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Immediate Inhibition of Spinal Secretory Phospholipase A_2 Prevents the Pain and Elevated Spinal Neuronal Hyperexcitability & Neuroimmune Regulatory Genes that Develop with Nerve Root Compression

Julia C. Quindlen-Hotek[1], **Sonia Kartha**[1], **Beth A Winkelstein**[1,2]

[1]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19146

[2]Department of Neurosurgery, University of Pennsylvania, Philadelphia, PA 19146

Abstract

Cervical nerve root injury induces a host of inflammatory mediators in the spinal cord that initiate and maintain neuronal hyperexcitability and pain. Secretory phospholipase A_2 ($sPLA_2$) is an enzyme that has been implicated as a mediator of pain onset and maintenance in inflammation and neural injury. Although $sPLA_2$ modulates nociception and excitatory neuronal signaling in vitro, its effects on neuronal activity and central sensitization early after painful nerve root injury are unknown. This study investigated whether inhibiting spinal $sPLA_2$ at the time of nerve root compression modulates the pain, dorsal horn hyperexcitability, and spinal genes involved in glutamate signaling, nociception, and inflammation that are seen early after injury. Rats underwent a painful C7 nerve root compression injury with immediate intrathecal administration of the $sPLA_2$ inhibitor thioetheramide-PC (TEA-PC). Additional groups underwent either injury alone or a sham surgery. One day after injury, behavioral sensitivity, spinal neuronal excitability, and spinal cord gene expression for glutamate receptors (mGluR5, NR1) and transporters (GLT1, EAAC1), the neuropeptide substance P, and pro-inflammatory cytokines (TNFα, IL1α, IL1β) were assessed. Treatment with the $sPLA_2$ inhibitor prevented mechanical allodynia, attenuated neuronal hyperexcitability in the spinal dorsal horn, restored the proportion of spinal neurons classified as wide dynamic range, and reduced genes for mGluR5, substance P, IL1α, and IL1β to sham levels. These findings indicate spinal regulation of central sensitization after painful neuropathy and suggest that spinal $sPLA_2$ is implicated in those early spinal mechanisms of neuronal, perhaps via glutamate signaling, neurotransmitters, and/or inflammatory cascades.

Keywords

radiculopathy; injury; neuronal hyperexcitability; sPLA2; inflammation; pain

Corresponding Author: Beth A. Winkelstein, PhD, Department of Bioengineering, University of Pennsylvania, 240 Skirkanich Hall, 210 S. 33rd Street, Philadelphia, PA 19104-6321, USA. winkelst@seas.upenn.edu.

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INTRODUCTION

The cervical nerve root is a common source of painful neuropathy resulting from neck trauma and/or disc disease [1]. Nerve root compression induces a host of inflammatory mediators in the spinal cord that initiate and maintain neuronal hyperexcitability and pain [2].. Spinal glutamate signaling has a role in synaptic plasticity and is altered after painful nerve root compression in the rat, with increased expression of the metabotropic glutamate receptor mGluR5 and decreased astrocytic glutamate transporter GLT-1 expression [3;4]. Although spinal hyperexcitability has been reported at later times after nerve root injury when pain persists and is attenuated by pain-relieving neuromodulatory drugs [4], it is unknown if spinal hyperexcitability is established early after painful injury paralleling when behavioral sensitivity, spinal cytokine upregulation, and spinal microglial proliferation are all evident [2;5].

The enzyme phospholipase A_2 (PLA_2) hydrolyzes the *sn*-2 position of membrane glycerophospholipids, which catalyzes the release of arachidonic acid from cell membrane phospholipids and initiates the synthesis of the inflammatory agents [6]. Secretory PLA_2 ($sPLA_2$) has been implicated as an early mediator of pain from inflammation and neural injury and is upregulated in the spinal cord as early as 4 hours after spinal cord injury in the rat [6]. Injecting $sPLA_2$ in the rat induces mechanical allodynia and activates spinal astrocytes and microglia within hours and increases TNFα expression [7;8]; its inhibition prevents hyperalgesia and attenuates inflammatory responses [9]. Beyond inducing inflammation, $sPLA_2$ potentiates excitatory glutamate signaling and is cytotoxic in neuronal culture [6;10]. Although $sPLA_2$ is implicated in neuronal excitotoxicity and excitatory signaling in vitro [10], the mechanisms through which $sPLA_2$ modulates spinal neuronal activity after painful root injury are unknown.

Since $sPLA_2$ modulates behavioral sensitivity [7;9], inflammatory cascades implicated in central sensitization [6;7;9;11], and neuronal excitability [9;10], this study investigated whether inhibiting spinal $sPLA_2$ at the time of a painful root injury can prevent pain and central sensitization that occur early after injury. The effects of $sPLA_2$ inhibition on mechanical hyperalgesia, dorsal horn neuron excitability, and spinal gene expression were evaluated one day after \ root compression.

METHODS

All experimental procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and carried out under the guidelines of the International Association for the Study of Pain [12]. Male Holtzman rats (306–462g) were housed under USDA- and AAALAC-compliant conditions.

Compression of the right C7 nerve root was performed with rats under inhalation isoflurane anesthesia (Fig. 1A) [4;13]. A C6/C7 hemilaminectomy were performed on the right side. The C7 dorsal nerve root was exposed and compressed with a 10-gf microvascular clip (World Precision Instruments; Sarasota, FL, USA) to apply a nerve root compression (NRC) injury for 15-mins [13]. Immediately after compression, the $sPLA_2$ inhibitor thioetheramide-

PC (0.25mg/mL; TEA-PC; Cayman Chemical Co., Ann Abor, MI, USA) in phosphate-buffered saline (PBS; 40–60μL) was administered by lumbar puncture (*NRC+sPLA$_2$ inh*; n=11) at a dose known to reduce pain [11;14]. Additional groups of rats underwent compression only (*NRC*; n=11) or a sham surgery (*sham*; n=10) where the nerve root was exposed but not compressed.

Mechanical hyperalgesia was measured in the right forepaw both before (baseline; day 0) and one day after surgery (Fig. 1A). The right forepaw was stimulated with von Frey filaments of increasing strengths (1.4g-26g) as previously described [13] to obtain the lowest strength filament to elicit a response (paw withdrawal threshold; PWT). A repeated-measures ANOVA with post-hoc Tukey HSD test compared PWTs between groups over time.

In a subset of rats (*NRC* n=3; *NRC+sPLA$_2$ inh* n=4; *sham* n=3), extracellular electrophysiological recordings were acquired in the dorsal horn of the cervical spinal cord on day 1, as previously described [4] (Fig. 1A). Electrophysiology recordings were also obtained from a naive rat. Rats were anesthetized intraperitoneally (i.p.) with sodium pentobarbital (45mg/kg) and given supplementary doses (5–10mg/kg, i.p.) as needed [4]. The C6/C7 spinal cord was exposed and a glass-insulated tungsten electrode (FHC; Bowdoin, ME, USA) was lowered into the ipsilateral dorsal horn to record evoked extracellular potentials using Spike2 software (CED; Cambridge, UK) [4].

Mechanoreceptive neurons in the spinal laminae were identified by brushing the plantar surface of the forepaw with four von Frey filaments (1.4g,4g,10g,26g) [4]. Voltage recordings were spike-sorted using Spike2 (CED) to isolate and count spikes evoked by each stimulus for individual neurons [4]. Neuron phenotypes were classified by their evoked responses [4]: wide dynamic range (WDR) neurons responded in a graded manner to increasing stimuli, low-threshold mechanoreceptive (LTM) neurons responded more to non-noxious (1.4g,4g) filaments, and nociceptive specific (NS) neurons responded only to noxious (10g,26g) filaments. Evoked responses for each stimulus were compared between groups using separate ANOVAs with a post hoc Tukey's HSD test. Differences in the proportion of neuron phenotypes were assessed between groups through Pearson χ^2 tests.

In a subset of rats (*NRC* n=6; *NRC+sPLA$_2$ inh* n=6; *sham* n=6), the ipsilateral C6 spinal cord was harvested to evaluate the effects of sPLA$_2$ inhibitor treatment on genes involved in neuronal signaling and inflammation (Fig. 1A). Spinal levels of genes for glutamate receptors (mGluR5, NR1) and transporters (GLT1, EAAC1), substance P, and pro-inflammatory cytokines (TNFα, IL1α, IL1β) (Fig. 1B) were quantified using quantitative reverse transcription polymerase chain reaction (RT-qPCR) methods [2;15–18]. Samples were run in duplicate and the relative target gene expression was analyzed using the comparative ΔΔCt method [2]. Gene expression was normalized to the housekeeping gene cyclophilin-A (CyA) and calculated as fold-change over normal (n=1). mRNA levels for each gene were compared between groups using ANOVAs with a post hoc Tukey's HSD test.

Inhibitor effectiveness was evaluated in spinal tissue at day 1 using a commercially-available $sPLA_2$ Assay Kit (Cayman Chemical; Ann Arbor, MI, USA) [8] (Fig. 1A). Ipsilateral C6 spinal cord was harvested (*NRC* n=5; *NRC+sPLA$_2$ inh* n=5; *sham* n=4) and $sPLA_2$ activity was measured as per assay instructions. $sPLA_2$ activity was also measured in un-operated rats (n=2). Activity rates were compared between groups using an ANOVA with a post hoc Tukey's HSD test.

RESULTS

Blocking spinal $sPLA_2$ immediately after *NRC* prevents the development of mechanical hyperalgesia that is evident at day 1 (Fig. 2). PWT after an NRC is significantly lower than its baseline ($p<0.001$) (Fig. 2). In contrast, the PWT with $sPLA_2$ inhibitor treatment remains at baseline ($p=0.86$) and is significantly higher than *NRC* alone ($p=0.002$) (Fig. 2). Although the PWT after *NRC* is significantly lower than *sham* on day 1 ($p<0.004$), there is no difference between *sham* and *NRC+sPLA$_2$ inh* ($p=1$) at that time (Fig. 2).

$sPLA_2$ inhibitor treatment prevents the spinal neuronal hyperexcitability that is observed at day 1 after *NRC* (Figs. 3A & 3B). Neuron activity was recorded from a total of 107 neurons (*NRC* n=20; *NRC+sPLA$_2$ inh* n=37; *sham* n=34; *normal* n=16) at 533±238μm below the pial surface. At day 1 after *NRC*, the number of evoked action potentials is significantly higher than that in a normal rat ($p<0.04$) for all filaments, and over the number of spikes in *sham* rats for all filaments except 1.4g ($p<0.02$) (Fig. 3B). However, $sPLA_2$ inhibition maintains neuron firing for all stimuli at *sham* and *normal* levels (Fig. 3B), with significant reductions compared to *NRC* for all filaments except 1.4g ($p<0.04$). $sPLA_2$ inhibition also prevents the increase in neurons classified as WDR in the dorsal horn that is produced by *NRC* (Fig. 3C). In the *NRC+sPLA$_2$ inh* group, 43% of neurons is classified as WDR, which is significantly less ($p<0.02$) than after *NRC* (78%) (Fig. 3C). The phenotypic distribution for the *NRC+sPLA$_2$ inh* group is not different ($p>0.74$) than that in *normal* or *sham* (Fig. 3C).

Spinal $sPLA_2$ inhibition reduces ($p<0.05$) spinal genes for mGluR5, substance P, and IL1 that are upregulated early after *NRC* (Figs. 4A–4C). Root compression significantly increases mGluR5 levels ($p<0.03$) and decreases glutamate transporter (GLT1, EAAC1) genes ($p<0.04$) compared to *sham* (Fig. 4A). However, NR1 is unchanged with injury ($p=0.88$) (Fig. 4A). $sPLA_2$ inhibition does not reverse the decrease ($p<0.01$) in glutamate transporters that is evident after compression (Fig. 4A). A painful compression also induces elevated substance P ($p<0.05$) and TNFα and IL1α ($p<0.02$) over *sham* (Figs. 4B & 4C). Although $sPLA_2$ inhibition also decreases TNFα levels that are elevated after *NRC* ($p<0.05$), that reduction is not significant ($p=0.07$) (Fig. 4C). $sPLA_2$ activity significantly increases after *NRC* compared to *sham* ($p<0.0004$) (Fig. 4D), and this increase is prevented by $sPLA_2$ inhibitor treatment ($p<0.003$) (Fig. 4D).

DISCUSSION

A single dose of the anti-inflammatory $sPLA_2$ inhibitor given at the time of a painful nerve root compression suppresses inflammatory responses (Fig. 4C), interrupts glutamate

signaling regulation (Fig. 4A), and prevents elevated spinal neuron activity (Fig. 3B). $sPLA_2$ inhibition also maintains substance P at sham levels (Fig. 4B), accompanying a similar suppression of spinal hyperexcitability (Fig. 3). Elevated spinal substance P mRNA after painful injury supports its role as a regulator in the development of central sensitization through its activation of post-synaptic neurokinin receptors [19]. Increased pro-inflammatory cytokines (Fig. 4C) can modulate neuronal activity by activating their receptors in the spinal cord or promoting the release of nociceptive mediators or excitatory glutamate from glial cells [20], which may be responsible for preventing increases in mGluR5 genes (Fig. 4A). Since both increased cytokine mRNA and neuronal hyperexcitability are attenuated by $sPLA_2$ inhibition immediately after injury (Figs. 3B & 4C), findings support its role in neuroinflammatory processes that facilitate spinal hyperexcitability with neuropathic pain [11].

The novel finding of dorsal horn neuronal excitability at an early time after a painful transient root injury parallels the quick development of pain in this model [5]. Central sensitization contributes to pain onset after neuropathic injury via the early functional alterations of neuromodulators [21] like substance P and mGluR5 which are upregulated at day 1 (Figs. 4A & 4B) and also at later times after this injury when spinal neuron hyperexcitability and phenotypic shifts are also observed [4;22]. $sPLA_2$ inhibition may prevent the neuron phenotypic switch via neuropeptide-mediated mechanisms. Substance P release from low threshold Aβ fibers after injury enables those non-nociceptive fibers to signal pain [23]; if decreased spinal substance P mRNA is taken as a proxy for decreased substance P release by Aβ afferents, $sPLA_2$ inhibition may also prevent hyperexcitability of post-synaptic spinal neurons.

$sPLA_2$ potentiates glutamatergic synaptic function and enhances glutamate-induced excitotoxicity [10]; its downstream cleavage product arachidonic acid inhibits synaptic glutamate uptake [14]. Excess synaptic glutamate resulting from increased activation of glutamate receptors and reduced transporter expression enhances nociceptive synaptic transmission [21]. $sPLA_2$ inhibition reduces spinal mGluR5 levels (Fig. 4A), but does *not* restore glutamate transporter genes to sham levels (Fig. 4A), suggesting $sPLA_2$ may mediate pain and hyperexcitability through mechanisms other than spinal glutamate uptake.

This is the first study demonstrating that a single intrathecal dose of $sPLA_2$ inhibitor given immediately after a neuropathic injury is sufficient to prevent the pain and spinal cellular respsonses that develop otherwise by day 1. Intracerebroventricular injection of the $sPLA_2$ inhibitor 12-epi-scalaradial immediately after carrageenan injection prevents mechanical hyperalgesia, suggesting that blocking the $sPLA_2$ pathway directly in the central nervous system *after* an insult may also relieve pain [24]. Pain and central sensitivity suppression with $sPLA_2$ inhibition in this study (Figs. 3 & 4) suggest the specific blockade of $sPLA_2$ may be a potential therapeutic approach for painful neuropathy that is upstream of the targets of traditional treatments like non-steroidal anti-inflammatory drugs.

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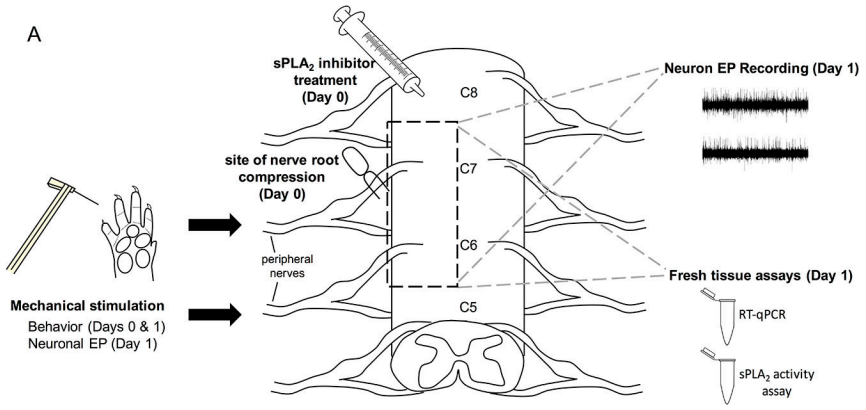

B

Gene	Sequence
mGluR5	F: 5′-GTAACCAAGACCAACCG-3′
	R: 5′-CGCATAGAACGTGCAA-3′
NR1	F: 5′-AGATGGCCCTGTCAGTGTGT-3′
	R: 5′-GTGAAGTGGTCGTTGGGAGT-3′
EAAC1	F: 5′-GGAATCACTGGCTGCTGCTC-3′
	R: 5′-GCTCACTGTGTCCTCGAACCAA-3′
GLT1	F: 5′-AACCGAGGGTGCCAACAA-3′
	R: 5′-AAGCAGCCCGCCACATAC-3′
SP	F: 5′-GACCAAATCAAGGAGGCAATG-3′
	R: 5′-TCTTCGGGCGATTCTCTGA-3′
TNFα	F: 5′-ATCATCTTCTCAAAACTCGAGTGACAA-3′
	R: 5′-CTGCTCCTCTGCTTGGT-3′
IL1α	F: 5′-AAGACAAGCCTGTGTTGCTGAAGG-3′
	R: 5′-TCCCAGAAGAAAATGAGGTCGGTC-3′
IL1β	F: 5′-CACCTCTCAAGCAGAGCACAG-3′
	R: 5′-GGGTTCCATGGTGAAGTCAAC-3′
CyA	F: 5′-TATCTGCACTGCCAAGACTGAGTG-3′
	R: 5′-CTTCTTGCTGGTCTTGCCATTCC-3′

Fig. 1. Schematic of the experimental protocol.

(A) Rats underwent a right C7 nerve root compression immediately followed by intrathecal treatment with an $sPLA_2$ inhibitor, no treatment, or a sham surgery. Behavioral testing was performed on the ipsilateral forepaw on days 0 and 1 and electrophysiology recording of the ipsilateral C6 or C7 spinal cord during mechanical stimulation of the ipsilateral forepaw was performed on day 1. C6 spinal cord tissue was used for either RT-qPCR or $sPLA_2$ activity analysis. **(B)** Genes for glutamate receptors and transporters, substance P (SP) and pro-inflammatory cytokines were assayed using published primer sequences [2;15–18].

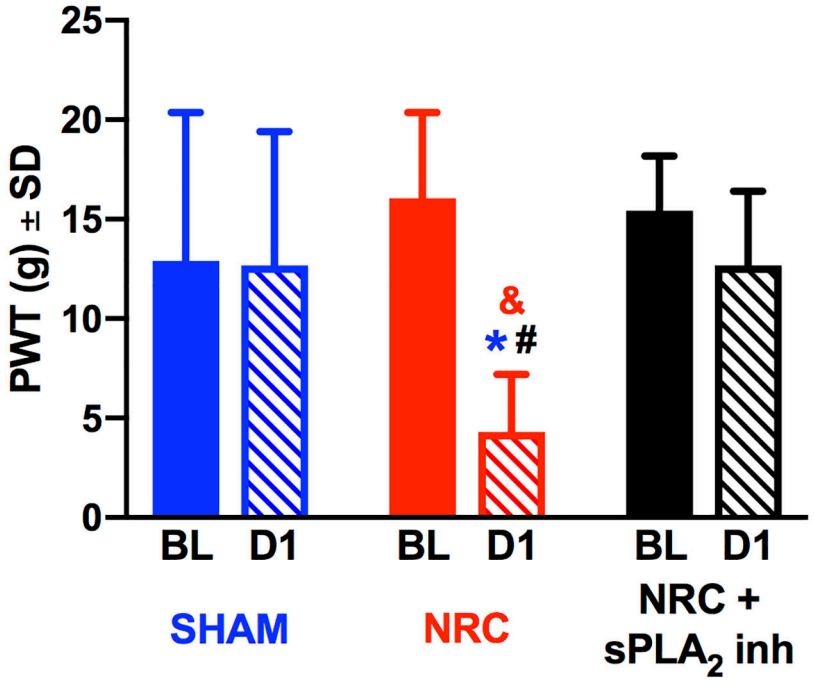

Fig. 2. Behavioral sensitivity.

Treatment with $sPLA_2$ inhibitor (*NRC+sPLA$_2$ inh*) prevents the reduction in PWT at day 1 (D1) that is evident after a root compression (*NRC)* (***p<0.003). At day 1 after *NRC*, PWTs decrease from baseline (BL) (**p<0.001) and are lower than *sham* levels at that day (*p<0.004). PWTs for *sham* and *NRC+sPLA$_2$ inh* are not different from each other.

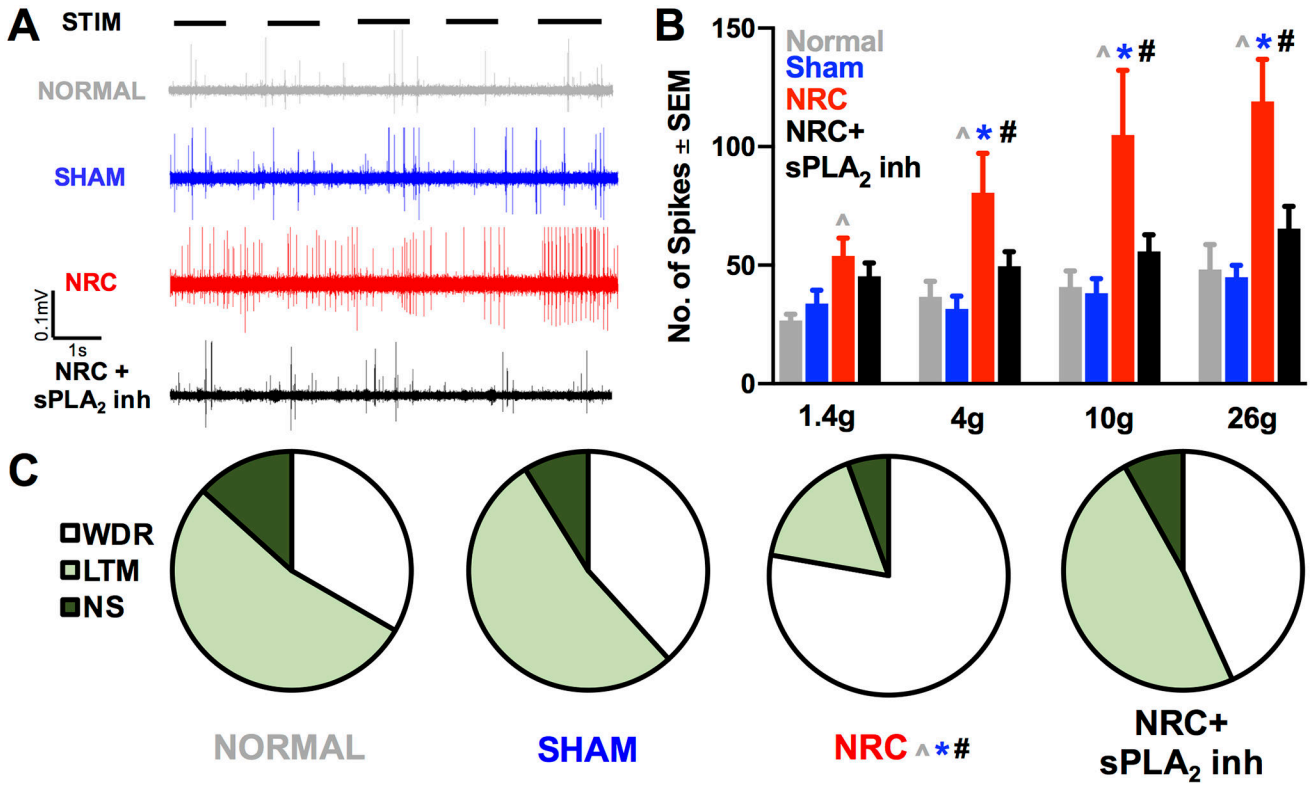

Fig. 3. Extracellular spike activity and neuronal classifications in the spinal dorsal horn.

(A) Representative recordings during noxious 26g filament stimulus (STIM) showing a reduction in evoked activity with $sPLA_2$ inhibitor compared to NRC. **(B)** Evoked activity after *NRC* is increased over *normal* levels for all stimuli (*p<0.04) and over *sham* levels for all filaments except the nonnoxious 1.4g filament (**p<0.02). $sPLA_2$ inhibitor treatment (*NRC+sPLA$_2$ inh*) prevents that increase compared to *NRC* in neuron firing for all stimuli except the 1.4g filament (***p<0.04). **(C)** There is a shift in the distribution of neuron phenotypes (WDR, LTM, NS) after *NRC* compared to *normal* (*p<0.02), *sham* (**p<0.02), and *NRC+sPLA$_2$ inh* (***p<0.03) with no differences between any other groups.

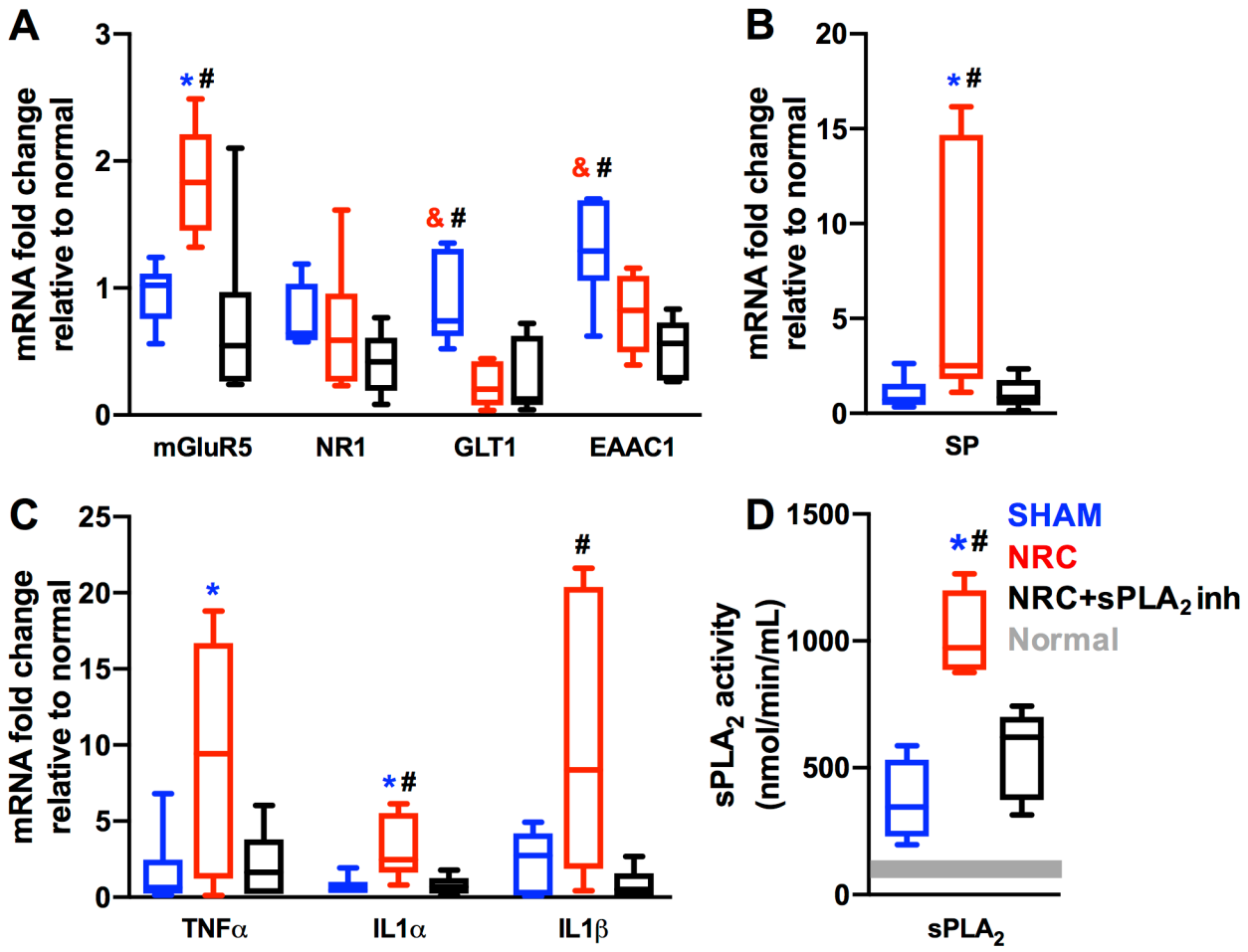

Fig. 4. Assays of mRNA and sPLA$_2$ activity in C6 ipsilateral spinal cord tissue.

(A) mGluR5 mRNA is elevated after *NRC* relative to *sham* (*p<0.03), and treatment with sPLA$_2$ inhibitor (*NRC+sPLA$_2$ inh*) prevents this increase (**p<0.01). NR1 mRNA levels are not different in any group. Both GLT1 and EAAC1 mRNA levels are reduced after *NRC* relative to *sham* (***p<0.04). Inhibitor treatment does not prevent this decrease as *NRC +sPLA$_2$ inh* mRNA levels are lower than *sham* (**p<0.01) and not different from *NRC* for both GLT1 and EAAC1. **(B)** Substance P (SP) mRNA is elevated after *NRC* relative to both *sham* (*p<0.05) and *NRC+sPLA$_2$ inh* (**p<0.05). **(C)** TNFα mRNA is elevated after *NRC* relative to *sham* (*p<0.02). IL1α mRNA is elevated after *NRC* relative to both *sham* (*p<0.01) and *NRC+sPLA$_2$ inh* (**p<0.02). IL1β mRNA is elevated after *NRC* relative to *NRC+sPLA$_2$ inh* (**p<0.03), but not *sham*. **(D)** Spinal sPLA$_2$ activity is significantly elevated after *NRC* relative to *sham* (*p<0.0004) and treatment with sPLA$_2$ inhibitor (*NRC +sPLA$_2$ inh*) prevents this increase (**p<0.003). sPLA$_2$ activity levels in *sham* and *NRC +sPLA$_2$ inh* are not different. Grey bar represents sPLA$_2$ activity in *normal* tissue.