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SMALL MOLECULE INHIBITORS OF PSD95–nNOS PROTEIN–PROTEIN INTERACTIONS SUPPRESS FORMALIN-EVOKED Fos PROTEIN EXPRESSION AND NOCICEPTIVE BEHAVIOR IN RATS

LAWRENCE M. CAREY,[a,b] WAN-HUNG LEE,[c] TANNIA GUTIERREZ,[a] PUSHKAR M. KULKARNI,[d] GANESH A. THAKUR,[d] YVONNE Y. LAI[a,e] AND ANDREA G. HOHMANN[a,b,c,f]*

[a] *Department of Psychological and Brain Sciences, Indiana University, Bloomington, IN, United States*

[b] *Program in Neuroscience, Indiana University, Bloomington, IN, United States*

[c] *Interdisciplinary Biochemistry Program, Molecular and Cellular Biochemistry Department, Indiana University, Bloomington, IN, United States*

[d] *Center for Drug Discovery, and Department of Pharmaceutical Sciences, Northeastern University, Boston, MA, United States*

[e] *Anagin, Inc., Indianapolis, IN, United States*

[f] *Gill Center for Biomolecular Science, Indiana University, Bloomington, IN, United States*

Abstract—Excessive activation of NMDA receptor (NMDAR) signaling within the spinal dorsal horn contributes to central sensitization and the induction and maintenance of pathological pain states. However, direct antagonism of NMDARs produces undesirable side effects which limit their clinical use. NMDAR activation produces central sensitization, in part, by initiating a signaling cascade that activates the enzyme neuronal nitric oxide synthase (nNOS) and generates the signaling molecule nitric oxide. NMDAR-mediated activation of nNOS requires a scaffolding protein, postsynaptic density protein 95 kDa (PSD95), which tethers nNOS to NMDARs. Thus, disrupting the protein–protein interaction between PSD95 and nNOS may inhibit pro-nociceptive signaling mechanisms downstream of NMDARs and suppress central sensitization while sparing unwanted side effects associated with NMDAR antagonists. We examined the impact of small molecule PSD95–nNOS protein–protein interaction inhibitors (ZL006, IC87201) on both nociceptive behavior and formalin-evoked Fos protein expression within lumbar spinal dorsal horn of rats. Comparisons were made with ZL007, an inactive analog of ZL006, and the NMDAR antagonist MK-801. IC87201 and ZL006, but not ZL007, suppressed phase 2 of formalin-evoked pain behavior and decreased the number of formalin-induced Fos-like immunoreactive cells in spinal dorsal horn regions associated with nociceptive processing. MK-801 suppressed Fos protein expression in both dorsal and ventral horns. MK-801 produced motor ataxia in the rotarod test whereas IC87201 and ZL006 failed to do so. ZL006 but not ZL007 suppressed paclitaxel-induced mechanical and cold allodynia in a model of chemotherapy-induced neuropathic pain. Co-immunoprecipitation experiments revealed the presence of the PSD95–nNOS complex in lumbar spinal cord of paclitaxel-treated rats, although ZL006 did not reliably disrupt the complex in all subjects. The present findings validate use of putative small molecule PSD95–nNOS protein–protein interaction inhibitors as novel analgesics and demonstrate, for the first time, that these inhibitors suppress inflammation-evoked neuronal activation at the level of the spinal dorsal horn.

Key words: *N*-methyl-D-aspartate receptor, neuronal nitric oxide synthase, postsynaptic density protein 95 kDa, protein–protein interaction inhibitor, central sensitization, dorsal horn.

INTRODUCTION

Intense activation of peripheral nociceptors can drive neuroplastic alterations in neuronal circuitry within the central nervous system (CNS) leading to increased excitability and synaptic efficacy, a phenomenon known as central sensitization (Ji and Woolf, 2001). Sensitization of CNS neuronal circuitry can produce exaggerated responses to both noxious and non-noxious stimulation, resulting in hyperalgesia and allodynia, respectively. The increased reactivity of spinal neuronal circuitry initiated by central sensitization may contribute to the development of a wide range of pathological pain states including neuropathic and inflammatory pain conditions (see Latremoliere and Woolf, 2009 for review). Glutamatergic signaling through the *N*-methyl-D-aspartate receptor (NMDAR) is an important mechanism involved in the generation of central sensitization (Woolf and Thompson, 1991; Ma and Woolf, 1995; South et al., 2003). NMDAR antagonists produce antinociceptive efficacy in various animal models of pain (see Zhou et al., 2011 for review). However, the clinical use of direct NMDAR antagonists is problematic as they produce deleterious side effects including learning and memory deficits, cognitive

*Correspondence to: A. G. Hohmann, Department of Psychological & Brain Sciences, Indiana University, 1101 East 10th street, Bloomington, IN 47405-7007, USA. Fax: +1-813-856-7187.
E-mail address: hohmanna@indiana.edu (A. G. Hohmann).

Abbreviations: AUC, area under the curve; CNS, central nervous system; CPS, composite pain scores; eNOS, endothelial nitric oxide synthase; FLI, Fos-like immunoreactive; i.p., intraperitoneal; NMDAR, *N*-methyl-D-aspartate receptor; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PSD95, postsynaptic density 95 kDa.

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dysfunction, motor impairment, dissociation from reality and abuse liability (Krystal et al., 1994; Pal et al., 2002).

Activation of NMDARs leads to calcium influx in the postsynaptic cell, an event that activates signaling pathways involved in the induction of plasticity implicated in pathological pain states (Ji et al., 2003). Following entry via NMDARs, calcium binds to calmodulin, which in turn activates the enzyme neuronal nitric oxide synthase (nNOS), which generates the signaling molecule nitric oxide (NO). Excess NO production is involved in pain signaling and the development of central sensitization (Kitto et al., 1992; Aley et al., 1998; Wu et al., 2001; Miclescu and Gordh, 2009). NMDAR-mediated production of NO relies on the tethering of nNOS to NMDARs via the scaffolding protein postsynaptic density 95 kDA (PSD95). Thus, decreasing PSD95 expression with antisense oligonucleotides attenuates NMDAR-mediated production of NO and cell death (Sattler et al., 1999) and knockdown of PSD95 expression within the spinal cord also decreases NMDA-induced thermal hyperalgesia (Tao et al., 2000), and delays the onset of mechanical and thermal hyperalgesia induced by spinal nerve ligation (Tao et al., 2001). These observations collectively suggest that the NMDAR–PSD95–nNOS complex may play a key role in the development of central sensitization.

Small molecules capable of disrupting PSD95–nNOS protein–protein interactions have recently been developed and show efficacy in preclinical models assessing pain behavior (Florio et al., 2009; Lee et al., 2015), depression-like behavior (Doucet et al., 2013), and neuroprotection following cerebral ischemia (Zhou et al., 2010). IC87201, a first in class PSD95–nNOS inhibitor, displays antinociceptive efficacy in rodent models of inflammatory and neuropathic pain when administered intrathecally (Florio et al., 2009) or when administered systemically in mice (Lee et al., 2015). Our recent studies showed that IC87201 and ZL006, a related molecule, disrupt binding of PSD95 and nNOS *in vitro*, suppress glutamate-induced excitotoxicity and attenuate inflammatory and neuropathic pain behavior in mice (Lee et al., 2015). Furthermore, IC87201 and ZL006 did not alter basal nociceptive thresholds in the absence of pain or produce motor ataxia (Lee et al., 2015) or memory impairments characteristic of the non-competitive NMDAR antagonist MK-801 (Smith et al., 2016). Thus, disruption of PSD95–nNOS protein–protein interactions may represent a novel, therapeutic mechanism for managing pathological pain that lacks problematic side effects associated with direct NMDAR antagonists. However, *in vivo* studies relying on assessments of pain behavior are not sufficient to conclude that PSD95–nNOS inhibitors act at a neural level to suppress the processing of nociceptive information. This conclusion requires the demonstration that PSD95–nNOS inhibitors disrupt the processing of nociceptive information.

Whether IC87201 and ZL006 suppress nociceptive processing and CNS sensitization associated with pathological pain is unknown. ZL006, but not a related analog, ZL007, disrupts co-immunoprecipitation of nNOS and PSD95 in hippocampal slice cultures (Zhou et al., 2010), suggesting that ZL007 may be a useful inactive analog to confirm mechanism of action of active PSD95–nNOS inhibitors. However, to our knowledge, the *in vivo* profile of this compound has never been characterized and whether or not ZL006 shows antinociceptive efficacy in rats is unknown. We, therefore, used IC87201, ZL006 and the putative inactive analog ZL007, to ask whether small molecule inhibitors of PSD95–nNOS protein–protein interactions alter neurochemical markers of inflammation-evoked neuronal activity at the level of the lumbar spinal dorsal horn in rats. Importantly, we evaluated whether systemically administered IC87201 and ZL006 decrease formalin-evoked nociceptive behavior and formalin-evoked Fos-like immunoreactivity in the lumbar spinal dorsal horn within the same subjects. We also evaluated whether IC87201 and ZL006, administered systemically, produce motor ataxia in rats using the rotarod test. Comparisons were made with the non-competitive NMDAR antagonist MK-801 and the putative inactive analog ZL007. Next we used ZL006, which has previously been shown to disrupt interactions between PSD95 and nNOS (but not NMDAR subunit NR2B-PSD95 (Zhou et al., 2010) or ERB4-PSD95 interactions (Lee et al., 2015) and maximally reduced formalin-evoked nociceptive behavior and dorsal horn Fos protein expression in our studies, to test the hypothesis that ZL006 but not ZL007 would reduce paclitaxel-induced neuropathic pain in rats. Finally, using co-immunoprecipitation, we evaluated whether paclitaxel-induced neuropathic pain promotes the formation of the PSD95–nNOS complex in the spinal cord *in vivo*, and whether systemic treatment with ZL006 could prevent the association of nNOS and PSD95 in lumbar spinal cord tissue. The results of our study demonstrate, for the first time, that ZL006 produces antinociception in rats whereas ZL007 is ineffective. We also show that IC87201 produces antinociception in rats following systemic administration. Finally, our studies show that the PSD95–nNOS complex is present in the lumbar spinal cord and also show that PSD95–nNOS protein–protein interaction inhibitors concomitantly suppress noxious stimulus-evoked increases in neuronal activation within spinal cord regions implicated in nociceptive processing as well as pain behavior in a manner that is selective for active but not inactive analogs.

EXPERIMENTAL PROCEDURES

Subjects

One hundred and eleven male Sprague–Dawley rats (285–446 g; Envigo, Indianapolis, IN, USA) were used in these experiments. All procedures were approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC) of Indiana University. Animals were housed in a temperature-controlled facility with free access to food and water in their home cages on a regular 12-h light/dark cycle.

Drugs and chemicals

ZL006, IC87201 and ZL007 were synthesized in the laboratory of Dr. Ganesh Thakur (by P.M.K.) at the

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Northeastern University Center for Drug Discovery (Boston, MA, USA). MK-801 and formaldehyde (37% in H_2O) were purchased from Sigma Aldrich (St. Louis, MO, USA). All drugs were dissolved in a vehicle of 20% dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO, USA), with the remaining 80% consisting of 95% ethanol (Sigma Aldrich, St. Louis, MO, USA), emulphor (Alkamuls EL 620L; Solvay) and 0.9% saline (Aquilite System; Hospira, Inc, Lake Forest, IL, USA) at a ratio of 1:1:8, respectively for intraperitoneal (i.p.) administration and administered at a volume of 1 ml/kg. Formalin was diluted from formaldehyde stock (100% formalin) in sterile saline to a final concentration of 2.5% and administered in a volume of 50 μl. Paclitaxel was purchased from Tecoland (Irvine, CA, USA) and was dissolved in a vehicle consisting of cremophor EL (Sigma–Aldrich, St. Louis, MO, USA), 95% ethanol (Sigma Aldrich, St. Louis, MO, USA) and saline at a ratio of 1:1:18 respectively and was administered at a volume of 1 ml/kg.

Formalin test

Rats received a single i.p. injection of ZL006 (4 or 10 mg/kg) ZL007 (10 mg/kg), IC87201 (4 or 10 mg/kg), MK-801 (0.1 mg/kg) or vehicle 30 min prior to intraplantar (i.pl.) injection of formalin. Animals were placed in an elevated, clear Plexiglas observation chamber immediately after i.p. injection and allowed to habituate to the observation chamber for 30 min. Following habituation, rats received a unilateral intradermal injection (50 μl) of 2.5% formalin into the superficial plantar surface of the hind paw. Nociceptive behaviors were video recorded over 60 min immediately following formalin injection and quantified from videotapes by a single rater (LMC) who was blinded to the experimental conditions. Composite pain scores (CPS) were calculated for every 5 min bin for the total duration of 60 min using the following scoring criteria: no behavior was scored 0, lifting was scored 1, and shaking/biting/flinching was scored as 2. The area under the curve (AUC) of pain behavior was calculated for both the early (phase 1, 0–10 min) and the late (phase 2, 10–60 min) for each subject as described in our previous work (Guindon et al., 2011).

Rotarod test

Motor performance was assessed in separate groups of rats using an accelerating Rotarod (IITC Life Science) (4–40 rpm, 300 s cutoff time) as performed in our previous work (Rahn et al., 2011). Rats were trained over two consecutive days and on the third day baseline latencies to descend from the rotating drum were measured. Following acquisition of baseline measurements, rats received a single i.p. injection of ZL006 (10 mg/kg), IC87201 (10 mg/kg), MK-801 (0.1 mg/kg) or vehicle. At 30 and 60 min following i.p. injection, rats were placed on the accelerating rotarod and the descent latency was recorded in duplicate. Animals that did not meet exclusion criteria (i.e. ability to stay on rotating drum for at least 30 s on baseline day) were removed from the study and did not receive any pharmacological treatments. The experimenter was always blinded to the experimental conditions.

Tissue preparation for immunohistochemistry

Immunohistochemical procedures were carried out in the same subjects used to assess antinociception in the formalin test. Immediately following conclusion of the formalin behavioral assay (i.e. one hour post-i.pl. formalin injection), rats were deeply anesthetized with 25% urethane, then transcardially perfused with 0.1% heparinized 0.1 M phosphate-buffered saline (PBS) followed by ice cold 4% paraformaldehyde. A separate group of rats (n = 3) received intraplantar injections of saline (50 μl) in lieu of formalin and were perfused 60 min later. Spinal cord tissue was extracted and kept in the same fixative for 24 h and then cryoprotected in 30% sucrose for 3 days prior to sectioning.

Immunohistochemistry

Immunohistochemical experiments were conducted as previously described (Tsou et al., 1996; Nackley et al., 2003a,b; Carey et al., 2016). Transverse sections (30 μm) of the L4–L5 lumbar spinal cord were cut on a cryostat and maintained in an antifreeze solution (50% sucrose in ethylene glycol and 0.1 M PBS) prior to immunostaining. Tissue was collected so that every fourth section would be processed for immunohistochemistry to ensure that the same cell could not be inadvertently counted twice in adjacent sections. Free-floating sections were washed three times in 0.1 M PBS, and then immersed in 0.3% H_20_2 for 30 min. To prevent non-specific binding, sections were pretreated for one hour with blocking buffer consisting of 5% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS, followed by incubation with rabbit polyclonal Fos protein antibody (1:1500, Santa Cruz Biotechnology, Dallas, TX, USA) for 24 h at 4 °C. Fos-like immunoreactivity was visualized using the avidin–biotin peroxidase method using diaminobenzidine as the chromagen. Three sections per animal displaying the highest levels of Fos-like immunoreactivity were selected and cells were counted from digitized images using a Retiga 1300 digital camera and a Leica DMLB microscope by an investigator blinded to the treatment conditions. The number of FLI cells was counted in each subdivision of the spinal cord as defined by Presley et al. (1990) and averaged to produce a single mean for each spinal cord region for each animal. Statistical analyses were performed to compare the number of FLI cells in each spinal cord region (averaged across animals, not across sections) as described in our previously published work (Tsou et al., 1996; Nackley et al., 2003a,b; Carey et al., 2016; see also Fig. 1D). The subdivisions used were the superficial laminae (laminae I and II), the nucleus proprius (laminae III and IV), the neck of the dorsal horn (laminae V and VI) and the ventral horn (laminae VII, VIII, IX, and X) (Presley et al., 1990). Intraplantar injection of saline in lieu of formalin (n = 3) did not induce appreciable expression of Fos-like immunoreactivity, consistent with the results of our previously published studies (Tsou et al., 1996; Nackley et al., 2003a,b; Carey et al., 2016) (data not shown).

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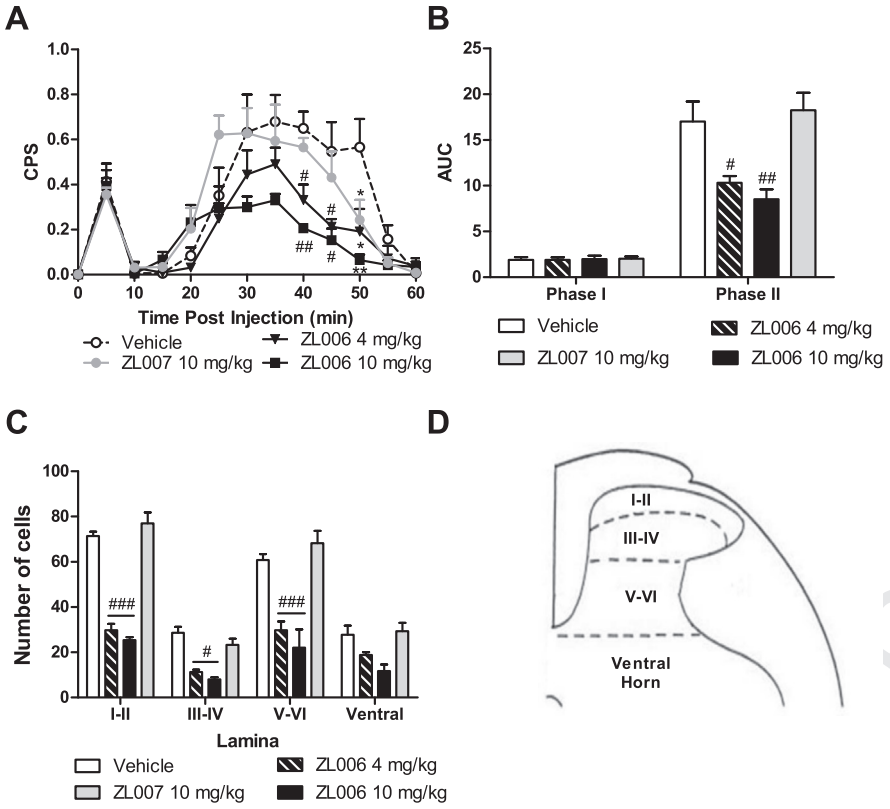

Fig. 1. ZL006 suppresses formalin-evoked nociceptive behavior and Fos-like immunoreactivity in lumbar spinal dorsal horn. (A) ZL006 (4 and 10 mg/kg i.p.) decreased composite pain scores (CPS) in a time-dependent manner. ZL006 (4 and 10 mg/kg i.p.) decreased formalin-induced CPS relative to ZL007 (10 mg/kg i.p.) and vehicle at 40 and 45 min post-formalin, while all groups showed lower CPS at 50 min post-formalin relative to vehicle. (B) ZL006 reduced the AUC of phase 2 but not phase 1 pain behavior relative to ZL007 and vehicle treatment. (C) ZL006 (4 and 10 mg/kg i.p.) decreased the number of Fos-like immunoreactive cells in the superficial dorsal horn, the nucleus proprius, and the neck region of the dorsal horn, whereas post hoc analyses did not reveal any differences between groups in the ventral horn. (D) Diagram of a lumbar spinal cord hemisection showing laminar subdivisions used to quantify formalin-evoked Fos protein expression (adapted from (Nackley et al., 2003b)). Data are expressed as mean ± SEM (n = 4–6 per group). $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$ vs. ZL007 and vehicle; $^{**}p < 0.01$, $^{*}p < 0.05$ vs. vehicle. One-way ANOVA Newman–Keuls post hoc. CPS: composite pain score, AUC: area under the curve.

Paclitaxel-induced peripheral neuropathic pain

Paclitaxel was used to produce chemotherapeutic-induced neuropathic pain in rats as described previously by our laboratory (Rahn et al., 2008, 2014; Deng et al., 2012, 2016). Rats were injected on four alternate days with paclitaxel (2 mg/kg, i.p.; cumulative dose 8 mg/kg i.p.) or its cremophor-based vehicle on day 0, 2, 4, and 6 following initiation of paclitaxel/cremophor vehicle dosing. The impact of small molecules and vehicle treatment on paclitaxel-induced mechanical and cold responsiveness was assessed during the maintenance phase of chemotherapy-induced neuropathy, when neuropathic pain was fully established, maximal and stable. We previously reported that paclitaxel-induced neuropathic pain is maximal and stable from day 12 post initiation of paclitaxel/cremophor vehicle dosing and was maintained throughout an 88-day observation interval (Deng et al., 2016). Following acquisition of baseline (pre-drug) levels of mechanical and cold responsiveness, rats received a single i.p. injection of ZL006 (2 or 4 mg/kg i.p.), ZL007 (4 mg/kg i.p.) or vehicle (i.p.). The highest dose of ZL006 evaluated in this study maximally suppressed formalin-evoked Fos protein expression and pain behavior and did not suppress Fos in the ventral horn. Mechanical and cold responsiveness were measured 30, 90 and 180 min post-drug.

Assessment of mechanical allodynia

Paw withdrawal thresholds to mechanical stimulation were measured using an electro von Frey anesthesiometer (IITC Life Science Inc., Woodland Hills, CA, USA) as described previously by our group (Rahn et al., 2008, 2014; Deng et al., 2012, 2016). Rats were placed on an elevated mesh platform underneath clear plastic observation chambers. Paw withdrawal thresholds were measured in duplicate in each paw, at each time point. Data are reported as the mean of these duplicate recordings averaged across paws.

Assessment of cold allodynia

Paw withdrawal frequencies to cold stimulation were measured using the acetone method as described previously by our group (Deng et al., 2012, 2016; Rahn et al., 2014). Rats were placed on an elevated mesh platform underneath clear plastic observation chambers, and a bubble of acetone was applied to the plantar surface of the hind paw using a blunt 1-ml syringe. Acetone was applied to each paw five times with 3 min intervals between stimulations. Animals were observed for 20 s after acetone application, and paw withdrawals were judged to be present if animals displayed one or more types of unilateral nocifensive behavior (licking, shaking, withdrawing, repetitive stepping on stimulated paw) during a given trial, whereas trials where animals did not display any form of unilateral nocifensive behavior were scored as zero. Paw withdrawal frequencies are reported as the percentage of trials wherein paw withdrawals occurred out of the 10 trials performed (5 trials per paw).

Generation of lumbar spinal cords for co-immunoprecipitation

A separate group of rats were treated with paclitaxel (2 mg/kg, i.p. on day 0, 2, 4 and 6 following initiation of paclitaxel dosing) or its cremophor-based vehicle and used to examine the impact of ZL006 versus vehicle treatment

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on co-immunoprecipitation of nNOS and PSD95 in the lumbar spinal cord. Paclitaxel-treated rats were injected (i.p.) with vehicle or ZL006 (10 mg/kg) during the maintenance phase of chemotherapy-induced neuropathic pain (i.e. day 16 following initiation of paclitaxel dosing), when mechanical and cold hypersensitivity are maximal (Deng et al., 2012, 2016; Rahn et al., 2014). Comparisons were made with a separate group of rats that received the cremophor-based vehicle in lieu of paclitaxel and was subsequently injected with the vehicle (i.e. day 16 following initiation of cremophor vehicle dosing on day 0, 2, 4 and 6). All rats were killed by rapid decapitation without anesthesia 30 min post-injection of small molecule or vehicle under identical conditions on the same day and were not subjected to behavioral testing. Lumbar spinal cord tissue was rapidly dissected and fast frozen in isopentane and stored at −80 °C until use.

Co-immunoprecipitation from spinal cord lysate followed by immunoblotting

Lumbar spinal cord tissue was homogenized with low-salt buffer (25 mM Tris–Cl, pH7.5, 70 mM NaCl, 1 mM EDTA, pH 8.0, 1% Ipegal CA-630) supplemented with Halt protease inhibitor cocktail and Halt phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA, USA), and precleared at 13,000 k/4 °C for 10 min. nNOS antibody at 1 μg per 100 μg of total protein lysate input (Santa Cruz, Santa Cruz, CA, USA A-11) was added to each lysate. Lysate-antibody mixtures were rotated for one hour at 4 °C. Magnetic protein G beads (Bio-rad, Hercules, CA, USA) were then added, and rotated for another 2 h. Beads were washed 5 times with the same buffer, and drained beads were heated at 70 °C for 10 min in LDS buffer for immunoblotting. All samples were processed concurrently. Samples were loaded to 8% Bis-Tris Gel (ThermoFisher Scientific, Waltham, MA, USA) and transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked with protein-free blocking buffer (ThermoFisher Scientific, Waltham, MA, USA) and incubated with mouse anti-nNOS (Santa Cruz, Santa Cruz, CA, USA A-11, 1:10,000), rabbit anti-PSD95 (ThermoFisher Scientific, Waltham, MA 700902, USA, 1:1,000), rabbit anti-cofilin (Abcam, Cambridge, UK, ab42824, 1:1000), and detected using goat anti-rabbit-HRP (Santa Cruz, Santa Cruz, CA, USA, 1:10,000), and goat anti-mouse-HRP (Santa Cruz, Santa Cruz, CA, USA, 1:10,000). Antibodies were diluted in PBS-Tween 20 (0.05%) with 5% goat serum. Blots were developed using SuperSignal West Femto Maximum Sensitivity substrate (ThermoFisher Scientific, Waltham, MA, USA) and visualized with a ChemiDoc touch imaging system (Bio-rad, Hercules, CA, USA). Images were quantified using Image Lab software (Bio-rad, Hercules, CA, USA).

Data analysis

Data were analyzed by repeated measures Analysis of Variance (ANOVA) and one-way ANOVA, as appropriate. Post hoc comparisons were performed using Newman–Keuls Post hoc test. Graphpad Prism Version 5.02 statistical software for windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com) was used to perform the aforementioned statistical analyses. Statmate2 (GraphPad Software, San Diego, CA, USA; www.graphpad.com) was used to calculate statistical power of the co-immunoprecipitation experiments using the sample sizes, standard deviations and observed difference between group means.

RESULTS

ZL006 reduces formalin-evoked nociceptive behavior and Fos-like immunoreactivity in spinal dorsal horn regions associated with nociceptive processing

Intraplantar formalin increased CPS in a biphasic manner ($F_{12,18}$ = 32.36, p < 0.0001, Fig. 1A) in rats treated (i.p.) with the PSD95–nNOS inhibitor ZL006, the putative inactive analog ZL007 and vehicle. ZL006 treatment decreased CPS ($F_{3,18}$ = 8.666, p < 0.001, Fig. 1A), and the interaction between time and drug treatment was significant ($F_{36,18}$ = 2.512, p < 0.0001, Fig. 1A). Post hoc comparisons revealed that both the high (10 mg/kg i.p.) and the low (4 mg/kg i.p.) dose of ZL006 decreased phase 2 of formalin-induced pain behavior relative to both ZL007 and vehicle treatments at 40 (p < 0.01, 0.05) and 45 min (p < 0.05) post-formalin. Pain scores were higher in vehicle-treated rats compared to all other groups at 50 min (p < 0.05 for each comparison) post-formalin.

Formalin injection also increased the AUC of pain behavior in a phase-dependent manner ($F_{1,38}$ = 209.4, p < 0.0001, Fig. 1B). ZL006 decreased the AUC of formalin pain ($F_{3,38}$ = 9.044, p < 0.0001, Fig. 1B) selectively during phase 2 of nociceptive behavior ($F_{3,38}$ = 8.931, p < 0.0001, Fig. 1B). Both the low (p < 0.05) and the high (p < 0.01) dose of ZL006 decreased the AUC of phase 2 pain behavior relative to rats treated with vehicle or ZL007.

ZL006 decreased formalin-evoked Fos-like immunoreactivity ($F_{2,36}$ = 110.3, p < 0.0001, Fig. 1C) in a lamina-dependent manner ($F_{3,36}$ = 48.55, p < 0.0001, Fig. 1C), and the interaction between drug and spinal cord region was significant ($F_{6,36}$ = 7.602, p < 0.0001, Fig. 1C). Both the high and low doses of ZL006 decreased the number of formalin-evoked Fos-like immunoreactive (FLI) cells relative to treatment with vehicle or ZL007 in the superficial dorsal horn (p < 0.001), the nucleus proprius (p < 0.05), and the neck region of the dorsal horn (p < 0.001). Pharmacological manipulations altered FLI cells in the ventral horn (p < 0.05), but post hoc comparisons failed to reveal significant differences between vehicle and pharmacological treatments in the ventral horn. Example photomicrographs are shown in Fig. 2.

IC87201 reduces formalin-evoked nociceptive behavior and Fos-like immunoreactivity in spinal dorsal horn regions associated with nociceptive processing

Intraplantar administration of formalin increased CPS in a biphasic manner ($F_{12,14}$ = 15.48, p < 0.0001, Fig. 3A) in

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rats receiving systemic injections of IC87201 or vehicle. IC87201 reduced formalin-evoked nociceptive behaviors ($F_{2,14}$ = 8.86, p < 0.01, Fig. 3A), and the interaction between time and IC87201 treatment was significant ($F_{24,14}$ = 3.229, p < 0.0001, Fig. 3A). Post hoc comparisons revealed that the high dose of IC87201 (10 mg/kg i.p.) facilitated the onset (at 15 (p < 0.05) and 20 min (p < 0.01)) and resolution (from 40–50 min (p < 0.05 for each time point)) of formalin-induced pain scores. The low dose of IC87201 (4 mg/kg i.p.) reliably reduced formalin-evoked CPS at 45 (p < 0.05) minutes post-formalin relative to vehicle.

Intraplantar (i.pl) formalin altered the AUC of formalin-induced pain behavior in a phase-dependent manner in these same subjects ($F_{1,28}$ = 110.2, p < 0.0001, Fig. 3B). IC87201 reduced the AUC of formalin pain ($F_{2,28}$ = 6.536, p < 0.01, Fig. 3B), selectively decreasing phase 2 pain behaviors ($F_{2,28}$ = 5.792, p < 0.01, Fig. 3B). Both the low and the high dose of IC87201 decreased the AUC of phase 2 formalin pain (p < 0.05 for each comparison) relative to vehicle-treated rats. By contrast, the AUC of phase 1 formalin pain did not differ between any of the treatment groups (p > 0.1).

IC87201 also reduced formalin-evoked Fos-like immunoreactivity ($F_{2,56}$ = 29.14, p < 0.0001, Fig. 3C) in a lamina-dependent manner ($F_{6,56}$ = 4.811, p < 0.001, Fig. 3C), and formalin also induced FLI in a lamina-dependent manner ($F_{3,56}$ = 38.47, p < 0.0001, Fig. 3C). Both the low and high doses of IC87201 decreased Fos-like immunoreactivity in the superficial dorsal horn (p < 0.0001 for each dose). The high dose of IC87201 also reduced Fos-like immunoreactivity in the nucleus proprius (p < 0.05) and the neck region of the dorsal horn (p < 0.05). By contrast, the number of FLI cells did not differ between groups in the ventral horn (p > 0.4). Example photomicrographs show the impact of IC87201 treatment on formalin-evoked FLI cells in Fig. 4.

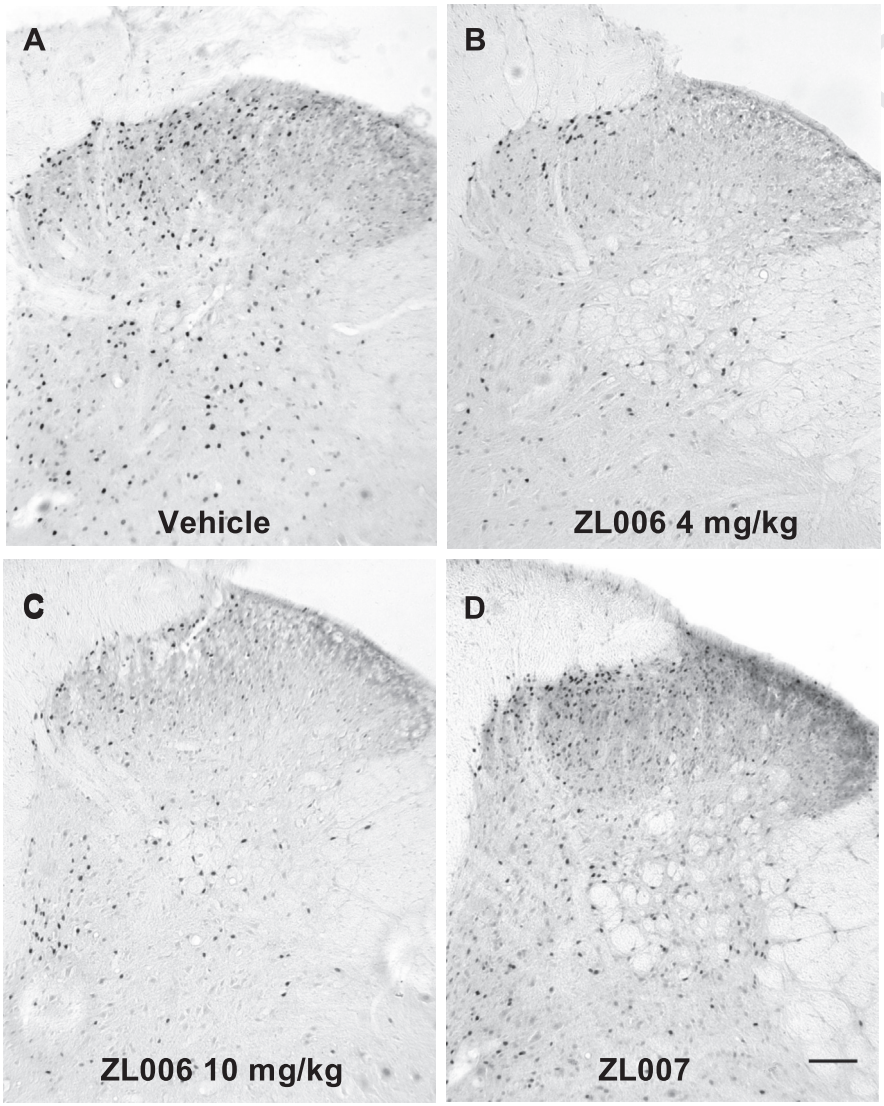

Fig. 2. Example photomicrographs taken at 10x magnification show formalin-evoked Fos-like immunoreactive cells in lumbar dorsal horn of rats treated with vehicle (A), ZL006 (4 mg/kg i.p.) (B), ZL006 (10 mg/kg i.p.) (C), and ZL007 (10 mg/kg i.p.) (D). Scale bar = 100 μm. Fos protein expression was largely absent in the dorsal horn contralateral to the formalin-treated paw. Fos protein was not induced by intraplantar injection of saline (data not shown).

IC87201 and ZL006 produce similar levels of antinociception and reductions of formalin-evoked spinal Fos protein expression

We compared the efficacy of the maximally efficacious doses of IC87201 (10 mg/kg i.p.) and ZL006 (10 mg/kg i.p.) with the NMDAR antagonist MK-801 (0.1 mg/kg) and vehicle in suppressing formalin-induced pain behavior and Fos-like immunoreactivity. Formalin evoked a biphasic pattern of nociceptive behavior in these groups ($F_{12,19}$ = 22.01, p < 0.0001, Fig. 5A). Drug treatment altered formalin-evoked nociceptive behavior ($F_{3,19}$ = 14.93, p < 0.0001, Fig. 5A) in a time-dependent manner ($F_{36,19}$ = 23.33, p < 0.0001, Fig. 5A). MK-801 and IC87201 decreased CPS at 10 min post-formalin relative to vehicle or ZL006 (p < 0.01). Both PSD95–nNOS inhibitors produced an apparent leftward shift in the time course of phase 2 pain behavior, as revealed by facilitation of both the onset and the resolution of pain during this phase. IC87201 facilitated the onset (from 15–20 min post-formalin (p < 0.05)) and the resolution of phase 2 pain (from 30–50 min post-formalin relative to vehicle (p < 0.05)) behavior. Similarly, ZL006 facilitated both the onset (i.e. at 20 min post-formalin

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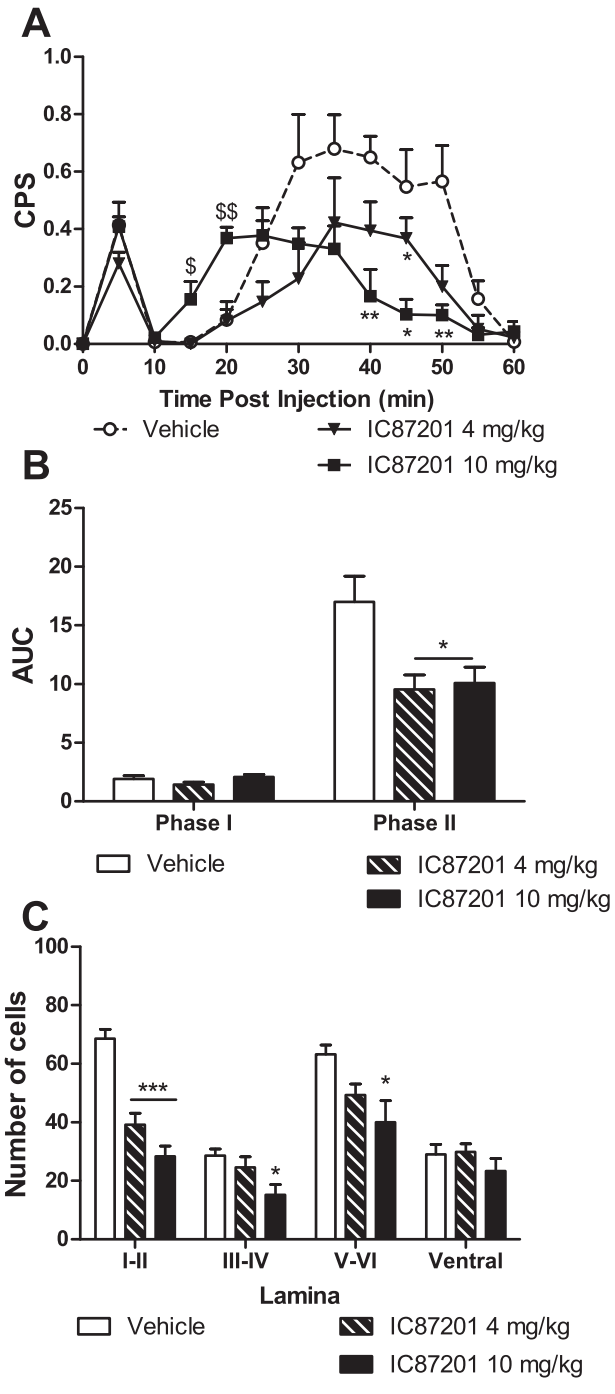
($p < 0.05$)) as well as the resolution of phase 2 (from 30–50 min post formalin ($p < 0.05$) pain behavior. MK-801 reduced CPS at 25 min relative to all other groups ($p < 0.05$). Effects of IC87201, ZL006 and MK801 did not differ from each other at any other phase 2 time point. MK-801 reliably suppressed pain behavior relative to vehicle from 30–50 min post formalin ($p < 0.01$ for each comparison).

Formalin increased the AUC of pain behavior in a phase dependent manner ($F_{1,38} = 78.4$, $p < 0.0001$, Fig. 5B). Drug treatment reduced the AUC ($F_{3,38} = 14.42$, $p < 0.0001$, Fig. 5B) preferentially during in phase 2 of formalin-evoked pain ($F_{3,38} = 13.15$, $p < 0.0001$, Fig. 5B). ZL006 ($p < 0.01$) and IC87201 ($p < 0.05$) and MK-801 ($p < 0.001$) all reduced the AUC of phase 2 formalin-evoked pain relative to vehicle whereas MK-801 produced a greater suppression of the AUC of phase 2 pain behavior relative to IC87201 and ZL006 ($p < 0.05$).

Treatment with either IC87201, ZL006 or MK-801 also reduced the number of formalin-evoked Fos protein-like immunoreactive cells ($F_{3,76} = 70.37$, $p < 0.0001$, Fig. 5C) in a lamina-dependent manner ($F_{9,76} = 6.261$, $p < 0.0001$, Fig. 5C) and the number of FLI cells also differed as a function of spinal cord region ($F_{3,76} = 32.82$, $p < 0.0001$, Fig. 5C). IC87201 ($p < 0.01$), ZL006 ($p < 0.01$) and MK-801 ($p < 0.001$) reduced formalin-evoked Fos protein expression relative to vehicle in the superficial dorsal horn, the nucleus proprius and the ventral horn. MK-801 produced a greater suppression of the number of FLI cells relative to IC87201 and ZL006 in the superficial dorsal horn and neck region of the dorsal horn ($p < 0.01$ for each comparison). In the ventral horn, only MK-801 ($p < 0.01$) reliably reduced formalin-evoked Fos-like immunoreactivity relative to vehicle. Example photomicrographs show the impact of maximally efficacious doses of PSD95–nNOS inhibitors ZL006 and IC87201 and the NMDAR antagonist MK-801 on formalin-evoked Fos protein expression in the lumbar dorsal horn (Fig 6).



ZL006 and IC87201 do not impair motor performance in the rotarod test in rats, whereas MK-801 induces motor ataxia

Rotarod performance did not differ between groups prior to pharmacological manipulations (baseline) ($p > 0.5$). MK-801 reduced the rotarod descent latency ($F_{3,18} = 3.07$, $p \leq 0.05$, Fig. 7) at 30 min post-injection relative to vehicle ($p < 0.05$) and relative to all other groups ($F_{3,18} = 3.604$, $p < 0.05$, Fig. 7) at 60 min post injection. The PSD95–nNOS inhibitors ZL006 and IC87201 did not impair rotarod performance in rats at any time point.

Fig. 3. IC87201 suppresses formalin-evoked nociceptive behavior and Fos-like immunoreactivity in lumbar spinal dorsal horn. IC87201 differentially altered the time course of formalin-induced composite pain scores. IC87201 (10 mg/kg i.p.) facilitated the onset of CPS 15–20 min post-formalin relative to all other groups and enhanced resolution of phase 2 pain behavior; CPS were reduced by IC87201 from 40–50 min post-formalin relative to vehicle treated-rats (A). IC87201 (4 mg/kg i.p.) reduced formalin-evoked CPS at 45 min post-injection relative to vehicle (A). IC87201 (4 and 10 mg/kg i.p.) reduced the AUC of phase 2 formalin-evoked pain (B). IC87201 (4 and 10 mg/kg i.p.) reduced the number of Fos-like immunoreactive cells in the superficial dorsal horn, whereas only the high dose reduced Fos protein expression in the nucleus proprius and the neck region of the dorsal horn (C). Data are expressed as mean ± SEM (n = 5–6 per group). $^{\$\$}p < 0.01$, $^{\$}p < 0.05$ IC87201 10 mg/kg vs. all other groups; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. vehicle. One-Way ANOVA, Newman-Keuls post hoc. CPS: composite pain score, AUC: area under the curve.

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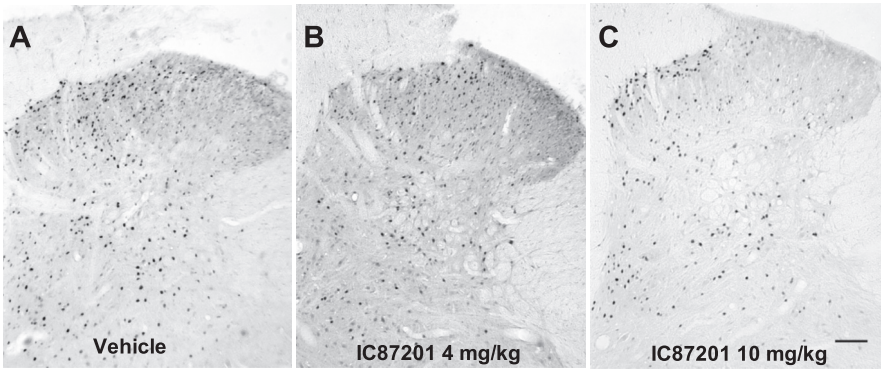

Fig. 4. Example photomicrographs taken at 10x magnification show formalin-evoked Fos-like immunoreactive cells in lumbar dorsal horn of rats treated with vehicle (A), IC87201 (4 mg/kg i.p.) (B) and IC87201 (10 mg/kg i.p.) (C). Scale bar = 100 μm. Fos protein expression was largely absent in the dorsal horn contralateral to the formalin-treated paw.

ZL006 reduces paclitaxel-induced mechanical and cold allodynia in rats

Paclitaxel produced mechanical ($F_{3,66}$ = 27.22, p < 0.0001; Fig. 8A) and cold allodynia ($F_{3,66}$ = 34.15, p < 0.0001; Fig. 8B) that was differentially impacted by ZL006, ZL007 and vehicle treatment. ZL006 reduced paclitaxel-induced mechanical hypersensitivity ($F_{3,66}$ = 29.48, p < 0.0001; Fig. 8A), and the interaction was significant ($F_{9,66}$ = 11.43, p < 0.0001; Fig. 8A). The high dose of ZL006 (4 mg/kg i.p.) elevated mechanical paw withdrawal thresholds at 30 min post-injection relative to all other groups (p < 0.001). ZL006 remained efficacious in producing antinociception at 90 min post-injection relative to rats treated with ZL007 (4 mg/kg i.p.) and vehicle (i.p.) (p < 0.001). The low dose of ZL006 (2 mg/kg i.p.) elevated mechanical paw withdrawal thresholds at 30 (p < 0.01), 90 (p < 0.05), and 180 (p < 0.05) minutes post injection relative to rats treated with ZL007 (4/mg/kg) or vehicle. ZL006 also decreased cold allodynia ($F_{3,66}$ = 17.35, p < 0.0001; Fig. 8B), and the interaction was significant ($F_{9,66}$ = 13.67, p < 0.0001; Fig. 8B). The high dose of ZL006 (4 mg/kg i.p.) reduced the frequency of withdrawing to the acetone-stimulated paw at 30 (p < 0.001) and 90 (p < 0.05) minutes post-injection relative to ZL007- (4 mg/kg i.p.) and vehicle-treated rats. The low dose of ZL006 (2 mg/kg i.p.) reduced paclitaxel-induced cold responsiveness at 30 (p < 0.001) minutes post-injection relative to ZL007- and vehicle-treated rats.

The PSD95/nNOS complex is present in the lumbar spinal cord of paclitaxel-treated rats

Paclitaxel produced mechanical hypersensitivity ($F_{1,10}$ = 377.4, p < 0.0001; Fig. 9A) that was dependent upon the treatment phase ($F_{1,10}$ = 213.8, p < 0.0001; Fig. 9A), and the interaction was significant ($F_{1,10}$ = 196.1, p < 0.0001; Fig. 9A). While neither group differed at baseline (p > 0.6), paclitaxel decreased mechanical paw withdrawal thresholds relative to rats treated with cremophor-based vehicle (t_{10} = 18.72, p < 0.0001; Fig. 9A). PSD95 and nNOS proteins and the nNOS/PSD95 complex were also present in the lumbar spinal cord (Fig. 9B). However, paclitaxel did not reliably increase association of PSD95 with nNOS in the lumbar spinal cord tissue (p > 0.2, one-tailed t-test). ZL006 (p = 0.19, one-tailed t-test) did not reliably reduce levels of nNOS/PSD95 interaction in lumbar spinal cord relative to vehicle treatment in paclitaxel-treated rats in all subjects. The observed power of the nonsignificant unpaired t-test comparing impact of ZL006 versus vehicle treatment on the association of PSD95 and nNOS in paclitaxel-treated lumbar spinal cords was 20%. A sample size of 30 per group would be required to detect a ZL006-induced disruption of PSD95–NOS association based upon the observed standard deviation, sample size and magnitude difference between means.

DISCUSSION

NMDARs are a critical link in the development and maintenance of central sensitization (Woolf and Thompson, 1991). NMDAR-mediated production of NO is of particular importance mechanistically as an effector pathway in the generation of persistent pathological pain (Meller and Gebhart, 1993). Therefore, reducing NMDAR-mediated production of NO represents a promising mechanism to alleviate chronic pain conditions. However, the problematic side effects associated with direct NMDAR antagonists and NOS inhibitors indicate the need for alternate mechanisms to suppress NMDAR-dependent NO production (Rees et al., 1989; Kobayashi et al., 1991; Bohme et al., 1993; Krystal et al., 1994; Holscher et al., 1996; Pal et al., 2002; Rickard and Gibbs, 2003; Koylu et al., 2005; Yildiz Akar et al., 2007). Because the scaffolding protein PSD95 tethers nNOS to NMDARs, protein–protein interactions between PSD95 and nNOS are required for NMDAR-mediated production of NO (Sattler et al., 1999). We, therefore, hypothesized that selective disruption of PSD95–nNOS protein–protein interactions would suppress CNS sensitization associated with pathological pain without the unwanted side effects of NMDAR antagonists. Our studies using the formalin test suggest that putative small molecule inhibitors of the protein–protein interaction between PSD95 and nNOS suppress NMDAR-dependent pain behavior as well as inflammation-evoked neuronal activation in the spinal dorsal horn without unwanted side effects of NMDAR antagonists.

In the present work, two pharmacological inhibitors of PSD95–nNOS protein–protein interactions, IC87201 and ZL006, selectively suppressed both formalin-evoked activation of spinal dorsal horn neuronal circuitry and formalin-evoked pain behavior in the same subjects. In our study, IC87201 and ZL006, but not the inactive analog ZL007, reduced formalin-evoked pain behavior

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and Fos protein expression in the lumbar dorsal horn. These observations are important because they support the hypothesis that antinociceptive effects of PSD95–nNOS inhibitors reflect a suppression of nociceptive processing at the level of the CNS. Moreover, PSD95–nNOS inhibitors selectively suppressed phase 2 of formalin pain, which is specifically linked to CNS sensitization (Hylden et al., 1989; Lebrun et al., 2000), similar to the NMDAR antagonist MK-801. Phase 1 of formalin pain, which has been linked closely to primary afferent activation (Puig and Sorkin, 1996), was unaffected by PSD95–nNOS inhibitors. Moreover, we show that both ZL006 and IC87201, administered systemically, produced antinociception in rats whereas ZL007 was ineffective. Similarly, at the dose that maximally reduced formalin-evoked nociceptive behavior and Fos protein expression, ZL006, which has not previously shown efficacy in rats in any prior publication, reduced paclitaxel-induced mechanical and cold hypersensitivity whereas ZL007 was ineffective. Our study is the first to demonstrate that the small molecule protein–protein interaction inhibitor ZL006 produces antinociception in rats. ZL006, has been reported to exhibit limited blood–brain barrier penetration (Wang et al., 2015) but efficacy exhibited by this compound in models of post-traumatic stress disorder (Shekar et al., 2012) and depression (Doucet et al., 2013) argue for central sites of action of ZL006. By contrast, ZL007 was ineffective in suppressing either formalin-evoked pain behavior or formalin-evoked Fos protein expression in rats. This observation is noteworthy because ZL007 failed to disrupt co-immunoprecipitation of nNOS and PSD95 in mouse hippocampal slice cultures observed with ZL006 (Zhou et al., 2010) but disrupted binding between purified PSD95 and nNOS interactions in our Alphascreen biochemical assay (unpublished data). The present study extends previous reports from our lab indicating that IC87201 and ZL006 specifically disrupt the interaction between purified nNOS and PSD95 proteins *in vitro*, reduce glutamate-induced excitotoxicity in cultured cortical neurons, and produce antinociceptive efficacy in mouse models of inflammatory and neuropathic pain in

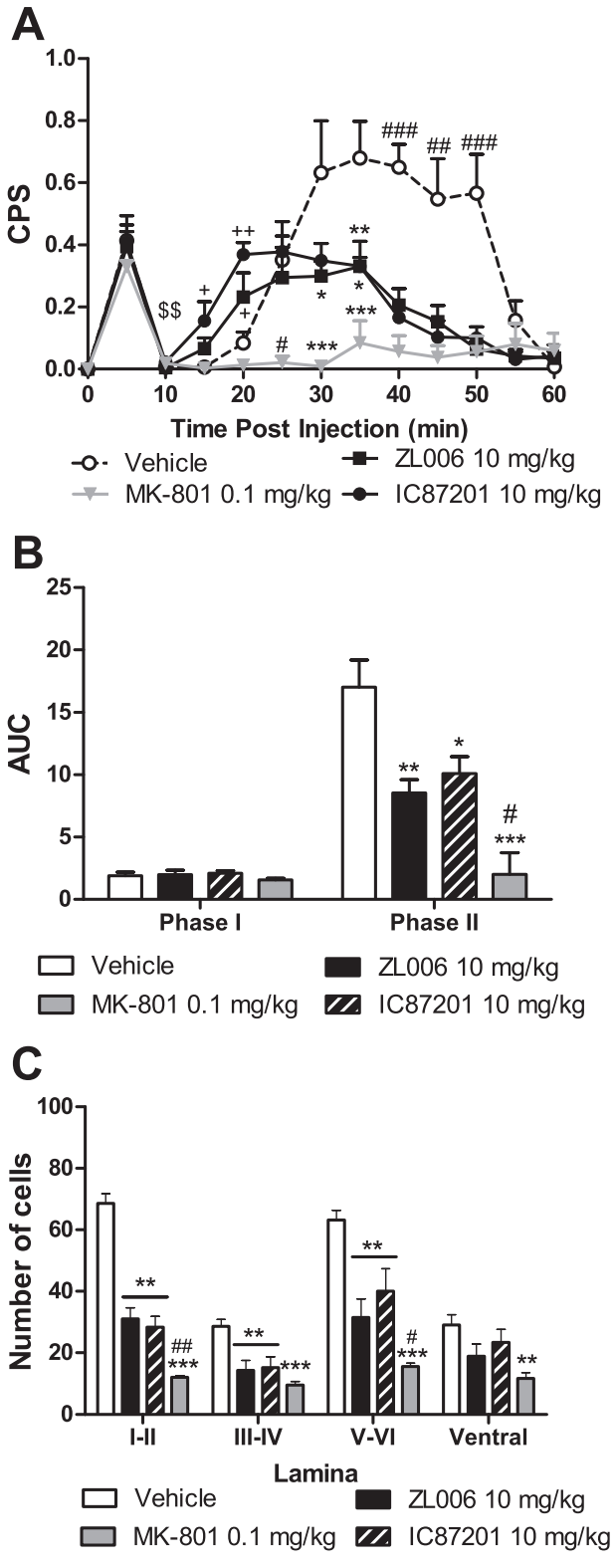

Fig. 5. ZL006 and IC87201 produce comparable suppressions of formalin-evoked pain behavior and Fos-like immunoreactivity in spinal dorsal horn. IC87201 decreased composite pain scores at 10 min post-formalin relative to rats treated with ZL006 or vehicle. IC87201 facilitated the onset (15–20 min) of formalin-induced CPS relative to vehicle or MK-801 treatment as well as the resolution of phase 2 pain (decreasing CPS from 40–50 min post-formalin) relative to vehicle (A). ZL006-treated rats displayed a transient increase in CPS at 20 minutes post-formalin relative to rats treated with MK-801 or vehicle, and decreased CPS at 30–50 minutes post-formalin relative to vehicle-treated rats (A). MK-801 decreased CPS at 10 minutes post-formalin relative to rats treated with ZL006 or vehicle, at 25 min post-formalin relative to all other groups, and 30–50 minutes post-formalin relative to vehicle-treated rats (A). MK-801 reduces AUC relative to all groups, whereas ZL006 and IC87201 reduce the AUC of phase 2 formalin pain relative to vehicle (B). ZL006, IC87201 and MK-801 reduced Fos-like immunoreactivity in the superficial dorsal horn, the nucleus proprius, and the neck region of the dorsal horn relative to vehicle (C). MK-801 produced a greater suppression of the number of FLI cells relative to ZL006 and IC87201 in the superficial dorsal horn and the neck region of the dorsal horn (C). Only MK-801 reliably suppressed the number of FLI cells in the ventral horn (C). Data are expressed as mean ± SEM (n = 5–6 per group). $^{\$\$}p < 0.01$ MK-801 and IC87201 vs. ZL006 and vehicle; $^{+}$ $^{++}p < 0.01$, $^{+}p < 0.05$ vs. vehicle and MK-801; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. vehicle; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ vs. all other groups. One-way ANOVA, Newman–Keuls post hoc. CPS: composite pain score, AUC: area under the curve.

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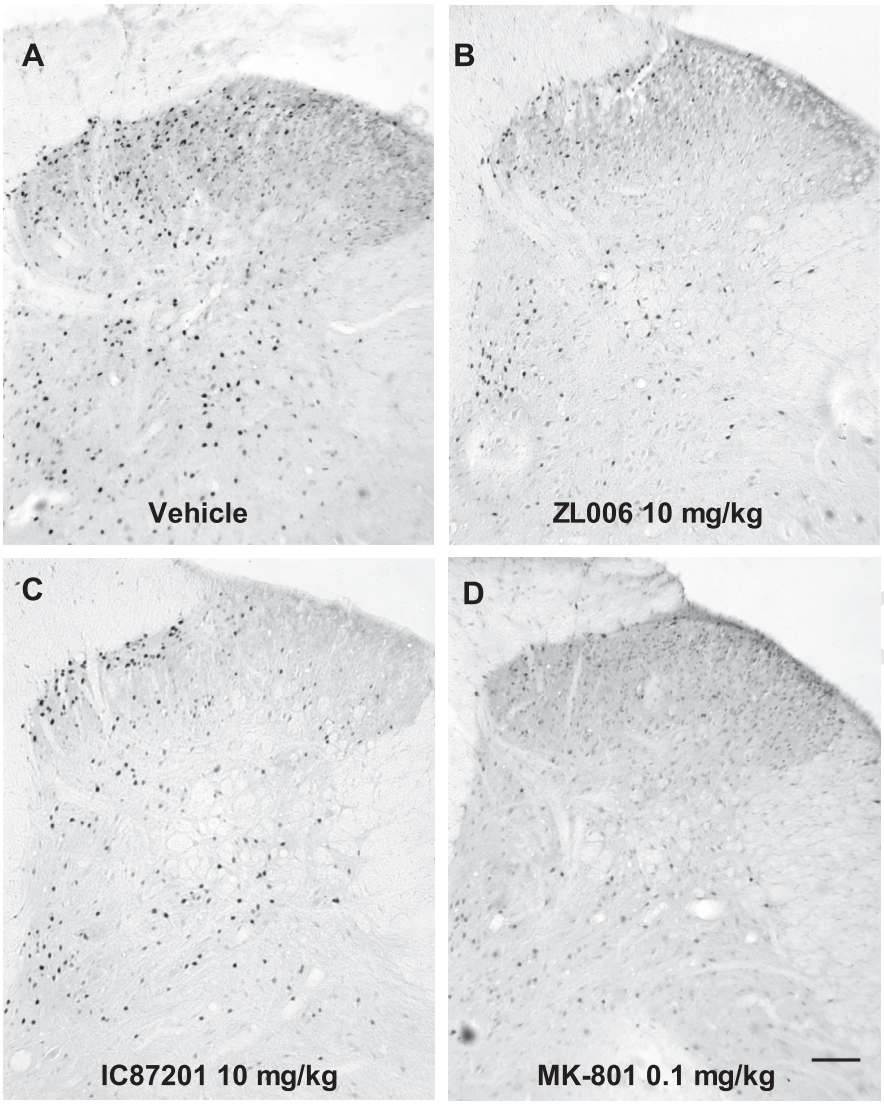

Fig. 6. Example photomicrographs taken at 10x magnification showing formalin-evoked Fos-like immunoreactive cells in lumbar dorsal horn of rats treated with vehicle (A), ZL006 (10 mg/kg i.p.) (B), IC87201 (10 mg/kg i.p.) (C), or MK-801 (0.1 mg/kg) (D). Scale bar = 100 μm. Fos expression was largely absent in the dorsal horn contralateral to the formalin-treated paw

mice (Lee et al., 2015). We previously verified that both IC87201 and ZL006 suppress NMDA-stimulated cGMP formation, a marker of NO production, in cultured hippocampal neurons (Smith et al., 2016). Thus, we have used validated pharmacological tools to test the hypothesis that PSD95–nNOS inhibitors suppress formalin-evoked neuronal activation and pain behavior in *vivo* using the same subjects. Nonetheless, caution must be exerted in extrapolating effects of ZL007 from *in vitro* to *in vivo* levels and/or across species (mice vs. rats). Moreover, because we evaluated a clinically relevant systemic route of drug administration more work is necessary to determine the site of action of small molecule PSD95–nNOS inhibitors by using intrathecal, intraventricular and site-specific intracranial injections in multiple brain regions. The observed pattern of antinociceptive efficacy during phase 2 (but not phase 1) of formalin pain, and in suppressing formalin-evoked protein expression, is consistent with suppression of CNS sensitization by PSD95–nNOS inhibitors.

Our studies also demonstrated that the putative PSD95–nNOS inhibitor ZL006, but not the inactive analog ZL007, suppressed mechanical and cold allodynia induced by treatment with the chemotherapeutic agent paclitaxel. We, therefore, examined whether the association of nNOS and PSD95 was increased in the lumbar spinal cord during the maintenance phase of paclitaxel-induced neuropathic pain at a time point when ZL006, but not ZL007, produced antinociception. ZL006 was specifically used for our studies because this ligand was shown previously to spare NMDAR subunit NR2B-PSD95 interactions (Zhou et al., 2010) and a structurally similar inactive analog (ZL007) was synthesized by us to confirm specificity of mechanism of action. Zhou et al. (2010) previously reported that the association of nNOS and PSD95 is increased in brain tissue in rodent models of cerebral ischemia and was disrupted by ZL006 with sample sizes of three per group. Paclitaxel, rather than unilateral formalin injection, was used for our co-immunoprecipitation studies because toxic challenge with a systemically administered chemotherapeutic agent can be expected to impact the spinal cord bilaterally rather than unilaterally and insufficient protein levels in native tissue would be expected to limit sensitivity of the detection method in dorsal horn hemisections derived from formalin-treated rats. While treatment with the chemotherapeutic agent paclitaxel produced allodynia, a reliable increase in the association of nNOS and PSD95 during the maintenance phase of paclitaxel-induced neuropathic pain (i.e. 16 days following onset of treatment) was not observed in lumbar spinal cord tissue using our validated co-immunoprecipitation methods. More work is necessary to determine whether supraspinal sites of antinociceptive action and descending mechanisms could contribute to the pattern of antinociceptive efficacy and results obtained here. Our studies, nonetheless, provide the important demonstration that ZL006, but not ZL007, attenuated both mechanical and cold hypersensitivity induced by paclitaxel treatment in rats, confirming utility of ZL007 as an inactive analog for *in vivo* manipulations in rats. However, while systemic treatment (i.p.) with ZL006 decreased the association of nNOS and PSD95 in paclitaxel-treated rats relative to paclitaxel-treated rats

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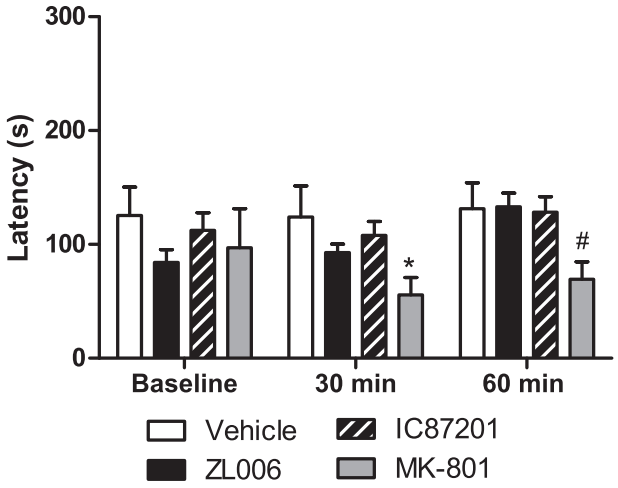

Fig. 7. The NMDAR antagonist MK-801 impairs rotarod performance in rats whereas PSD95–nNOS inhibitors ZL006 and IC87201 do not. MK-801 reduced descent latency in the rotarod test at 30 min post-injection relative to vehicle and 60 min post-injection relative to all other groups. ZL006 and IC87201 did not impair rotarod performance. Data are expressed as mean ± SEM (n = 5–6 per group). $^{*}p < 0.05$ vs. vehicle; $^{\#}p < 0.05$ vs. all other groups. One-way ANOVA, Newman–Keuls post hoc.

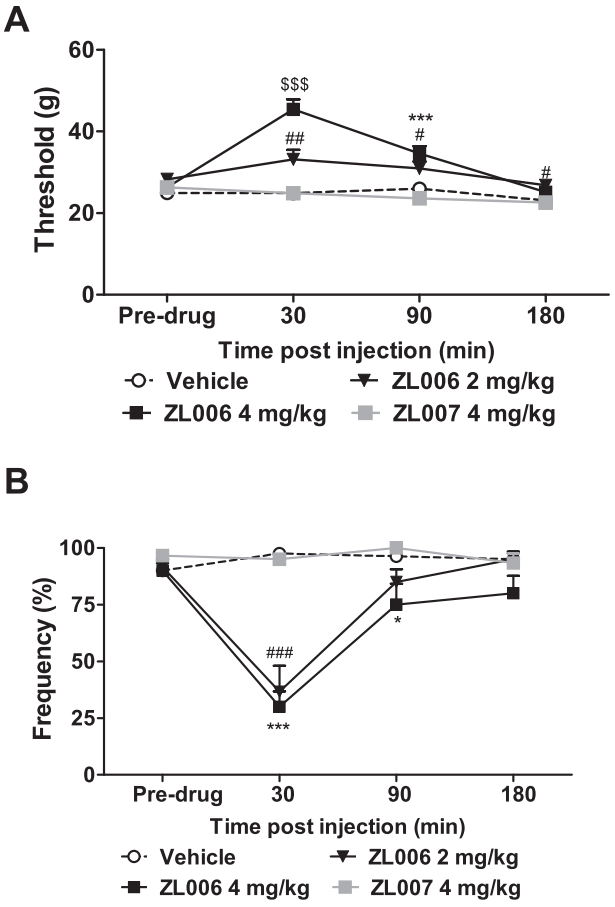

Fig. 8. ZL006 attenuates paclitaxel-induced mechanical and cold allodynia. ZL006 (4 mg/kg i.p.) increased the threshold for paw withdrawal to mechanical stimulation in paclitaxel-treated rats relative to vehicle and ZL007 (4 mg/kg i.p.) treatment at 30 and 90 min post-injection. The high dose of ZL006 produced a greater antinociceptive effect compared to the low dose of ZL006 at 30 min post injection (A). ZL006 (2 mg/kg i.p.) reduced mechanical hypersensitivity at 30, 90 and 180 min post-injection relative to rats treated with ZL007 or vehicle (A).In paclitaxel-treated rats, ZL006 (2 mg/kg i.p.) reduced the frequency of paw withdrawal to cold stimulation (cold allodynia) at 30 min post-injection relative to ZL007- and vehicle-treated rats (B). ZL006 (4 mg/kg i.p.) reduced cold allodynia at 30 and 90 min post-injection relative to ZL007 (4 mg/kg i.p.) and vehicle (B). Data are expressed as mean ± SEM (n = 6–8 per group). $^{\$\$\$}p < 0.001$ ZL006 4 mg/kg vs. all other groups; $^{***}p < 0.001$, $^{*}p < 0.05$ ZL006 4 mg/kg vs. ZL007 and vehicle; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ ZL006 2 mg/kg vs. ZL007 and vehicle. Two-way ANOVA, one-way ANOVA, Newman–Keuls post hoc.

receiving vehicle (i.p.), this reduction failed to reach statistical significance. The failure to observe a reliable decrease in the association of nNOS and PSD95 in the present co-immunoprecipitation experiments could be attributed to a number of factors. Power analyses performed on the data derived from our paclitaxel-treated spinal cords indicate that our study was underpowered to detect significant mean differences between ZL006 and vehicle treatment, with observed power being only 20%. Moreover, large sample sizes (n = 30 per group) would be necessary to achieve 80% power to detect the observed 0.28 magnitude difference between means with alpha set at 0.05. The previous report by Zhou et al. demonstrating that ZL006 disrupts nNOS-PSD95 association *in vivo* used a different route of administration (i.v. vs. i.p.), a different tissue type (mouse cortex vs. rat lumbar spinal cord), and a more robust model of pathology (middle cerebral arterial occlusion vs. chemotherapy-induced neuropathic pain). Moreover, because the entire lumbar spinal cord (i.e. including dorsal and ventral horn and surrounding white matter), was processed to ensure uniformity of samples between subjects, it is also possible that, the sampling methods employed may have diluted the magnitude of observed changes and sensitivity of the methods used to detect disruption of the PSD95–nNOS complex. Because PSD95 binds many proteins, it is also possible that ubiquity of PSD95 relative to nNOS may limit ability to detect changes in the PSD95–nNOS complex by co-ip. It is also possible that changes in downstream signaling can occur with only transient changes in the PSD95–nNOS complex. Finally, supraspinal as well as spinal sites of action could contribute to the antinociceptive effects of systemically administered PSD95–nNOS inhibitors. Such factors could limit the ability to detect robust increases in the PSD95–nNOS complex and a reliable decrease in their association produced by ZL006 treatment in lumbar spinal cord. Additional work is necessary to evaluate the site of action of PSD95–nNOS inhibitors and to determine whether other CNS regions (i.e. punches derived from yet to be identified brain regions) provide adequate protein levels for the detection of the PSD95–nNOS complex and determine whether other pathological pain states produce a measurable increase in the association of nNOS and PSD95 and. Nonetheless, it is important to emphasize that the PSD95–nNOS complex was reliably detected in lumbar spinal cord of paclitaxel-treated rats. Moreover, ZL006 was effective at reducing the behavioral manifestations of two mechanistically distinct types of pain, as well as in suppressing noxious stimulus-evoked neuronal

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activation within spinal dorsal horn nociceptive circuitry, whereas ZL007 was inactive under the same conditions.

Formalin-evoked nociceptive behavior occurs in two distinct phases, with an immediate primary nocifensive phase lasting 0–5 min thought to reflect primary afferent activation (Puig and Sorkin, 1996), and a second phase beginning 15–20 min post-formalin injection, which is thought to reflect sensitization occurring within the spinal cord and facilitation of responses to otherwise innocuous stimulation (Hylden et al., 1989; Lebrun et al., 2000). The time period between phases of formalin-evoked pain (i.e. 5–15 min post-injection) is normally characterized by a state of quiescence and disappearance of nociceptive behavior attributed to transient reductions in activity of primary afferent nociceptors (Puig and Sorkin, 1996) and descending inhibitory controls (Franklin and Abbott, 1993; Kaneko and Hammond, 1997). Both doses of IC87201 and ZL006 reduced overall nocifensive behavior in the second phase of formalin-evoked pain in rats, as demonstrated by quantification of the AUC of phase 2 pain behavior. However, IC87201, and to a lesser extent, ZL006 also produced an apparent leftward shift in both the onset *and* resolution of phase 2 nociceptive behaviors. While NO is typically thought of as a proinflammatory mediator, differences in peripheral vs. central activity of NO exist could account for this effect. NOS inhibitors have been reported to exacerbate and prolong the resolution of inflammation when administered locally to sites of inflammation, whereas systemic administration of these agents attenuated inflammation (Paul-Clark et al., 2001). NO modulates neuronal function at spinal and supraspinal sites in a complex manner (see Prast and Philippu, 2001 for review). It is possible that the apparent increase in pain observed in animals treated with the highest doses of IC87201 and ZL006 during the interphase pain reflects a leftward shift in the onset of phase 2 pain behavior that could have occurred due to modulation of descending inhibitory control circuitry. It is important to note that while both the onset and the resolution of phase 2 pain behavior was facilitated by PSD95–nNOS inhibitors, the total amount of phase 2 pain behavior, as defined by the AUC, was *markedly* diminished by PSD95–nNOS inhibitor treatment with either active analog. Likewise, all compounds were administered systemically, and PSD95–nNOS inhibitors would, hypothetically, only target postsynaptic neurons that contain NMDARs, PSD95 and nNOS. Thus, NOS inhibitors, which nonspecifically inhibit nNOS as well as endothelial nitric oxide synthase (eNOS), (see Vitecek et al., 2012 for review) could be expected to produce a different pattern of *in vivo* effects on nociceptive responding. For example, NOS inhibitors or NO application can produce pro- or antinociceptive effects varying with local vs. systemic application by altering peripheral nociceptive responding while lowering

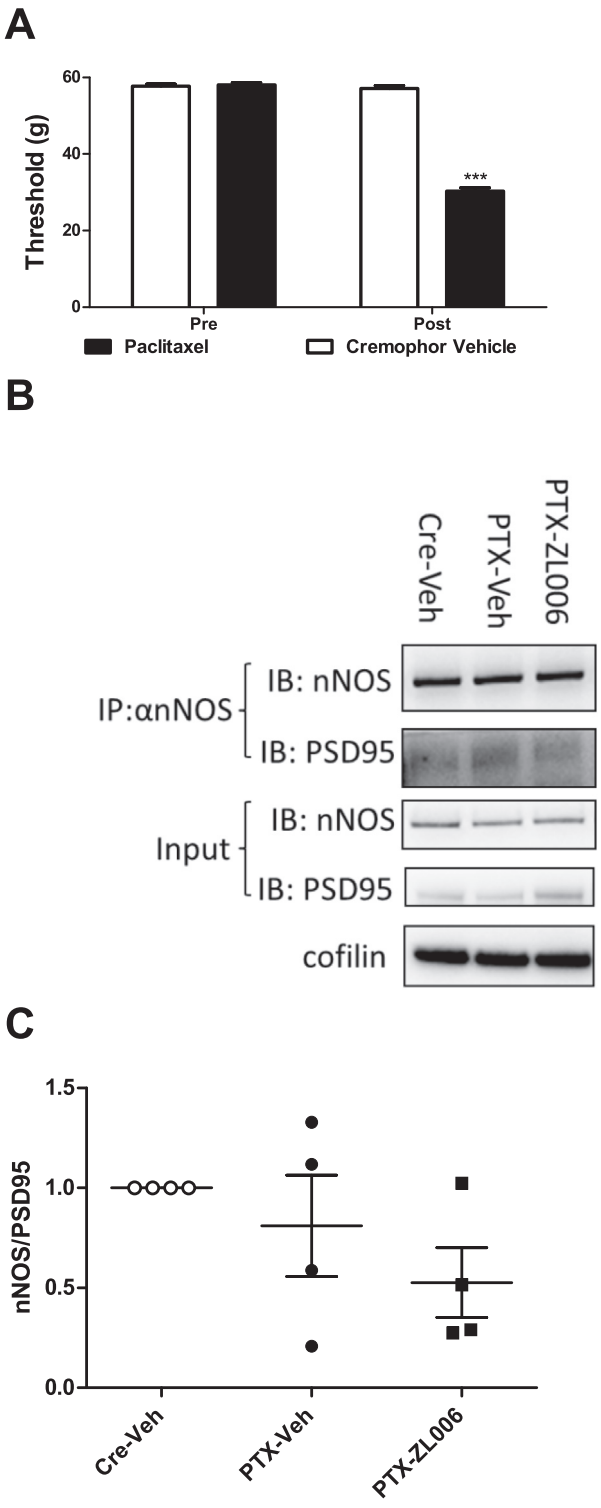

Fig. 9. The PSD95–nNOS complex is present in the spinal cord of the paclitaxel-treated rats. Paclitaxel treatment decreased paw withdrawal thresholds to mechanical stimulation relative to rats treated with cremophor-based vehicle and relative to pre-injection baseline responding (A). Data are mean ± SEM (n = 4–8 per group). $^{***}p < 0.0001$ paclitaxel-treated rats vs. cremophor-treated rats. Two-way ANOVA, two-tailed paired t-test. Representative western blots showing coimmunoprecipitation of PSD95 with nNOS in the lumbar spinal cord of rats treated with either paclitaxel (PTX) or cremophor-based vehicle (B). Paclitaxel treatment did not reliably alter the association of nNOS and PSD95 in lumbar spinal cord (C). nNOS/PSD95 levels in cremophor-treated rats receiving vehicle (Cre-Veh) and paclitaxel-treated rats receiving vehicle (PTX-Veh) or ZL006 (10 mg/kg i.p) (PTX-ZL006) (C). Data are mean ± SEM with scatterplot showing individual subjects data (n = 4 per group).

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central sensitization driven by peripheral nociceptors. Presumably, PSD95–nNOS protein–protein interaction inhibitors require interaction with postsynaptic proteins (i.e. PSD95) that would specifically alter CNS sensitization. Future studies could examine whether PSD95–nNOS protein–protein interaction inhibitors lack efficacy following local injections in the hind paw that would target peripheral mechanisms. Nonetheless, both IC87201 and ZL006 were effective overall at reducing pain behaviors *throughout* the second phase of formalin-evoked pain in its entirety. ZL007, an analog of ZL006, failed to produce antinociceptive efficacy in rats in the current study, and, likewise, failed to decrease formalin-evoked Fos-like immunoreactivity in lumbar spinal dorsal horn. While ZL006, IC87201 and MK-801 all effectively reduced pain behavior in phase 2 of formalin-evoked pain and Fos-like immunoreactivity in spinal dorsal horn regions associated with nociceptive processing. PSD95–nNOS inhibitors were maximally efficacious in suppressing formalin-evoked Fos protein expression in the superficial dorsal horn of the spinal cord. Only MK-801 reliably decreased Fos expression in the ventral horn. Thus it is noteworthy that although MK-801 has well-established antinociceptive properties in the formalin test (Vaccarino et al., 1993; Chaplan et al., 1997), the dose used in the current study also produced modest motor impairment in the rotarod test in our study but did not prevent rats from navigating a radial arm maze used to assess spatial memory performance in our previous work (Smith et al., 2016).

An important observation of our study was that PSD95–nNOS disruptors suppressed formalin-evoked pain behavior and spinal neuronal activation in the same animals and at doses that did not produce motor impairment characteristic of the non-competitive NMDAR antagonist MK-801. Neither IC87201 nor ZL006 produced motor impairment associated with the noncompetitive NMDAR antagonist MK-801 in rat subjects. ZL006 has previously been reported to not impair spatial memory in mice whereas the nNOS inhibitor 7-nitroindazole decreased spatial memory (Zhou et al., 2010). However, lack of observable impairments in spatial memory tasks is not sufficient to conclude that memory impairment is absent following pharmacological manipulations. Previous work by our group showed that MK-801 (at the same dose employed in the present study) eliminated memory for source of origin of information (i.e. source memory) under conditions in which spatial memory was spared (Smith et al., 2016). By contrast, ZL006 and IC87201, at the highest efficacious doses evaluated in the present study, did not impair spatial or source memory in rats under identical testing conditions (Smith et al., 2016). Even at supramaximal doses (30 mg/kg), IC87201 and ZL006 did not produce motor impairment or alter basal nociceptive thresholds in mice in our previous work (Lee et al., 2015). These observations collectively suggest that disruption of PSD95–nNOS interactions may exhibit better therapeutic ratios compared to NMDAR antagonists. PSD95–nNOS inhibitors are also likely to exhibit limited side effect profiles compared to NOS inhibitors because NOS inhibitors are not selective for nNOS but inhibit other NOS isoforms (e.g. eNOS), that similarly translates into unwanted side effects (Rees et al., 1989; Kobayashi et al., 1991). Thus, non-specific amino acid NOS inhibitors (Bohme et al., 1993; Rickard and Gibbs, 2003; Koylu et al., 2005), selective indazole nNOS inhibitors (Holscher et al., 1996; Yildiz Akar et al., 2007) and genetic deletion of nNOS (Weitzdoerfer et al., 2004) also produce impairments in cognitive functioning, learning and memory. In conclusion, the pattern of antinociceptive efficacy, suppression of inflammation-evoked neuronal activation in lumbar spinal dorsal horn, and lack of side effects produced by ZL006 and IC87201 (see also (Lee et al., 2015; Smith et al., 2016)) suggest that small molecule nNOS/PSD95 protein–protein interaction inhibitors should exhibit a superior therapeutic index compared with NMDAR antagonists and may represent a safer, effective mechanism to alleviate chronic pain.

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CONFLICT OF INTEREST

YYL is partially employed at Anagin, LLC.

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