediately frozen at −80°C until assayed. The concentration of PGE_2 was determined using a commercially available enzyme-linked immunosorbent assay kit (Cayman Chemical, Ann Arbor, MI).

Testing. Mechanical allodynia was quantified by determining the 50% withdrawal threshold using von Frey filaments.[37] Briefly, rats were placed in a plastic cage with a wire mesh bottom to allow access to the plantar surface of the left hind paw. Thresholds were measured after a 20-min acclimatization period. Testing was performed during the daylight portion of the circadian cycle (0800–1800 h), and allodynia was defined as a withdrawal threshold of no more than 4 g. The investigators were blinded to the identity of the treatments throughout the study.

Drugs. All drugs were dissolved in 100% dimethyl sulfoxide and diluted with normal saline at the time of injection to yield a final concentration of 50%. Intrathecal drugs were injected using a Hamilton syringe and a hand-operated microsyringe pump. All equipment was sterilized with 70% alcohol before injection and thoroughly rinsed with 0.9% sterile saline. Intrathecal drugs were injected to conscious, unrestrained rats in a volume of 5 μl followed by 8 μl sterile saline. The intrathecal catheter was immediately resealed with the stainless steel plug. S(+)-ibuprofen, the active isomer of the nonselective cyclooxygenase inhibitor, and R(−)-ibuprofen, the inactive cyclooxygenase inhibitor, were purchased from Research Biochemicals International (Natick, MA); SC-236, a selective cyclooxygenase-2 inhibitor, and SC-

560, a selective cyclooxygenase-1 inhibitor, were generous gifts from Searle (Skokie, IL).

In Vitro *Experiments*

Tissue Preparation. Rats were anesthetized with urethane (1.5 g/kg intraperitoneally) and killed by decapitation. The spinal cord was removed immediately using hydraulic expulsion. After careful removal of the dural and arachnoid membranes, the lumbar region was visually identified, excised, and mounted on cutting blocks. The tissue was submersed in sucrose-modified artificial cerebrospinal fluid aerated with 95% O_2 and 5% CO_2 and sectioned using a Vibratome. Spinal cord slices (600–800 μm) were placed in artificial cerebrospinal fluid aerated with 95% O_2 and 5% CO_2 and stored at room temperature.

Determination of Glutamate Concentration. The basal and PGE_2-evoked release of glutamate from spinal cord slices was determined using a modified enzymatic assay.[38,39] Briefly, the slices were transferred from the storage buffer to a 1 × 1-cm cuvette containing (mM): NaCl (120), KCl (3.1), NaH_2PO_4 (1.25), HEPES (25), glucose (4), $MgCl_2$ (1), $CaCl_2$ (2), glutamate dehydrogenase (40 U/ml; BioVectra, Charlottetown, Canada), and nicotinamide adenine dinucleotide phosphate (1) (pH 7.4). The cuvette was placed in a temperature-controlled Shimadzu RF-1501 spectrofluorometer. The contents of the cuvette were continuously mixed using a magnetic stirrer, and the temperature was held at 37°C. HEPES buffer containing the tissue slice was oxygenated with 100% O_2 throughout the experiment. Glutamate released from the tissue was immediately oxidized to α-ketoglutarate by glutamate dehydrogenase, thereby preventing neuronal reuptake of glutamate.[39,40] The reduced form of nicotinamide adenine dinucleotide phosphate generated from this reaction was quantitated using spectrofluorometry (excitation, 335 nm; emission, 430 nm; delay, <1 s). PGE_2 (Biomol) was initially dissolved in ethanol and evaporated under 100% nitrogen gas. It was then dissolved in normal saline, diluted with the same to yield the desired concentrations, and added directly to the cuvette using a microsyringe. Each concentration of PGE_2 was tested using a separate slice so that a full PGE_2 concentration–response curve was determined in each animal. Standard curves were prepared on each day of analysis.

Statistical Analysis

All data are reported as the mean ± SEM. Paw withdrawal thresholds are presented in grams. The concentration of PGE_2 in microdialysis samples ($[PGE_2]_{dialysate}$) is presented in pmol/ml or as the percent of baseline. Glutamate release is reported as pmol · min^{-1} · mg^{-1} protein. Statistical testing was performed using Sigmastat® 2.03 for Windows® (SPSS, Inc., Chicago, IL). Pretreatment and posttreatment values were compared within each treatment group using one-way repeated-measures ANOVA followed by the Newman–Keuls test. Comparisons were also made across all drug- and vehicle-treated groups at each time point using one-way completely randomized ANOVA followed by the Newman–Keuls test. Concentration–response analysis was performed using methods from Tallarida and Murray.[41] $P < 0.05$ was considered to be statistically significant.

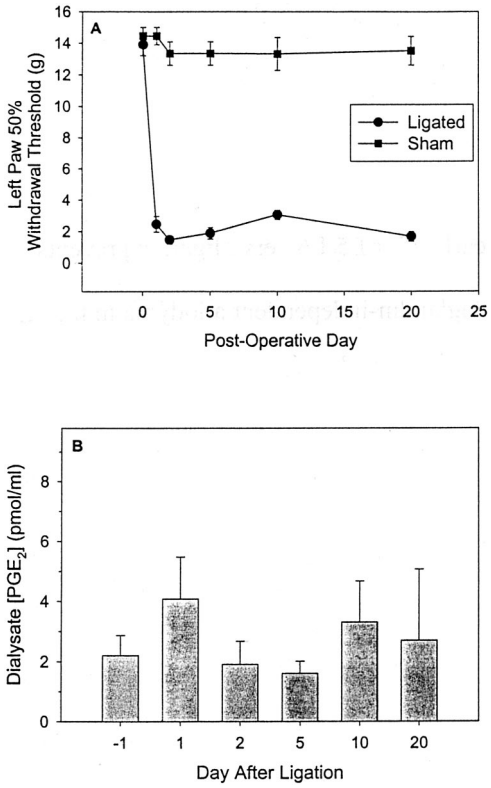

Fig. 1. (*A*) Left hind paw withdrawal thresholds of animals used in the microdialysis experiments. All values for the ligated group are significantly different from preligation values ($P < 0.05$). Each *point* represents the mean ± SEM of six to eight animals. (*B*) Basal prostaglandin E_2 concentrations in spinal dialysate before and after nerve ligation. Spinal dialysate samples were collected immediately before brushing the left hind paw. Each *bar* represents the mean ± SEM of six to eight animals. There were no significant differences among the groups ($P > 0.05$).

Results

Rats undergoing L5–L6 spinal nerve ligation showed a significant decrease in paw withdrawal threshold, from ≥ 15 g to ≤ 4 g, beginning 1 day later (fig. 1A). This enhancement in mechanical sensitivity remained stable for at least 20 days and was confined to the plantar surface of the hind paw ipsilateral to nerve ligation. In addition, the affected hind paw was often kept in an elevated and cupped position, minimizing contact with the cage floor. These rats were otherwise healthy and showed normal feeding behavior and regular weight gain. The paw withdrawal thresholds of all sham-oper-

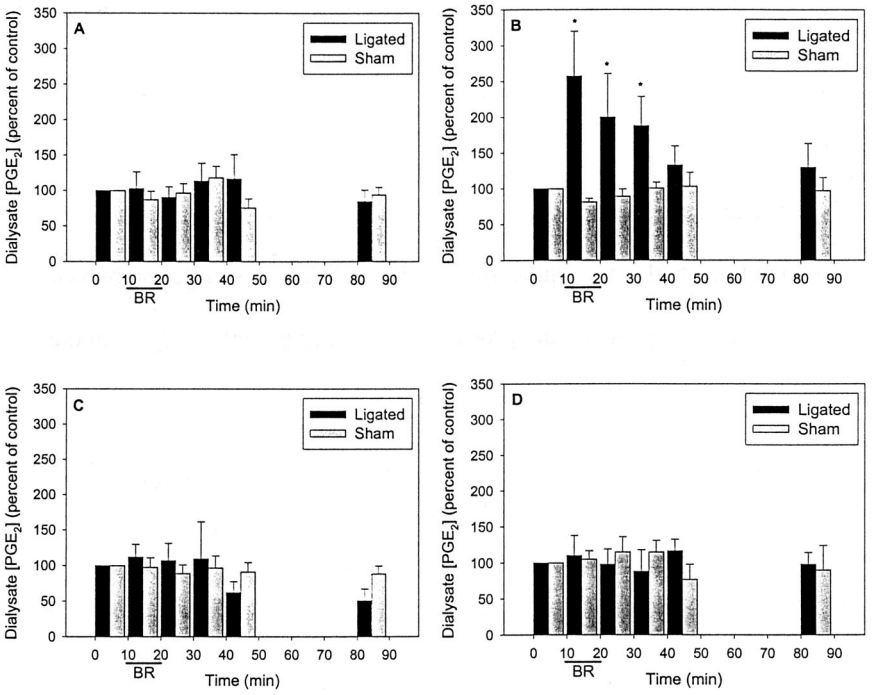

Fig. 2. Prostaglandin E_2 concentration in spinal dialysate ($[PGE_2]_{dialysate}$) before and after brushing (BR) the left hind paw of nerve-ligated or sham-operated rats (*A–C*) or the contralateral (right) hind paw of nerve-ligated or sham-operated rats (*D*). (*A*) Before ligation, brushing the plantar surface of the left hind paw evoked no detectable increase in the $[PGE_2]_{dialysate}$. The same stimulus on the left hind paw evoked a significant increase in $[PGE_2]_{dialysate}$ compared with baseline and the corresponding sham controls 2 days after nerve ligation (*B*) (*$P < 0.05$) but was without effect by day 10 after ligation (*C*). Brushing the contralateral hind paw had no effect on $[PGE_2]_{dialysate}$ compared with baseline or sham-operated controls ($P > 0.05$) 5 days after ligation (*D*). Each *bar* represents the mean ± SEM of six or seven animals.

ated rats remained unchanged from presurgical values (fig. 1A). The basal concentration of PGE_2 in spinal dialysate samples, which ranged from 1.6 ± 0.4 pmol/ml to 4.1 ± 1.4 pmol/ml, was unchanged from preligation values up to 20 days after nerve ligation (fig. 1B).

Before nerve ligation, brushing the plantar surface of the left hind paw had no effect on $[PGE_2]_{dialysate}$ (fig. 2A), nor did it evoke any protective or nocifensive-like behavioral responses. In contrast, the same stimulus evoked a significant increase in $[PGE_2]_{dialysate}$ (257 ± 62% of baseline) (fig. 2B) beginning 1 day after ligation and nocifensivelike behaviors, including protection of the affected hind paw, abrupt paw withdrawal, vocalization, and avoidance of the stimulus applicator. The $[PGE_2]_{dialysate}$ peaked during brushing and declined gradually (up to 30 min) thereafter (fig. 2B). This effect persisted for at least 5 days (data not shown). By day 10, brushing the left hind paw was without effect on the $[PGE_2]_{dialysate}$ (fig. 2C), although the animals were still allodynic (paw withdrawal threshold, ≤ 4 g) (fig. 1A). Brushing the contralateral (right) hind paw of nerve-ligated or sham-operated rats had no effect on the $[PGE_2]_{dialysate}$ or behavior up to 5 days after ligation (fig. 2D).

To test the relevance of spinal prostanoid synthesis to allodynia, separate groups of rats were treated with 100 μg of intrathecal S(+)-ibuprofen, R(−)-ibuprofen, or vehicle beginning 2 h after spinal nerve ligation (figs. 3 and 4). Rats treated with intrathecal S(+)-ibuprofen during an 8-h period (four intrathecal injections of 100 μg given every 2 h) exhibited normal posture (no cupped hind paw) and paw withdrawal thresholds (≥ 15 g) (fig. 3A) for at least 24 days after nerve injury. Brushing the ipsilateral hind paw had no effect on the $[PGE_2]_{dialysate}$ in these same animals (figs. 4A and B). In contrast, ligated rats treated with intrathecal S(+)-ibuprofen for 4 h (100 μg every 2 h for a total of two injections) (fig. 3A), R(−)-ibuprofen for 4 h or 8 h (100 μg given every 2 h for a total of two or four injections, respectively) (fig. 3B), or vehicle using the same treatment schedules (fig. 3C) developed allodynia that was indistinguishable from untreated, ligated rats in terms of paw withdrawal threshold (fig. 1) and brush-evoked changes in behavior and $[PGE_2]_{dialysate}$ (figs. 4A–C). Area-under-the-curve analysis showed no difference ($P = 0.65$) in the brush-evoked $[PGE_2]_{dialysate}$ of the R(−)-ibuprofen treated group (fig. 4B) *versus* ligated, untreated rats (fig. 2B). The ability of brushing to increase $[PGE_2]_{dialysate}$ in the R(−)-ibuprofen (allodynic) group was lost by day 10 (fig. 4C), similar to that in ligated, untreated (allodynic) rats (fig. 2C).

To determine whether allodynic animals also show pharmacodynamic changes to spinal prostanoids, separate groups of rats undergoing spinal nerve ligation were treated with intrathecal R(−)- or S(+)-ibuprofen for 8 h (100 μg given every 2 h for a total of four injections). The concentration–response effect of PGE_2 on glutamate release was compared using spinal cord slices prepared from allodynic and nonallodynic animals. PGE_2 evoked the release from the slices of sham-operated (nonallodynic) rats in a concentration-dependent manner (fig. 5) (table 1). Whereas the PGE_2 concentration–response curves of ligated (untreated) and ligated (R[−]-ibuprofen treated) rats showed dramatic leftward shifts from control, there was no difference between sham-operated (nonallodynic) and ligated (S[+]-ibuprofen [8 h] treated, nonallodynic) rats (fig. 5) (table 1).

To determine whether this protective effect resulted

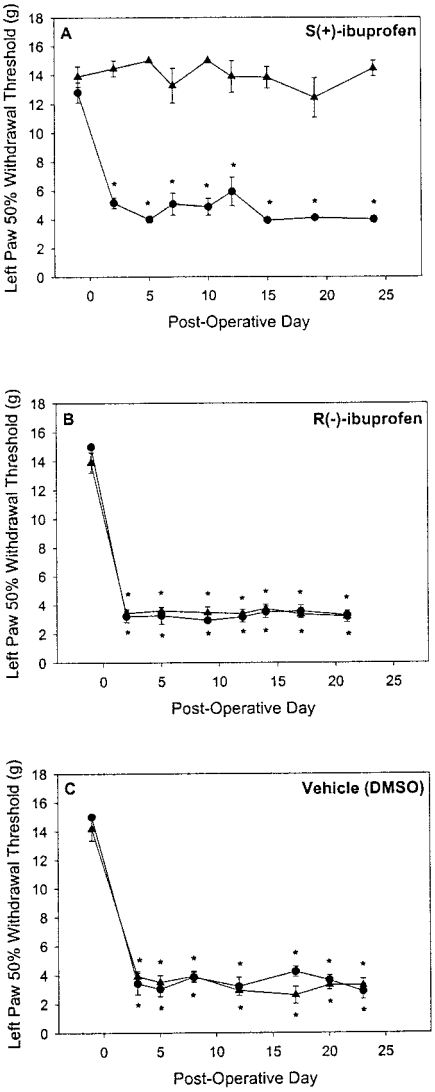

Fig. 3. Left hind paw withdrawal thresholds up to 24 days after spinal nerve ligation. Animals received two (4-h treatment) (*solid circles*) or four (8-h treatment) (*solid triangles*) postoperative doses of 100 μg S(+)-ibuprofen (*A*), 100 μg R(−)-ibuprofen (*B*), or 5 μl dimethyl sulfoxide (*C*) given intrathecally beginning 2 h after spinal nerve ligation. Each *point* represents the mean ± SEM of six animals. All values for the 4-h S(+)-ibuprofen treatment group are significantly different from preligation values and the corresponding 8-h treatment group. No value for the 8-h S(+)-ibuprofen treatment group was different from the preligation value. All values for the groups treated with R(−)-ibuprofen and dimethyl sulfoxide (4 h and 8 h) were significantly different from preligation (*$p < 0.05$).

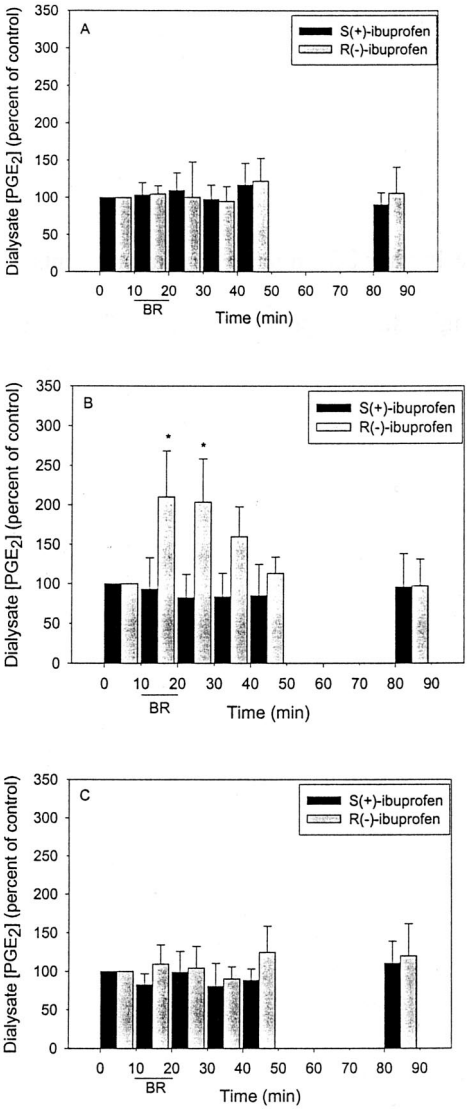

Fig. 4. Prostaglandin E_2 (PGE_2) concentration in spinal dialysate before and after brushing (BR) the left hind paw of nerve-ligated rats. (*A*) Before ligation, brushing of the plantar surface of the left hind paw evoked no detectable increase in the PGE_2 concentration in spinal dialysate. (*B*) In contrast, on day 2 after ligation, the same stimulus applied to the left hind paw of rats treated postoperatively with R(−)-ibuprofen evoked a significant increase in the PGE_2 concentration compared with baseline or the corresponding S(+)-ibuprofen-treated animals (*$P < 0.05$); no detectable increase was observed in rats treated postoperatively with S(+)-ibuprofen. (*C*) Brushing had no effect on day 10 in either group. Each *bar* represents the mean ± SEM of six or seven animals.

from early cyclooxygenase-1 or cyclooxygenase-2 inhibition, rats were treated with intrathecal SC-560 or SC-236 (100 μg) beginning 2 h after spinal nerve ligation. Paw withdrawal thresholds in ligated rats treated with an 8-h regimen of intrathecal SC-560 were slightly, but not significantly, decreased as compared to preligation values for up to 20 days (fig. 6A). For the 4-h treatment, paw withdrawal thresholds on days 2 and 5 were statistically different from preligation values but never decreased below 9 g. In contrast, animals treated with intrathecal SC-236 (4- or 8-h regimen) showed a time course of paw withdrawal threshold (fig. 6B) unchanged from ligated, untreated rats (fig. 1A); all time points were significantly different from preligation values.

Discussion

Ligation of the left L5–L6 spinal nerves produced a robust sensitization to innocuous mechanical stimula-

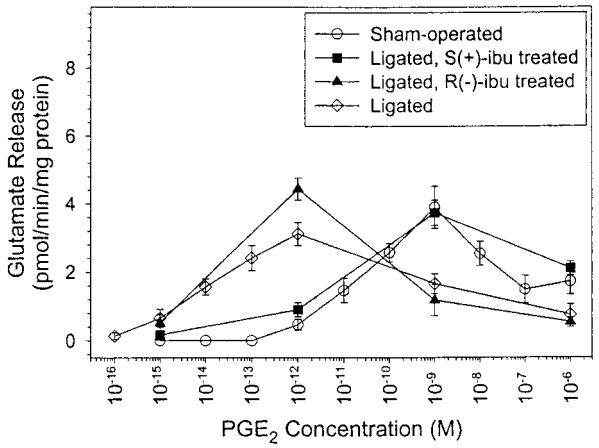

Fig. 5. Prostaglandin E_2 (PGE_2)–evoked glutamate release using spinal cord slices from sham-operated and nerve-injured rats. The addition of PGE_2 produced concentration-dependent glutamate release from spinal cord slices of sham-operated (nonallodynic) animals (see table 1 for EC_{50} and 95% CI of each group). This effect was significantly enhanced (leftward shift) in slices from ligated (allodynic) rats. The concentration–response curve using slices from ligated rats treated with S(+)-ibuprofen (four intrathecal doses of 100 μg every 2 h, beginning 2 h after ligation), which prevented allodynia (fig. 3), was not significantly different from the sham-operated group. In contrast, slices from ligated rats treated with R(−)-ibuprofen, which had no effect on allodynia (fig. 3), yielded a concentration–response curve that was not significantly different from ligated, untreated (allodynic) rats. Each *point* represents the mean ± SEM of at least five animals (5–14 slices).

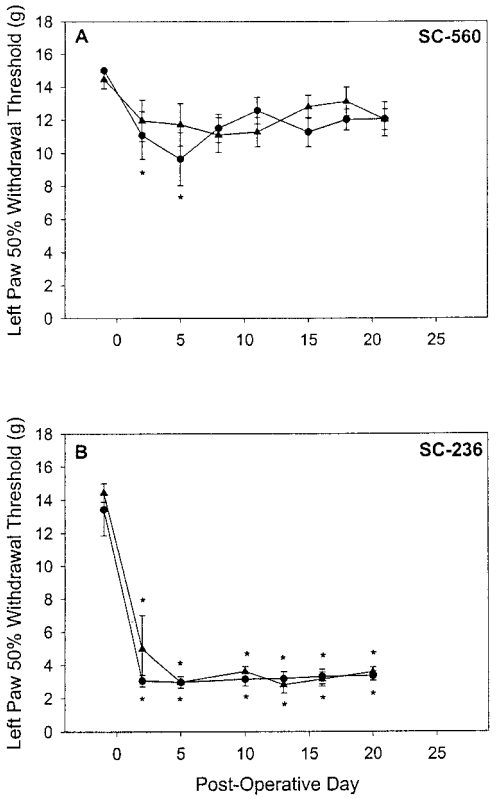

Fig. 6. Left hind paw withdrawal thresholds up to 21 days after spinal nerve ligation. Animals received two (4-h treatment) (*solid circles*) or four (8-h treatment) (*solid triangles*) postoperative doses of 100 μg SC-560 (*A*) or 100 μg SC-236 (*B*) given intrathecally, beginning 2 h after spinal nerve ligation. Each *point* represents the mean ± SEM of six animals. *Asterisks* indicate significant difference from preligation values ($P < 0.05$).

tion on the plantar surface of the left hind paw beginning 1 day later. This was indicated by the marked decrease in the paw withdrawal threshold (≤ 4 g), brush-evoked nociceptive-like behavior, and concurrent stimulus-evoked increase in $[PGE_2]_{dialysate}$. The area of sensitization remained highly circumscribed over time and was absent in sham-operated controls. Brushing the hind paws of sham-operated animals or outside the affected dermatomes, including the contralateral hind paw, of ligated rats was without effect. These results indicate that prostanoid synthesis is recruited in the spinal cord of animals that underwent L5–L6 nerve ligation, a response that is spatially and temporally linked to a stimulus that elicited allodynia in the same animals during the prostaglandin-dependent phase of allodynia. The brush-evoked release of PGE_2, which is in decline by day 5,[26] was absent by day 10, even though brush-evoked allodynia persisted for up to 20 days independent of any detectable change in $[PGE_2]_{dialysate}$.

The connection between allodynia and spinal prostaglandins appears to involve more than just the emergence of brush-evoked prostanoid synthesis. There was also a marked decrease in the EC_{50} of PGE_2-evoked glutamate release in slices prepared from the affected (L5–L6) segments of allodynic rats compared with thoracic slices from the same animals (data not shown) or slices of L5–L6 spinal cord from sham-operated controls. Spinal glutamate has a recognized role in neuropathic states, including the NMDA receptor–coupled activation of phospholipase A_2, nitric oxide synthase, cyclooxygenase, and various kinases,[42–44] many of whose products diffuse into the extracellular space to enhance neurotransmitter release. The mechanisms underlying the increased potency of PGE_2 are currently being investigated but could include the amplification of spinal prostaglandin synthesis in nerve-injured segments in which increased expression of cyclooxygenase-2 has been shown to occur.[25] To our knowledge, a marked pharmacodynamic

Table 1. EC_{50} Values (95% CIs) of PGE_2 on Glutamate Release from Spinal Cord Slices

Sham-operated (nonallodynic)	S(+)-ibuprofen treated (nonallodynic)
2.4×10^{-11} (9.3×10^{-12}–6.1×10^{-11}) M	1.1×10^{-11} (5.0×10^{-12}–2.5×10^{-11}) M
Ligated (allodynic)	R (−)-ibuprofen treated (allodynic)
8.9×10^{-15} (4.3×10^{-15}–1.9×10^{-14}) M	2.0×10^{-14} (1.1×10^{-14}–3.7×10^{-14}) M

All animals underwent L5–L6 spinal nerve ligation except the sham-operated group.

change in the affected spinal segments of demonstrably allodynic animals has not been previously reported.

Allodynia and spinal prostaglandins appear to be functionally linked in the early postinjury period. Brush-evoked spinal PGE_2 release and allodynia were significantly attenuated by intrathecal S(+)-ibuprofen, a cyclooxygenase-1 and cyclooxygenase-2 inhibitor, but not R(−)-ibuprofen, in the spinal nerve ligation model.[26] The relevance of spinal prostaglandins to the development of allodynia is further strengthened by the results of the current study. When given early after ligation, all rats treated with 100 μg S(+)-ibuprofen developed none of the characteristic features of allodynia for up to 24 days. Paw withdrawal thresholds remained normal (≥ 15 g); brushing had no effect on the $[PGE_2]_{dialysate}$; and there was no change in PGE_2-evoked glutamate release from spinal cord slices, compared with sham-operated controls. In contrast, R(−)-ibuprofen, an inactive cyclooxygenase inhibitor and control treatment in these experiments, had no protective effect whatsoever. R(−)-ibuprofen has been shown to inhibit the transcription factor, NFκB *in vitro*,[45,46] a factor known to regulate the expression of cyclooxygenase-2 in macrophages and human gingival fibroblasts.[47,48] To the extent that upregulation of cyclooxygenase-2 after injury is important in the establishment of allodynia, a factor yet to be confirmed, the inhibition of NFκB by R(−)-ibuprofen would be predicted to attenuate allodynia. No such effect was observed, but further studies are required to address this possibility. Overall, the results of these experiments support the hypothesis that spinal prostaglandins are critical factors in the initiation of changes (*e.g.*, central synaptic excitability and neuronal sensitization) leading to prostaglandin-dependent and prostaglandin-independent allodynia. The protective effect of S(+)-ibuprofen also depended on the duration of treatment (only the 8-h regimen was effective), thereby suggesting that prostaglandin synthesis must be inhibited for a minimum period of time after ligation to effectively prevent or interrupt the events leading to allodynia. The results of this study are in general agreement with and build on the preliminary work of Zhao *et al.*,[25] who showed that the nonselective cyclooxygenase inhibitor, indomethacin, given spinally 2 h after spinal nerve ligation, partially attenuated the development of allodynia for up to 4 weeks in the rat.

To extend this work, cyclooxygenase-1- and cyclooxygenase-2-selective inhibitors were used to provide information about the isozymes catalyzing spinal prostaglandin synthesis in the nerve-injured cord. The doses of SC-236 (cyclooxygenase-2-selective) and SC-560 (cyclooxygenase-1-selective) were chosen from previous dose–response studies in our laboratory,[26] in which we showed a dramatic difference in the inhibitory effect of SC-236 and SC-560 on established allodynia using near equimolar intrathecal doses suggesting that isozyme selectivity is maintained at the doses used. In the current study, the contrast in effects with SC-560 and SC-236 was also striking. Whereas SC-560 (4- and 8-h treatments) prevented the emergence of all features of allodynia, an effect comparable with that of S(+)-ibuprofen (8-h treatment), SC-236 (4- or 8-h treatment) was totally ineffective. These results indicate that cyclooxygenase-1, known to be constitutively expressed in the spinal cord,[49] catalyzes the synthesis of prostaglandins in the period immediately after ligation. It is this early synthesis that appears necessary for the development of prostaglandin-dependent and prostaglandin-independent allodynia. In contrast, inducible cyclooxygenase-2, which is known to be upregulated 24 h after ligation,[25] appears to be important in maintaining prostaglandin-dependent allodynia, a stage when cyclooxygenase-1 inhibitors are largely ineffective.[26] Thus, cyclooxygenase-2 may need to achieve sufficient expression over time. The differential localization of constitutive cyclooxygenase-1 and constitutive cyclooxygenase-2 reported in primary afferent terminals of the rat[8] may also explain the early sensitivity to cyclooxygenase-1 inhibitors.

An obvious question arising from these experiments is whether allodynia would have developed without drug treatment (*e.g.*, unsuccessful nerve ligation). This outcome is unlikely because the protective effect was stereospecific, selective for cyclooxygenase-1, and dependent on treatment duration. Moreover, more than 90% of naive animals undergoing L5–L6 spinal nerve ligation in our laboratory develop allodynia within 1 day of surgery, a result comparable with that reported by Chaplan *et al.*[37] Thus, the uniform failure of nerve ligation within an experimental group is improbable and inconsistent with the results of this study. These data indicate that treatment with spinal cyclooxygenase inhibitors early after ligation prevents or, at the least, significantly delays (> 25 days), the emergence of prostaglandin-dependent and prostaglandin-independent allodynia.

Figure 7 is a proposed model of the early (synaptically mediated) events effecting prostaglandin-dependent and prostaglandin-independent allodynia after L5–L6 spinal nerve ligation. Glutamate, released from the central terminals of primary afferent fibers beginning immediately after nerve injury,[50,51] activates postsynaptic NMDA receptors, an essential step in central sensitization and allodynia.[52,53] Subsequent intracellular events, including an increase in Ca^{2+} concentration and the release of arachidonic acid *via* PLA_2, trigger the early synthesis of spinal prostaglandins by constitutive enzymes (cyclooxygenase-1 more so than cyclooxygenase-2). After diffusion to the extracellular space, spinal prostaglandins enhance the excitability of adjacent cells[8,13] and feed back to reinforce glutamate release.[14,15,54] Sustained NMDA receptor activation initiates the expression of inducible cyclooxygenase-2,[49] thereby enhancing the capacity for stimulus-evoked prostaglandin synthesis in the spinal cord (cyclooxygenase-2 more so than cyclooxy-

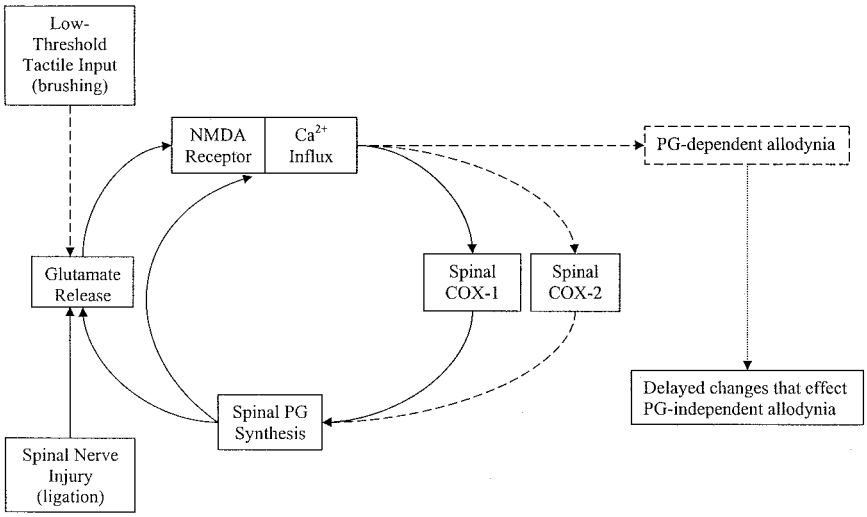

Fig. 7. A proposed model of the early (synaptically mediated) events in the spinal cord causing allodynia after L5–L6 spinal nerve ligation. Glutamate, released from the central terminals of primary afferent fibers beginning immediately after ligation, activates postsynaptic NMDA receptors. Subsequent intracellular events (omitted for clarity), including an increase in the Ca^{2+} concentration and activation of PLA_2, trigger the early synthesis of spinal prostaglandins by constitutive activity (cyclooxygenase-1 > cyclooxygenase-2). In the extracellular space, spinal prostaglandins diffuse to adjacent cells to enhance glutamate release and cell excitability (postsynaptic effect). Sustained NMDA receptor activation initiates the delayed expression of inducible cyclooxygenase-2 in the spinal cord (*dashed arrow*). Within 24 h of ligation, central sensitization, combined with the enhanced capacity for prostaglandin synthesis (cyclooxygenase-2 > cyclooxygenase-1), results in the emergence of temporary, prostaglandin-dependent allodynia. In the next few weeks (*dotted arrow*), prostaglandin-dependent allodynia recedes and leaves long-term allodynia independent of spinal prostaglandins. This may reflect phenotypic changes in primary afferents, sprouting of primary afferents into the outer laminae, altered gene regulation, and changes in expression of critical receptors, ion channels, or enzymes.

genase-1) and the emergence of prostaglandin-dependent allodynia.[26] During the next few weeks, prostaglandin-dependent allodynia recedes and leaves long-term, prostaglandin-independent allodynia. The latter may reflect phenotypic changes in primary afferents, sprouting of primary afferents into outer laminae, and altered gene regulation.[55–59] The ability of cyclooxygenase inhibitors to prevent the emergence of prostaglandin-dependent and prostaglandin-independent allodynia strongly suggests that these two states are related.

In conclusion, the results of this study provide solid evidence that spinal prostaglandins, synthesized immediately after spinal nerve ligation, are critical in the development of prostaglandin-dependent and prostaglandin-independent allodynia, and that their early pharmacologic disruption affords protection against this neuropathic state. They build on and are consistent with previous work showing the prostaglandin-dependent nature of allodynia in this model,[25,26] but are seemingly at odds with anecdotal reports that nonsteroidal antiinflammatory drugs provide little, if any, benefit to patients with allodynia. How can this apparent contradiction be resolved? Our data indicate that the timing of treatment, the type of cyclooxygenase inhibitor used, and perhaps the route of administration are critical factors influencing the effectiveness of nonsteroidal antiinflammatory drugs, not in relieving neuropathic pain *per se*, but in preventing its emergence. They suggest that nonsteroidal antiinflammatory drugs would need to be given early (*i.e.*, hours) after injury, after which their clinical value would decrease. The latter prediction is seemingly consistent with current clinical experience. Although further research is needed, the potential implications of this research for clinical neuropathic pain are exciting.

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