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Original Article

Cellular sources of cyclooxygenase-1 and -2 up-regulation in the spinal dorsal horn after spinal nerve ligation

by

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Key words: Neuropathic pain; spinal cord; microglia; astrocyte; neuron; COX

Abstract

Aims: Recent studies suggested that the development of neuropathic pain associated with neural injury may be partly due to up-regulation of cyclooxygenase (COX) in the CNS. However, the cellular sources of COX-1 and COX-2 up-regulation following nerve injury are unclear. Methods: We investigated the spinal cellular sources of COX-1 and COX-2 in association with allodynia following L5 spinal nerve ligation (SNL). Results: Post-SNL pain-related behaviour was shown by increased sensitivity to mechanical stimulation. There was a significant increase in both COX-1 and COX-2 immunoreactivity \((p<0.01)\) on the ipsilateral side of spinal dorsal horn. Double immunofluorescence labeling demonstrated that COX-1 immunoreactive cells co-localized chiefly with dorsal horn neuronal nuclei and microglia, whereas COX-2 was expressed in neuronal cytoplasm. Conclusion: These findings demonstrate that while spinal dorsal horn neurons are important source of COX-1 and COX-2 after nerve injury, microglia also contribute to the pathogenesis of neuropathic pain, partly by producing additional COX-1.
**List of abbreviations**

<table>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>COX</td>
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<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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<td>IR</td>
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<td>PLP</td>
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Introduction

Neuropathic pain caused by damage to or dysfunction of the nervous system is characterized by spontaneous pain and allodynia in which normally innocuous tactile stimuli can cause pain. Accumulating evidence in recent studies shows that glial cells in the central nervous system (CNS) in particular in the spinal cord play somewhat important roles in the development of neuropathic pain [1]. After nerve injury, glial cells in particular microglia may proliferate and become activated [2, 3], and they may release inflammatory mediators such as cytokines and prostanoids, which may in turn activate and/or enhance the sensitivity of primary afferents and spinal cord neurons [4]. Among other molecules and mediators, cyclooxygenases COX-1 and COX-2 that are the enzymes involved in the production of prostaglandins (PGs) via arachidonic acid pathways appear to play certain functional roles in inflammation and pain processing [5, 6].

COX-2 may play an important role in neuropathic pain. COX-2 immunoreactivity increases at the site of nerve injury in the partial sciatic nerve ligation model [7] and in the spinal dorsal horn in the spinal nerve ligation (SNL) model [8]. COX-2 can enhance the conversion of PGs from arachidonic acid to increase the excitability of nociceptive neurons to peripheral stimulation, resulting in central sensitization [9, 10].
Relatively little is known about the involvement of COX-1 in neuropathic pain. Nevertheless, the classical view that COX-1 is constitutive, in contrast to COX-2 induced in inflammation, has now been challenged, as both isoforms are present in the CNS, and both can be induced and/or up-regulated in the spinal cord after nerve injuries [8, 11-15]. The involvement of COX-1 in neuropathic pain was indirectly supported by the fact that COX-1 selective inhibitor (SC-560) administration in the early stages could significantly reverse the development of mechanical allodynia after spinal nerve injury [16].

Despite the clear up-regulation of COX-1 and COX-2 after nerve injuries and their roles in the pathogenesis of neuropathic pain, a question that remains to be answered is where COX-1 and COX-2 are produced. Microglia (and some other glial cells) may be a culprit since many previous studies have revealed microglial involvement in neuropathic pain [17].

In this study we set out to identify the cellular sources for the up-regulation of COX in the rats following spinal nerve injury by measuring COX-1- and COX-2 immunoreactivities (IRs) in the spinal dorsal horn. The cellular and subcellular
co-localization of COX-1 and COX-2 proteins was determined by double immunofluorescence labeling of COX and specific cellular markers. The hypothesis to be tested was that glial cells, in particular microglia, may be an important additional source of COX up-regulation in neuropathic pain.

**Materials and Methods**

**Animal model of mechanical allodynia**

Twenty male Sprague-Dawley (SD) rats were randomly divided into two groups to serve as SNL or sham control. L5 spinal nerve was ligated according to the procedure described [18]. Briefly, under deep anaesthesia with sodium pentobarbital (Nembutal, 50mg/kg, i.p., Boehringer Mannheim), the rats’ L5 spinal nerve was isolated and tightly ligated with 6-0 silk thread in half (5) of the animals. Similar procedure for sham control was performed for the remaining half (5) animals but their spinal nerve was not ligated. All the experimental protocols and animal handling procedures were approved by the ethics committee on the use of human and animal subjects in teaching and research ethics, Hong Kong Baptist University, and all effects were made to ensure that both animal numbers and suffering were minimal.

**Neuropathic mechanical hypersensitivity**
Behavioural tests were performed to assess mechanical allodynia by measuring paw withdrawal threshold (PWT) one day before SNL as baseline control and then the measurement was repeated on day 3 and 7 after surgery. The detailed methods were described elsewhere [8, 19].

**Immunohistochemistry**

At the end of experiment (post-surgery Day 7), the rats were overdosed with pentobarbital and perfused intracardially with cold saline, followed by paraformaldehyde-lysine-periodate (PLP) solution that consisted of 2% paraformaldehyde, 75mM L-lysine and 0.01mM periodate in phosphate buffer (pH 7.4). The lumbar enlargement around L5 spinal segment was collected from the perfused animals. Tissues were postfixed overnight in PLP fixative, cryopreserved in 30% sucrose for at least 48 h, snap frozen in dry ice-cooled isopentane and stored at -80°C until used.

Transverse sections (30µm) were cut by cryostat and processed for immunohistochemical staining as previously described [8]. Sections were blocked with 5% goat serum in 0.3% Triton X-100 for 1 hour at room temperature and then incubated overnight at 4°C with primary antibody against COX-1 (1:300, Cayman Chemical, Ann Arbor, MI, USA) or COX-2 (1:150, Cayman Chemical, Ann Arbor,
MI, USA). The sections were then incubated with Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (1:1000, Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature.

For double immunofluorescence staining, spinal sections were incubated overnight at 4°C with a mixture of rabbit anti-COX-1 or anti-COX-2 antibody and mouse anti-neuronal nuclei marker (NeuN, 1:1000, Chemicon Millipore, Temecula, CA, USA), mouse astrocyte marker GFAP (1:1000, Chemicon Millipore, Temecula, CA, USA) or mouse microglia marker anti-CD11b/OX-42 (1:100, Serotec, Oxford, UK) antibodies. Afterwards the sections were incubated with a mixture of Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (1:1000, Invitrogen, Carlsbad, CA, USA) and Alexa Fluor® 568 goat anti-mouse IgG secondary antibody (1:1000, Invitrogen, Carlsbad, CA, USA). The sections were mounted with fluorescent mounting medium (DakoCytomation, Carpinteria, CA, USA) that contains 4,6-diamino-2-phenylindole (DAPI, Biotium, Hayward, CA, USA) for nuclear staining. Slides where primary antibody was omitted served as a negative control to ensure no autofluorescence from the secondary antibody in the reaction sequences of the labeling experiments.

*Image analysis*
Similar to previously described [19], five slides (30µm) of L5 segments were selected randomly from all animals in each group for the measurement of immunoreactivity in the dorsal horn. To determine the levels of immunoreactivity of COX-1-IR and COX-2-IR, digital images were captured by fluorescence microscope with same parameters. The intensity of immunoreactivity per section was measured and analyzed using Image J NIH software, with an average derived from 5 sections for each animal and expressed as mean ± S.E.M.

**Statistical analysis**

Data were compared by means of Student’s paired $t$ test or one-way ANOVA followed by Dunnett’s post hoc comparisons to assess group differences. $P < 0.05$ was considered significant.

**Results**

**Mechanical allodynia**

Baseline measurement of paw withdrawal thresholds (PWTs) on both hind-paws did not show any significant difference between sham-control and SNL groups prior to
the operation. In naïve control, no significant difference was found (data not shown). After SNL, mechanical hypersensitivity was observed since the PWTs on the ipsilateral hind-paw significantly decreased at 3 (19.46±1.51g) and 7 (21.77±1.04g) days after SNL in comparison with those of the sham-control group at corresponding days and site (31.59±2.28g and 33.38±2.25g, respectively; ***p<0.001) (Fig. 1; Table 1), as well as when compared with the PWTs of the corresponding pre-operation baseline (31.53±0.57g, ###p<0.001). These results demonstrated that the ipsilateral hind-paw of SNL rats developed a marked allodynia to mechanical stimulation after SNL.

**Up-regulation of COX-1 and COX-2 in dorsal horn**

The immunoreactivities (IR) for COX-1 and COX-2 were examined in the L5 spinal dorsal horn collected 7 days after SNL (Fig. 2). In the superficial (particularly laminae I-II) dorsal horn of SNL rats, there was an increase in COX-1-IR (Fig. 2A) and COX-2-IR (Fig 2B) in the ipsilateral side when compared with that of the contralateral side. Both COX-1 and COX-2 IR were most prevalent in the superficial dorsal horn instead of the deeper laminae. The semi-quantitative analysis results illustrated that both COX-1-IR and COX-2-IR increased significantly in the spinal dorsal horn ipsilateral to SNL (p<0.01, Student’s paired t test). To confirm that
antibody labeling was specific, control experiments where the primary antibody was omitted were carried out. No immunofluorescence was detected when the primary antibody was absent (Data not shown).

**Cellular and subcellular sources of COX-1 and COX-2**

To identify the specific cell types that expressed COX-1 and COX-2 after SNL, double immunostaining was performed for the cells expressing COX-1-IR or COX-2-IR with cell-specific markers: NeuN for neurons, GFAP for astrocytes and OX-42 for microglia.

COX-1 immunoreactive cells in the spinal dorsal horn at Day 7 post-SNL were predominantly co-localized with neuronal marker NeuN (Fig. 3c) and microglia marker OX-42 (Fig. 3f) but not the astrocyte marker GFAP (Fig. 3g-i). Although the methods we used did not allow us to quantify the COX-1 source separately from neurons and microglia, it appears that neurons in particular their nuclei were a dominant source (Fig. 3a-c) whereas microglia cells were an additional important source of COX-1 (Fig. 3d-f).
For COX-2, almost all COX-2 immunoreactive cells in the spinal dorsal horn were co-localized with the neuronal marker NeuN (Fig. 4c) but not with OX-42-positive (Fig. 4f) nor with GFAP-positive cells (Fig. 4i). In contrast to COX-1 that appeared to be expressed in both neurons and microglia, COX-2 could only be observed in neurons.

Since the double immunostaining demonstrated that COX-1 was particularly expressed by neuronal nuclei (Fig. 3a-c), nuclear marker DAPI was then added to confirm subcellular localization of COX-1. The results revealed that COX-1 was indeed predominantly expressed in the nucleus of neurons (Fig. 5A), with only trace amount detectable in the cytoplasm. In contrast, COX-2 was expressed only in the cytoplasm of neurons as there was no co-localization with DAPI (Fig. 5B).

**Discussion**

In this study, we examined COX up-regulation and its sources in the spinal dorsal horn after SNL. Up-regulation of COX-1 and COX-2 was found in the ipsilateral lumbar superficial dorsal horn 7 days after SNL along with the development of mechanical allodynia. For the first time, we demonstrated that COX-2 was
prominently in neuronal cytoplasm, whereas COX-1 was from neuronal nuclei and microglia in the superficial dorsal horn.

This study is in line with previous findings in that both COX-1 and COX-2 can be induced and/or up-regulated in the spinal cord after nerve injuries [8, 11-15]. In contrast to COX-2 that has been well shown to be involved in the development of mechanical allodynia [8, 11-15], relatively little is known about the role of COX-1 in neuropathic pain. Nevertheless, COX-1 must be involved in the pathogenesis of neuropathic pain, as evidenced by the facts that prolonged COX-1 expression has been observed in spinal microglia during central sensitization [20], COX-1 deficient mice show an increased threshold to nociceptive stimuli, and that the COX-1 selective inhibitor (SC-560) could significantly reverse the development of mechanical allodynia after spinal nerve injury [16]. A recent study from Kanda demonstrated that prostaglandin D2 (PGD2), a potent inflammatory mediator, produced by microglia is COX-1 dependent, and neurons in the spinal cord can receive PGD2 from microglia following peripheral nerve injury. The intrathecal injection of COX-1 inhibitor significantly attenuated the mechanical allodynia in nerve injury [21]. The fact that COX-1, in contrast to COX-2, is found predominantly in neuronal nuclei and in microglia is perhaps a reflection that COX-1 is more constitutional while COX-2 is
more induced, and COX-1 up-regulation in the spinal cord is largely attributable to microglia activation.

Previously Durrenberger et al. observed an increase in COX-2 expression in human injured nerve and in rat chronic constriction injury (CCI) model, and later showed the time course of COX-2 up-regulation [22, 23]. Interestingly, in contrast to the injured nerve, spinal COX-2 in CCI rats was found to be decreased at all time points in their latter study. They suggested that the COX-2 up-regulation was due to macrophage infiltration as there was an increase in the number of microglial-like COX-2 immunoreactive cells in the peripheral nerve and dorsal root ganglia. In this study, we used the SNL model and demonstrated that spinal dorsal horn neurons are important source of COX-1 and COX-2 while microglia also contribute to the pathogenesis of neuropathic pain, partly by producing additional COX-1. In our study, COX-2 was found to be co-localized predominantly in the spinal dorsal horn neurons after SNL. Although this finding is not consistent with some previous reports [22, 23], several previous studies also demonstrated the expression of COX-2 protein in rat spinal dorsal and ventral horn neurons [24-26], while some other studies also showed the presence of COX-2 in radial glia [27], astrocytes [24], or endothelia [28]. Though we also observed trace COX-2 in some of the radial glia in the white matter, its role in neuropathic pain remains unknown and remains to be further invested. Furthermore, it
remains to be investigated whether treatment with COX inhibitors, including specific and non-selective COX inhibitors, could inhibit spinal nerve ligation-induced mechanical hypersensitivity.

One of the important findings of this study is COX-1 up-regulation in the spinal dorsal horn. We found that COX-1 is not only from neurons but also from microglia. However, due to the limitation of the methods used in this study, we could not quantify the COX-1 up-regulation from neurons and microglia separately, neither could we identify whether COX-1 up-regulation was attributable to activation of the endogenous microglia, newly proliferated cells, or macrophages migrated into the nervous tissue, since most microglia were clustered together with their processes tangled (Fig. 3f). However, a previous study [3] by means of more sophisticated methods has shown that axonal injuries could induce microglia proliferation, and lesion-reactive microglia accounted for the vast majority of proliferating cells in the dentate gyrus after damaging projecting axons to the region, and 24% of all microglial cells were proliferating 3 days post-lesion. As they found that actively proliferating microglial cells are clustered, and our study also showed that COX-1 expression was particularly strong on clustered OX-42 positive microglial cells (Fig. 3f), it appears that COX-1 up-regulation is chiefly from these proliferating microglia.
apart from neurons, and microglia may contribute to the pathogenesis by COX-1 expression apart from making other new cytokines and proteins [17].

In this study, we found that the dorsal horn neurons were a major source for both COX-1 and COX-2. It appears that damaged neurons may play an important role and interplay with microglia to up-regulate COX in the pathogenesis of neuropathic pain. The damaged neurons may perhaps work as an initiator by releasing prostaglandins, nitric oxide, substance P, excitatory amino acids etc for other chain reactions including activation of glial cells in the CNS. A number of studies have shown some functional changes of spinal dorsal horn neurons in mechanical allodynia caused by traumatic nerve injury [29-31]. Takeda et al. [32] suggested that the spinal PG surge following COX-2 up-regulation may directly or indirectly activate spinal glia, hence contributing to the neuropathic hypersensitivity. After a peripheral nerve injury, sensitization occurs which is characterized by increased spontaneous activity of neurons, a lowered threshold and increased response to a given stimulus.

Whether and how the pathophysiological and functional changes are related to COX up-regulation have been investigated. A previous study revealed that COX-2 may act as a multifunctional neuronal modulator [33]. In rodents, COX-2 is up-regulated in spinal cord and brain in response to nerve injury, leading to increased spinal cord prostaglandin E2 (PGE2) synthesis, and hence to central sensitization, allodynia, and
hypersensitivity, whereas COX-2 inhibition prevented the increase in spinal cord PGE2 concentrations caused by the injury and attenuated the pain response [34-37]. The COX-2 inhibitor, meloxicam, exerts antiallodynic effects on established neuropathic pain in diabetic mice, which indicates that COX-2 inhibitor can acts as a prominent drug for treatment of diabetic neuropathic pain [38].

This study showed that microglia were an important source of COX-1 apart from neurons (Fig. 3i,f). There is increasing evidence supporting a role for microglia in the pathogenesis in pain and in the maintenance of neuronal homeostasis [17, 39]. In animal models of mechanical allodynia, activation of microglia was consistently observed. Spinal microglia contribute to the development and maintenance of mechanical hypersensitivity after a variety of peripheral nerve injuries, including SNL [40] and chronic constriction nerve injury [41]. A microglia inhibitor, minocycline, may reduce mechanical allodynia, possibly by suppressing p38 activation [42]. Recently, the injection of activated microglia into the dorsal horn has been shown to induce mechanical allodynia, while depletion of activated microglia has been found to block pain induction [43, 44].

In this study we only selected 7th day after SNL for COX investigation. This was based on our hypothesis that indigenous glia cells are involved in neuropathic pain by producing COXs, and our preliminary investigation showed that the activation of glial
cells reaches a peak at Day 7 after SNL. This was consistent with some previous findings. For example, Tanga et al showed the markers of microglial activation (ITGAM, TLR4, and CD14) increased significantly after nerve transaction, peaked at 4 to 7 days and began to decline afterward [45]. In the study by Zhu et al. (2003), the number of COX-1-IR cells increased in cells with glial morphology in the superficial laminae of the ipsilateral spinal cord 4 days after SNL but decreased in the rest of the ipsilateral spinal cord 4 weeks after PSNT and 2 weeks after SNL. However, different studies have presented rather confusing results previously. For example, in spared nerve injury (SNI) model, an increase in COX-2 protein was observed in the dorsal horn at 24 hours post surgery which then returned to sham levels at 72 hours [46]. Similar findings were also reported by Zhao [14]. In rat chronic constriction injury (CCI) model, in contrast to the up-regulation of COX-2 in the injured peripheral nerve, spinal COX-2 was found to be decreased at all time points at days 4, 21, and 30 after the nerve injury in the rats [22, 23]. In L5 SNL model, COX-2 positive cells were co-expressed with the Schwann cell marker at Day 1 and followed by co-expression with macrophage marker 7 days post-surgery [47]. COX-1 protein was found to be increased 4 days after SNL and decreased 2 weeks after SNL [15]. In contrast, COX-1 protein was not detectable on 1, 3 and 14 days after L5/L6 ligation [14].
It was puzzling that in the study by Durrenberger et al (2006) that, in contrast to their earlier report [22], COX-2 was down-regulated at all time points 4, 21 and 30 days after constrictive nerve injury in the rat, but our previous studies showed that at least COX-2 was increased and still significantly up-regulated 38 days after SNL [8]. It still remains to be studied how long this COX-2 (and COX-1) up-regulation may last and whether it is associated with neuropathic pain development and maintenance in long term. In our opinion, dynamic COX changes in the spinal cord could be due to recruitment of macrophages or fluctuation of a cocktail of inflammatory mediators that can be influenced by many factors.

Experimental data on the cellular localization of COX-1 and COX-2 remain controversial. Early studies indicated, in different tissue types though, that in 3T3 (fibroblast) cells, both COX-1 and COX-2 localized in the nuclear envelope and endoplasmic reticulum [48]. In vascular endothelial cell, both COX-1 and COX-2 have nuclear and cytoplasmic localization, and COX-2 travels between the nucleus and the cytoplasm upon IL-1β stimulation [49]. This study revealed, for the first time in the spinal cord, that COX-1 up-regulation in the spinal dorsal horn is chiefly from neuronal nuclei and microglia, whereas COX-2 up-regulation is from neuronal cytoplasm. The exact contribution of these two isoforms to neuropathic pain remains unclear, but the distinct distribution pattern of the two suggests that COX-1 and
COX-2 may play somewhat different roles in the pathogenesis of neuropathic pain.

Despite COX-1 and COX-2 share similar structure and catalytic properties, they use different pools of arachidonic acid [50] and have distinct function in cell proliferation and differentiation [51-53]. Researchers have proposed that COX-1 and COX-2 may acquire arachidonic acid from different phospholipases [54, 55]. It has also been shown that COX-1 and COX-2 exhibit differences in their subcellular localization and ability to metabolize arachidonic acid by working in independent prostanoid biosynthetic systems [56], and each COX enzyme has been reported to have a distinct subcellular localization and a functional coupling to constitutive and inducible membrane-associated prostaglandin E₂ synthase enzymes, the enzymes responsible for the final conversion of PGH₂ to PGE₂ [57, 58]. Thus, it appears that COX-1 and COX-2 may play somewhat different roles in pain mechanisms at different locations.

Nevertheless, COX-1 in neuropathic pain is relatively less investigated, perhaps partly due to its constitutive nature in other body tissues, such as the gastrointestinal tract, and inhibition of it could be associated with severe side effects. It is noted that in Fig 5 that COX-1 is present in the cytoplasm of some but not all of the neurons. This was perhaps due to existence of different subgroups of neurons and COX-1 up-regulation may be related to receptors in different subpopulations of neurons. In this connection, Chopra et al. [59] reported that COX-1 is a marker for a subpopulation of putative...
nociceptive neurons in rat dorsal root ganglia. In this regard, it is perhaps worth investigating further in future studies what types of neurons (e.g. small vs big) at what locations (e.g. superficial vs deep laminae) would have the potential for what subtypes of COX up-regulation, and what receptors are involved, respectively.

In summary, our findings demonstrated that there was an up-regulation of COX-1 and COX-2 expression in the spinal cord of rats along with the development mechanical allodynia after SNL. The SNL induced up-regulation of spinal COX-1 and COX-2 proteins was co-localized in different cell types. COX-2 was predominantly expressed in the cytoplasm of dorsal horn neurons, whereas COX-1 up-regulation appeared chiefly from the nuclei of dorsal horn neurons but also from microglia that appeared to be activated and proliferating after the nerve injury.

**Competing interests**

We declare that there are no competing interests with other people or organizations

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participated the behavioral and immunohistochemical experiments. YM conducted most parts of the experiment and SC participated the image acquisition and statistical analysis. YM, AL and HQ interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

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Cyclooxygenase-1 is a marker for a subpopulation of putative nociceptive neurons in 

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Figure legends

Figure 1. Mechanical hypersensitivity after SNL. PWTs were measured before, and on 3 and 7 days after SNL. PWTs on ipsilateral side of injury were significantly decreased following L5 SNL (mean ± SEM; n = 5 for each group; one-way ANOVA followed by Dunnett’s post hoc test). ***p<0.001 when compared with PWTs of the sham control group; ###p<0.001 when compared with baseline PWTs of the same group obtained on the pre-operative day.

Figure 2. The fluorescent photomicrographs demonstrating an induction and up-regulation of COX-1 and COX-2 expression in spinal cord at day 7 after SNL. (A) There was a significant increase in COX-1-IR, in particular in superficial laminae I-II, in spinal dorsal horn ipsilateral to the nerve injury. (B) There was an increase in COX-2-IR in the spinal dorsal horn ipsilateral to the nerve injury (scale bar=200 µm). Quantitative analysis of COX-1-IR and COX-2-IR was shown on the right where the data were presented as mean COX-IR ± S.E.M for comparisons of COX-1 (up) and COX-2 (low) between the ipsilateral and contralateral sides of SNL rat spinal dorsal horn. ** and ## represent statistically significant difference with p <0.01 (Student’s paired t test) for COX-1-IR and COX-2-IR respectively.
Figure 3. Co-labeling to identify the cellular sources of COX-1 in the spinal dorsal horn of SNL rats. Double immunofluorescence for COX-1 (a, d, g, green) and NeuN, a neuronal marker (b, red), OX-42, a microglia marker (e, red) and GFAP, an astrocyte marker (h, red). Double immunofluorescence indicated COX-1 positive cells co-localized with NeuN and OX-42 (arrows) in the spinal dorsal horn 7 days after SNL (magnification x400; scale bar=50µm applies to all).

Figure 4. Double immunofluorescence showing COX-2 (a, d, g, green) co-localized with NeuN (b-c, red) but not with OX-42 (e-f, red) or GFAP (h-i, red), in the spinal dorsal horn 7 days after SNL. Arrows in (c) point to COX-2/NeuN co-localized neurons (magnification x400; scale bar=50µm applies to all).

Figure 5. Nuclear staining with DAPI to identify the subcellular sources of COX-1 and COX-2 in the neurons of spinal dorsal horn. (A) COX-1 (i, green) was localized predominantly within the nuclei (iii, iv, blue) of NeuN-positive cells (ii, red). The arrows indicated the location of COX-1-positive nuclei in neurons. (B) COX-2 (i, green) was absent in the nucleus (iii, iv, blue), but localized mainly in the cytoplasm of neuron (ii, iv, red). The arrows indicated the location of COX-2 in the cytoplasm of
neurons. Nuclei were counterstained with DAPI (blue) (magnification x600; scale bar=50μm applies to all).

**Table**

Table 1. Mechanical hypersensitivity on different days after SNL

<table>
<thead>
<tr>
<th>Time after SNL (days)</th>
<th>Paw Withdrawal Threshold (gm)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 3</td>
<td>Day 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ipsi</td>
<td>Control</td>
<td>Ipsi</td>
<td>Control</td>
</tr>
<tr>
<td>SNL</td>
<td>31.53±0.57</td>
<td>32.55±0.97</td>
<td>19.46±1.51</td>
<td>34.25±1.63</td>
</tr>
<tr>
<td>Sham</td>
<td>32.79±0.70</td>
<td>33.73±0.95</td>
<td>31.59±2.28</td>
<td>33.83±2.25</td>
</tr>
</tbody>
</table>

Significant differences are indicated by ***p<0.001 when SNL group compared with the sham control group (n = 5 for each group; one-way ANOVA)
Fig. 1

![Graph showing paw withdrawal threshold (g) over different time points after surgery.](image)

- SNL ipsi
- Sham ipsi
- SNL contral
- Sham contral

**Bars represent the mean ± standard error.***

### Days after surgery:
- **Before**
- **SNL**
- **Day 3**
- **Day 7**

Significance:
- *****:** p < 0.001
- **###:** p < 0.0001

Note: Additional details and analysis would be included in the full text.
Fig. 2

A

Ipsilateral

COX-1

Contralateral

B

Ipsilateral

COX-2

Contralateral

Bar

7 days after injury

COX-1 IR

COX-2 IR

SINL ipsi

SINL Contral

**

###

7 days after injury
Fig. 5

A

i

COX-1

ii

NeuN

iii

COX-1/DAPI

iv

COX-1/NeuN/DAPI

B

i

COX-2

ii

NeuN

iii

COX-2/DAPI

iv

COX-2/NeuN/DAPI