Inhibiting spinal secretory phospholipase A$_2$ after painful nerve root injury attenuates established pain and spinal neuronal hyperexcitability by altering spinal glutamatergic signaling

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Abstract

Neuropathic injury is accompanied by chronic inflammation contributing to the onset and maintenance of pain after an initial insult. In addition to their roles in promoting immune cell activation, inflammatory mediators like secretory phospholipase A$_2$ (sPLA$_2$) modulate nociceptive and excitatory neuronal signaling during the initiation of pain through hydrolytic activity. Despite having a known role in glial activation and cytokine release, it is unknown if sPLA$_2$ contributes to the maintenance of painful neuropathy and spinal hyperexcitability later after neural injury. Using a well-established model of painful nerve root compression, this study investigated if inhibiting spinal sPLA$_2$ 7 days after painful injury modulates the behavioral sensitivity and/or spinal dorsal horn excitability that is typically evident. The effects of sPLA$_2$ inhibition on altered spinal glutamatergic signaling was also probed by measuring spinal intracellular glutamate levels and spinal glutamate transporter (GLAST and GLT1) and receptor (mGluR5, GluR1, and NR1) expression. Spinal sPLA$_2$ inhibition at day 7 abolishes behavioral sensitivity, reduces both evoked and spontaneous neuronal firing in the spinal cord, and restores the distribution of neuronal phenotypes to those of control conditions. Inhibiting spinal sPLA$_2$ also increases intracellular glutamate concentrations and restores spinal expression of GLAST, GLT1, mGluR5, and GluR1 to uninjured expression with no effect on NR1. These findings establish a role for spinal sPLA$_2$ in maintaining pain and central sensitization after neural injury and suggest this may be via exacerbating glutamate excitotoxicity in the spinal cord.

Introduction

Neuropathic pain from neural tissue damage occurs in approximately 10% of the US population,[1] with the cervical dorsal nerve roots especially vulnerable to trauma.[2–5] Nerve root compression is associated with robust neuroimmune activation cascades including increased production of inflammatory mediators and spinal glial activation that can initiate and maintain pain.[6–14] Although increased inflammatory responses[13–16] alter spinal neuronal hyperexcitability,[11,14,17–21] much is still unknown about how inflammatory mediators promote chronic pain states.

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Among the robust inflammatory mediators involved in spinal neuroimmune activation is the family of phospholipase A_2 enzymes[22,23] and, in particular, the cytosolic phospholipase A_2 ($cPLA_2$) and secretory phospholipase A_2 ($sPLA_2$) isoforms that contribute to the production of downstream inflammatory proteins like prostaglandins and leukotrienes.[24–30] Both enzymatic isoforms hydrolyze the sn-2 ester bond of glycerophospholipids, releasing free fatty acids, like arachidonic acid (AA) and lysophospholipids, that act as secondary signaling molecules to enhance spinal nociceptive cascades.[25,27] Despite being constitutively active, increased $sPLA_2$ and $cPLA_2$ expression occurs concurrent with immune activation early after neural injury.[29,30] Secretory PLA_2 is upregulated as early as 4 h after spinal cord injury.[23] Its direct spinal application in naive rats induces behavioral sensitivity as soon as within 1 h and microglial activation within 5 h,[31] suggesting that early increases in spinal $sPLA_2$ may initiate pain and inflammation. In fact, inhibiting spinal $sPLA_2$ immediately after painful root trauma prevents the onset of pain and spinal hyperexcitability.[21] Although spinal $sPLA_2$ expression remains elevated 7 days after a painful root compression,[13,32] it is unknown if inhibiting spinal $sPLA_2$ at that late time, when spinal hyperexcitability and glial activation are already established[32–34] can attenuate existing pain.

Elevated phospholipases generate bioactive lipid mediators that can further exacerbate spinal inflammation and potentiate nociceptive signaling.[35–39] AA concentrations increase in pain states, including preferentially only in the dorsal horn ipsilateral to neural injury.[40] Moreover, inhibiting AA production by the $cPLA_2$ inhibitor, AACOCF3, after chronic sciatic nerve constriction prevents the onset of pain.[25] Together, these findings suggest that inhibiting generation of bioactive lipids could provide pain relief; however, since both the $sPLA_2$ and $cPLA_2$ isoforms hydrolyze phospholipids, with $cPLA_2$ implicated for primary AA production,[25,41,42] it is unknown if inhibiting either or both isoforms attenuates neuropathic pain.[42] The extent of cross-talk between isoforms has only been probed in cultured mammalian renal mesangial cells and findings suggest $sPLA_2$ may regulate $cPLA_2$ generation of AA in a protein kinase C- and extracellular signaling kinases 1/2 (ERK1/2)-dependent manner.[43] Despite this relationship between isoforms, whether spinal $sPLA_2$ regulates $cPLA_2$ activity in persistent pain states is unknown.

Lipid mediators directly and indirectly contribute to neuronal hyperexcitability,[17–20,44,45] including enhancing spinal glutamate excitotoxicity by increasing receptor activity and inhibiting glutamate uptake in glia.[46–49] Increases in spinal AA concentration competitively bind to the glutamate-binding pocket of the transporter proteins glutamate aspartate transporter 1 (GLAST1) and glutamate transporter-1 (GLT1), reducing glutamate uptake with glutamate spillover into the extracellular synaptic space.[49–52] Unlike glutamate transporters, AA and other lipid mediators have not been shown to directly bind to metabotropic or ionotropic glutamate receptors; therefore, altered expression of the metabotropic

glutamate receptor 5 (mGluR5), N-methly-d-aspartate receptor 1 (NR1) or glutamate receptor GluR1[53,54] following AA or lipid mediator application may be due to indirect activation via spinal glial glutamate uptake disruption. Both decreased spinal GLT1 and increased spinal mGluR5 accompany pain and spinal neuronal hyperexcitability at day 7 after root injury.[9,33] The concurrent increase in lipid mediator production through increased $sPLA_2$[13,32] and hydrolytic activity may further contribute to this altered spinal glutamatergic signaling. Although immediate inhibition of spinal $sPLA_2$ reduces spinal mGLUR5 transcript levels, but does not alter glutamate transporter expression 1 day after a painful nerve root injury,[21] it is unknown if inhibiting spinal $sPLA_2$ later after painful injury restores altered spinal glutamate signaling or reduces pain and/or hyperexcitability.

Since $sPLA_2$ modulates pain, neuronal hyperexcitability,[22,55] and the inflammatory cascades implicated in central sensitization after neuropathic injury,[23,24,31,56] this study investigated if inhibiting spinal $sPLA_2$ with thioetheramide-PC (TEA-PC) at day 7 after a painful root compression in the rat reduces existing behavioral sensitivity and/or spinal hyperexcitability[14,33,57] via altered spinal glutamatergic signaling. Although $sPLA_2$ expression increases at 1 day after painful root compression and remains elevated through day 7,[13,32] spinal $sPLA_2$ activity persistence has not been measured. Therefore, spinal $sPLA_2$ activity was first measured at day 7 after a painful root compression to establish its relevance as a target.

Although immediate intrathecal TEA-PC administration prevents increased spinal $sPLA_2$ activity at day 1,[21] the duration of active inhibition is not known nor are the effects when given after spinal neuroimmune responses and pain are established (at day 7).[13,34,58] Furthermore, the effects of spinal $sPLA_2$ inhibition by TEA-PC at that dose on the catalytic activity of the $cPLA_2$ isoform and glutamate signaling that is involved in establishing pain and spinal hyperexcitability[59-62] have not been measured for later times. Understanding if and/or how spinal $sPLA_2$ inhibition mediates these processes that maintain pain later after neuropathic injury may provide useful insights into how to effectively treat neuropathic pain once established.

Materials and methods

Surgical procedures and inhibitor treatment

All procedures were approved by our institution's Institutional Animal Care and Use Committee and performed under the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.[63] Spinal $sPLA_2$ activity at day 7 after a painful nerve root compression was first characterized using two groups of rats ($377 \pm 21g$) undergoing either a painful nerve root compression (*NRC*; $n = 4$) or a sham surgery (*sham*; $n = 3$) to serve as surgical controls. Under inhalation anesthesia (4% induction, 2% for maintenance) male Holtzman rats underwent

a unilateral C7 nerve root compression (NRC) that induces behavioral sensitivity.[21,32,33,57,64] Briefly, following a midline incision between the C2 and T2 vertebrae, a C6/C7 hemilaminectomy and partial facetectomy were performed on the right side to expose the C7 right dorsal nerve root. A 10-gf microvascular clip (World Precision Instruments; Sarasota, FL) was inserted through a small incision in the dura and placed around the nerve root to apply compression (NRC) for 15 min.[14,21,32,33] Rats undergoing a sham surgery received identical surgical procedures with the nerve root exposed but not compressed. After each procedure, surgical wounds were closed using 3–0 polyester sutures and surgical staples and monitored while rats recovered in room air.

To assess the effectiveness of spinal $sPLA_2$ inhibition against established neuropathic pain, in a second set of rats (452 ± 38 g) separate groups received an intrathecal injection of either the $sPLA_2$ inhibitor TEA-PC (NRC+$sPLA_2$ inhibitor $n = 8$) or a vehicle injection of phosphate buffered saline (PBS) (NRC+vehicle $n = 8$) 7 days after a painful nerve root compression. Sham procedures were also included to control for the effects of surgery and intrathecal administration; that additional group of rats received a sham surgery in which the nerve root was exposed but not compressed, and an intrathecal injection of PBS was administered at day 7 following surgery (sham+vehicle $n = 4$).

Rats underwent either a painful nerve root compression or sham exposure and on post-operative day 7, rats were randomly assigned to receive an intrathecal injection of either TEA-PC or PBS. All drug solutions were administered via syringe in the intrathecal space between L4 and L5 via a lumbar puncture to the rats under anesthesia.[14,33] The treatment group (NRC+$sPLA_2$ inhibitor $n = 8$) received a single 40–60 µL injection administered slowly over 1–2 min of the $sPLA_2$ inhibitor TEA-PC (0.25 mg/mL) dissolved in PBS. In the vehicle treatment group (NRC+vehicle $n = 8$), rats received a 40–60 µL injection of PBS, corresponding to the weight-matched volume of the inhibitor administration. The same vehicle treatment was administered to rats that underwent sham procedures (sham+vehicle $n = 4$).

Behavioral sensitivity measurements

To assess if inhibiting spinal $sPLA_2$ at day 7 after a painful nerve root compression attenuates established behavioral sensitivity, mechanical hyperalgesia in response to mechanical stimulation was measured in the forepaw ipsilateral to the injury.[65] Behavioral sensitivity was measured prior to any surgical procedures on day 0 (baseline) and daily thereafter through day 14. During testing, the plantar surface of the forepaw was stimulated by a series of weighted von Frey filaments of increasing strengths (1.4–26 g) to identify the withdrawal threshold to forepaw stimulation.[14,21,66] An observer, blinded to each group, applied each filament five times before advancing to the next highest strength and if two consecutive filaments both induced a withdrawal response exhibited by licking or lifting of the paw, the lower strength

filament was recorded as the withdrawal threshold. On each assessment day, rats were acclimated to the testing environment and tester for at least 15 min, followed by three rounds of testing with 10 min of rest between each round. Paw withdrawal thresholds were averaged across rounds for each rat on each day. Differences in the ipsilateral forepaw thresholds between groups were compared using a two-way repeated measures ANOVA with post-hoc Tukey's test.

Spinal phospholipase activity rate assay

In the first set of rats, spinal cord tissue was harvested at day 7 (NRC $n = 4$; sham $n = 3$) to evaluate $sPLA_2$ activity after injury. In the second set of rats (NRC+$sPLA_2$ inhibitor $n = 8$; NRC+vehicle $n = 8$; sham+PBS $n = 4$), spinal cord was assayed at day 14 to evaluate whether the $sPLA_2$ inhibitor stopped $sPLA_2$ cleavage activity. Spinal $sPLA_2$ activity was measured in the ipsilateral C6 spinal cord using a $sPLA_2$ Activity Kit (Cayman; Ann Arbor, MI) as previously described.[21] Rats were transcardially perfused with 250 mL of chilled PBS. Spinal tissue was then homogenized in 50 mM HEPES buffer, pH 7.4 and centrifuged at 10,000g for 15 min at 4°C. Supernatant was removed and incubated with both $cPLA_2$ and $iPLA_2$ inhibitors to isolate $sPLA_2$ activity, and then incubated with the substrate arachidonoyl thio-PC, at room temperature as per assay instructions. In the same set of rats (NRC+$sPLA_2$ inhibitor $n = 8$; NRC+vehicle $n = 8$; sham+PBS $n = 4$), spinal $cPLA_2$ activity was assessed at day 14 using a $cPLA_2$ Activity Kit (Cayman; Ann Arbor, MI).[67] Similar to the $sPLA_2$ activity assay, homogenized samples were centrifuged at 10,000g and the supernatant was incubated with $sPLA_2$ and $iPLA_2$ inhibitors to isolate $cPLA_2$ activity before incubation with the arachidonoyl thio-PC substrate at room temperature for 1 h in assay buffer.

For both $sPLA_2$ and $cPLA_2$ activity assays, fluorescent measurements were made every minute over 10 min using a fluorescent plate reader (Tecan InfinitePro; Mannedorf, Switzerland). The reaction rate for each of $sPLA_2$ and $cPLA_2$ activity for each sample was separately calculated by measuring absorbance readings over time after subtracting absorbances from non-enzymatic controls. Rates of $sPLA_2$ activity on day 7 were compared separately between NRC and sham groups using a Student's t-test. For spinal tissues on day 14, $sPLA_2$ and $cPLA_2$ activity rates were separately compared between inhibitor- and vehicle-treated groups using a one-way ANOVA (group) with post-hoc Tukey's test.

Spinal intracellular glutamate measurements

Intracellular glutamate was also assayed in the ipsilateral C6 spinal cord tissue of rats in the inhibitor- (NRC+$sPLA_2$ inhibitor $n = 8$) and PBS-treated (NRC+vehicle $n = 8$; sham+PBS $n = 4$) groups using the tissues homogenized for the phospholipase A_2 activity assays at day 14. Using a Glutamate Glo Assay Kit (Promega; Madison, WI) as

described,[59] homogenized spinal tissue was further permeabilized (1% Triton X-100, 500 mM NaCl, 5 mM HEPES, pH 7.4) and combined with a luciferin detection solution consisting of glutamate hydrogenase, NAD, reductase, and the reductase substrate for 1 h.[59] Luminescence readings were then collected on a fluorescent plate reader (Tecan InfinitePro; Mannedorf, Switzerland) with the extent of luminescence corresponding to the concentration of glutamate in the sample. Relative luminescence in spinal tissue samples was compared across the inhibitor-treated (NRC+sPLA$_2$ inhibitor $n = 8$) and PBS-treated (NRC+vehicle $n = 8$; sham+PBS $n = 4$) groups using a one-way ANOVA (group) with Tukey's post-hoc test.

Spinal electrophysiological recordings

On day 14 before the tissue harvest, electrophysiological recordings of the spinal neurons were acquired from the C6–C7 dorsal horn on the side ipsilateral to the surgery from rats in the inhibitor-treated (NRC+sPLA$_2$ inhibitor $n = 8$) and PBS-treated (NRC+vehicle $n = 8$; sham+PBS $n = 4$) groups. For electrophysiology recordings, rats received an intraperitoneal (i.p.) injection of sodium pentobarbital (45 mg/kg) and anesthesia was maintained with supplementary doses (5–10 mg/kg i.p.) for the duration of the recording procedures.[33,68,69] Rats also received a tracheotomy and were connected to a mechanical ventilator (40–50 cycles/min) and CO_2 monitor (CWE, Inc.; Ardmore, PA). Prior to recording, the spinal cord was exposed and the dura around the C6/C7 spinal levels was removed to allow insertion of the recording electrode. Rats were immobilized on a stereotactic frame (David Kopf Instruments; Tujunga, CA); core body temperature was maintained at 35–37°C using a heating plate and monitored using a rectal probe (Physiotemp; Clifton, NJ).

Extracellular voltage potentials were recorded using a glass insulated tungsten electrode (FHC; Bowdoin, ME) that was lowered into the superficial (I–II) and deep laminae (III–IV) of the spinal cord via a micropositioner.[4,5,12-14] Signals were digitally sampled at 25 kHz (Micro1401; CED; Cambridge, UK) and recorded using Spike2 software (CED; Cambridge, UK).[33,68,69] Mechanosensitive neurons were identified by brushing the plantar surface of the forepaw;[33,68–70] following identification, a series of non-noxious and noxious mechanical stimuli was applied to the forepaw ipsilateral to the injury consisting of a light brush, von Frey filaments (1.4, 4, 10, and 26 g) and a noxious pinch (60 g vascular clip).[33,68–70] Before any stimulus was applied to the forepaw there was a 2-s baseline period, stimulation began with a light brushing (applied for 10 s), followed by application of a series of non-noxious and noxious von Frey filaments (1.4 g, 4 g, 10 g, and 26 g) each applied for five stimulations of 1-s followed by 1-s of recovery, and application of a noxious pinch (applied for 10-s), as previously described.[33,68–70] In total, the non-noxious and noxious stimuli were applied for 30-s intervals.

Voltage recordings from each neuron were spike-sorted using Spike2 (CED; Cambridge, UK) to identify individual neurons. Both evoked and spontaneous activity were recorded since both types of neuronal activity are hallmarks of central sensitization.[62,71,72] The number of evoked spikes was summed over the continuous 10-s stimulus period for each of the brush and pinch stimuli. For stimulation by the von Frey filaments, the number of evoked spikes was summed from the initial application of the filament until 1-s after the last application.[33,68–70] For each evoked stimulus, the baseline firing in the 1-s period prior to stimulus application was quantified and subtracted from the total evoked spike count. Spontaneous activity was evaluated by totaling the number of spikes in the 2-s baseline period prior to the brush stimulation.[73] Neurons were classified based on their spontaneous activity during the baseline period before the application of the brushing: neurons exhibiting no baseline firing (0 spikes/sec), or active neurons exhibiting some level of spontaneous activity either at less than 1 spike/sec or at or more than 1 spike/sec. The distribution of spontaneously active neurons was compared between groups using a Pearson χ^2 tests (4×3 contingency table) with Tukey post-hoc test.

Since a hallmark of central sensitization is an increase in the number of neurons responding to a wide range of inputs,[68,70] each neuron for which recordings were made was phenotypically classified according to their evoked responses from von Frey stimulation. Wide dynamic range (WDR) neurons were taken as those that respond in a graded manner to stimuli of increasing strength; neurons were classified as low-threshold mechanoreceptive (LTM) neurons if they responded more to non-noxious filaments (1.4 g and 4 g) than noxious filaments (10 g and 26 g) and nociceptive specific (NS) neurons were those responding only to noxious filaments.[73] The evoked spike counts and spontaneous activity were also analyzed separately for the WDR and NS neuronal populations since both of these phenotypes respond differently to prolonged noxious stimulation having varied contributions to central sensitization.[74,75] Evoked responses were compared between groups using a two-way nested ANOVA (group × stimulus), with neurons nested within rats and rats nested within groups, with Tukey post-hoc test.[33,68–70] Pearson χ^2 tests (4×3 contingency table) with Tukey post-hoc test evaluated differences in the distribution of neuron phenotypes in each group as well as the differences in distribution of spontaneously firing neurons in each group, separately for each neuron phenotype.

Spinal western blot and analyses

The C7 and C8 spinal cord tissue ipsilateral to procedures was collected on day 14 from the inhibitor-treated (NRC+sPLA$_2$ inhibitor $n = 8$) and PBS-treated (NRC+vehicle $n = 8$; sham+PBS $n = 4$) groups to assess glutamate transporter (GLT1, GLAST) and glutamate receptor (mGluR5, GluR1, NR1) expression. Spinal cord tissue from a normal unoperated

rat ($n = 1$) was included in each blot to serve as a control. Tissue was homogenized in lysis buffer (50 mmol/L TrisHCl, 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L ethylenedia-minetetraacetic acid (EDTA, pH 8.0)) in the presence of protease and phosphatase inhibitors (Sigma-Aldrich Corp.; St Louis, MO).[69,70] Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed by loading spinal cord protein (37 µg per well) on a polyacrylamide gel (Invitrogen; Carlsbad, CA) and running for 75 min at 150 V. Protein was transferred from the poly-acrimide gel onto a polyvinylidene difluoride (PVDF) membrane using an iBlot (Invitrogen; Carlsbad, CA). Gels were divided based on their high-molecular weight (75–150 kDa; mGluR5, NR1, GluR1) or low-molecular weight (25–75 kDa; GLT1, GLAST) protein targets.

Membranes were then blocked for 1 h using an Intercept Blocking Buffer (Li-Cor Biosciences; Lincoln, NE) and incubated overnight at 4°C with primary antibodies for the secondary signaling molecule, the ionotropic glutamate receptors NMDA subunit NR1 (rabbit, 1:1000; Millipore; Billerica, MA) and GluR1 (rabbit, 1:1000; Millipore; Billerica, MA), the metabotropic glutamate receptor mGluR5 (rabbit, 1:1250; Millipore, Billerica, MA), the astrocytic glutamate transporters GLAST (rabbit, 1:2000; Abcam, Cambridge, MA) and GLT1 (rabbit, 1:500, Abcam, Cambridge, MA), or β-tubulin as a loading control (mouse, 1:2000; Covance, Princeton, NJ). The PVDF membrane was washed in tris-buffered saline (TBS) with 0.1% Tween, followed by a 2-h incubation at room temperature with goat anti-rabbit 800 and goat anti-mouse 680 fluorescent secondary antibodies (1:10,000; Li-Cor Biosciences, Lincoln, NE). Each membrane was imaged using an Odyssey Imaging System (Li-Cor Biosciences; Lincoln, NE). The fluorescence intensity of each target protein band was analyzed using the Odyssey 2.1 software and normalized to the corresponding β-tubulin fluorescence to control for the amount of protein loaded. Protein expression was calculated as fold-changes over corresponding protein levels in normal tissue and each

protein of interest was compared between groups using separate one-way ANOVAs with a post hoc Tukey's test.

Results

Spinal $sPLA_2$ activity at day 7 after a painful nerve root compression (962.25 ± 175.30 µmol/min/mL) is significantly elevated ($p = 0.005$) over activity levels for a sham procedure (397.60 ± 193.96 µmol/min/mL) (Figure 1(a)).

Inhibiting spinal $sPLA_2$ 7 days after a nerve root compression injury attenuates the behavioral sensitivity that is typically evident over 14 days after that injury (Figure 2). After nerve root compression, the withdrawal thresholds for the ipsilateral paw are significantly lower ($p < 0.0001$) than baseline (day 0) and corresponding sham thresholds ($p < 0.0002$) through day 7, and thresholds after a sham procedure are not different from baseline on any day tested (Figure 2). Following behavioral assessments on day 7, the withdrawal thresholds are significantly elevated ($p < 0.0004$) with inhibitor treatment over thresholds for the vehicle-treated group starting on day 8, returning to sham levels through day 14 (Figure 2). In contrast, thresholds with vehicle treatment on day 7 remain significantly lower than both their corresponding baseline ($p < 0.0001$) levels and the sham ($p < 0.014$) levels, as well as those for the inhibitor treatment ($p < 0.0004$) (Figure 2).

Both spinal $sPLA_2$ and $cPLA_2$ activity are modulated at day 14 after a painful root compression and a single intrathecal dose of TEA-PC at day 7 is sufficient to inhibit spinal $sPLA_2$ and alter $cPLA_2$ activity in the spinal cord (Figures 1 and 3). Spinal $sPLA_2$ activity in the vehicle-treated NRC group is significantly higher ($p = 0.035$) than sham activity levels at day 14 (Figure 1(b)). However, spinal $sPLA_2$ activity after inhibitor treatment at day 7 is reduced to sham levels and is significantly lower ($p = 0.026$) than corresponding levels in the vehicle-treated group (Figure 1(b)). Inhibitor treatment also brings spinal $cPLA_2$ activity to sham levels (Figure 3(a)), which are significantly lower ($p = 0.008$) than the activity in
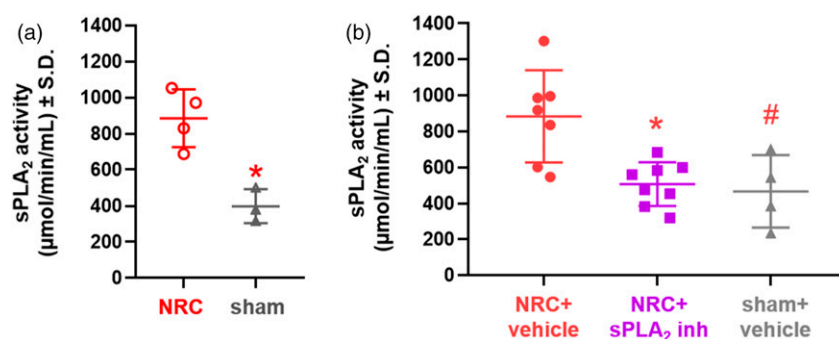

Figure 1. Spinal $sPLA_2$ activity is elevated at days 7 and 14 after a painful nerve root compression and is attenuated at day 14 following spinal $sPLA_2$ inhibition given at day 7. **(a)** Spinal $sPLA_2$ activity at day 7 after a painful nerve root compression (NRC) is significantly elevated ($p = 0.005$) over sham control levels. **(b)** Inhibitor treatment at day 7 significantly reduces (*$p = 0.026$) spinal $sPLA_2$ activity to sham levels on day 14. Spinal $sPLA_2$ activity after NRC with vehicle treatment is also significantly higher (#$p = 0.035$) than for sham.
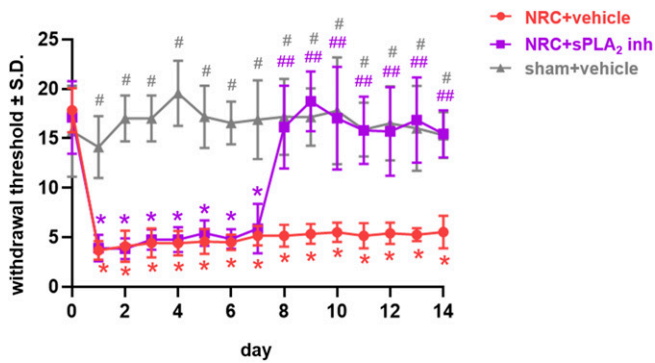
Figure 2. Spinal sPLA$_2$ inhibition at day 7 after painful nerve root compression abolishes existing sensitivity through day 14. Painful nerve root compression (NRC) significantly decreases withdrawal thresholds from baseline (*p < 0.0001) and relative to corresponding sham thresholds (#p < 0.0002) through day 7. Treatment with the PBS vehicle on day 7 does not alter withdrawal thresholds, which remain significantly lower than baseline (*p < 0.0001) and sham (#p < 0.014) through day 14. But, after intrathecal treatment with the sPLA$_2$ inhibitor, thresholds are significantly elevated (##p < 0.0004) over thresholds for the vehicle treatment group and return to sham levels as early as day 8 lasting through day 14.

the corresponding vehicle-treated injury group (Figure 3(a)). Spinal cPLA$_2$ activity levels after NRC and vehicle treatment are also significantly elevated (p = 0.008) over those of the sham group. Spinal sPLA$_2$ inhibition significantly increases (p = 0.006) spinal intracellular glutamate concentrations as compared to vehicle treatment (Figure 3(b)). Intracellular glutamate concentrations after vehicle treatment are also significantly lower (p = 0.022) than sham levels (Figure 3(b)). There are no differences in intracellular glutamate levels detected between the sham and inhibitor-treated NRC groups (Figure 3(b)).

In addition to increasing intracellular glutamate concentration to sham levels (Figure 3(b)), spinal sPLA$_2$ inhibition restores glutamate transporter expression and decreases expression of two of the glutamate receptors probed (Figures 4 and 5). Specifically, GLAST expression (60 kDa) is significantly lower (p = 0.034) in the inhibitor-treated group compared to the vehicle-treated group after NRC and is the same as sham levels, which are also significantly (p = 0.027) lower than the untreated group (Figure 4(b)). Those same groups track together for GLT1 (62 kDa) expression, with both the inhibitor-treated and sham control groups having significantly more (p < 0.0009) GLT1 than the group with NRC and vehicle treatment (Figure 4(b)). Of note, for treatment with vehicle, GLT1 expression in several samples is at normal levels at day 14 (Figure 4(b)). Expression of both the mGluR5 and GluR1 receptors is greater than normal levels with vehicle treatment (Figure 5) and is significantly higher (p = 0.01 mGluR5; p = 0.035 GluR1) than levels in the sham control group (Figure 5(b)). Spinal sPLA$_2$ inhibition significantly reduces both mGluR5 (p = 0.002) and GluR1
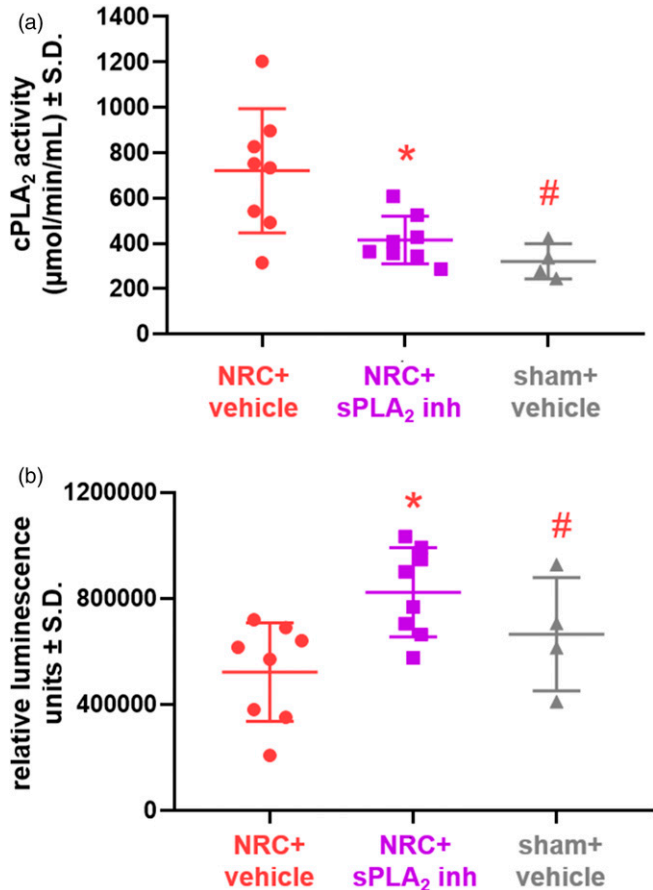

Figure 3. Intrathecal administration of the sPLA$_2$ inhibitor reduces cPLA$_2$ activity in the spinal cord and increases intracellular glutamate concentration after a painful nerve root compression (NRC). **(a)** Spinal cPLA$_2$ activity in the vehicle treated group is significantly elevated over (#p = 0.008) sham activity levels at day 14; but, sPLA$_2$ inhibition after NRC significantly reduces (*p = 0.018) spinal cPLA$_2$ activity to sham levels. **(b)** Intracellular glutamate levels in spinal tissues after a painful NRC are significantly lower with vehicle treatment than for NRC with inhibitor treatment (*p = 0.006) and sham (#p = 0.022).

(p = 0.0004) expression when compared to vehicle-treated levels (Figure 5(b)) and keeps them at sham levels. There were no differences in NR1 expression detected between groups.

Recordings were made at day 14 from a total of 126 spinal neurons at an average depth of 442 ± 270 µm across all groups. Although a painful nerve root compression has 34.5% of the spinal neurons classified as LTM, 55.2% as WDR and 10.4% as NS at day 14, spinal sPLA$_2$ inhibition restores the proportion of spinal neurons classified as WDR neurons to sham levels (Figure 6(a)). The distribution of neuronal phenotypes after inhibitor treatment is significantly different (p = 0.005) from the proportion of phenotypes in the vehicle-treated group (Figure 6(a)). The distribution of neuronal phenotypes in the vehicle-treated painful compression group is also significantly different (p = 0.027) from
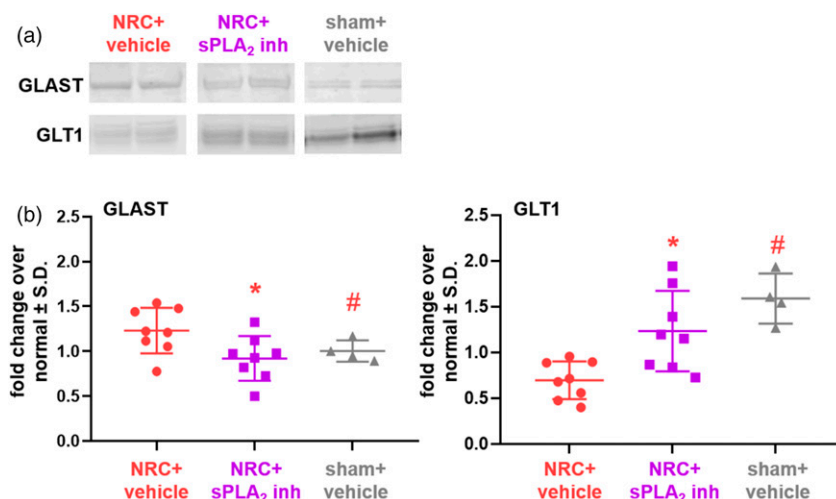
Figure 4. Spinal sPLA$_2$ inhibition restores glial transporter expression at day 14 after nerve root injury. **(a)** Representative blots show spinal glutamate transporter expression for all groups. **(b)** Spinal GLAST expression with vehicle treatment after a painful root compression (NRC) is higher than levels of expression for sham (#p = 0.027), but inhibitor treatment after NRC significantly reduces (*p = 0.034) GLAST expression to sham levels. Conversely, vehicle treatment significantly downregulates GLT1 compared to sham control levels (#p < 0.0006), but GLT1 protein levels for NRC with inhibitor treatment are significantly (*p <0.009) higher than levels for untreated NRC and are not different from sham.
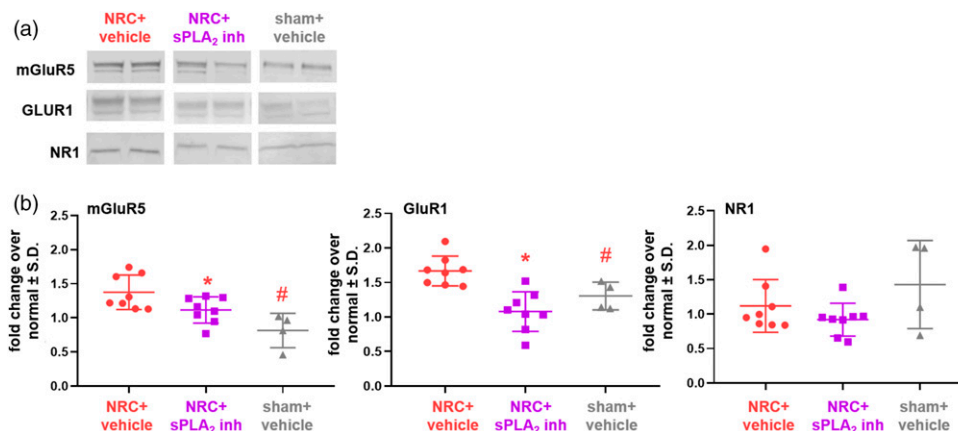

Figure 5. At day 14, painful nerve root compression (NRC) increases mGluR5 and GluR1 expression that is reduced with spinal sPLA$_2$ inhibition. **(a)** Representative blots show spinal mGluR5, GluR1, and NR1 expression. **(b)** Spinal sPLA$_2$ inhibition significantly reduces both mGluR5 (*p < 0.01) and GluR1 (*p < 0.035) expression to sham levels, as compared to expression in vehicle-treated spinal cord. Whereas expression in the vehicle-treated NRC group for both receptors is significantly greater (#p = 0.002 mGluR5; #p = 0.0004 GluR1) than sham expression levels. NR1 expression is not different between any groups.

that of the sham group, with no differences observed in the distribution between the inhibitor-treated and sham groups (Figure 6(a)). In addition, spinal sPLA$_2$ inhibition blocks the increase in evoked spikes for each neuronal phenotype that is usually evident and keeps activity at sham levels (Figure 6(b)). In particular, stimulation of the affected forepaw with both non-noxious (1.4, 4 g) and noxious (10 g, 26 g) filaments evokes significantly fewer spikes in the inhibitor-treated group and the nonpainful sham control compared to responses following a painful compression with vehicle treatment for all neuronal phenotypes, including LTM (p < 0.0005), WDR (p < 0.038), and NS (p < 0.029) (Figure 6(c)).

In contrast, vehicle treatment with a painful root compression induces significantly more spikes (LTM p < 0.0007; WDR p < 0.039; NS p < 0.015) for each neuronal phenotype compared to the evoked responses in the sham group, for all filaments tested (Figure 6(c)). There are no differences in the evoked responses between the inhibitor-treated compression and sham groups for any filament.

In addition to preventing the increases in activity and shift in the distribution of neuronal phenotypes (Figure 6), inhibiting spinal sPLA$_2$ at day 7 also attenuates the overall dorsal horn neuronal hyperexcitability that is evident 14 days after a painful nerve root compression (Figure 7). Although
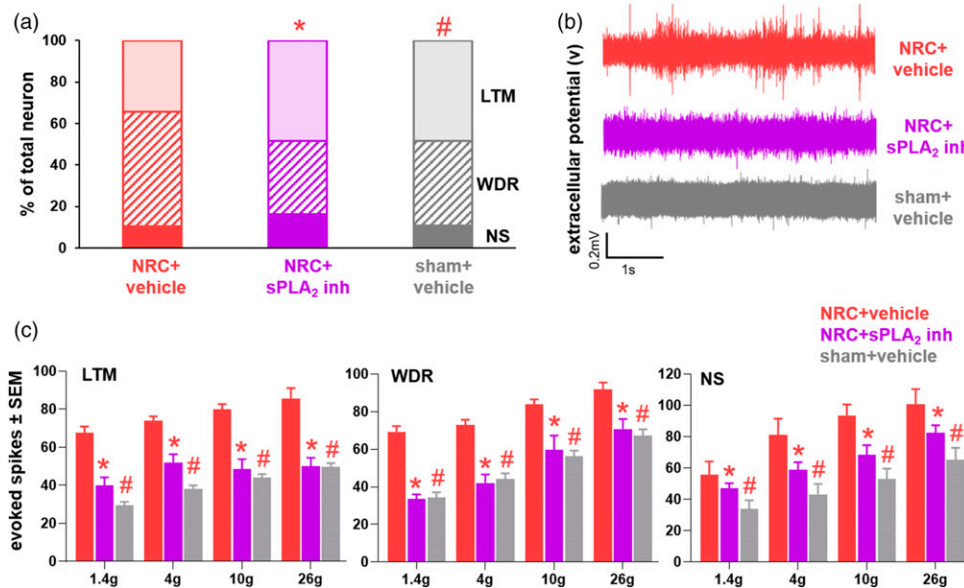
Figure 6. Spinal neuronal phenotype distribution and evoked activity at day 14 with a nerve root compression (NRC) are restored to sham levels with spinal sPLA$_2$ inhibitor treatment at day 7. **(a)** The proportion of WDR neurons is greatest with for NRC with vehicle treatment and the distribution of LTM, WDR and NS neurons is significantly (#p = 0.027) different from sham and NRC with inhibitor treatment (*p = 0.005). Inhibitor treatment restores the phenotypic distribution to sham levels. **(b)** Representative voltage tracings from WDR neurons show inhibitor treatment reduces neuronal activity following 1.4 g stimulation as compared to vehicle. **(c)** sPLA$_2$ inhibitor treatment significantly reduces evoked activity in all neuronal phenotypes (LTM *p < 0.0005; WDR *p < 0.038; NS *p < 0.029) compared to activity in the NRC with vehicle treatment, with no differences between the inhibitor treated and sham groups. Evoked activity in the vehicle-treated group is also significantly elevated (LTM #p < 0.0007; WDR #p < 0.039; NS #p < 0.015) for all filaments compared to sham for every neuronal phenotype.

there are no differences in neuronal firing evoked by the non-noxious light brush among groups (Figure 7(a)), neuronal activity elicited by a noxious pinch is significantly lower for the compression with inhibitor treatment at day 7 (p = 0.005) and sham (p = 0.0001) than the responses for the group undergoing a painful compression with vehicle treatment at day 7 (Figure 7(a)). Inhibitor treatment also significantly lowers (p < 0.026) evoked responses to sham levels in spinal dorsal horn neurons for stimulation with both the non-noxious and noxious filaments as compared to response of the vehicle-treated group (Figure 7(b)). The neuronal firing evoked in the group receiving compression with vehicle treatment at day 7 is significantly higher (p < 0.004) than that for the sham control group for all filaments tested (Figure 7(b)).

In addition to regulating spinal neuron activity evoked by paw stimulation, neurons similarly exhibit different spontaneous activity responses across the groups (Figure 7(c) and (d)). Spinal sPLA$_2$ inhibition reduces the increased spontaneous activity that is observed in the dorsal horn at day 14 with a painful nerve root compression (Figure 7(d)), with a greater proportion of neurons with no baseline firing or <1 spike/sec, as well as fewer active neurons with spontaneous activity (>1 spike/sec) (Figure 7(d)). The distribution of spontaneous firing rates among neurons is significantly different (p = 0.033) in the compression with inhibition

treatment compared to the painful vehicle-treated group, but there are no differences in distribution compared to the sham control group (Figure 7(d)). The distribution of spontaneously active neurons in the sham control group is also significantly lower (p = 0.007) than for compression with vehicle treatment (Figure 7(d)). Spinal sPLA$_2$ inhibition also selectively reduces spontaneous activity in the WDR neurons, with the proportion of inactive neurons (0 spikes/sec) increasing to 71.0% with treatment as compared to 55.2% in the vehicle-treated group. Similarly, the proportion of spontaneously active neurons (with any activity greater than 0) decreases to 29.0% after inhibitor treatment as opposed to 44.8% with vehicle treatment. The distribution of spontaneous activity responses following inhibitor treatment is significantly different (p = 0.038) than the distribution observed in the vehicle-treated groups and there are no differences (p > 0.05) in the distribution of spontaneous neurons between the inhibitor-treated and sham groups. The distribution of WDR neurons exhibiting spontaneous activity in the vehicle-treated injury group (70%) is also significantly higher (p = 0.006) than the distribution for the WDR neurons in the inhibitor-treated group (40%). There are no differences in the proportion of neurons exhibiting spontaneous activity among groups for either the LTM or NS neurons.
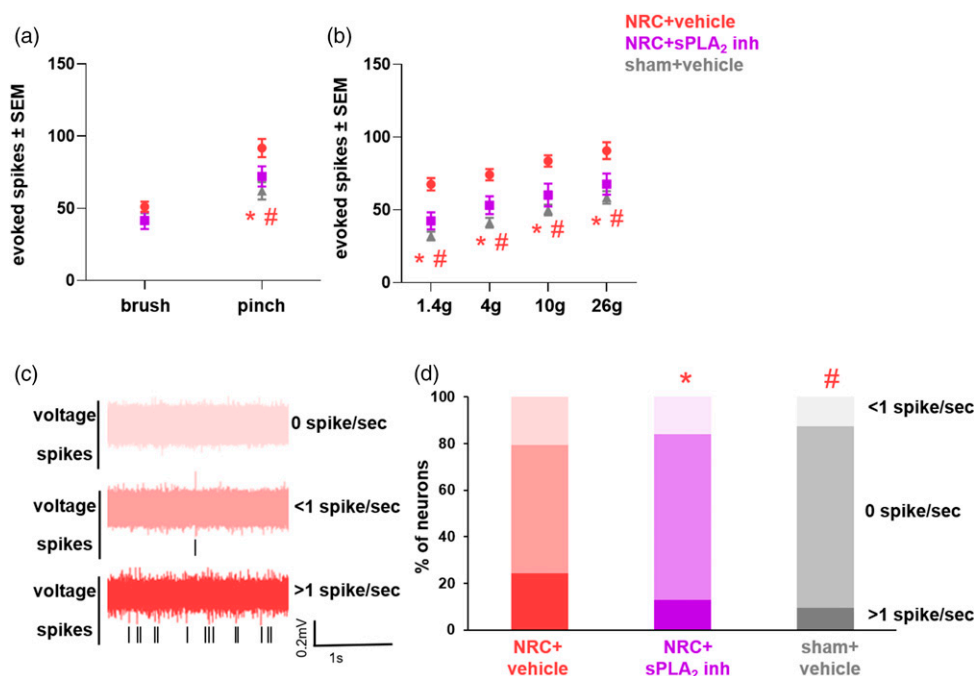
Figure 7. Spinal $sPLA_2$ inhibition at day 7 returns evoked and spontaneous dorsal horn activity to sham levels on day 14 after painful nerve root injury. **(a)** Differences in dorsal horn evoked activity are only observed after a noxious pinch, with both the inhibitor-treated (*p = 0.005) and sham (#p = 0.0001) groups exhibiting significantly fewer evoked spikes than the vehicle-treated nerve root compression (NRC) group. **(b)** Both non-noxious and noxious filament stimulation also elicit significantly more evokes spikes for NRC with vehicle treatment than sham (#p < 0.004); yet, inhibitor treatment significantly reduces (*p < 0.026) evoked activity to sham levels. **(c)** Example voltage traces from vehicle-treated neurons that display no baseline firing (0 spikes/sec) or active firing (either <1 or ≥1 spike/sec). **(d)** Spinal $sPLA_2$ inhibition also restores the proportion of neurons that exhibit spontaneous activity to sham levels and is significantly different (*p = 0.038) than the proportion of neurons observed after NRC with vehicle treatment. At day 14, the proportion of neurons with spontaneous activity is also significantly different (#p = 0.006) in the vehicle NRC group than that in the sham control group.

Discussion

Inhibiting spinal $sPLA_2$ after painful neuropathic injury, at a time when both pain and abberant neuronal activity are established,[9,64] attenuates both that behavioral sensitivity and neuronal hyperexcitability, in part by restoring the spinal glutamatergic signaling that is altered after a painful nerve root compression (Figures 2–7). The abolishment of behavioral sensitivity (Figure 2) occurs in conjunction with increased spinal intracellular glutamate concentration (Figure 3(b)) suggesting spinal $sPLA_2$ may contribute to the excitotoxicity and glutamate spillover that promotes the neuronal hyperexcitability that maintains neuropathic pain.[62,76] Since mounting evidence in neuronal cultures shows bioactive lipids modulate glutamatergic neurotransmission,[55,77,78] inhibiting spinal $sPLA_2$ may act by mechanisms that attenuate bioactive lipid generation. In fact, the decrease in $cPLA_2$ activity in the spinal cord at day 14 with $sPLA_2$ inhibition (Figure 1(b) and 3(b)) suggests that inhibiting $sPLA_2$ alone may be sufficient to regulate hydrolysis of phospholipid membranes and liberation of bioactive lipids. This purported decrease in hydrolytic activity and likely generation of AA with $sPLA_2$ inhibition may restore glial glutamate transporter expression (Figure 4), thereby restoring spinal glutamate uptake and increasing intracellular glutamate concentrations (Figure 3(b)) and reducing excitotoxicity. Spinal $sPLA_2$ inhibition also reduces mGluR5 and GluR1 expression in conjunction with decreasing spinal neuronal hyperexcitability (Figures 5–7) which supports its neuromodulatory role in maintaining neuropathic pain.

Abolishing behavioral sensitivity is consistent with other reports that inhibiting spinal phospholipase activity reduces established neuropathic pain.[25,32] However, inhibiting downstream proteins in the phospholipase A_2 cascade, like cyclooxygenase (COX), is not sufficient to reduce such established pain.[25] Although spinal $sPLA_2$ is elevated at day 7 after injury,[13,23,32] spinal $sPLA_2$ hydrolysis activity is also upregulated at both days 7 and 14 after a painful nerve root compression (Figure 1), suggesting that $sPLA_2$-mediated lipid generation continues even after the initial injury and inflammatory response. Similarly, spinal $cPLA_2$ activity is elevated at day 14 but that activity returns to sham levels with administration of the $sPLA_2$ inhibitor (Figure 3(a)). Although the $sPLA_2$ and $cPLA_2$ isoforms primarily hydrolyze extracellular and intracellular phospholipids, respectively,[42,79] there is substantial cross-talk between both isoforms and in vitro studies suggest the actions of $cPLA_2$ depend on coordination

with sPLA$_2$.[41,79,80] Studies in granulocytes[81] and mast cells[82] point to sPLA$_2$-mediated release of lysophospholipids as amplifying cPLA$_2$ activation, leading to increased synthesis of lipid mediators in inflammation. Inhibiting spinal sPLA$_2$ is sufficient to *also* reduce spinal cPLA$_2$ activity (Figure 3(b)) and attenuate pain (Figure 2). Interestingly, intrathecal cPLA$_2$ inhibition by AACOCF3 after chronic constriction injury only attenuates pain with *repeated twice daily* dosing.[25] The difference in effective dosing may be attributed to the complex actions of sPLA$_2$ outside the cell, including the release of not only AA but also saturated, monounsaturated, and polyunsaturated fatty acids (PUFAs) like docosahexaenoic acid (DHA) and platelet activating factor (PAF), which are all precursors to anti-inflammatory lipid mediators involved in the pathogenesis of neuropathic pain.[42,82] Together with the regulation of intracellular cPLA$_2$ activity, production of lipid mediators that contribute to neuropathic pain, and the relative ease of accessing extracellular sPLA$_2$ versus intracellular cPLA$_2$, inhibiting spinal sPLA$_2$ is a more attractive treatment target.

Neuropathic pain is characterized by progressive down-regulation of spinal glutamate transporter expression,[25,33,83] contributing to the accumulation of extracellular glutamate that potentiates neuronal hyperexcitability.[84] Reduced spinal glutamate uptake is further mediated by endogenous lipids, including AA[85,86] and DHA,[87] through direct competitive inhibition of the glutamate binding site in GLT1 and GLAST. Although AA and DHA concentrations were not assayed here, the decreased spinal sPLA$_2$ and cPLA$_2$ activity with spinal sPLA$_2$ inhibition suggests decreased lipid mediator generation and increased intracellular glutamate uptake. The concurrent restoration of spinal GLAST and GLT1 transporter expression (Figure 4) implies a likely restoration of spinal glutamate uptake as well. Since GLT1 is responsible for the majority of glutamate uptake in the spinal cord,[85] the increase in GLAST after painful root compression may be a compensatory mechanism.[33,88] GLT1 expression at day 14 after injury (Figure 4(b)) is not downregulated to the same extent as observed at day 7 in this same injury model by immunohistochemistry.[33] Despite differences in techniques, spinal GLT1 expression may depend on activation of the transcription factor, kappa B motif-binding phosphoprotein (KBBP) in astrocytes, which is regulated by pre-synaptic signaling of mGluR and GluR receptors.[89] Increased spinal mGluR5 and GluR1 at day 14 (Figure 5(b)) may increase pre-synaptic neurotransmitter and glutamate release[35] activating KBBP and initiating the translation of more GLT1 protein. For conditions with GLT1 at normal levels (Figure 5(b)) KBBP activation may be involved; probing the contribution of pre-synaptic inputs to the production of new astrocytic GLT1 after injury would test this hypothesis.

Increasing intracellular glutamate may also reduce activation of spinal glutamate receptors (Figure 3). These findings support the hypothesis that spinal sPLA$_2$ indirectly contributes to glutamate receptor activation and neurotransmission by exacerbating excitotoxicity. Glutamate receptor activation is sensitive to extracellular glutamate levels, with mGluR5 and NR1 displaying tonic activation of receptors between 1–20 μM,[90] below the estimated excitotoxic glutamate concentration (20–100 μM) causing neural injury. Conversely, GluRs mediate most of the fast-excitatory neurotransmission and require the highest concentrations of extracellular glutamate (100–1000 μM).[90] Although intracellular glutamate levels serve as a proxy measurement for extracellular glutamate here, decreases in both spinal mGluR5 and the less-sensitive GluR1 receptor further supports sPLA$_2$'s role in increasing excitotoxic glutamate concentration and modulating glutamatergic signaling with painful injury. NR1 expression at day 14 is unaltered with painful root compression (Figure 5(b)) which differs from a report with a painful chronic sciatic nerve constriction.[91] However, since altered NR1 expression was reported in synaptosome-extracted tissues from a model of inflammatory pain rather than using whole tissue lysates,[92] the lack of changes in NR1 observed here may be due to tissue preparation techniques. In addition to increased NR1 expression, phosphorylation and activation of other NMDAR subunits are also implicated in the pathogenesis of central sensitization,[30] neither of which were evaluated here. Measuring the effects of inhibiting spinal sPLA$_2$ on synaptic-level changes in NMDAR subunits and phosphorylation states of glutamate receptors would test whether spinal sPLA$_2$ inhibition mediates the synaptic plasticity that maintains central sensitization.

Spinal sPLA$_2$ inhibition decreases the proportion of spinal WDR neurons after injury (Figure 6(a)). The increase in WDR neurons after a painful nerve root compression[33,68] (Figure 6) is a hallmark of central sensitization,[62] indicating a reorganization of high-threshold Aδ and C fibers synapsing with dorsal horn neurons.[93,94] The increased response to graded stimulation (Figure 6(a)) also suggests expansion of WDR neurons' receptive fields, which could account for the shift in neuronal phenotypic distribution after injury.[95] Restoring spinal excitatory signaling after painful root compression with the GLT1-agonist, ceftriaxone, similarly restores the proportion of WDR neurons after injury,[33] suggesting that increasing spinal glutamate uptake (Figures 3 and 4) may also reset the spinal reorganization that occurs with central sensitization.[62] The effects of the downstream effectors of phospholipase A$_2$, including COX and prostaglandin E2 (PGE2), sensitize spinal dorsal horn neurons.[96–98] PGE$_2$ inhibition reduces evoked responses from WDR neurons by reducing EP2 receptor activation,[96] which may contribute to the reduced evoked activity after spinal sPLA$_2$ inhibition (Figure 7). WDR neurons increase spontaneous activity after painful neuropathic injury,[71,72] likely driving central sensitization after neuropathy.[99] Interestingly, spinal application of PGE$_2$ alone is not sufficient to elicit spontaneous action potentials;[96] yet, inhibiting spinal glutamate uptake increases spontaneous action potentials in response to non-noxious and noxious stimulation.[100] These reports suggest that the reduced spontaneous activity observed with spinal sPLA$_2$ inhibition (Figure 7) is likely due to its

modulation of glutamate transporters, independent of the actions of its downstream inflammatory mediators.

Overall, inhibiting spinal sPLA$_2$ provides a robust treatment approach for attenuating neuropathic pain and spinal neuronal hyperexcitability after painful nerve root injury. It is important to note that the effects of spinal sPLA$_2$ inhibition on neuropathic pain were evaluated using only reflexive measurements and only in male Holtzman rats. Given growing evidence of the affective components of pain[101] and differences in pain processing between males and females,[102] further assessment of sensory and affective painful behaviors is needed in both genders in order to better define and translate sPLA$_2$ inhibitors to the clinic. Similarly, despite the reduction observed in sPLA$_2$ activity (Figure 3(a)), both the sPLA$_2$ expression following administration of TEA-PC as well as the pharmacokinetic profile, including half-life, of the inhibitor were not tested in this study. Moreover, any conclusion is based on the concentration and formulation used here. Although this study used same dose as was found to prevent pain and/or spinal hypersensitivity, neither that work[21] nor this study explicitly tested specificity of the TEA-PC for sPLA$_2$. Therefore, it is unknown exactly how the current effects occurred or how long they will last after a *single* intrathecal injection and will need to be further characterized in separate studies. It is possible that lipid conjugation of the prodrug (sPLA$_2$ inhibitor, TEA) to the lipid phosphorylcholine (PC) may extend the half-life of the drug as observed in.[103-105] In particular, the lipid prodrug of indomethacin, DP-155, was shown to improve half-life in brain tissue to 94 h compared to 24 h with indomethacin alone.[105] However, lipid products also were not directly measured here. Future studies are needed to determine the exact half-life extension of lipid conjugation to TEA since it will be needed to inform future dosing paradigms.

While a neuromodulatory role of sPLA$_2$ after painful injury has been speculated based on in vitro findings and early intervention in vivo,[21,32,55,77] current findings suggest spinal sPLA$_2$ contributes to glutamatergic neurotransmission as well as exacerbates spinal excitotoxicity. Although the roles of lipid mediators like PAF, which stimulates glutamate release from pre-synaptic neurons and astrocytes,[106,107] were not investigated, decreased activity of both PLA$_2$ isoforms suggests that sPLA$_2$ inhibition likely reduces generation of lipid mediators. As an emerging area of interest in understanding and treating pain, defining the neuromodulatory contributions of bioactive lipids may provide insights into novel mechanisms and therapeutic targets for pain. Nevertheless, these findings suggest spinal sPLA$_2$ may be a novel and robust neuromodulatory target to treat persistent neuropathic pain.

Author Contributions

The study design and conceptualization was performed by SK and BAW. SK and PG performed our experimental techniques and data analysis. SK, PG, and BAW all contributed to the interpretation of findings and manuscript drafting. Final manuscript was put together by BAW.

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