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Spinal Expression of Hippo Signaling Components YAP and TAZ Following Peripheral Nerve Injury in Rats

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Abstract

Previous studies have shown that the morphology and number of cells in the spinal cord dorsal horn could change following peripheral nerve injury and that the Hippo signaling pathway plays an important role in cell growth, proliferation, apoptosis, and dendritic remodeling. In the present study, we examined whether the expression of YAP and TAZ, two critical components regulated by Hippo signaling, in the spinal cord dorsal horn would be altered by chronic constriction sciatic nerve injury (CCI). We found that 1) YAP was mainly expressed on CGRP- and IB4-immunoreactive primary afferent nerve terminals without noticeable expression on glial cells, whereas TAZ was mainly expressed on spinal cord second order neurons as well as microglia; 2) upregulation of YAP and TAZ expression followed two distinct temporal patterns after CCI, such that the highest expression of YAP and TAZ was on day 14 and day 1 after CCI, respectively; 3) there were also unique topographic patterns of YAP and TAZ distribution in the spinal cord dorsal horn consistent with their distinctive association with primary afferents and second order neurons; 4) changes in the YAP expression were selectively induced by CCI but not CFA-induced hindpaw inflammation; and 5) the number of nuclear profiles of TAZ expression was significantly increased after CCI, indicating translocation of TAZ from the cytoplasm to nucleus. These findings indicate that peripheral nerve injury induced time-dependent and region-specific changes in the spinal YAP and TAZ expression. A role for Hippo signaling in synaptic and structural plasticity is discussed in relation to the cellular mechanism of neuropathic pain.

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Conflict of interest statement

There is no conflict of interest in this project.

Keywords

Neuropathic pain; Hippo signaling pathway; spinal cord; dorsal horn; proliferation; dendrite; synaptic plasticity; structural plasticity

Introduction

Neuropathic pain often results from complicated neurological disorders such as neuropathy, spinal cord injury, multiple sclerosis and stroke (Cruccu et al., 2010). The current mainstay of neuropathic pain treatment relies heavily on medications that provide only symptomatic management. An alternative approach to improving neuropathic pain treatment is to better understand disease processes causing neuropathic pain in order to shift the strategy from symptomatic management to targeting the underlying mechanism of neuropathic pain (Finnerup et al., 2010).

To date, a number of mechanisms of neuropathic pain have been proposed, including ectopic discharges, sensitization of nociceptors, phenotypic switching, disinhibition, and neuroinflammation (Berger et al., 2011; Caterina et al., 2000; Devor, 2009; Eijkelkamp et al., 2010; Hughes et al., 2007; Wei et al., 2010). However, it is well known that peripheral nerve injury can change the shape, size and number of dendritic spines. Cell apoptosis has also been associated with neuropathic pain following nerve injury. For instance, although sciatic nerve lesion alone failed to produce neuronal cell death in laminae I–III of the rat's spinal dorsal horn, a combination of sciatic nerve lesion and stimulation of myelinated fibers resulted in neuronal cell death in superficial layers of the spinal cord dorsal horn (Coggeshall et al., 2001). At least one of the consequences of neuronal cell death is the loss of GABAergic inhibitory interneurons following peripheral tissue injury (Mao et al., 2002; Scholz et al., 2005).

Recently, the Hippo signaling pathway has been shown to play an important role in neuronal development and diseases (Emoto, 2011). Activation of mammalian sterile 20-like 2 (MST2), a core component of Hippo signaling, induced neuronal cell death (Liu et al., 2012). On the other hand, Yes kinase-associated protein (YAP) is a downstream of Hippo signaling, which serves as a positive regulator of cell proliferation but a negative regulator of cell differentiation during mammalian neurogenesis (Zhang et al., 2012a), possibly by regulating sonic hedgehog homolog (shh) signaling (Fernandez et al., 2009; Lin et al., 2012). Loss of function of key Hippo signaling components leads to changes in cell proliferation, cell survival, tissue overgrowth, as well as cell shape and organ size (Boggiano and Fehon, 2012; Hipfner and Cohen, 2004; Justice et al., 1995; Udan et al., 2003; Zhang et al., 2012b). Furthermore, transcriptional coactivator with PDZ-binding motif (TAZ) is a transcriptional coactivator of YAP. The sequence of TAZ is similar to that of YAP despite their differences in the N-terminal proline-rich domain, second WW domain, and SH3 binding motif (Zhao et al., 2008). These findings suggest a similar functional role for YAP and TAZ because both are negatively regulated by the Hippo signaling pathway in mammals (Hao et al., 2008; Oka et al., 2008; Zhang et al., 2008; Zhao et al., 2007).

Therefore, it is possible that both YAP and TAZ as transcriptional coactivators play a role in cell growth and proliferation following peripheral nerve injury. In this study, we examined whether the expression of YAP and TAZ in the spinal cord dorsal horn would be altered following chronic constriction sciatic nerve injury (CCI) (Bennett and Xie). We found, for the first time, that 1) YAP and TAZ are differentially expressed in neuronal and glial cells with distinct topographic distribution patterns, 2) their expression was upregulated after CCI

but at different time points, and 3) the YAP expression appeared to be selectively induced by nerve injury and was unchanged after CFA-induced inflammation.

Results

Expression of YAP and TAZ in the spinal cord dorsal horn

To identify the pattern of YAP and TAZ expression in the spinal dorsal horn in the absence of peripheral nerve injury, we first examined whether YAP and TAZ would be expressed on CGRP-immunoreactive and IB4-immunoreactive primary afferent nerve terminals as they represent peptide and non-peptide sensory neurons, respectively. We found that YAP (Fig. 1A, B) was extensively expressed on CGRP- as well as IB4- immunoreactive primary afferent nerve terminals. Specifically, YAP was co-expressed with CGRP-positive primary afferents in both lamina I and the outer layer of lamina II, as well as IB4-positive primary afferents in the inner layer of lamina III. In contrast, TAZ expression (Fig. 2A, B) was primarily associated with spinal cord second order neurons with little or no expression on primary afferent nerve terminals. The results indicate that YAP is expressed in both large and small primary sensory neurons, whereas TAZ is present primarily in spinal second order neurons.

Expression of YAP and TAZ on glial cells

We then examined whether YAP and TAZ would be expressed on glial cells in the spinal cord dorsal horn. At the L4–L5 level, both YAP and TAZ showed little co-expression with the astrocyte marker GFAP (Fig. 1C, 2C). However, TAZ was double-labeled with the microglial marker Iba1 (Fig. 1D, 2D), whereas no co-expression was detected between YAP and Iba1 (Fig. 2). Thus, only TAZ was expressed in microglial cells, and neither YAP nor TAZ in astrocytes, within the spinal cord dorsal horn.

Topographic distribution patterns of YAP and TAZ expression after CCI

Nociceptive threshold for both mechanical and thermal withdrawal on ipsilateral hindpaw was significantly decreased on post-CCI day1 and remained low up to at least post-CCI day14, as compared with sham-operated rats (Fig 3A,B, $n = 8$; $*P < 0.05$). There were no differences in withdrawal threshold on the contralateral hindpaw (Fig 3 A,B, $n = 8$; $P > 0.05$). After CCI, topographic distribution patterns of YAP and TAZ expression were noticeably different in the ipsilateral spinal cord dorsal horn (Fig. 4). Specifically, YAP was expressed only in lamina I–II, whereas TAZ expression was diffusely distributed across the spinal cord dorsal horn (Fig. 4). These distribution patterns are consistent with the basal expression of YAP (primary afferents) and TAZ (second order neurons) in the spinal cord dorsal horn, indicating that peripheral nerve injury enhanced the YAP and TAZ expression but did not alter their respective expression patterns.

Temporal patterns of YAP and TAZ expression after CCI

YAP expression was increased on day1, whereas TAZ expression was increased on both day1 and day7 but returned to baseline on day14 after CCI (Fig. 4B, C, D). In the ipsilateral spinal cord dorsal horn, the highest level of YAP expression was detected on day14 after CCI (Fig. 5A, $n=8$, $*P < 0.05$ on day1 and day7, $**P < 0.01$ on day14). In contrast, the highest level of TAZ expression occurred on day 1 after CCI (Fig. 5C, $n=8$, $**P < 0.01$ on day1, $*P < 0.05$ on day7, $P > 0.05$ on day14). In the contralateral spinal cord dorsal horn, neither YAP nor TAZ expression was significantly different after CCI, as compared with sham-operated rats (Fig. 5B, 5D, $n=8$, $P > 0.05$). These results demonstrate different temporal patterns of YAP and TAZ expression in the spinal cord dorsal horn following CCI.

Lack of changes in YAP expression after CFA-induced hindpaw inflammation

We then examined whether a similar change in the spinal YAP expression would be induced in rats with hindpaw inflammation induced by injecting 50 μ l complete Freund's adjuvant (CFA) into an ankle joint. Different from the change in YAP expression observed in CCI rats, CFA induced hindpaw inflammation but did not change YAP expression in the ipsilateral dorsal horn when examined on day 7. We chose this time point to examine the YAP expression after CFA because rats with CFA showed the most significant behavioral change on day 7. The western blot analysis did not detect a significant change in the level of spinal YAP expression on day 7 as compared with sham rats (Fig. 6, $n=4$, $P > 0.05$). The results indicate that the spinal YAP expression was selectively induced by peripheral nerve injury.

Nuclear translocation of TAZ expression after CCI

YAP and TAZ are regulated by core components of the Hippo signaling pathway, which could lead to their translocation to the nucleus (Zhao et al., 2008). Since TAZ was extensively expressed in the spinal cord dorsal horn, we examined whether the number of nuclear profiles of TAZ expression would be increased following CCI. The analysis was carried out by comparing co-expression of TAZ with DAPI in the ipsilateral spinal dorsal horn between sham and CCI rats (day 1). The number of nuclear profiles of TAZ expression was significantly increased after CCI, as compared with sham rats (Fig. 7A, B, $n=3$, $**P < 0.01$). Consistent with its expression on primary afferent nerve terminals, there was no noticeable co-expression of YAP with DAPI in the spinal cord dorsal horn after CCI (Fig. 7C). These results indicate that significant translocation of TAZ expression from the cytoplasm to nucleus occurred after peripheral nerve injury.

Discussion

We have demonstrated, for the first time, that two critical downstream components of the Hippo signaling pathway display different temporal and topographic patterns of expression within the spinal cord dorsal horn following peripheral nerve injury. It is widely accepted that nociceptive signals are transmitted from the periphery to the spinal cord dorsal horn through both peptidergic and non-peptidergic primary sensory afferents (Hunt and Rossi, 1985). CGRP is a neuropeptide consisting of 37 amino acids, which is used as a marker for nerve growth factor (NGF)-dependent primary sensory neurons (Averill et al., 1995). CGRP-immunoreactive terminals were abundant in lamina I and II with a lesser presence in lamina III, V and X of the spinal cord dorsal horn (Gibson et al., 1984). Electrophysiological experiments demonstrate CGRP-induced sensitization of spinal cord dorsal horn neurons (Leem et al., 2001; Sun et al., 2004; Yu et al., 2002). On the other hand, IB4-binding glycoprotein is considered a marker for glial cell line-derived neurotrophic factor (GDNF)-dependent small primary sensory neurons (Molliver et al., 1997). IB4-positive neurons appear to possess characteristic electrophysiological features (Stucky and Lewin, 1999) and frequently innervate GABA-positive interneurons in the spinal cord dorsal horn (Ribeiro-da-Silva, 2004). These previous findings suggest that both CGRP- and IB4-positive primary sensory afferents are contributory to nociceptive transmission (Chen et al., 2010b; Guo et al., 2012; Yu et al., 2009).

In the present study, we found that YAP, but not TAZ, was co-expressed with CGRP-positive primary afferents in both lamina I and the outer layer of lamina II as well as IB4-positive primary afferents in the inner layer of lamina II, suggesting that YAP may be primarily related to regulation of nociceptive transmission in the spinal cord dorsal horn. In contrast, TAZ expression was mainly associated with spinal cord second order neurons with

litter or no expression on primary afferent terminals, suggesting that TAZ is likely to take part in spinal segmental modulation of nociceptive transmission.

Glial cells include astrocytes, oligodendrocytes and microglia (Rocheffort et al., 2002). A number of studies have implicated astrocytes and microglia in the mechanism of chronic pain conditions including neuropathic pain (Garrison et al., 1991; McMahon and Malcangio, 2009; Milligan and Watkins, 2009; Ren and Dubner, 2010; Shi et al., 2012). Our result showed that only TAZ was expressed on microglia but at a much lower level as compared with its neuronal expression. Neither YAP nor TAZ was noticeably expressed on astrocytes, suggesting a possible role for TAZ in the modulation of neuro-glial interaction following peripheral nerve injury.

Of interest is that there are two different temporal patterns of YAP and TAZ expression following peripheral nerve injury, such that the highest level of YAP and TAZ expression occurred on day 14 and day 1, respectively. In addition, the YAP expression was persistently increased throughout the entire experimental period, whereas the TAZ expression began to subside after day 7 of CCI. These unique temporal patterns of YAP and TAZ expression may indicate that TAZ would be involved in initiating a spinal process through a neuro-microglial crosstalk, whereas YAP may play a role in sensitization of primary afferent neurons. Both processes are likely to contribute to the cellular mechanism underlying acute and chronic phases of neuropathic pain (McMahon and Malcangio, 2009; Tanga et al., 2004; Zhang and De Koninck, 2006).

In the present study, we demonstrated a relationship between changes in YAP/TAZ expression and behavioral signs of hyperalgesia and allodynia. However, we were unable to independently confirm a functional correlation between blocking YAP/TAZ expression and improvement of nociceptive behavior in CCI rats, because there are currently no effective *in vivo* tools available to modulate YAP and TAZ function. Nonetheless, the temporal relationship between the onset and maintenance of hyperalgesia and allodynia and the YAP/TAZ expression suggests an important role for these two transcriptional coactivators in this process.

There are at least two possibilities that YAP and TAZ could contribute to the cellular mechanism of neuropathic pain. First, once activated, YAP and TAZ are translocated to the nucleus and bind to the transcription factor TEAD family to promote cell growth and proliferation (Chen et al., 2010a; Mahoney et al., 2005). A possible role for TAZ activation is to promote proliferation of glial cells in the spinal cord dorsal horn, as *de nova* proliferation of glial cells has been demonstrated in animal models of tissue injury (Echeverry et al., 2008; Liu et al., 2000; Milligan and Watkins, 2009). This notion is also supported by the literature showing both changes in microglial morphology (McMahon and Malcangio, 2009) and the density of glial cells (Saur et al., 2013) in response to peripheral tissue injury or physical exercise. Second, YAP expression may regulate the function of IB4-immunoreactive primary afferent neurons, which is likely to innervate GABAergic inter-neurons in the spinal cord dorsal horn. It also has been reported that dendritic fields are essential for neuronal circuit formation and function (Emoto et al., 2004), which is regulated by the Hippo signaling pathway (Emoto et al., 2006). In this regard, a possible role for TAZ expression is to shape the morphology of dendrites of GABAergic inter-neurons, leading to malfunction of GABAergic inter-neurons. This notion would be consistent with the role of altered spinal inhibitory synaptic transmission in chronic pain conditions including neuropathic pain (Todd, 2010). It is also possible that loss of GABAergic inhibitory inter-neurons after nerve injury might serve as a positive feedback for TAZ to be translocated to the nucleus of spinal inter-neurons (Zhao et al., 2007), further influencing their morphology.

A growing body of evidence indicates that there are both similarities and differences in the mechanisms of neuropathic versus inflammatory pain (Alexander et al., 2012; Belkouch et al., 2011; Guan et al., 2010; Ikeda et al., 2012; Kumar et al., 2010; Zhang et al., 2010). In this study, changes in YAP expression were selectively associated with peripheral nerve injury but not a predominately inflammatory condition (CFA-induced hindpaw inflammation). One limitation of the current study is that we only compared YAP but not TAZ expression between CCI and CFA rats. Future studies should examine TAZ expression in CCI and CFA rats to determine whether two Hippo components would play different roles under different pain conditions. Another limitation is that we investigated the translocation of TAZ expression from the cytoplasm to nucleus using immunofluorescence histochemistry. In future experiments, Western blot could be used separately to examine the cytoplasmic and nuclear components of TAZ expression to further confirm its translocation.

To our knowledge, this is the first demonstration of changes in Hippo signaling in relation to neuropathic pain in rats. Future studies may explore the functional importance of the Hippo signaling pathway in chronic pain conditions using both molecular biology and pharmacological tools as they become available.

Methods

Experimental animals

Adult male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 250–300 g were used. Animals were housed in cages with water and food pellets available *ad libitum* and under controlled temperature ($21^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and relative humidity ($50\% \pm 10\%$). The animal room was artificially illuminated from 7 am to 7 pm. The experimental protocol was approved by our Institutional Animal Care and Use Committee and was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

CCI Model

A total of 60 rats underwent CCI or sham operation of the right sciatic nerve as previously described (Bennett and Xie, 1988). Briefly, the sciatic nerve was exposed in the mid-thigh under anesthesia (sodium pentobarbital, 50 mg/kg, i.p.). Using a 4-0 chromic gut, three ligatures were made loosely around the nerve with a 1.5 mm interval between each ligature. The wound was closed with wound clips. Sham-operated rats underwent the same procedure but no ligatures were applied to the sciatic nerve. After surgery, all rats were monitored until they recovered from anesthesia and no antibiotics were administered.

Behavioral Test

Animals were habituated to the test environment once daily (a 60-min session) for two consecutive days before the baseline testing. After habituation to the test environment, the response to thermal and mechanical stimulation was determined on the ipsilateral and contralateral hindpaw before surgery (baseline) and on postoperative days 1, 7 and 14. For thermal hyperalgesia test, paw withdrawal latency was measured using an Analgesia Meter (Model 390, IITC Life Science, Inc.). The radiant heat source was focused on the plantar surface of each hindpaw and light intensity was preset to obtain a baseline latency of approximately 12 s. A cut-off time was set at 20 s to avoid tissue damage. Each rat underwent two trials with a 5-min interval and the mean value of two trials was used as the withdrawal latency. For mechanical threshold test, each rat was placed into a plastic cage with a wire mesh bottom. Mechanical threshold was measured using a von Frey filament set with a calibrated range of bending force. A single filament was applied to the plantar surface for five times with an interval of 5 s. A positive response was defined as at least one clear

withdrawal response out of five applications. Filaments were applied in an up-or-down order according to a prior negative or positive response to determine the threshold withdrawal force.

Immunofluorescence histochemistry

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffer (PB, 0.1M; pH 7.2 to 7.4; 4 °C). Lumbar spinal cords were dissected, post-fixed overnight, and changed to 30% sucrose solution until sample blocks sank to the bottom. Tissues were mounted using Cry O-Z-T embedding medium and transverse spinal cord sections (25 μ m). A free-floating method was used for the staining processes. After being washed 3 times (5 minutes each) with 1 $\times$ PBS, the sections were blocked in 0.1M PBS containing 5–7% goat serum, 1% bovine serum albumin (BSA), and 3% Triton X-100 for 1 hour at room temperature. For double-immunostaining of YAP or TAZ with calcitonin gene-related peptide (CGRP), isolectin B4 Conjugates (IB4), glial fibrillary acidic protein (GFAP), or ionized calcium binding adaptor molecule 1 (Iba1), sections were incubated for 24 hours at 4 °C with first primary antibody YAP (1:2000, Novus, Littleton, CO), TAZ (1:400, Novus, Littleton, CO), CGRP (1:1000, Abcam, Cambridge, MA), IB4 (1:2000, Invitrogen, CA), GFAP (1:400, Santa Cruz, Santa Cruz, CA), or Iba1 (1:100, Abcam, Cambridge, MA). The sections were rinsed 3 times and then incubated with 1:500 cyanine 3-conjugated or FITC-secondary antibody (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) for 1 hour at room temperature. Blue fluorescent DAPI (1:500; Invitrogen) was used to stain the nucleus for 15 minutes. Spinal sections were examined using an Olympus fluorescence microscope, recorded with a digital camera, and processed with Adobe Photoshop CS4 (Adobe System Incorporated, San Jose, CA).

Western Blot

Rats were sacrificed under pentobarbital anesthesia. The L4–L5 spinal dorsal horn was separated and immediately stored on dry ice and kept at –80 °C until use. Separated by each side, samples were homogenized in a buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1mM sodium orthovanadate, 0.01% Triton-X100, 0.01% NP-40) containing a mixture of proteinase inhibitors (Sigma). Tissue homogenates were centrifuged at 7000 rpm for 10 minutes at 4 °C. Protein samples (50 μ g) were loaded and separated on SDS-PAGE gel (12%) and transferred to polyvinylidene difluoride filters (Millipore, Bedford, MA). Membranes were blocked with 1%–5% milk for 1 hour at room temperature and then incubated overnight at 4 °C with a primary YAP antibody (1:2000, Cell Signaling, Danvers, MA), TAZ antibody (1:1000, Santa Cruz, Santa Cruz, CA). This process was followed by 3 washes in PBS and incubation for 1 hour with a HRP-conjugated secondary antibody (goat anti-rabbit 1:5000, Santa Cruz, Santa Cruz, CA) at room temperature on a rocker. The blots were visualized in ECL solution (Thermo Scientific, Waltham, MA) for 5 minutes and exposed onto x-ray films (Kodak, Rochester, NY) for 10–20 minutes. The membranes were then incubated in a stripping buffer (Thermo Scientific, Waltham, MA) for 10 minutes at room temperature, re-probed with a β -actin antibody (1:5000, Sigma, St. Louis, MO), washed in PBS $\times$ 3 and again incubated for 1 hour with a HRP-conjugated secondary antibody (donkey anti-rabbit 1:5000, Santa Cruz, Santa Cruz, CA) as a loading control. The density of each band was measured by Quantity One software (Bio-Rad, Hercules, CA) and normalized against a corresponding loading control band.

Statistical analysis

All results are expressed as mean $\pm$ standard error (SEM). For western blot analysis, the protein expression in a sham group was normalized as 1, and the relative density of other

groups was calculated proportionately. Differences were compared using one way-ANOVA followed by a post hoc test (Turkey) among four groups. For immunofluorescence histochemistry, the number of nuclear profiles was counted under each magnified field ($\times 40$) and compared using unpaired Student's t-tests. Differences with a probability (P) less than 0.05 were considered statistically significant.

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Highlights

1. This study first indicates the potential roles for Hippo signaling components YAP and TAZ in rat peripheral nerve injury.
2. YAP and TAZ are differentially expressed in neuronal and glial cells with distinct topographic distribution patterns.
3. Their expression was upregulated after CCI but at different time points.
4. The YAP expression appeared to be selectively induced by nerve injury and was unchanged after CFA-induced inflammation.

Fig.1. Immunostaining of YAP with CGRP- or IB4-immunoreactivity in the spinal cord dorsal horn

Samples were taken from the ipsilateral L4–L5 spinal cord segments of sham-operated or CCI rats. (A) Co-expression of YAP and CGRP-immunoreactive on primary afferent nerve terminals. (B) Co-expression of YAP and IB4-immunoreactive on primary afferent nerve terminals. (C, D) No noticeable YAP expression with astrocytes or microglia in the spinal cord dorsal horn. Scale bars: 200 μ m.

Fig.2. Immunostaining of TAZ in the spinal cord dorsal horn

Samples were taken from the ipsilateral L4–L5 spinal cord segments of sham-operated or CCI rats. (A, B) TAZ expression was primarily associated with second order neurons in the spinal cord dorsal horn. There was no noticeable co-expression of TAZ with CGRP-immunoreactive or IB4-immunoreactive primary afferent nerve terminals. (C) Little expression of YAP on astrocytes. (D) Co-expression of YAP and Iba-1 (a microglial marker) in the spinal cord dorsal horn. Scale bars: 200 μ m.

Fig. 3. Behavioral changes after CCI

(A) There was a decrease in withdrawal threshold to von Frey filaments on the ipsilateral but not contralateral hindpaw. (B) There was also a decrease in withdrawal latency (PWL) in response to heat stimulation on the ipsilateral but not contralateral hindpaw. * $P < 0.05$, as compared with sham rats on the same side ($n = 8$).

Fig. 4. Distribution of spinal YAP and TAZ expression after CCI

(A) Immunofluorescence image showing the distribution of YAP and TAZ on the ipsilateral dorsal horn of sham rats. (B, C, D) Immunofluorescence images showing the distribution of YAP and TAZ expression in the ipsilateral dorsal horn on day 1 (B), day 7 (C), and day 14 (D). Scale bars: 500 μ m.

Fig. 5. Time-dependent changes in spinal YAP and TAZ expression after CCI

(A) The YAP expression (Western blot) was persistently increased from day1 to day14 in the ipsilateral dorsal horn after CCI. The highest expression was on day14 ($n=8$, $*P < 0.05$, $**P < 0.01$ vs. sham rats). (B) No significant changes in the YAP expression were detected on the contralateral side ($n=8$, $P > 0.05$). (C) The TAZ expression (Western blot) was increased in the ipsilateral dorsal horn after CCI. The highest expression day was on day1 but no significant increases were detected on day 14 ($n=8$, $*P < 0.05$, $**P < 0.01$ vs. sham rats). (D) No significant changes in the TAZ expression were detected on the contralateral side ($n=8$, $P > 0.05$).

Fig. 6. Lack of changes in YAP expression after CFA-induced hindpaw inflammation
Western blot analysis showed that YAP expression was not changed after CFA on day7 (n=4, $P > 0.05$ vs. Control rats).

Fig. 7. Nuclear translocation of TAZ after CCI

(A, B) The number of TAZ-positive nuclear (DAPI) profiles per section (arrows) was increased in the ipsilateral spinal cord dorsal horn on day 1 after CCI (n=3, ** $P < 0.01$ vs. sham rats). (C) YAP was not expressed in the nucleus of spinal cord dorsal horn neurons on day 14 after CCI. Scale bars: 200 μ m.