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Cyclooxygenase-1 in the Spinal Cord Is Altered after Peripheral Nerve Injury

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Background: **The mechanisms underlying neuropathic pain are incompletely understood and its treatment is often unsatisfactory. Spinal cyclooxygenase-2 (COX-2) expression is upregulated after peripheral inflammation, associated with spinal prostaglandin production leading to central sensitization, but the role of COX isoenzymes in sensitization after nerve injury is less well characterized. The current study was undertaken to determine whether COX-1 was altered in this model.**

Methods: **Male rats underwent partial sciatic nerve transsection (PSNT) or L5–L6 spinal nerve ligation (SNL). Four weeks after PSNT and 4 h, 4 days, or 2 weeks after SNL, COX-1 immunohistochemistry was performed on the L2–S2 spinal cord.**

Results: **COX-1 immunoreactivity (COX-1-IR) was unaffected 4 h after SNL. In contrast, 4 days after SNL, the number of COX-1-IR cells increased in the ipsilateral spinal cord. COX-1-IR increased in cells with glial morphology in the superficial laminae, but decreased in the rest of the ipsilateral spinal cord 4 weeks after PSNT and 2 weeks after SNL. These changes in immunostaining were greatest at the L5 level.**

Conclusion: **These data suggest that COX-1 expression in the spinal cord is not static, but changes in a time- and laminar-dependent manner after nerve injury. These anatomic data are consistent with observations by others that spinally administered specific COX-1 inhibitors may be useful to prevent and treat neuropathic pain.**

NEUROPATHIC pain is a physically and emotionally debilitating condition for which treatment is often inadequate. The mechanisms that underlie neuropathic pain are poorly understood, but several animal models have been developed to probe these mechanisms. Partial sciatic nerve transsection (PSNT) and L5–L6 spinal nerve ligation (SNL) in rats, two widely used models, result in hyperalgesia (*i.e.*, increased pain intensity in response to noxious stimuli) and allodynia (*i.e.*, pain in response to normally innocuous stimuli).[1,2]

Prostaglandins are synthesized in the spinal cord during acute nociceptive stimulation,[3] peripheral inflammation,[4–7] intrathecal injection of substance P[8] or kainic acid,[9] and mechanical injury to the spinal cord.[10,11] Cyclooxygenase (COX), the rate-limiting enzyme in prostaglandin synthesis, is constitutively expressed as isoenzymes COX-1 and COX-2 in the spinal cord.[4,12,13] Accumulating evidence indicates that COX-2 plays an important role in hypersensitivity induced by peripheral inflammation.[5,12,14–17] Thus, COX-2 but not COX-1 mRNA and protein are increased in spinal cord homogenates after peripheral inflammation, and hypersensitivity is prevented or treated by intrathecal injection of selective COX-2 but not COX-1 inhibitors.

The role of COX-1 and COX-2 in neuropathic pain remains unclear and has received little attention. Intrathecal injection of the nonselective COX inhibitor, indomethacin, near the time of peripheral nerve injury delays for many days the onset of hypersensitivity,[18] and intrathecal injection of a selective COX-1 inhibitor at the time of nerve injury permanently inhibits the development of hypersensitivity. In addition, intrathecal injection of a COX-1-preferring inhibitor, ketorolac, is effective in attenuating thermal hyperalgesia and cold allodynia induced by sciatic nerve injury in rats[19] and reverses tactile allodynia induced by partial sciatic nerve ligation.[20] Based on these observations, we tested whether COX-1 expression is altered in the spinal cord in animal models of neuropathic pain. We previously observed no change in spinal COX-1 expression after SNL,[18] but that result was in tissue homogenates. The purpose of the current study was to examine whether localized changes in COX-1 expression occur after PSNT or SNL injury, using immunohistochemistry.

Materials and Methods

Male Sprague-Dawley rats (220–260 g) were used in this study with the approval of the Animal Care and Use Committee of Wake Forest University.

Rats were placed in clear plastic cages on an elevated mesh floor and allowed to accommodate for 30 min. Paw withdrawal threshold in response to probing with von Frey filaments was measured using the up-and-down method as previously described.[21] One group of rats was then anesthetized with 2–3% halothane and the left sciatic nerve partially transected using a slightly modified procedure of that previously described.[1,2] Briefly, the sciatic nerve was exposed at the midthigh level, and the medial half of the nerve was transected with finely tipped scissors at a point just proximal to the branch running to the musculus biceps femoris. The skin was then sutured and the animals recovered in their cages.

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Another group of rats underwent L5–L6 SNL as described by Kim and Chung.[22] The left L5 and L6 spinal nerves were isolated and ligated tightly with 5-0 silk suture. Sham control animals received the same anesthesia and incision in the skin and muscles, without any manipulation of the nerves. Three or four rats were used in each group, and a group of three normal rats was also studied.

Because tactile allodynia develops in rats within 1 week after L5–L6 SNL[22] and 2 weeks after PSNT[1] surgery, and remains stable for 4 weeks, rats with PSNT or sham operation were perfused 4 weeks after surgery. Rats with SNL or sham operation were perfused 4 h, 4 days, and 2 weeks after surgery to determine the time course of changes. Before perfusion, paw withdrawal threshold to von Frey filaments was measured. Rats were then deeply anesthetized with pentobarbital and perfused transcardially with 0.01 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in ice-cold 0.1 M phosphate buffer (PB) (pH 7.4). The caudal lumbar spinal cords were removed and postfixed in the same fixative for 3 h. After cryoprotection in 30% sucrose in 0.1 M PB at 4°C for 24 h, these tissues were cut on a cryostat at a 40 μm thickness.

A series of one of every 10 sections were collected in PBS. After pretreatment with 0.3% H_2O_2, sections were blocked with 5% normal goat serum, incubated for 24 h at 4°C in a mouse monoclonal antiovine COX-1 antibody (1:2000; Cayman, Ann Arbor, MI) diluted in PBS containing 0.3% Triton-X 100 (PBS+T), and 5% normal goat serum. Subsequently, the sections were sequentially incubated with secondary goat anti-mouse antibody and ABC reagent (Vectastain ABC; Vector, Burlingame, CA) according to the instructions of the manufacturer. Between incubations, sections were washed twice in PBS+T for 10 min. Finally, the immunoprecipitates were developed by 3,-3' diaminobenzidine and enhanced by nickel. After immunostaining, sections were dehydrated in ethanol and cleared xylene, cover-slipped, and examined by light microscopy.

For quantification of COX-1-immunoreactive cells (COX-1-IR) in the spinal cord of normal and SNL rats, four sections at the L5 level were chosen from each rat and digitally imaged. The L5 level was chosen because changes in immunostaining were greatest at this level, tapering to no change two or three dermatomes cephalad and caudad. An area with the same size was specified in the superficial laminae (I, II, and III) and deep laminae (IV, V, and VI) in spinal dorsal horns. By using Sigma Scan (Jandel Scientific, Carpinteria, CA), the COX-1-IR cells were automatically counted using a fixed threshold for all sections from the normal, 4-h, and 2-week groups. The threshold for the 4-day group was reduced to include the lightly stained cells, because at this time point, all the positively stained cells were lightly stained. Each data point is compared to that in the baseline (normal) rats. Data are presented as mean ± SEM and were analyzed using one-way ANOVA followed by a Tukey test. $P < 0.05$ was considered to be significant.

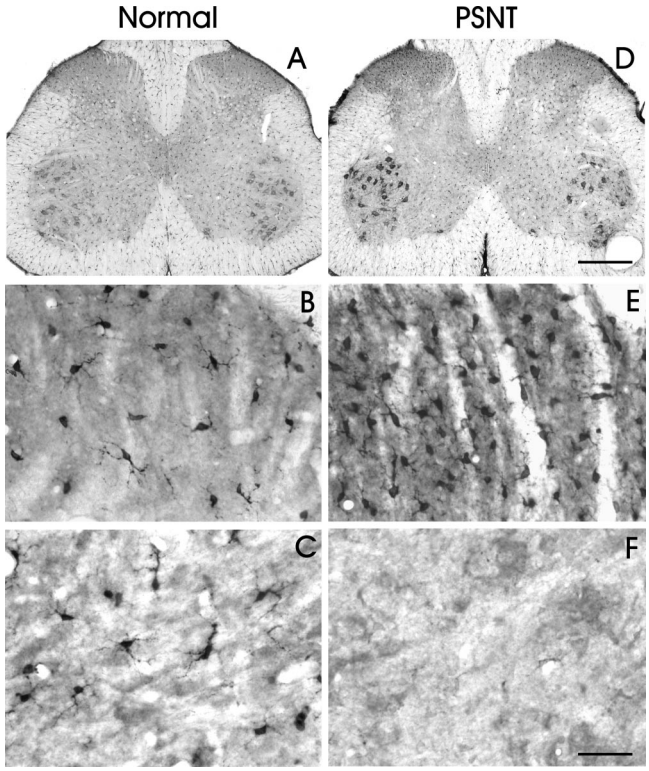

Fig. 1. Cyclooxygenase-1 (COX-1) immunoreactivity in spinal cords of normal rats and those that underwent partial sciatic nerve transsection (PSNT) 4 weeks after surgery. (*A, D*) Low magnification of COX-1 staining in spinal cords of normal and PSNT rats. (*B, E*) High magnification of COX-1 staining in superficial and deep spinal dorsal horn of normal rat. (*C, F*) High magnification of COX-1 staining in superficial and deep spinal dorsal horn of PSNT rat. *Scale bar*: *A, D,* 200 μm; *B, C, E,* and *F,* 20 μm.

Results

Paw withdrawal threshold decreased from 37.33 ± 0 g before surgery to less than 4 g 4 weeks after PSNT and 2 weeks after SNL. Four days after surgery, rats with SNL showed slight allodynia with a paw withdrawal threshold of 17.11 ± 6.58 g.

COX-1 was constitutively expressed in the spinal cord in normal rats (fig. 1A). COX-1-IR was located in cells with glial morphology throughout the spinal cord gray and white matter and in cells with motor neuron morphology in the ventral horn. High magnification of the glial-like profiles showed that COX-1-IR extended from the cell body cytoplasm to the processes (fig. 1B and C).

Partial sciatic nerve transsection dramatically increased the number of COX-1-IR glial-like profiles in the superficial laminae of the ipsilateral spinal dorsal horn of L4–L6 spinal cord (fig. 1D). High magnification showed that these COX-1-IR profiles showed the same morphology as

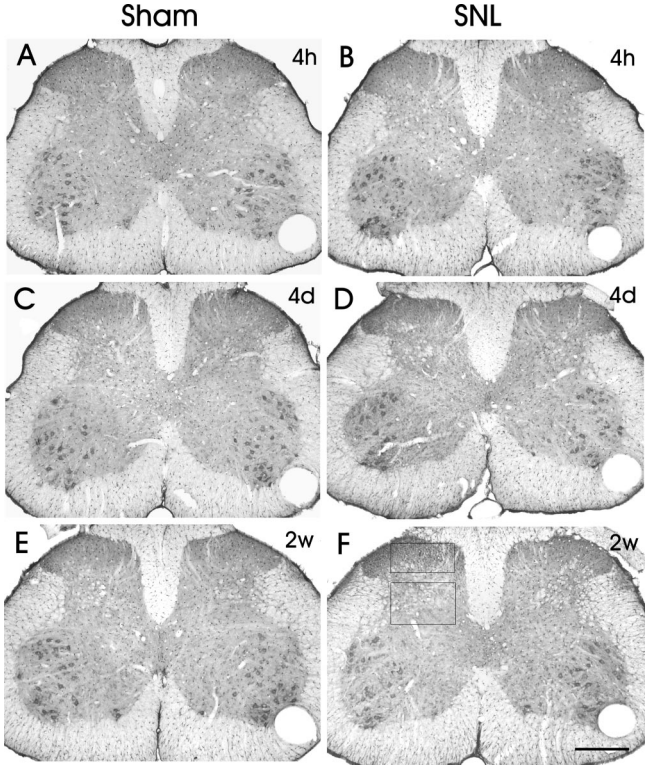

Fig. 2. Cyclooxygenase-1 (COX-1) immunoreactivity change over time in the spinal cords from sham-operated rats and those that underwent L5–L6 spinal nerve ligation (SNL). (*A, B*) Sham-operated and SNL rats 4 h after surgery. (*C, D*) Sham-operated and SNL rats 4 days after surgery. (*E, F*) Sham-operated and SNL rats 2 weeks after surgery. Contralateral sides are marked by a *hole* in the *ventral white matter*. The area of quantification in the superficial and deep laminae is indicated by the *upper* and *lower rectangles* in *F*. *Scale bar*, 200 μm.

those in normal controls (fig. 1E). Interestingly, PSNT concomitantly decreased COX-1-IR in the deep dorsal horn, ventral horn, and white matter. Indeed, after PSNT, there were no COX-1-IR profiles in the deep dorsal horn (fig. 1D and F). PSNT did not affect COX-1-IR staining in the spinal cord contralateral to injury (fig. 1D).

Spinal nerve ligation resulted, 2 weeks after injury, in a pattern of change in COX-1-IR similar to that observed after PSNT. Four hours after SNL, COX-1-IR was no different than normal and sham-operated rats (fig. 2A and B compared with fig. 1A). Four days after SNL, the number of COX-1-IR cells increased diffusely throughout the entire spinal cord ipsilateral to injury. These cells were lightly stained, and no intensely stained cells were observed (fig. 2C and D). Two weeks after SNL, the pattern of change in COX-1-IR in the ipsilateral spinal cord was the same as that seen in the rats with PSNT. COX-1-IR profiles in the superficial dorsal horn ipsilateral to nerve injury were increased in number compared with those in sham-operated rats, and at this time, the intensity of staining was greater than that 4 days after injury. In the deep dorsal horn, COX-1-IR disappeared, and in the ventral horn and white matter, COX-1-IR was dramatically decreased (figs. 1D and 2D–F). Quantification of

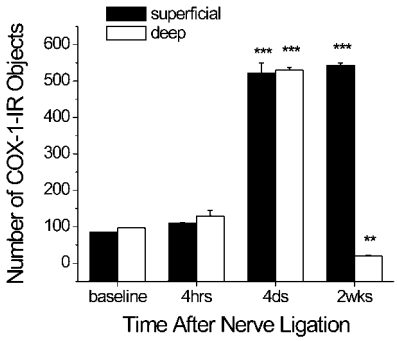

Fig. 3. Quantification of cyclooxygenase-1 (COX-1) immunoreactivity in the superficial laminae (I, II, and III) (*filled bar*) and deep laminae (IV, V, and VI) (*open bar*) in the ipsilateral dorsal horn of L5–L6 spinal nerve ligation rats over time. Each *bar* represents the mean, and *error bars* indicate the SEM. Significant differences between the each time point and baseline are indicated by $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA) (n = 3 in each group).

areas of immunostaining for COX-1 in the spinal cord showed statistical significance for these changes (fig. 3). High magnification showed the COX-1-IR profiles in sham-operated and SNL rats at all the time points tested had the same morphology as that in normal rats (figs. 1B and C, and 4). The contralateral spinal cord showed no

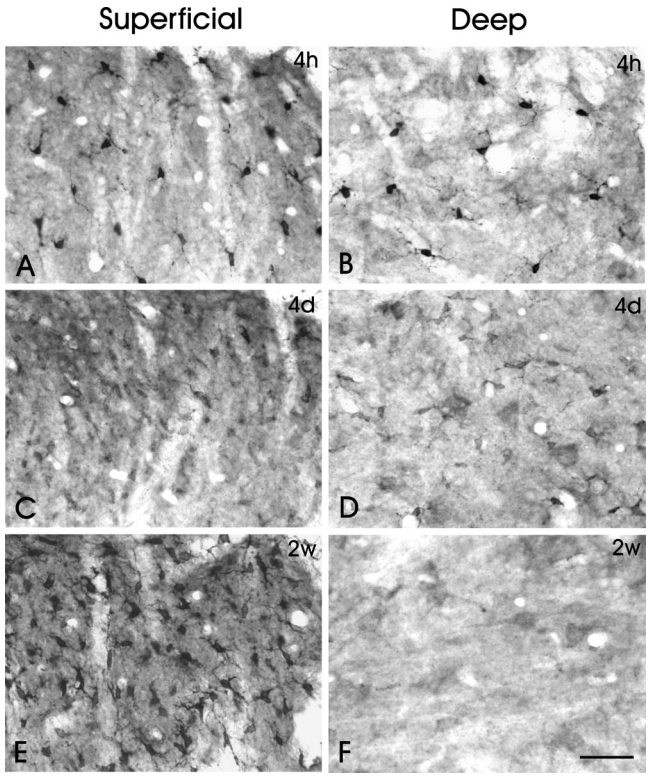

Fig. 4. High magnification of cyclooxygenase-1 (COX-1) immunoreactivity over time in the ipsilateral spinal dorsal horn of rats that underwent L5–L6 spinal nerve ligation. (*A, B*) Superficial and deep laminae of the ipsilateral spinal dorsal horn 4 h after surgery. (*C, D*) Superficial and deep laminae of the ipsilateral spinal dorsal horn 4 days after surgery. (*E, F*) Superficial and deep laminae of the ipsilateral spinal dorsal horn 2 weeks after surgery. *Scale bar*, 20 μm.

difference from normal and sham-operated rats (figs. 1D, 2B and D, and F).

Discussion

Consistent with previous investigations,[4,12,13,23,24] the current study shows that COX-1 is constitutively expressed in the spinal cord. Aside from the large motor neuron COX-1-IR profiles, the remaining small COX-1-IR cells are evenly distributed in the gray and white matter. This distribution and cell morphology suggest that these cells are glia, in agreement with previous observations of COX-1-IR in mouse spinal cord[23] and colocalization of COX-1-IR in rat spinal cord with markers for microglia (unpublished data).

An emerging series of observations suggest there are fundamental differences in spinal COX isoenzymes involved in different pain states. A dominant, perhaps exclusive, role for spinal COX-2 occurs with peripheral inflammation.[5,12,14–17] In contrast, COX-1-IR is increased after incisional surgery, and tactile hypersensitivity after this surgery is blocked by selective COX-1 inhibitors, but unaffected by intrathecal injection of selective COX-2 inhibitors.[25] Hypersensitivity from nerve injury is delayed[18] or prevented by intrathecal injection of nonisoenzyme-selective, or COX-1-selective inhibitors but not by COX-2-selective inhibitors. The current study shows a long-term change in COX-1 in the spinal cord after nerve injury, consistent with the hypothesis that COX-1 is important in the development of hypersensitivity induced by nerve injury. Should this model be predictive of the human experience, it may lead to new treatment strategies to prevent neuropathic pain when the time of injury is known, such as amputation or thoracic surgery.

The current study suggests COX-1 may also be important in the maintenance of hypersensitivity after nerve injury. In contrast, previous studies[5,12,14,24] have shown that COX-1 does not play a role in inflammatory pain. This may be the result of the different mechanisms involved in these two types of pain. PSNT and SNL induce stable neuropathic hypersensitivity in rats, which lasts 6 to 8 weeks.[1] Four weeks after PSNT or 2 weeks after SNL, when rats had stable hypersensitivity, the number of COX-1-IR cells dramatically increased in the ipsilateral superficial laminae compared with normal or sham control. Because superficial laminae are the sites for the termination of nociceptive Aδ and C fiber primary afferents, prostaglandins produced by COX in the superficial laminae may be important in maintaining hyperalgesia from altered neurotransmission in this region. This is supported by the behavioral studies that show that intrathecal ketorolac, a COX-1-preferring inhibitor, restores morphine efficacy in rats that underwent SNL.[26] Intrathecal ketorolac alone also effectively attenuates thermal hyperalgesia and cold allodynia induced by sciatic nerve injury in rats[19] and reverses tactile allodynia induced by partial sciatic nerve ligation.[20] We are currently completing safety trials of intrathecal ketorolac in patients with neuropathic pain, to be followed by controlled trials to examine its efficacy.

The pattern of COX-1-IR increase in the dorsal horn ipsilateral to nerve injury is different from that in the postoperative pain model of paw incision. The latter shows a diffuse increase in the entire ipsilateral spinal dorsal horn, with a more prominent increase in the medial region.[25] In the current study, the increase of COX-1-IR is located only in the ipsilateral superficial laminae. These COX-1-IR glial cells only increase in number, without changing in morphology, unlike the altered morphology observed after surgery.[25] Whether this reflects differences in timing (days in the postoperative model and weeks in the nerve injury models) or activation mechanisms specific to the injury is unknown.

The decrease in the current study in COX-1-IR in the deep laminae of the spinal cord ipsilateral to the nerve injury was striking. We suspect that previous studies that have failed to observe changes in total COX-1 protein in the spinal cord after nerve injury missed important regional anatomic changes lost when using tissue homogenates. Peripheral nerve injury has been shown to cause a considerable degree of anatomic plasticity in the spinal cord. Using transganglionic tracers, Shortland and Fitzgerald[27] showed that transsection of the sciatic nerve in neonate rats caused the A and C fibers of the intact saphenous nerve to extend their arborizations into the territory of the transected sciatic nerve, whereas the sciatic afferents that survived neonatal axotomy retained a normal terminal distribution. It is noteworthy that the Aβ fibers that had sprouted into the sciatic territory, and the surviving sciatic Aβ fibers, had sprouted dorsally into the superficial laminae. In contrast, the labeled C fibers had not sprouted into the deeper laminae occupied by Aβ fibers and indeed showed a reduced intensity of terminal labeling. This type of change could occur, probably to a lesser extent, after PSNT and SNL in adult rats. In this case, spontaneous firing in the Aβ and C fibers would concentrate on the neurons in the superficial laminae. Nonetheless, it not clear whether upregulation of COX-1 in the superficial laminae and decrease in COX-1 in the deep laminae cause Aβ and C fibers sprouting or *vice versa* or whether these two are caused by a third factor.

In summary, two types of peripheral nerve injury associated with hyperalgesia and allodynia show similar changes in COX-1 expression weeks after injury. COX-1-IR increases in the superficial dorsal horn ipsilateral to injury and decreases in the deeper dorsal horn, ventral horn, and white matter. These data support a change in COX-1 in the spinal cord after nerve injury, unlike find-

ings with peripheral inflammation, and suggest targeting spinal COX-1 inhibition could relieve neuropathic pain.

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