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N-methyl-D-aspartate receptor activation is downstream coupled to pannexin 1 opening by Src kinase in dorsal horn neurons: an essential link for mechanical hyperalgesia in nerve-injured rats

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Abstract

A well-recognized molecular entity involved in pain-related neuroplasticity is the N-methyl-D-aspartate receptor (NMDAR), which is crucial for developing chronic pain. Likewise, the pannexin 1 (Panx1) channel has been described as necessary for initiating and maintaining neuropathic pain, driving nociceptive signals dependent on spinal NMDAR through several possible mechanisms. Through behavioral, pharmacological, and molecular approaches, our study in male rats has revealed several key findings: (1) neurons located in spinal cord laminae I and II express functional Panx1 channels in both neuropathic and sham rats. These channels can open (indicated by YOPRO-1 uptake) through the stimulation of NMDARs with intrathecal NMDA; (2) intrathecal NMDA leads to increased expression of pSrc and pPanx1 in dorsal horn neurons. This elevation exacerbates existing mechanical hyperalgesia in nerve-injured rats; (3) inhibition of Src with intrathecal PP2 or blockade of Panx1 with intrathecal ^{10}Panx effectively mitigates NMDA-induced effects and reduces the spontaneous mechanical hyperalgesia of nerve-injured rats. Notably, while ^{10}Panx successfully alleviates hyperalgesia, it does not alter pSrc expression; and (4) NMDA-stimulated YOPRO-1 uptake in neurons of laminae I-II of spinal cord slices were prevented by the NMDAR antagonist D-AP5, the Src inhibitor PP2 (but not PP3), as well as with the ^{10}Panx and carbenoxolone. Therefore, NMDAR activation in dorsal horn neurons triggers an NMDAR-Src-Panx1 signaling pathway, where Panx1 acts as an enhancing effector in neuropathic pain. This implies that disrupting the NMDAR-Panx1 communication (eg, through Src inhibitors and/or Panx1 blockers) may offer a valuable strategy for managing some forms of chronic pain.

Keywords: NMDA receptor, Src protein kinase, Pannexin 1, Neuropathic pain, Mechanical hyperalgesia, Spinal cord

1. Introduction

Chronic pain represents a pathological condition recognized as a public health challenge, impacting over 30% of individuals globally across various age groups.[14] It exerts profound effects not only on the patients but also extends to their families, the broader social and professional spheres.[21] Systematic studies indicate that the prevalence of chronic pain varies between 11% and over 40%, with a prevalence of 20.5 to 21.8% in the United States[56] and up to 43.5% in the United Kingdom.[22]

Neuropathic and inflammatory pain represent common forms of chronic pain characterized by an abnormal state of responsiveness or heightened sensitivity of the nociceptive system, known as central sensitization.[2,9] Most neurons and nociceptive pathways circuits engaged in central sensitization across the neuroaxis undergo changes in their functional status. These alterations include increased spontaneous activity, decreased activation threshold, suprathreshold stimulation response, and an enlargement of their receptive fields.[23,37,73] A well-recognized receptor participating in chronic pain is the N-methyl-D-aspartate receptor (NMDAR), a glutamate-gated receptor channel widely expressed in both the peripheral and central nervous system (CNS).[28,36,42] Under pathological conditions, such as chronic pain, uncontrolled glutamate release leads to persistent activation of NMDARs, resulting in loss of Ca^{2+} homeostasis and increased sensitivity of the spinal cord and brain pathways that process sensory information, particularly pain related.[42,71] N-methyl-D-aspartate receptor has been proposed to possess both canonical (Ca^{2+}-dependent) and noncanonical (metabotropic) signaling pathways, which are partially regulated by the nonreceptor tyrosine kinase Src, a crucial regulatory hub where multiple intracellular signaling cascades converge to modulate NMDAR activity.[3,59,71] In chronic pain, Src plays an essential role in

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regulating intracellular signal transduction and enhances NMDAR single-channel gating, leading to NMDAR-mediated synaptic currents in neurons by phosphorylating NMDAR subunits.[53,59,69] However, the interaction between NMDAR and Src is reciprocal, as NMDAR excitation has been observed to be downstream coupled with increased Src activity.[71,70]

Recent studies indicate that NMDARs interact with various receptors and channels, including pannexin 1 (Panx1) channels.[40,45,74] Remarkably, in hippocampal brain slices, NMDAR subunit NR1 forms a signaling complex with Src and Panx1, where the disruption of this complex has shown neuroprotective effects in ischemia.[13] Pannexin 1 is a nonselective large-pored plasma membrane channel permeable to small molecules such as ions and neurotransmitters.[39] Pannexin 1 exerts significant effects on various types of pain, including neuropathic pain, trigeminal hypersensitivity, and migraine.[9,29,34] Moreover, Panx1 functionally interacts with NMDARs in the pathological conditions of epilepsy and orofacial ectopic pain, and its involvement in chronic pain requires NMDAR activation.[8,40,79] Recently, we observed Panx1 expression in intrinsic dorsal horn neurons of nerve-injured rats and increased mechanical hyperalgesia triggered by NMDARs stimulation requires the activation of neuronal Panx1 channels to initiate and maintain nociceptive signaling in nerve-injured rats.[8] Therefore, we hypothesized that after NMDAR activation, Panx1 acts as an effector downstream of Src in neuropathic pain, contributing to the mechanical hyperalgesia observed in nerve-injured rats.

2. Methods

2.1. Animals

A total of 90 adult male Sprague–Dawley rats (225-250 g) were used in all experiments conducted in this study. Rats were obtained from the breeding facility of the Faculty of Chemistry and Pharmacy of the University of Chile, Santiago, Chile. The decision to use only male rats was made to mitigate the influence of hormonal fluctuations and other factors associated with sex disparities in pain and analgesia in both rodents and humans.[49,72] Therefore, we cannot exclude the possibility that our findings might differ if female rats were used, especially given previously reported sex-related differences in the physiological responses to Panx1 knockout[24] and conditional Panx1 deficiency.[1] While altered sexually dimorphic responses in mice with global or conditional inactivation of Panx1 do not necessarily imply similar results with pharmacological blockade of Panx1, it is clear that our data cannot be directly generalized to females. Animals were housed under a controlled environment with 12 h light–dark cycles, temperature-controlled (24°C) and ad libitum access to food and water. All animal experiments were adhered to the ARRIVE guidelines[35] and conducted per the Guide for the Care and Use of Laboratory Animals.[51] Approval for this study was obtained from the Institutional Animal Care and Use Committee of the University of Santiago of Chile (protocol #321). This study involved the use of in vivo (algesimetry), ex vivo (dye uptake in spinal cord slices), and *in vitro* (immunofluorescence and western blotting of spinal cord proteins) experiments, along with pharmacological manipulations in both nerve-injured animals and controls.

2.2. Induction of nerve injury (neuropathic rat model)

The spared nerve injury (NI) neuropathic model was induced as previously described.[7,8,54] Briefly, rats were anesthetized with 3% isoflurane, the right hind limb was shaved, a 1.5-cm skin incision and blunt dissection of the underlying subcutaneous connective and biceps femoris muscle were performed. The sciatic nerve and its 3 terminal branches were then exposed, and the sural nerve was transected distal to the sciatic nerve, leaving the common peroneal and tibial nerves intact. The overlying tissues were sutured in layers. Postsurgery rats were administered 3 mg/kg of the nonsteroidal anti-inflammatory drug ketoprofen and 5 mg/kg of the antibiotic enrofloxacin subcutaneously for 2 days. Controls (sham surgery) involved exposure of the sciatic nerve and its branches without inducing any lesion (**Fig. 1**).

2.3. Drugs and treatments in in vivo experiments

Rats received intrathecal (i.t.) administration of drugs using a previously described method.[48,64] A 25-μL Hamilton syringe with a 25 Ga × 1-inch needle was inserted into the L5-6 lumbar space. About 10 μL i.t. injection of vehicle, selective Panx1 antagonist ^{10}Panx 300 μM,[7,8] scrambled control peptide Scr^{10}Panx 300 μM, Src inhibitor PP2 10 μM,[12] or inactive Src inhibitor PP3 10 μM. An hour later, rats receive i.t. administration of the selective NMDAR agonist N-methyl-D-aspartate (NMDA) 600 μM.[75] ^{10}Panx was purchased from GenScript (Piscataway, NJ), while scrambled control peptide Scr^{10}Panx (FSVYWAQADR, Cat. No.3708), PP2 (Cat. No.1407), PP3 (Cat. No.1407), and NMDA (Cat. No.0114) were purchased from Tocris (Bio-Techne Corporation, Minneapolis, MN). ^{10}Panx, Scr^{10}Panx, and NMDA were dissolved in saline (0.9% NaCl), while PP2 and PP3 were dissolved in dimethyl sulfoxide (DMSO) to make stock concentrations of 50 mM, which were appropriately diluted with saline immediately before administration. Control animals were given 10 μL i.t. of the corresponding vehicle used to dissolve each drug.

2.4. Behavioral assessment of mechanical hypersensitivity

To assess mechanical hyperalgesia, we measured the hind paw withdrawal threshold using an analgesymeter (Ugo Basile, Varese, Italy) described by Randall and Selitto.[55] Briefly, the rat's right hind paw was placed under uniformly increasing pressure over time until withdrawn and/or a vocalization response occurred; at this time, the pressure value recorded in the algesimeter was considered the withdrawal threshold (g/cm^2). To prevent paw injury, the maximum pressure exerted was limited to 480 g/cm^2 (cut-off value). The algesimetric test was performed in both sham and nerve-injured rats before the surgery and at 1, 3, and 5 days after surgery to confirm hyperalgesia induction. On the experiment day, the baseline hind paw withdrawal threshold was determined for each animal prior to the first intrathecal injection of drug or vehicle (−15 and 0 minutes). Algesimetric evaluation continued every 15 minutes until 180 minutes (**Fig. 1**). The second intrathecal injection of drug or vehicle was administered immediately after the algesimetric evaluation at min 60. Behavioral testing was performed with the experimenter blinded to the treatment regimen. The effects generated by the different treatments were evaluated by calculating the area under the curve (AUC) in the paw withdrawal threshold graph and its variation (ΔAUC) related to the control group treated with saline. A negative ΔAUC indicates a hyperalgesic effect, while a positive ΔAUC indicates a hypoalgesic effect.

2.5. Spinal cord slice preparation

Seven days after surgery (**Fig. 1**), rats were deeply anesthetized with isofluorane (3-4%) and euthanized by cardiac perfusion with PBS 1x supplemented with heparin. Laminectomy was

performed from the T12 to L5 vertebrae using fine scissors to expose the spinal cord. Nerve roots were carefully cut, and the lumbar enlargement (L3-L5 spinal cord section corresponding to T13-L2 vertebrae) was carefully lifted using a spinal cord hook and transferred to a dish containing artificial cerebrospinal fluid (aCSF; 212 mM sucrose; 2.5 mM KCl; 10 mM glucose; 25 mM $NaHCO_3$; 1.2 mM NaH2PO_4; 2 mM $CaCl_2$; 1.3 mM $MgSO_4$; 2 mM pyruvate; 400 μM sodium L-ascorbate; and 3 mM myo-inositol at pH 7.4) bubbled with carbogen (95% O_2/5% CO_2) at room temperature. The dura mater was carefully removed, avoiding any compression or damage to the lumbar spinal cord section. The tissue was embedded in a 4% (w/v) gel low-gelling-temperature agarose (Agarose Type IX-A, Ultra-low Gelling Temperature, Sigma, San Luis, MO) and glued onto the stage of a vibratome (Vibrating 7000smz-2 model microtome, Campden Instruments, Loughborough, UK). Transverse spinal cord slices (200 μm) were cut in ice-cold aCSF. The slices were transferred into aCSF at 37°C and treated according to each experiment.

2.6. YOPRO-1 dye uptake assay

YOPRO-1 is commonly used as a dye to assess Panx1 channel permeability into cells. Following established protocols previously described,[62,80] the dye uptake assay relied on the dye's ability to traverse the cytoplasmic membrane and accumulate intracellularly through Panx1. Transverse spinal cord slices were placed in tissue culture plates (12 wells) with 1.5 mL oxygenated aCSF solution (95% O_2 and 5% CO_2 at 37°C) and preincubated for 10 minutes with either vehicle alone or containing ^{10}Panx peptide (300 μM), Scr^{10}Panx1 peptide (300 μM), carbenoxolone (CBX, 100 μM), PP2 (10 μM), PP3 (10 μM), or D-AP5 (50 μM). Subsequently, the slices were challenged with NMDA (600 μM) for 20 minutes, followed by a 10 minutes incubation with a YOPRO-1 dye solution (5 μM). The spinal cord slices were washed 3 times for 5 minutes and fixed for 1 hour with 4% PFA. YOPRO-1, ^{10}panx, Scr^{10}Panx, and NMDA were dissolved in aCSF, while PP2 and PP3 were dissolved in DMSO (see above, drugs and treatments section, **Fig. 1**). The final concentration of DMSO did not exceed 0.1%. Spinal cord slices were incubated overnight with anti-NeuN rabbit monoclonal antibodies (1:1000 dilution; Abcam, EPR12763, Cambridge, UK) and then incubated 1 hour at room temperature with goat antirabbit Alexa Fluor 555 secondary antibody (1:1000, Thermo Fisher, Waltham, MA). Nuclei were stained by Hoechst 33342 (1: 3000, Invitrogen, Carlsbad, CA), and slices were mounted with Fluoromount-G (Thermofisher Scientific, eBioscience, 4958-02, Waltham, MA). YOPRO-1 uptake was analyzed by immunostaining of positive neuronal cells. Fluorescence was measured by confocal microscopy (Carl Zeiss, LSM 800, Oberkochen, Germany), and images of laminae I-II were taken with a 40x objective and analyzed with ImageJ software.

2.7. Expression of pannexin 1 protein in dorsal horn neurons

Animals were deeply anesthetized with isofluorane 3% and perfused transcardially with 0.1M PBS, followed by 4% paraformaldehyde, pH 7.4. We identified the lumbar spinal L3-L5 sections and harvested, followed by a postfixation at 4°C and transferred sequentially to 15% and 30% sucrose in 0.1M PBS for 24 hours. Transversal slices (20 μm thick) at the levels L3-L5 were made in a Minux FS800 cryostat (RWD Life Science, Houston, TX). Antigen retrieval was performed by exposing the slices to 10 mM Tris-EDTA buffer, pH:9.0, at 90°C for 10 minutes. The floating transverse sections were permeabilized with 0.3% Triton X-100 in PBS for 20 minutes and incubated in a blocking solution (5% normal goat serum and 0.5% Triton X-100 in 0.1M PBS) for 2 hours. Primary antibodies anti-Panx1 (1:50, rabbit polyclonal PA5-77358, Thermo Fischer Scientific) and anti-NeuN (1:5000, mouse monoclonal MAB377) were diluted in a blocking solution, and slices were incubated at 4°C overnight. Floating sections were washed 3 times for 5 minutes in PBS and mounted onto silane-coated slides. Sections were incubated at room temperature with antimouse Alexa 488 and antirabbit Alexa Fluor 555 for 2 hours, washed 3 times in PBS 1x and coverslipped with Hoechst 33342-Fluoromount G and visualized with a confocal microscope (SP5, Leica, Mannheim, Germany).

2.8. Western blotting in tissue from spinal cord dorsal sections

All analyses were performed on the L3-L5 spinal cord section prepared as described above. The spinal cord was cut vertically in the midline from rostral to caudal, and the right dorsal section (ipsilateral to sural nerve transection) was quickly lysed in RIPA synthesis buffer (Invitrogen) using an ultrasonic homogenizer. Total proteins were determined with BCA Protein Assay Kit (Cell Signaling Technology, 7780, Danvers, MA), and 20 μg was loaded in each SDS-PAGE well and transferred onto PVDF membrane. Membranes were blocked using 5% BSA in TBS-T (10 mM Tris, 100 mM NaCl, 1% Tween 20) for 1 hour at room temperature and incubated overnight at 4°C with anti-Panx1 (1: 500, rabbit polyclonal, #PA5-77358, Thermo Fischer Scientific), anti-pPanx1 phosphorylated at Tyr198 (1:100, rabbit polyclonal #ABN1681, Milipore Sigma), anti-Src (1:500, rabbit polyclonal, #2101s, Cell Signaling Technology), anti-pSrc416 (rabbit polyclonal, #2108, Cell Signaling Technology), or anti-GAPDH (rabbit polyclonal #PLA0125, Millipore Sigma), with gentle shaking. Blottings were then washed 3 times with TBS-T, incubated in Clarity Western ECL substrate/reagent according to the manufacturer's protocol, imaged, and quantified using ImageJ.

The reliability of the ABN1681 (anti-Panx1-pY198) antibody, used in the current study to detect Panx1 phosphorylation, has recently been questioned.[58] However, the study by Ruan et al.[58] 5 relied on cotransfection experiments conducted in heterologous expression systems (HEK293T and Neuro2A cells), which lack tissue-specific evidence. As a result, their findings may not fully apply to the complex signaling pathways present in spinal cord neurons. In contrast, the ABN1681 antibody has been successfully used in several prior studies to detect SRC-mediated phosphorylation of Panx1.[18,71,70]

2.9. Immunofluorescence quantification

The relative fluorescence quantification was assessed using ImageJ 1.54j (Fiji). Briefly, regions of interest (ROIs) were automatically selected by using the "Segmentation" plugging to recognize positive neurons stained by NeuN (channel 1). The selected NeuN-ROIs were transferred to the channels Pannexin1 or YOPRO-1 (channel 2), followed by adjusting threshold to remove the background. The relative fluorescence of Pannexin1 or YOPRO-1 on each positive neuron cells was obtained. Each "n" was built from 3 different images and composed by at least 40 neurons, and the data are expressed as "RawIntDen" (the sum of the values of the pixels in the ROI selected) present on each neuron cell.

2.10. Glutamate content release in the spinal cord

The glutamate supernatant content was measured using liquid chromatography-mass spectrometry (LC-MS), as previously

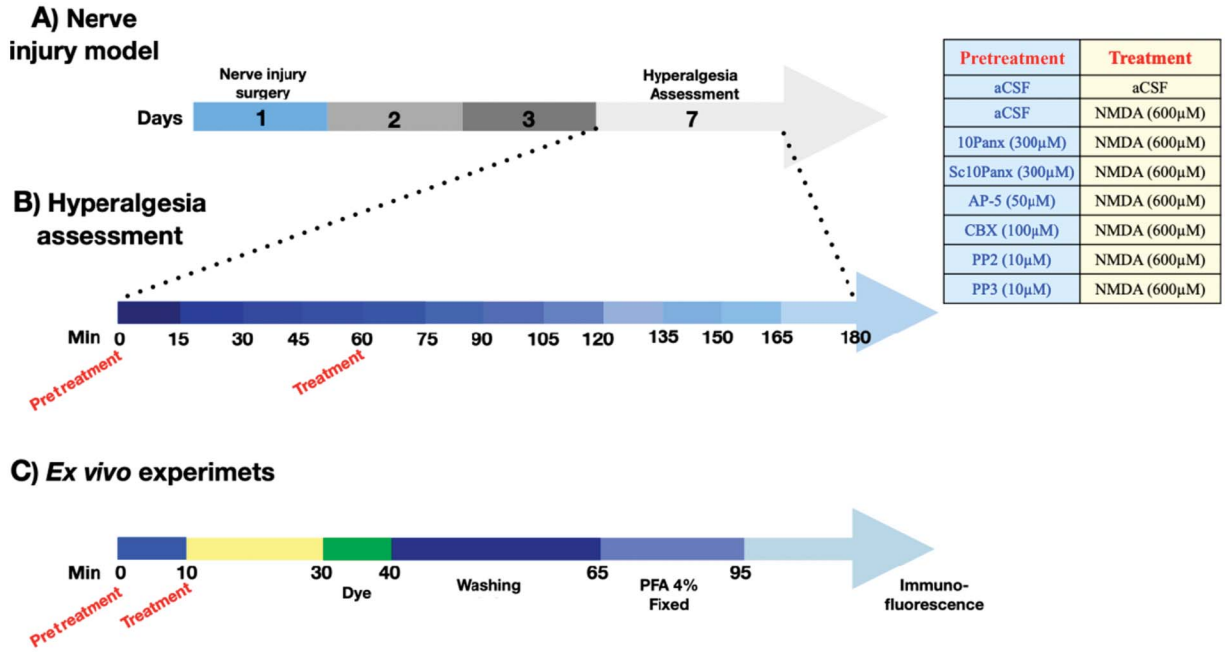

Figure 1. Scheme of nerve injury–induced pain, pretreatment and treatment administration, assessment of mechanical hypersensitivity, and YOPRO incorporation in sham and nerve-injured rats. (A) On day 1, the nerve injury surgery was induced. (B) On day 7 after surgery (time 0), the animals were administered intrathecally with the pretreatment, followed by the treatment at 60 minutes. Concurrently, assessment of mechanical hyperalgesia was conducted. (C) On day 7, coronal slices of the spinal sections were obtained from sham and nerve-injured rats from other experimental series. The slices then underwent pretreatment and treatment administration, incubation with the dye YOPRO-1, washes, slice fixation, immunofluorescence, image acquisition, and subsequent data analysis with the FiJi software.

reported.[17] Briefly, after cutting the spinal cord slices, the sections were recovered on 1 mL of aCSF for 30 minutes. The resulting recovery in aCSF was collected and diluted 1:30 (in water for HPLC) and an aliquot (20 μL) was mixed with 4 μL of borate buffer (pH 10.8) and derivatized by adding 4 μL of fluorogenic reagent (20 mg of ortho-phthaldialdehyde and 10 μL of β-mercaptoethanol in 5 mL of ethanol). Ninety seconds after derivatization, the samples were injected into an HPLC system with the following configuration: isocratic pump (model PU-4180, Jasco Co., Ltd., Tokyo, Japan), reverse-phase C-18 column (Kromasil 3-4.6, Bohus, Sweden), and a fluorescence detector (model FP-4020, Jasco Co). The mobile phase containing 0.1 M NaH_2PO_4 and 24.0% (v/v) CH_3CN (pH 5.7) was pumped at a flow rate of 0.8 mL/min. The retention time for glutamate was 1.8 minutes, and the detection limit was 5 fmol/μL. The peak area corresponding to glutamate was compared with a reference standard, and concentrations were calculated using a calibration curve created with a chromatographic program (ChromNAV 2.0, Jasco Co. Ltd).

2.11. Statistical analysis

All data were expressed as the mean ± SEM. Statistical analyses were performed using Prism 10 software (GraphPad Software Inc., San Diego, CA). Nonparametric statistics was chosen because animal groups were composed of 6 rats, a sample size that usually does not allow to demonstrate a Gaussian distribution of data group. Mann–Whitney test was used for the statistical analysis of one variable between 2 unpaired groups. Kruskal–Wallis test was used for intergroup statistics of 3 or more unpaired groups, followed by Dunn multiple comparisons test. Friedman test was used to analyze intragroup variations of matched data over time (ie, time-course effect), followed by Dunn multiple comparisons test. Finally, the one-sample Wilcoxon Signed-Rank test was used to calculate the area under a time-course curve (AUC) and its variation (ΔAUC) compared to a control group. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Pannexin 1 expression and dye uptake by dorsal horn neurons of the rat spinal cord

Using immunofluorescence confocal microscopy, we first investigated the expression of Panx1 and phosphorylated Panx1 (pPanx1) in the dorsal horn (laminae I-II) of both sham and nerve-injured rats. Coronal spinal cord slices (L3-5) from sham or nerve-injured rats were fixed and stained to visualize Panx1, pPanx1, neuronal marker NeuN, and nuclear marker Hoechst 33342 (**Figs. 2A and B**). Fluorescence intensity analysis of confocal images revealed similar expression levels of Panx1 in laminae I-II of the spinal cord in both control and nerve-injured rats (189.9 ± 12.0 and 198.9 ± 27.3, respectively, P = not significant) colocalizing with NeuN (**Figs. 2C and D**). In contrast, examination of phosphorylated Panx1 (pPanx1-Y198) expression showed a substantial increase of pPanx1 in nerve-injured rats (486.2 ± 33.7, $P < 0.05$) compared to control animals (174.6 ± 19.9) (**Figs. 2E and F**). These findings suggest that in neuropathy, changes in the expression level of Panx1 are not observed in the dorsal horn of the spinal cord compared to control rats. However, a significant increase of pPanx1-Y198 approximately 2.5-fold was found in nerve-injured rats compared to control animals, supporting that pPanx1 activity in dorsal horn neurons of nerve-injured rats may play a role in central sensitization.

Next, we investigated the functionality of Panx1 channels expressed in dorsal horn neurons of laminae I-II from sham and nerve-injured rats by measuring the incorporation of the

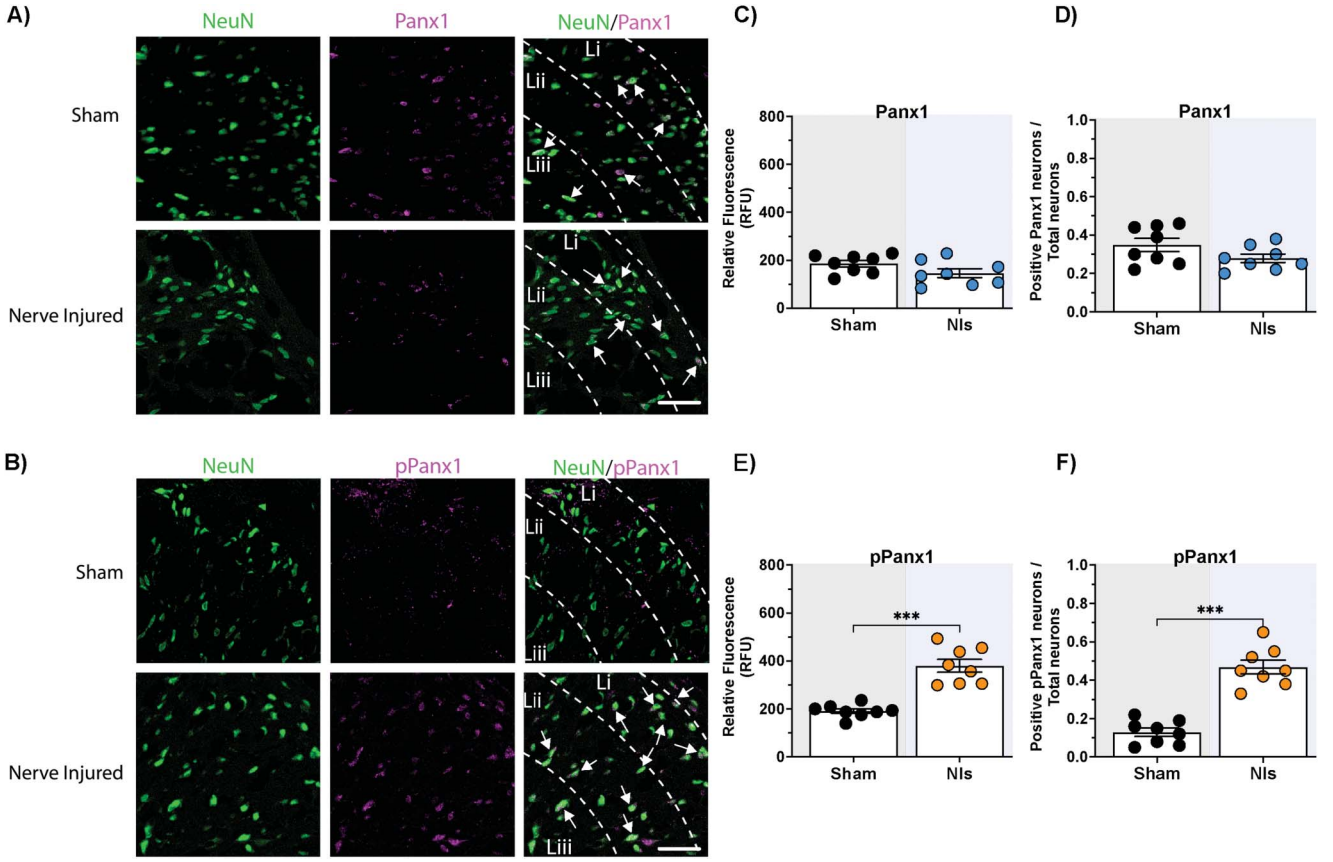

Figure 2. Expression of pannexin 1 in the spinal dorsal horn. Representative confocal immunofluorescence images depicting the expression of Pannexin 1 (Panx1) in spinal cord transverse slices spanning the L3-L5 region of sham and nerve-injured rats. (A) Panx1 and (D) pPanx1 expressions colocalized with NeuN in neurons located within the dorsal horn laminae I-II. Quantitative analysis of fluorescence intensity of Panx1 (B) and pPanx1 (E) in dorsal horn neurons. Ratio relationship of positive neurons expressing Panx1 (C) and pPanx (F) within total dorsal horn neurons. The data, presented as the average values from 8 sham and nerve-injured rats with 3 images captured per animal, are expressed as means ± SEM. Intergroup statistical significance (E and F) was determined using Mann–Whitney test, ***$P < 0.001$. Scale bar, 100 μm. The arrows indicate overlapping between NeuN/Panx1.

fluorescent molecule YOPRO-1 and comparing the spontaneous uptake with stimulated NMDAR activation. Ex vivo spinal cord slices from sham and nerve-injured rats were evaluated for YOPRO-1 incorporation after treatment with NMDA or with the NMDAR glutamate site antagonist D-AP5 (D-AP5-NMDA) (see Methods). Activation of NMDAR (by NMDA treatment) in slices from sham rats resulted in a significant increase in YOPRO-1 incorporation (**Figs. 3A and B**, $P < 0.05$). However, when NMDAR was inhibited by the selective NMDAR antagonist D-AP5 (AP5-NMDA treatment), it led to a significant reduction in YOPRO-1 incorporation (**Figs. 3A and B**, $P < 0.05$), with values similar to those observed in sham control slices incubated with the vehicle. In spinal cord slices from nerve-injured rats, higher YOPRO-1 incorporation was found in control slices (vehicle treatment) compared to control slices from sham rats (**Figs. 3B–D**). This higher YOPRO-1 incorporation in nerve-injured vehicle–treated slices could be explained by a higher glutamate supernatant content (sham 0.023 ± 0.001, nerve-injured 0.071 ± 0.011 μM). Meanwhile, NMDAR activation in slices from nerve-injured rats induces a substantial (but not significant) increase in YOPRO-1 incorporation. However, when D-AP5 inhibited NMDAR resulted in a significant reduction in YOPRO-1 incorporation (**Figs. 3B and D**, $P < 0.001$), with values lower than those obtained with control vehicle treatment. These findings indicate that Panx1 channels expressed in dorsal horn neurons are functional and sensitive downstream to NMDAR stimulation and inhibition.

3.2. N-methyl-D-aspartate receptor mediates mechanical nociception in nerve-injured rats through Src kinase

To investigate the roles of NMDAR and Src in nerve-injured pain using Randall–Selitto testing, 5 groups of rats with neuropathy resulting from surgical damage to the sural nerve, which had previously demonstrated mechanical hyperalgesia in the Randall–Selitto test, were used. Seven days after surgery (time 0, **Fig. 1**), intrathecal administration of vehicle, PP2 (a Src family tyrosine kinase inhibitor), or the negative control drug, PP3, was conducted, followed by i.t. saline or i.t. NMDA 60 minutes later.

Administration of vehicle did not alter the hind paw withdrawal threshold of nerve-injured rats (white circle). However, intrathecal administration of NMDA (600 μM, blue circle) 60 minutes later significantly increased the nociceptive behavior, as evidenced by a significant decrease of the hind paw withdrawal threshold from 160.9 ± 3.23 to 93.7 ± 3.8 gr cm^{-2} at t = 75 minutes ($P < 0.005$). This increase was sustained for approximately 60 minutes before returning to typical values observed in nerve-injured animals (**Fig. 4A**). Analysis of the AUC confirmed the increase in nociceptive behavior (**Fig. 4B**).

To assess the involvement of Src in neuropathic pain, PP2 or PP3 was intrathecally administered at t = 0, followed by a second intrathecal administration of vehicle or NMDA 60 minutes later. Administration of PP2 (green circle) induced a significant increase in the hind paw withdrawal threshold of nerve-injured rats (from 162.5 ±

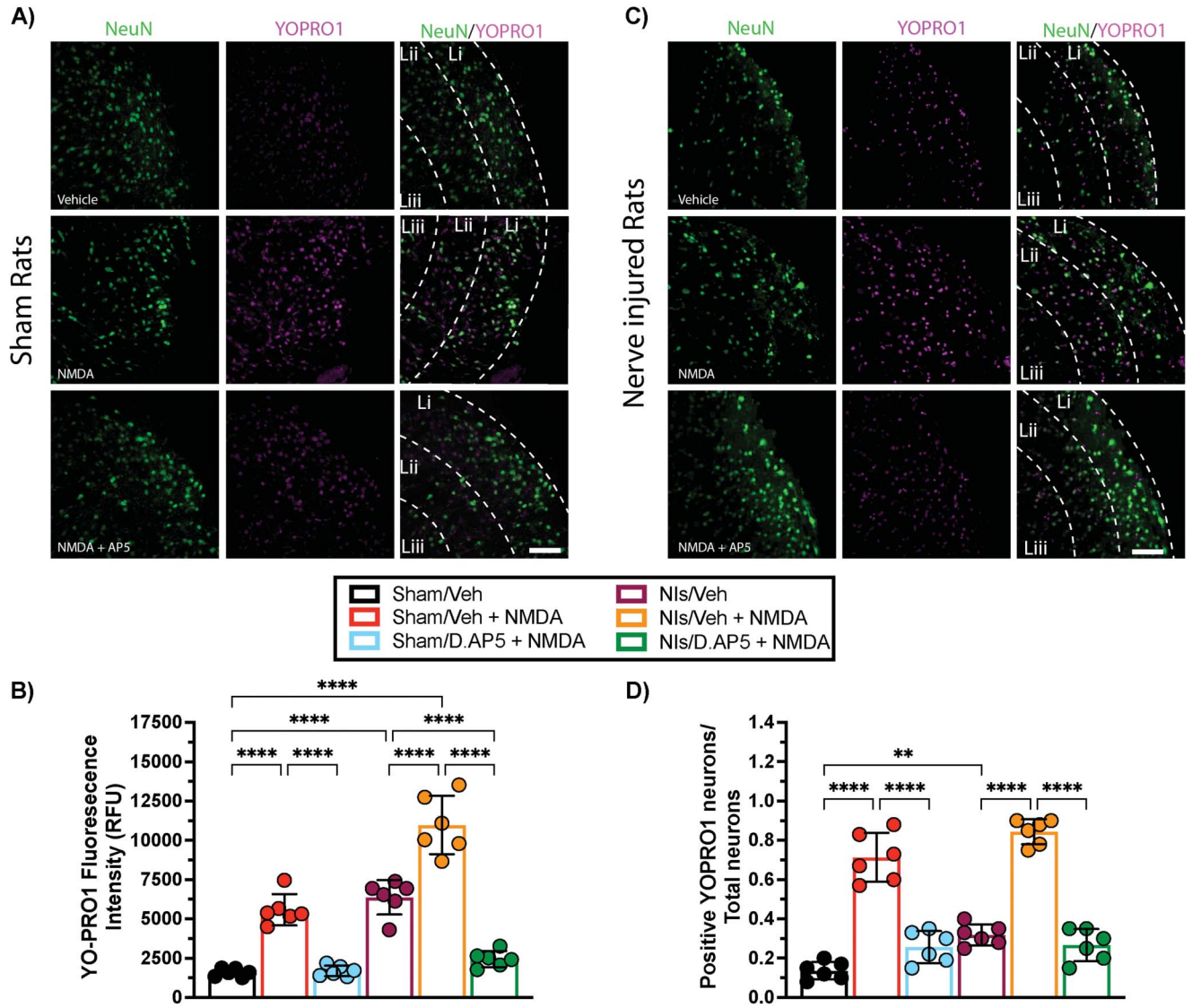

Figure 3. Functional expression of Pannexin 1 (Panx1) in dorsal horn neurons in laminae I-II. Ex vivo YOPRO-1 incorporation assay revealed the functional activity of Panx1 in neurons of the spinal cord laminae I-II, mediated by NMDAR activation both in sham and nerve-injured rats. Representative confocal images depict YOPRO-1 incorporation in neurons of the spinal cord laminae I-II in sham (A) and nerve-injured (B) rats treated with NMDA (600 μM) or preincubated with D-AP5 (50 μM) followed by NMDA poststimulation. (C) Quantitative analysis of YOPRO-1 fluorescence intensity incorporation in sham and nerve-injured rats. (D) Ratio of YOPRO-1 positive neurons to the total number of neurons in each condition. Data are expressed as means ±SEM, n= 6. Kruskal–Wallis test intergroup statistics followed by Dunn multiple comparisons test, $^{**}P < 0.005$, $^{****}P < 0.0001$ compared to vehicle treatment (C and D). Scale bar, 100 μm. NMDAR, N-methyl-D-aspartate receptor.

12.4 to 233.5 ± 17.73 at t = 30 minutes) for approximately 105 minutes (**Fig. 4A**). Area under the curve analysis confirmed this decrease in nociception mediated by Src inhibition (**Fig. 4B**). In contrast, administration of the inactive Src inhibitor PP3 followed by NMDA (yellow circle) resulted in a significant decrease in the hind paw withdrawal threshold, similar to that observed in nerve-injured animals treated only with NMDA (**Fig. 4A**). It is confirmed by AUC analysis (**Fig. 4B**). Furthermore, inhibition of Src by PP2 not only decreased mechanical hyperalgesia in nerve-injured rats (dark red circle) but also protected them from the increase in nociceptive behavior induced by NMDA administration (red circle) (**Figs. 4A and B**). These results suggest that NMDAR stimulation in nerve-injured rats increases mechanical nociception by activating Src downstream, or that NMDAR stimulation is ineffective when Src is inhibited.

To further understand Src's role in chronic pain in neuropathic animals, a similar drug administration scheme (i.t. PP2 or PP3 followed by i.t. NMDA) was applied to other groups of rats, both nerve-injured and sham. Sixty minutes after the last drug administration (i.t. NMDA), the L3-L5 segment of the spinal cord was removed, and the right dorsal section ipsilateral to sural nerve transection was dissected. Western blotting showed no significant change in total Src protein expression after NMDA administration (**Figs. 4C and D**). However, phosphorylated Src416 (Src phosphorylated at Tyr416 in the activation loop of the kinase domain[70]) was upregulated in nerve-injured animals stimulated with NMDA compared to either sham rats or nerve-injured rats without NMDA stimulation (**Figs. 4E and F**). This upregulation of pSrc416 mediated by NMDA did not occur when animals were pretreated with PP2 but not PP3. In addition, a group of rats receiving ^{10}Panx (a Panx-1 mimetic inhibitory peptide that blocks Pannexin-1 channels) showed no modification in Src (**Figs. 4C and D**) and pSrc416 (**Figs. 4E and F**) protein expressions, indicating no involvement of Panx1 inhibition in pSrc416 upregulation.

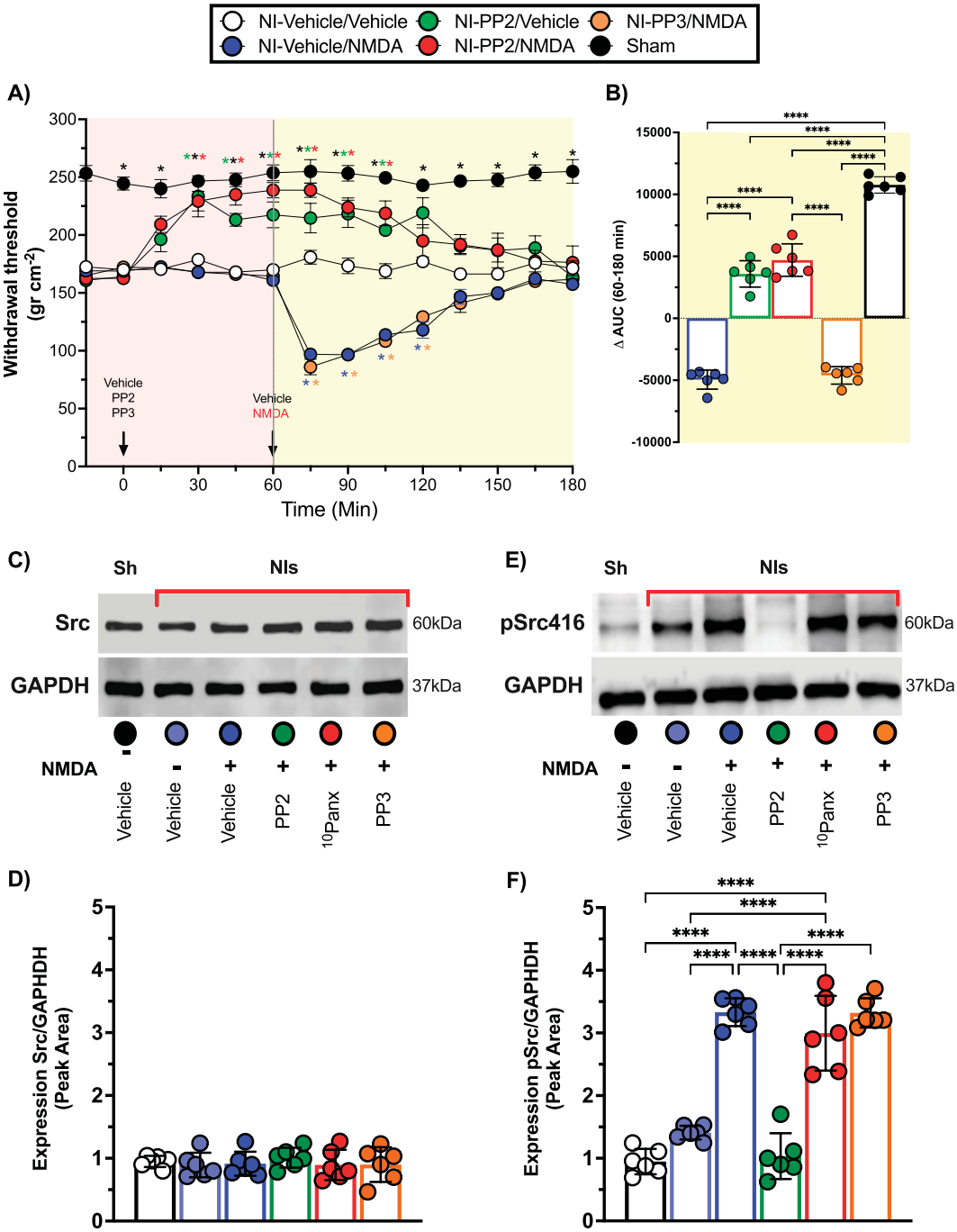

Figure 4. NMDAR activation enhances mechanical nociception in nerve-injured rats via Src kinase. Activation of NMDAR mediates and enhances mechanical nociception in nerve-injured rats through Src kinase. Inhibition of Src reduces mechanical hyperalgesia in nerve-injured rats. (A) Time course of intrathecal administration of PP2 (10 μM) or PP3 (10 μM) on mechanical nociception. (B) Integrated response as Δ area under the curve (ΔAUC) from 60 to 180 minutes. Representative western blot expression of total Src (C) and pSrc416 (D) in the spinal cord (L3-L5) of sham or nerve-injured rats with or without i.t NMDA (600 μM) administration, in presence of PP2, PP3, or ^{10}Panx (300 μM). Quantitative analysis of Src (E) and pSrc416 (F), normalized to GAPDH. Data are expressed as means ± SEM, n = 6. Statistical analysis was performed using (A) Friedman test intragroup statistics followed by Dunn multiple comparisons test, $^{*}P < 0.05$; (B) one-sample Wilcoxon Signed-Rank test, $^{*}P < 0.05$, $^{**}P < 0.01$; and (E and F) Kruskal–Wallis test intergroup statistics followed by Dunn multiple comparisons test, $^{****}P < 0.0001$. NMDAR, N-methyl-D-aspartate receptor.

3.3. Pannexin 1 blockade prevented N-methyl-D-aspartate receptor-Src–mediated mechanical nociception in nerve-injured rats

To investigate the role of Panx1 channels downstream of NMDAR-Src activation in neuropathic pain, Panx1 was blocked by i.t. injection of ^{10}Panx in rats undergoing Randall–Selitto testing, while scrambled ^{10}Panx (scr^{10}Panx) served as a negative control of ^{10}Panx. ^{10}Panx alone (300 μM) rapidly and significantly increased the hind paw withdrawal threshold ($P < 0.005$), effectively counteracting the hyperalgesic state existing in nerve-injured rats. Moreover, blocking Panx1 with ^{10}Panx prevented the increase in nociception induced by NMDA administration (**Figs. 5A and B**), mimicking the effect observed with Src inhibition under similar conditions of NMDAR stimulation (**Figs. 4A and B**). In contrast, the inactive molecule scr^{10}Panx had no impact on the

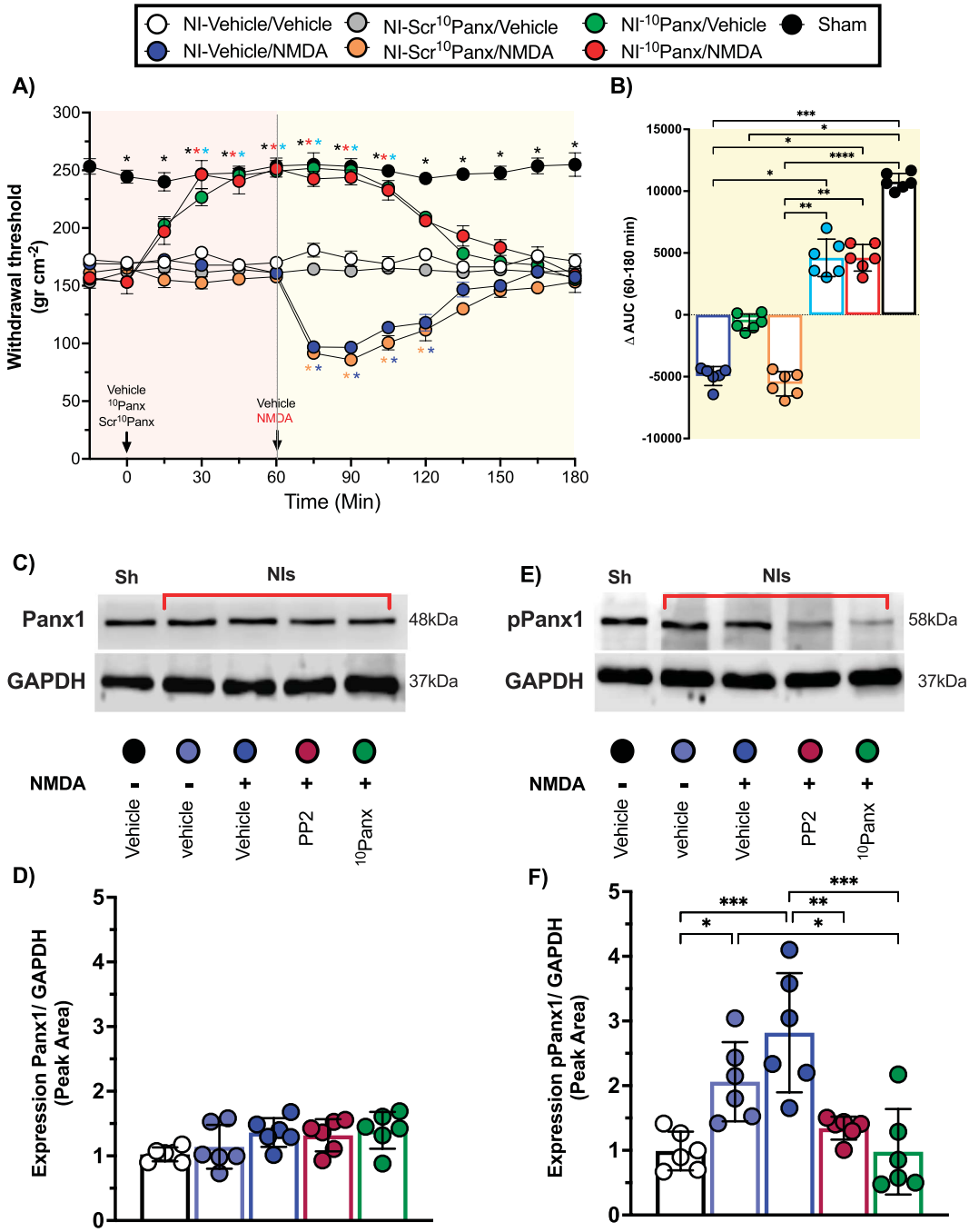

Figure 5. Pannexin 1 blockade prevents NMDAR-mediated mechanical nociception in nerve-injured rats. Panx1 blockade prevents NMDAR-mediated mechanical nociception in nerve-injured rats, leading to the recovery of mechanical hyperalgesia. (A) Time course of intrathecal administration of ^{10}Panx (300 μM) or Scr-^{10}Panx (300 μM) on mechanical nociception in nerve-injured rats. (B) Integrated response as the area under the curve (ΔAUC) from 60 to 180 minutes. Representative western blot expression of total Panx1 (C) and pPanx1 (D) in the spinal cord of sham or nerve-injured rats with or without i.t NMDA (600 μM) administration, in presence of PP2 or ^{10}Panx. (E and F) Panx1 and pPanx expression normalized to GAPDH. Data are expressed as means ± SEM, n= 6. Statistical analysis was performed using (A) Friedman test intragroup statistics followed by Dunn multiple comparisons test, $^{*}P < 0.05$; (B) one-sample Wilcoxon Signed-Rank test, $^{**}P < 0.01$, $^{***}P < 0.001$; and (E and F) Kruskal–Wallis test intergroup statistics followed by Dunn multiple comparisons test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NMDAR, N-methyl-D-aspartate receptor; Panx1, Pannexin 1.

hind paw withdrawal threshold and failed to protect against the nociception increase triggered by NMDA administration. These findings suggest that NMDAR stimulation in nerve-injured rats enhances mechanical nociception through downstream coupling to Panx1 channels.

In parallel rat groups subjected to a similar drug administration scheme as the previous algesimetric experiment, Western blotting analysis of proteins from the right dorsal section of the L3-L5 spinal cord segment revealed that total Panx1 expression remained unchanged in nerve-injured rats compared with sham controls, and also that neither ^{10}Panx, nor PP2, nor NMDA administered intrathecally were able to modify the expression of Panx1 in the spinal cord tissue (**Figs. 5C and D**). In contrast, i.t. NMDA significantly increases the expression of pannexin 1 phosphorylated at Y198 (pPanx1-Y198) in spinal cord tissue from nerve-injured rats. In addition, intrathecal administration of

^{10}Panx or PP2 previous to intrathecal NMDA downregulated pPanx1-Y198 expression in the spinal cord tissue from nerve-injured rats (**Figs. 5E and F**).

Taken together, these in vivo (algesimetry) and in vitro (Western blot) data indicate that in nerve-injured rats, NMDAR activation upregulates pSrc, which subsequently activates Panx1 channels to increase mechanical nociception during nerve-injured pain. The potential involvement of certain glial connexins that might be targeted by ^{10}panx during its attenuation of the hyperalgesic state in nerve-injured rats can likely be ruled out. This conclusion is based on the fact that the antihyperalgesic effects mediated by connexin targeting in the spinal cord are delayed, typically taking 8 hours to 5 days to manifest.[66,67,77] This contrasts with the nearly immediate effects observed with Panx1 inhibition by ^{10}panx in the present study, which is likely because of the extended time required to reduce the release of glial products and the subsequent glial-neuron interactions necessary to produce significant neuronal effects.

3.4. N-methyl-D-aspartate receptor-Src mediates pannexin 1 opening in spinal cord neurons of nerve-injured rats

The opening of Panx1 channels and their interaction with the NMDA-Src complex were further investigated ex vivo by measuring the incorporation of the fluorescent molecule YOPRO-1 into neurons of laminae I-II of spinal cord slices from nerve-injured rats. To assess whether NMDAR activated Panx1 through Src, slices were coincubated with the Src inhibitor PP2 in addition to NMDA. Incubation with NMDA alone induced a substantial increase in YOPRO-1 uptake. However, incubation with PP2-NMDA resulted in a significant decrease in YOPRO-1 incorporation into spinal neurons compared to vehicle or NMDA alone (**Fig. 6A**), indicating that a significant portion of YOPRO uptake into laminae I-II neurons depends on NMDAR-stimulated Src activity. Incubation with the inactive control drug PP3 plus NMDA did not reduce YOPRO-1 incorporation (**Figs. 6A and B**).

To demonstrate that NMDAR-Src activation led to YOPRO-1 incorporation through Pannexin 1 channels, Panx1 was inhibited using ^{10}Panx peptide. Results showed that adding ^{10}Panx to the incubation solution prior to NMDA led to a significant decrease in YOPRO-1 incorporation in spinal neurons slices compared to values of slices treated with vehicle or NMDA alone (**Fig. 6B**). This result was replicated by incubating the slices in NMDA in presence of carbenoxolone 100 μM, a well-known unspecific Panx1 blocker (**Fig. 6C**). Analysis of YOPRO-1 total fluorescence intensity and incorporation in neurons confirmed a major colorant incorporation mediated by NMDA, which was decreased by PP2 and ^{10}Panx (**Figs. 6B and D**). In contrast, the inactive peptide scr^{10}Panx did not decrease YOPRO-1 incorporation in spinal neurons after the addition of NMDA (**Figs. 6B and D**).

Our results indicate that a significant portion of NMDAR-stimulated YOPRO uptake into laminae I-II neurons occurs via Panx1 channels, which open downstream to NMDA-dependent Src activity. The involvement of some connexins in YOPRO-1 uptake, which are sensitive to the ^{10}panx peptide and CBX, can be ruled out because connexin type 36, which is the type of connexin eminently expressed in neurons,[15] including dorsal horn neurons,[5] is not permeable to YOPRO-1.[27]

4. Discussion

The present study was designed to investigate the involvement of Panx1 channels as downstream effector of Src-mediated signaling after NMDAR activation in dorsal horn neurons during nerve-injured pain, focusing on their contribution to mechanical hyperalgesia. Previous studies have demonstrated that laminae I-II neurons and interneurons receive fast-acting excitatory glutamatergic neurotransmission from nociceptive and thermoreceptive Aδ and C afferents, thereby warranting consideration as nociceptive neurons, while neurons situated in lamina III inner to VI are non-nociceptive sensory neurons that receive glutamatergic innervation from myelinated low-threshold mechanoreceptors.[20,65] Using a multifaceted approach encompassing behavioral, pharmacological, and molecular methodologies, we aimed to elucidate the intricate mechanisms underlying pain processing in the laminae I-II of the dorsal horn. As previously documented, Panx1 has emerged as a crucial effector in various pain types, including neuropathic pain, trigeminal hypersensitivity, and migraine.[7,29,34]

First, we demonstrated the presence of functional Panx1 channels in neurons located within laminae I and II of the dorsal horn in sham-operated and nerve-injured rats. This was evidenced through immunoassays targeting Panx1 and phosphorylated Panx1 (pPanx1) and by assessing the uptake of YOPRO-1 fluorescence using confocal microscopy. The expression of Panx1 channels had been previously reported in a variety of CNS neurons; however, investigations specifically focusing on its presence within dorsal horn neurons of the spinal cord have been limited. Prior work by Zappalà et al.[78] highlights Panx1 expression primarily in motoneurons within lamina IX at the rostral site of the dorsal horn. However, our recent findings provided novel insights, revealing expression of Panx1 with the neuronal marker NeuN in intrinsic neurons of the dorsal horn in nerve-injured rats.[8]

Furthermore, our results not only confirmed the existence of Panx1 channels in dorsal horn neurons of control and nerve-injured rats but also revealed their functionality. Upon NMDAR stimulation, Panx1 exhibited activity, allowing the influx of YOPRO-1 dye into neurons. This observation provides compelling evidence supporting the hypothesis of a downstream stimulatory interaction from NMDAR to Panx1 channels in dorsal horn neurons, thereby implicating Panx1 in the pathophysiology of neuropathic pain. We also explored the mechanism underlying NMDAR stimulation-induced activation of Panx1 channels in nerve-injured pain, examining both behavioral and molecular levels. Our findings revealed that NMDAR stimulation exacerbates mechanical hyperalgesia in nerve-injured rats by engaging Src tyrosine kinase. Indeed, inhibition of Src with i.t. PP2 not only mitigates the spontaneous mechanical hyperalgesia in nerve-injured rats but also attenuates the hyperalgesic response triggered by i.t. NMDA administration. In addition, i.t. NMDA led to an upregulation of Src phosphorylated at Tyr416 in dorsal horn neurons, an effect that was prevented by prior i.t. PP2 administration. Although PP2 did not modify nociceptive responses in normal rats,[11] i.t. NMDA elicited pronounced mechanical allodynia in intact mice, which was attenuated by i.t. PP2.[38]

In neurons, excitation of NMDAR can be downstream coupled to Src activity through either (1) a Ca^{2+}-dependent pathway involving Ca^{2+}-dependent protein kinase C (PKC)/PyK2/Src signaling or (2) a Ca^{2+}-independent metabotropic-like pathway. In the Ca^{2+}-dependent signaling, Ca^{2+} influx into neurons during NMDAR excitation activates conventional isoforms of Ca^{2+}-dependent PKC, among other calcium-sensitive intracellular proteins. This activation is reported to be linked to Src via the nonreceptor tyrosine kinase PyK2,[41,68,81] serving normal physiological function,[10,47,76] as well as maladaptive changes, including epilepsy[52] and cerebral ischemia.[13,26] In neuropathic

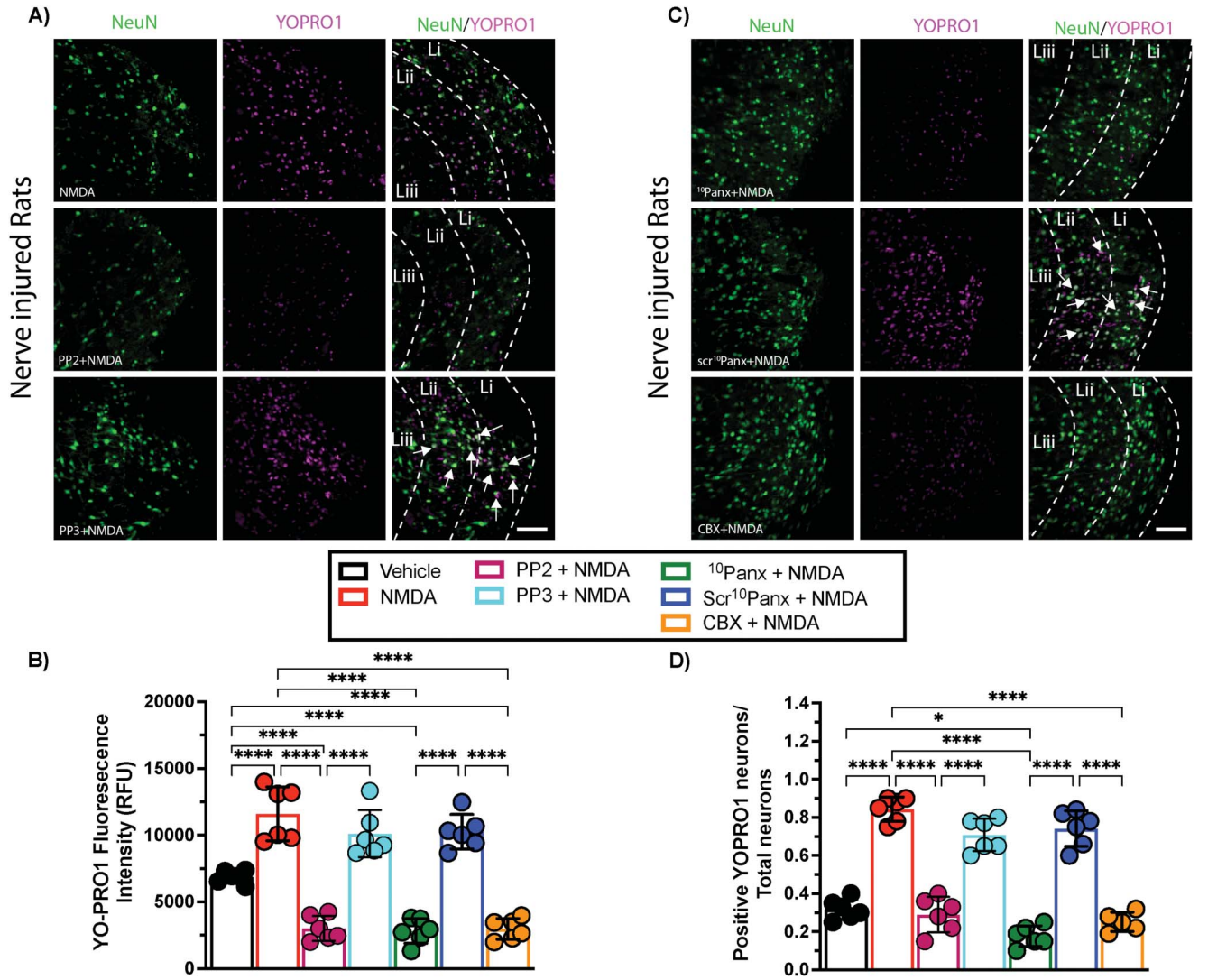

Figure 6. NMDAR-Src mediates pannexin 1 opening in spinal cord neurons. Activation of NMDAR-Src opens pannexin 1 channels in spinal cord neurons, facilitating YO-PRO-1 incorporation. Representative confocal images of YOPRO-1 incorporation in neurons of the spinal cord laminae I-II in nerve-injured rats treated with (A) vehicle, PP2 or PP3 (10 μM) and (B) ^{10}Panx, Scr^{10}Panx (300 μM) or CBX (100 μM), followed by NMDA (600 μM) stimulation. Quantitative fluorescence intensity analysis of YOPRO-1 incorporation in sham (C) and nerve-injured (D) rats. Data are expressed as means ± SEM, n = 6. $^{*}P < 0.05$, $^{****}P < 0.0001$, compared to vehicle treatment, Kruskal–Wallis test intergroup statistics followed by Dunn multiple comparisons test. Scale bar, 100 μm. The arrows indicate YO-PRO-1 incorporated in neurons. CBX, carbenoxolone; NMDAR, N-methyl-D-aspartate receptor.

pain, this pathway upregulates NMDAR, which is critical for pain hypersensitivity.[60] PyK2 has also been observed to be directly activated by intracellular free Ca^{2+} after NMDAR stimulation, which is associated with various neurological diseases.[16]

Another less studied, NMDAR-dependent Ca^{2+}-activated pathway coupled to Src activation is the nitric oxide (NO)/S-nitrosylation-activated Src pathway.[16,61] This pathway involves the stimulation of constitutive NO synthase by Ca^{2+}/calmodulin-dependent protein kinases, particularly CaMKII and CaMKIV, leading to the formation of NO from arginine in the CNS and initiating signaling in this pathway. Constitutive neuronal NO synthase is upregulated in the dorsal horn of monoarthritic[31] and nerve-injured rats,[33] although the downstream signaling pathways were not studied in those reports.

Regarding the role of a Ca^{2+}-independent metabotropic pathway, NMDAR can induce excitotoxicity and neuronal death in the hippocampus through a mechanism that does not require the receptor ion channel but instead operates via a Ca^{2+}-independent metabotropic NMDAR/Src kinase/Panx1 pathway. Previous studies have demonstrated that metabotropic NMDAR signaling drives long-term depression[50] and activity-induced dendritic spine shrinkage in hippocampal neurons, through Ca^{2+}-independent signaling.[63] Conductance-independent metabotropic signaling of NMDARs has been described in various neuronal types under both physiological and pathological conditions,[19] but not yet in dorsal horn neurons during neuropathic pain or other pain modalities.

The specific pathway by which NMDAR activation leads to Src activity in dorsal horn neurons of the spinal cord remains unclear. However, our findings demonstrated an upregulation in pSrc416 in the animals treated with NMDA, a phosphorylation site that drives the kinase domain activation loop.[57] This aligns with previous study showing that anoxia and exogenous NMDA can activate Src, as evidenced by increased Src phosphorylation at Y416 in CA1 pyramidal neurons of hippocampal slices,[70] supporting our results. Controversially, Src kinase not only acts upstream of Panx1 but also regulates NMDAR activity through GluN2 subunit phosphorylation, essential for synaptic plasticity.

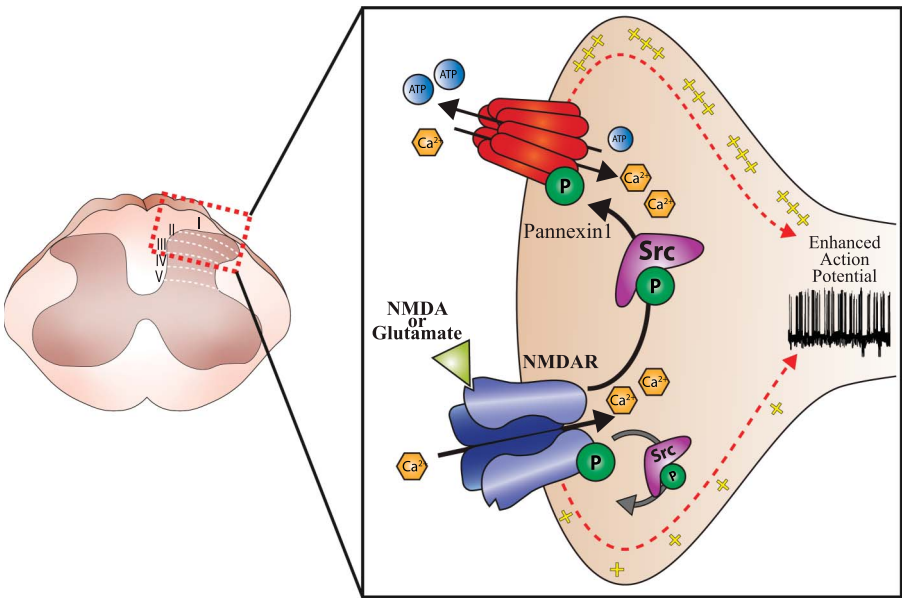

Figure 7. Pannexin 1 channel as a downstream effector of Src-mediated signaling after NMDAR activation (with NMDA or glutamate) in dorsal horn laminae I-II neurons during nerve-injured pain. Calcium influxes through NMDAR and pannexin 1 are indicated, leading to enhanced action potentials. NMDAR, N-methyl-D-aspartate receptor; P, phosphorylation.

This interplay is described as the NMDAR-PKC-PyK2-Src-pYGluN2 loop cascade,[25,40,46] suggesting that Src serves as a conduit for various non-NMDAR signaling mechanisms regulating GluN2 tyrosine phosphorylation and NMDAR function associated with synaptic plasticity. Indeed, well-established evidence indicates that NMDAR plays both a trigger and an effector role in central sensitization.[32,44] In particular, NMDAR in nociceptive neurons of the spinal and medullary dorsal horns are critical in nociceptive transmission and synaptic plasticity, recruiting of Src protein tyrosine kinase downstream of NMDAR and contributing to spinal sensitization.[4,30,44]

Our results further demonstrated that Src tyrosine kinase, activated downstream of NMDARs, mediates hyperalgesia in nerve-injured rats by opening Panx1 channels in spinal cord neurons (**Fig. 7**). Intrathecal injection of NMDA in animals resulted in both mechanical hyperalgesia and increased expression of pPanx1 phosphorylated at Tyr198 in dorsal horn neurons. Blockade of Panx1 by 10Panx reduced both hyperalgesia and pPanx1 expression, consistent with previous findings indicating that Panx1 activation can be regulated by Src tyrosine kinase activity through phosphorylation at Y198.[6,18] Whether NMDA antagonists further increase the withdrawal threshold in neuropathic rats treated with or without 10Panx or PP2 was not tested in the present study. However, the combined application of the NMDAR antagonist D-APV and the Panx1 blocker ^{10}Panx was shown to inhibit AD currents in CA1 pyramidal neurons to the same extent as either blocker alone,[70] thus supporting that Panx1 is a major downstream effector of NMDAR activation.

The NMDA-Src-Panx1 cascade had not been previously explored in the dorsal horn neurons of animals experiencing neuropathic chronic pain. Here, we elucidated that the opening of Panx1 channels in these neurons is likely a result of downstream activation of this excitatory pathway. Our findings suggest that activated NMDARs induce Src, which phosphorylates Panx1 at a consensus site in the intracellular loop, as observed in conditions like anoxia and orofacial ectopic pain.[40,70] In our study, we demonstrated that the incorporation of YOPRO-1 in dorsal horn neurons of spinal cord slices from nerve-injured rats was significantly increased compared to the sham group. In addition, treatment with the NMDAR antagonist D-AP5 significantly reduced YOPRO-1 uptake in both sham and nerve-injured rats, indicating a decrease in membrane permeation because of NMDAR inactivation. Furthermore, including the Src inhibitor PP2 significantly decreased YOPRO-1 uptake, implicating Src kinase in the plasma membrane's permeability to the dye. Moreover, our results showed that treatment with the Panx1 inhibitor ^{10}Panx or the channel blocker CBX reduced dye uptake to levels similar to those found in sham animals. This suggests that the change in plasma membrane permeation induced by NMDAR activation is combined with constitutive Panx1 activity or maintained by mechanisms other than those gated by NMDAR stimulation.

In conclusion, using a combination of behavioral, pharmacological, and molecular approaches, we demonstrated that (1) neurons of spinal cord laminae I and II exhibits functional Panx1 channels, in both nerve-injured and sham rats, which can open by stimulation of NMDARs via intrathecal NMDA; (2) intrathecal NMDA increased both pSrc and pPanx1 expression in dorsal horn neurons, exacerbating mechanical hyperalgesia in nerve-injured rats; (3) inhibition of Src with PP2 or blockade of Panx1 with ^{10}Panx attenuated the NMDA-induced effects and mitigated spontaneous mechanical hyperalgesia in nerve-injured rats; and (4) NMDA-triggered YOPRO-1 uptake in neurons of laminae I-II of spinal cord slices from nerve-injured rats was impeded by treatment with the NMDAR antagonist D-AP5, Src inhibitor PP2, and Panx1 blockers ^{10}Panx and carbenoxolone. Overall, NMDAR stimulation in dorsal horn neurons activates an NMDAR-Src-Panx1 signaling pathway, wherein Panx1 serves as an amplifying effector in neuropathic pain. This knowledge could contribute to the development of new therapeutic approaches for chronic pain based on disrupting the communication between NMDAR and Panx1, potentially through Src inhibitors and/or Panx1 blockers.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Data availability statement: The anonymized data collected are available as open data via the University of Santiago de Chile online data repository: https://doi.org/10.60547/USACH/F6CZ2S.

Comments: NMDA is a master receptor, ie, relevant in passing on the nociceptive information between the primary nociceptive neuron and the secondary projection neuron in the spinal dorsal horn. That means that the perception of acute and chronic pain disappears without NMDA activation in the spinal cord. The authors initially showed that Panx-1 is an essential canal for neuropathic pain perception because an inhibition Panx-1 produces analgesia in this chronic pain model. The present study showed that NMDAR activation in dorsal horn neurons triggers an NMDAR-Src-Panx1 signaling pathway, where Panx1 acts as an enhancing effector in neuropathic pain. Therefore, disrupting the NMDAR-Panx1 communication may offer a valuable strategy for managing some forms of chronic pain.

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