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Loose ligation of the rat sciatic nerve elicits early accumulation of Shank1 protein in the postsynaptic density of spinal dorsal horn neurons

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Abstract

Plasticity in the spinal dorsal horn may contribute to the development of pain following peripheral nerve injury. Shank proteins are a constituent family of the post-synaptic density (PSD), and they may play a role in synaptic plasticity through activity-dependent synaptic remodeling and growth. In this study we examined the early consequences of the loose ligation of the sciatic nerve on Shank1 protein and message levels in the PSD of spinal dorsal horn neurons.

Four hours after sciatic ligation, the protein levels of Shank1 increased in the ipsilateral PSD of ligated animals. In contrast, no changes were detected in the contralateral PSD of these ligated animals, or either the ipsilateral or contralateral PSD of sham-operated animals. Shank1 was linked to the PSD marker protein PSD-95 and the NR2B subunit of NMDA receptors. The ligated animals also exhibited two early signs of pain behavior, a shift in weight bearing and thermal hyperalgesia. There was no overall change in Shank1 message in either ligated or sham-operated animals. The accumulation of Shank1 in the PSD was abolished by intrathecal pre-treatment with anisomycin or Shank1 siRNA but not non-target siRNA. The same pre-treatment prevented both of the early signs of pain behavior. Intrathecal pre-treatment with either MK-801 or U0126 similarly prevented the Shank1 accumulation and alleviated both of the behavioral signs of pain.

The early accumulation of Shank1 in the PSD of dorsal horn neurons may be a necessary step in the injury-associated plasticity that in time leads to the development of persistent pain.

Keywords

Chronic constriction injury; Glutamate receptors; Neuropathic pain; Nociception; Structural proteins; Synaptic plasticity

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1. Introduction

Shank proteins are a constituent family of the post-synaptic density (PSD). They were initially characterized by different groups and this has lead to a diverse terminology. Shank (SH3 domain and ankyrin repeat containing protein) is used most often, but ProSAP (proline-rich synapse associated protein) is a common alternative although ProSAP1 and 2 correspond to Shank2 and 3 [2,9,10,17,22].

Shanks contain a number of protein interaction domains including N-terminal ankyrin (ANK) repeats, an SH3 (Src homology 3) domain, a PDZ (PSD-95/DLG/ZO1) domain, proline-rich clusters (Pro), a cortactin binding domain (ppI) and a SAM (sterile alpha motif) domain. These domains enable Shank to interact with multiple constituents of the PSD. For example, the PDZ domain of Shank directly interacts with the C-terminal QTRL motif of GKAP/ SAPAP/DAP-1, a protein that binds to the GK domain of the PSD-95 family of proteins (PSD-95/SAP90, SAP97, chapsyn-110/PSD-93, and SAP102). Similarly, the proline-rich region of Shank directly interacts with the EVH1 domain of Homer, and this allows for a potential link between NMDA and metabotropic glutamate receptor complexes. All Shank transcripts are subject to alternative splicing, and several alternative splice forms of Shank1 have been reported.

Shank proteins appear to play a central role in synaptogenesis and synaptic plasticity. The multiple protein interacting domains contained within their structure enable Shanks to link to other scaffolding proteins, ionotropic and metabotropic glutamate receptors and their associated signaling pathways, and cytoskeletal components. Also, given that Shanks are located further away from the PSD membrane than PSD-95 [23] they are well-positioned to promote activity-dependent synaptic remodeling by linking postsynaptic glutamate receptors with actin-regulatory molecules such as cortactin [2,9,10,17,22].

Plasticity in the spinal dorsal horn is thought to underlie at least in part pain behavior following peripheral nerve injury [15,21]. Previously we reported that increased protein levels of Shank1 in the PSD of spinal dorsal horn neurons are associated with neuropathic pain 7 days after loose ligation of the sciatic nerve [13]. In the present study we focused on the early consequences of the injury.

We hypothesized that sciatic ligation was associated with early increases in Shank1 protein and message, and we surmised that this increase may be an important first-step in the injury-associated plasticity that leads to pain behavior. We also sought to examine whether the protein synthesis inhibitor anisomycin or small interfering RNAs (siRNA) which specifically target Shank1 message would prevent changes in Shank1 protein expression and thus alleviate signs of early pain behavior. We further investigated whether changes in Shank1 protein expression were dependent on the activation of NMDA receptors and extracellular signal-regulated kinase 1 & 2 (ERK1/2). There is a well-established association between injury-associated plasticity in the spinal dorsal horn and the activation of NMDA receptors and ERK1/2 [15,21].

2. Materials and Methods

2.1. Animals

Male Harlan-Sprague-Dawley rats (~200-250g) were used. All experiments were conducted in accordance with guidelines accepted by the International Association for the Study of Pain [24]. The animal protocol was approved by the Animal Care and Use Committee of the School of Medicine and Public Health at the University of Wisconsin-Madison.

2.2. Behavioral Tests

A dual channel scale (Incapacitance Meter™, Stoelting, Chicago, IL), which separately measures the weight borne by each hind limb, was used for the weight-bearing test. While normal rats distribute weight about equally, animals with a unilateral injury will shift their weight from an injured to a non-injured limb. This shift is taken as a measure of discomfort in the injured limb. A 1s weighing period was used to average 20 measurements, and the average ratio of the injured over uninjured weight distribution was then calculated for each animal.

Thermal hyperalgesia was assessed with the well-established hind paw withdrawal latency test [7] using a plantar analgesia instrument (Stoelting, Chicago, IL). Animals were acclimated for 15-20 min. The ipsilateral, injured, paw was tested four times to obtain an average latency. Each of the four trials were separated by 5 min. Baseline withdrawal latencies were obtained for all animals before they were randomly assigned to control, sham-operated or ligated groups. Four hours after sciatic exposure or ligation the withdrawal latencies of all animals were obtained again (second latency test). The weight-bearing test preceded the second paw withdrawal latency test.

2.3. Intrathecal drug application

Shank1 (1μM) or non-target (control) siRNA (1μM) (both from Santa Cruz Biotechnology, Santa Cruz, CA) were injected intrathecally 2h before sciatic exposure or ligation in a volume of 10μl using the procedure of Mestre and colleagues [12] as described in detail previously [14]. Shank1 siRNA targets Shank1 mRNA specifically while non-target (control) siRNA does not have any known gene target in human, rat or mouse. The siRNAs were mixed with diluted transfection reagent (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated for 20-30 min at room temperature before application.

Anisomycin (125μg), MK-801 (20μg), U-01026 (5μg) or vehicle were injected 15 min before sciatic exposure or ligation in a volume of 10μl each. All drugs were from Sigma-Aldrich, St. Louis, MO. MK-801 was dissolved in saline, anisomycin and U-0126 in 10% DMSO. For all intrathecal injections animals were briefly anesthetized as described below.

2.4. Anesthesia, sciatic ligation and tissue collection

Animals were anesthetized with isoflurane. Body temperature was kept at 37°C with a homeothermic blanket system. Anesthesia was sufficiently deep to prevent arousal but light enough to permit spontaneous respiration. Adequate anesthesia was assessed by monitoring blink or ear reflexes, withdrawal to toe pinches, respiratory rate, and absence of spontaneous movements.

Loose ligation of the sciatic nerve (chronic constriction injury) was performed using the Bennett and Xie [1] procedure as described previously [13,14]. Briefly, the sciatic nerve was exposed and loosely ligated with 4 simple interrupted 4-0 chromic gut sutures placed about 1mm apart. In sham-operated animals the sciatic nerve was exposed but not ligated. Control animals were anesthetized but were not subject to surgery.

For tissue collection the animals were re-anesthetized and euthanized with an intracardiac injection of saturated potassium chloride. A laminectomy rapidly (<2 min) exposed the lumbar spinal cord at L5, and about 1cm of the cord was excised and cut into dorsal and ventral halves and the dorsal half further divided into ipsilateral and contralateral quadrants. All tissues were stored at -80°C until use.

2.5. Fractionation

Tissues were homogenized in 4 mM HEPES buffer containing 320mM sucrose, 10mM EDTA and 0.06% of a mixture of protease inhibitors [4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane, bestatin, leupeptin, and aprotinin], and centrifuged at 1000g at 4°C for 10 min to yield a pellet (P1) and supernatant (S1) fraction. The P1 fraction was re-suspended in sucrose buffer and centrifuged again at 1000g at 4°C for 10 min to yield the nuclear (P1′) and S1′ fraction. The S1 and S1′ fractions were combined and spun at 12,000g at 4°C for 15 min to yield the P2 and S2 fractions. The P2 fraction was washed in sucrose buffer and centrifuged at 13,000g at 4°C for 15 min to yield the crude synaptosomal P2′ pellet. This pellet was re-suspended in sucrose buffer and then hypotonically lysed with 9 volumes of cold water. The P2′ suspension was restored to 4mM HEPES and centrifuged at 30,300g at 4°C for 30 min to yield the synaptosomal vesicles and cytosolic fraction (LS1) and the heavy membrane pellet (LP1). The LP1 pellet was re-suspended in 20mM Tris HCl-buffer (pH 7.4) containing 100mM NaCl, 50mM NaF, 2 mM EDTA, 0.5% Triton X-100 and 0.04% of the protease inhibitor mixture. The LP1 fraction contains the PSD proteins.

2.6. Western immunoblots and immunoprecipitation

After assaying for total protein content the LP1 and S2 fractions were processed using the Western immunoblot procedure as described previously [13,14]. Shank1 was purchased from Sigma-Aldrich, St. Louis, MO or Santa Cruz Biotechnology, Santa Cruz, CA and used at a dilution of 1:600. Beta III tubulin served as the loading control (1:1000; Promega, Madison, WI). Protein levels were estimated from optical density measurements using the BioSpectrum 500 Image Analysis System (UVP, Upland, CA). Shank1 levels within a gel were expressed over the beta III tubulin levels, and then the levels in sham-operated or ligated animals were normalized to those in control, uninjured animals.

For immunoprecipitation, the Shank1 antibody was added to LP1 fractions and incubated overnight at 4°C. Pre-washed protein A agarose beads (Invitrogen, Carlsbad, CA) were then added and the mixture incubated for 2h at 4°C. The beads were separated by centrifugation (10,000g for 30s at 4°C) and washed five times in ice-cold phosphate-buffered saline before sample buffer was added and the mixture heated for 4 min. The beads were then removed by centrifugation (10,000g for 30s at 4°C) and the supernatants used in the immunoblot procedure. Antibodies directed against PSD-95 (1:1000) or the NR2B subunit of the NMDA receptor (1:200) were purchased from Millipore, Billerica, MA or Thermo-Scientific, Rockford, IL. Membranes were also processed for Shank1 as detailed above to ascertain immunoprecipitation.

2.7. RT-PCR

Shank1 mRNA levels were determined by the real-time polymerase chain reaction (RT-PCR) as described previously [16]. Briefly, total RNA was isolated from homogenized tissues with Trizol (Invitrogen, Carlsbad, CA) and cDNA was synthesized from 1μg of the total RNA. Quantitative RT-PCR was then performed with the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) by monitoring in real time the increase in fluorescence of SYBR-GREEN dye. Relative expression levels of Shank1 in each sample were determined using a standard curve of 3-fold serial dilutions. Average fold induction relative to control animals was determined after normalizing to the amount of 18S rRNA in each sample. A 2-fold or greater change was considered significant. Primer sequences are available upon request.

2.8. Statistical Analysis

ANOVA was used for the statistical data analysis. The main emphasis was on detecting differences in Shank1 protein or message levels between control, sham-operated and ligated animals, or vehicle and drug-treated animals. In the behavioral experiments the emphasis was on detecting differences in weight distribution or latencies between control, sham-operated and ligated or between the vehicle and drug-treated groups. Significant effects were further analyzed with Scheffe's post-hoc test, and statistical difference was inferred at $p \leq 0.05$. Each group consisted of 6 animals. All data are expressed as mean±SEM.

3. Results

We focused on the 4h post-ligation period based on our previous studies on the early consequences of the sciatic ligation on selected gene or protein expression [14,16]. In addition, this is the earliest time after surgery that the presence of pain behavior in ligated animals can be differentiated from the lack of pain behavior in sham-operated animals.

3.1. Increases in Shank1 protein in the PSD of dorsal horn neurons were associated with the sciatic ligation

The protein levels of Shank1 in the ipsilateral PSD-containing LP1 fraction of ligated animals were significantly higher than those in control animals, 156±12%, $F(4,25)=13.7$, $p<0.001$ (Fig. 1). In contrast, Shank1 levels in the contralateral fraction of these ligated animals (106±5%), or either the ipsilateral (108±6%) or contralateral (105±8%) LP1 fractions in sham-operated animals were not significantly different from controls.

To investigate whether Shank1 levels specifically accumulated in the PSD-containing LP1 fraction we also assayed for Shank1 protein content in the S2 fraction which excluded the PSD. Our data indicated that the content of Shank1 protein in the ipsilateral S2 fraction of the spinal dorsal horn of ligated animals was significantly lower when compared to controls, 81±7%, $F(4,25)=3.7$, $p<0.05$ (Fig. 1). In contrast, there were no changes in Shank1 protein levels in the S2 fraction of the contralateral dorsal horn in ligated animals (91±3%), or either side of the dorsal horn in sham-operated animals (91±2% and 95±3%). These data suggested a preferential accumulation of Shank1 in the ipsilateral PSD of ligated animals.

3.2. Signs of pain behavior accompanied the sciatic ligation and the accumulation of Shank1 in the PSD

Before the animals were euthanized and their tissues collected for the immunoblotting experiments, they were behaviorally tested to examine whether they exhibited signs of pain behavior.

Ligated animals placed less weight on their injured, ipsilateral limb 4h post-ligation as their ipsilateral to contralateral ratio was reduced to 0.58±0.02 (Fig. 2). In contrast, neither uninjured control, nor sham-operated animals showed this shift in weight distribution suggestive of pain behavior (0.98±0.02 and 0.91±0.02, respectively). ANOVA indicated a significant difference among groups, $F(2,15)=87.0$, $p<0.001$. Scheffe's post-hoc test confirmed that the difference was due to the weight-bearing ratio in ligated animals.

In the same ligated animals there was also a significant reduction in the 4h post-ligation withdrawal latency (second test latency) of the injured, ipsilateral limb (5.8±0.5s) when compared to the pre-surgery baseline (7.8±0.3s, Fig. 2). In contrast, there was no significant reduction in the second test withdrawal latency in either control or sham-operated animals (7.5 ±0.4s vs. 7.7±0.1s, and 7.8±0.4s vs. 7.1±0.2s, respectively). ANOVA confirmed no difference

among groups at baseline, F (2,15)=0.2, p<1, but a significant difference at 4h post-ligation, F(2,15)=13.6, p<0.01 which was due to the second test latency in ligated animals.

3.3. The sciatic ligation was not associated with an early change in Shank1 message

In these experiments we sought to establish whether the ligation-associated increase in Shank1 protein in the PSD was a reflection of an injury-elicited activation of the Shank1 gene.

Shank1 mRNA levels in the ipsilateral spinal dorsal horn of ligated animals were comparable to those of control animals, 1.2±0.1-fold (Fig. 3). Similarly, the Shank1 mRNA levels in the contralateral dorsal horn of ligated animals, or either side of the spinal dorsal horn in sham-operated animals were not different from controls. Statistical analysis confirmed that there were no significant differences among the groups, F(4,35)=2.2, p<0.1.

3.4. Intrathecal pre-treatment with either anisomycin or Shank1 siRNA prevented the ligation-associated increases in Shank1 protein in the PSD

Given that the sciatic ligation was not associated with activation of the Shank1 gene we hypothesized that at least some of the increases in Shank1 protein may have been a result of synthesis from existing Shank1 mRNA. We thus injected intrathecally the protein synthesis inhibitor anisomycin to assess its effects on the ligation-associated accumulation of Shank1 in the PSD.

In vehicle-treated ligated animals there was a substantial increase in Shank1 protein in the LP1 fraction of the ipsilateral spinal dorsal horn, 150±5% (Fig. 4). The increase was comparable to that seen previously (Fig. 1). Pre-treatment with anisomycin prevented this increase, 111 ±3%, F(2,15)=79.4, p<0.001, suggesting that at least some of the Shank1 accumulation resulted from existing message translation.

To further explore this possibility we injected intrathecally Shank1 siRNA in an attempt to silence the existing Shank1 message. In ligated animals pre-treated with non-target siRNA we detected the typical injury-associated accumulation of Shank1 protein in the ipsilateral LP1 fraction 4h post-ligation, 157±8% (Fig. 4). Pre-treatment with Shank1 siRNA abolished this increase, 115±3%, F(2,15)=39.9, p<0.001. Taken together these data suggested that at least some of the ligation-associated accumulation of Shank1 protein in the PSD was due to protein synthesis from existing Shank1 message.

3.5. A pre-emptive single intrathecal injection of either anisomycin or Shank1 siRNA alleviated early signs of pain behavior

Before euthanasia and tissue collection all animals were behaviorally tested to examine whether they exhibited early signs of pain behavior.

Anisomycin pre-treatment prevented a shift in weight-bearing behavior 4h post-ligation in ligated animals (0.84±0.03). This was significantly different from the behavior of vehicle-treated ligated animals (0.58±0.02, p<0.001) and was suggestive of pain relief (Fig. 5). The same anisomycin-treated ligated animals also did not exhibit thermal hyperalgesia 4h post-ligation (8.5±0.3s vs. 8.6±0.2s at baseline, Fig. 5). This was again significantly different from the behavior of the vehicle-treated ligated animals which exhibited thermal hyperalgesia (7.9 ±0.5s vs. 5.5±0.3s, p<0.05).

Pre-treatment with Shank1 siRNA similarly prevented both the ligation-associated shift in weight-bearing behavior (0.91±0.04) and the reduction in second test latency (8.9±0.6s vs. 9.3 ±0.6s at baseline, Fig. 5). In contrast, pre-treatment with non-target siRNA failed to modify

either the decreases in weight distribution ratio (0.67±0.3, $p<0.001$) or the second test latency (6.5±0.3 vs. 8.8±0.1s at baseline, $p<0.005$).

Neither the weight-bearing behavior nor the paw withdrawal latencies of control or sham-operated animals were modified by pre-treatment with vehicle, anisomycin, non-target siRNA or Shank1 siRNA (Fig. 5).

These results suggested that application of either anisomycin or Shank1 siRNA at a dose sufficient to prevent the ligation-elicited accumulation of Shank1 protein in the PSD of spinal dorsal horn neurons effectively relieved both of the early, injury-associated signs of pain behavior.

3.6. MK-801 and U0126 pre-treatment prevented the ligation-associated accumulation of Shank1 protein and the early pain behavior

In these experiments we sought to determine whether blockade of NMDA receptors with MK-801 or ERK1/2 activity with the MEK inhibitor U0126 would modify the changes in Shank1 protein levels. There is a well-established association between injury-elicited plasticity in the spinal dorsal horn and the activation of NMDA receptors and ERK1/2.

The injury-elicited accumulation of Shank1 protein in the ipsilateral LP1 fraction was significantly attenuated by pre-treatment with either MK-801 (114±5%) or U0126 (124±6%), $F(3,21)=23.1$, $p<0.001$ (Fig. 6).

The same pre-treatment with either MK-801 or U0126 effectively prevented the typical ligation-associated weight-bearing pain behavior in vehicle-treated animals, $F(8,45)=20.2$, $p<0.001$. Similarly, the ligation-associated thermal hyperalgesia was abolished by both MK-801 and U0126, $F(8,45)=9.7$, $p<0.005$. Neither MK-801 nor U0126 modified the weight-bearing behavior or the second test latency of control or sham-operated animals (Fig. 7).

3.7 Shank1 protein was linked to PSD-95 and NR2B in the ipsilateral PSD of spinal dorsal horn neurons in ligated animals

In an effort to further localize the ligation-associated changes in Shank1 protein to the PSD we sought to detect protein-protein interaction between Shank1 and the well-established PSD marker protein PSD-95 as well as the NR2B subunit of the NMDA receptor. We immunoprecipitated Shank1 in the ipsilateral PSD of the dorsal horn of ligated animals and then probed these samples with antibodies directed against PSD-95 or NR2B. Our results confirmed a protein-protein link between Shank1 and PSD-95 or NR2B in the ipsilateral LP1 fraction of ligated animals (Fig. 8). These data provided further support for the notion that the ligation-associated accumulation of Shank1 protein is localized to the PSD.

We previously reported that there was a significant increase in Shank1 protein in the ipsilateral LP1 fraction of spinal dorsal horn neurons 7 days after loose ligation of the sciatic nerve [13]. This is the time of the full expression of ligation-associated neuropathic pain behavior in our hands [13]. In order to assess whether the link between Shank1 and NMDA receptors is present at this time we similarly immunoprecipitated Shank1 in ipsilateral LP1 fractions from the spinal dorsal horn of animals 7 days post-ligation. Our results confirmed the link between Shank1 and PSD-95 and NR2B in the PSD at the time when neuropathic pain is fully developed (Fig. 8).

4. Discussion

In this study we examined the early consequences of the loose ligation of the sciatic nerve on the levels of Shank1 protein in the PSD of spinal dorsal horn neurons. Our data established

that there was an accumulation of Shank1 in the ipsilateral PSD of ligated animals exhibiting two signs of pain behavior, a shift in weight distribution and thermal hyperalgesia. There was no change in the overall level of Shank1 message in either ligated or sham-operated animals. The Shank1 accumulation was abolished by intrathecal pre-treatment with anisomycin or with Shank1 siRNA. The same pre-treatment eliminated both of the signs of pain behavior. Intrathecal pre-treatment with either MK-801 or U0126 similarly prevented the Shank1 accumulation and alleviated both early signs of pain behavior. The early accumulation of Shank1 in the PSD of dorsal horn neurons may be an important step in the injury-associated plasticity that leads to pain behavior. To our knowledge, this is the first demonstration of a nerve injury-associated increase in Shank1 protein levels in the PSD of spinal dorsal horn neurons which can be prevented to eliminate early signs of pain behavior.

We appreciate that while we have used a model of neuropathic pain in this study we only focused on the early consequences of the sciatic ligation. The pain behavior cannot thus be characterized as chronic, and ‘sub-chronic’ [15] is probably a more appropriate description. Nevertheless, even only a few hours after the sciatic ligation there seem to be important changes in nociceptive processing occurring in the spinal dorsal horn. This early spinal plasticity may then underlie at least to some extent the observable early pain behavior. It is of interest in this respect that in our hands at 4h post-surgery (but not before) the presence of pain behavior in ligated animals can be differentiated from the lack of pain behavior in sham-operated animals.

Several studies have confirmed the involvement of Shank in synapse development and plasticity in the brain. For example, increased levels of Shank elicited earlier synapse maturation and an increased size of dendritic spines in the hippocampus [20]. Shank3 over-expression was essential for the maintenance of spines and synapses in hippocampal cultures and was sufficient to induce the generation of spines in aspiny neurons in the cerebellum [19]. Patients with Shank3 gene mutations suffered severe mental retardation, delayed speech and autism [5]. Shank1 deficient mice exhibited smaller dendritic spines and impaired long term memory [8].

Little is known about Shank function in the spinal dorsal horn. We are aware of only one other study in which the role of Shank1 was examined [6]. These authors reported that Shank1 co-immunoprecipitated with PSD-95, NR2B and Src and concluded that a coupling between NMDA and metabotropic receptor cascades was necessary for the initiation of another type of persistent pain, Freund's adjuvant-elicited inflammatory hyperalgesia.

Shank1 may similarly contribute to pain behavior following peripheral nerve injury. The sciatic ligation may engender or enhance the pairing of NMDA and metabotropic glutamate receptor families through a link between Shank1 and Homer1b/c [4]. As we stated previously [13], this link would allow for increased coordinated activation of all the PSD molecules associated with the two glutamatergic receptor complexes even if only one of these complexes were activated. In other words, a low intensity peripheral stimulus that perhaps only activated metabotropic glutamate receptors could now influence NMDA-linked molecules even if the NMDA receptor remained inactive due to an insufficient degree of membrane depolarization because of the low intensity stimulus. Behaviorally, this may then be reflected in an exaggerated response to a peripheral stimulus, in other words allodynia and hyperalgesia.

More generally, however, Shank proteins may serve as “master scaffold” proteins [22] as they create a large and dense platform in the PSD by self-association via the SAM-domain. This platform forms a backbone to which multiple other protein complexes in the PSD are linked through the many protein interacting domains contained within the Shank structure. These complexes include other scaffolding proteins such as Homer and PSD-95, ionotropic and metabotropic glutamate receptors and associated signaling pathways, and cytoskeletal

components such as F-actin. Strong synaptic activity is accompanied by major changes in the PSD matrix, and it is clear now that dynamic regulation of the various constituents of the PSD plays an important role in the establishment of greater synaptic efficacy through activity-dependent changes in post-synaptic structure and function. Shank proteins may play a central role in this process [9,22].

This central role of Shanks is perhaps underscored by the presence of Shank1 mRNA in dendrites. This makes Shank one of a limited number of other PSD proteins with dendritically localized message. The dendritic transport of the Shank1 mRNA depends on a targeting element in its 3′ untranslated region, and the translation is probably initiated by a local stimulus [3].

Shank1 may play a similar central role in the spinal dorsal horn following peripheral nerve injury. We did not detect an overall change in Shank1 mRNA (Fig. 3) but we effectively prevented Shank1 accumulation in the PSD with an intrathecally delivered dose of anisomycin (125μg, Fig. 4) reported to block more than 90% of protein synthesis [18]. This suggests that perhaps some of the increase in Shank1 protein in the PSD was due to existing message translation. This notion is further supported by our data showing that Shank1 siRNA, but not non-target siRNA (Fig. 4), similarly prevented protein accumulation in the PSD (Fig. 4) again suggesting a block of the injury-associated translation due to silencing of the existing Shank1 mRNA.

Shank1 seems well-positioned to respond quickly to changes in afferent input to affect synapse re-organization within the locally affected dendritic region [3]. In our study, the increase in primary afferent activity due to the nerve injury may have elicited an activity-dependent accumulation of the Shank1 protein in affected synapses to set the stage for short and long-term enhancement of synaptic function. Like activity-dependent synaptic plasticity [11] persistent pain exhibits early and late phases of development [15,21]. Together with our previous results [13] the present data suggest that accumulation of Shank1 protein in the PSD may be an important component of both the early and the late phases. As such, Shank1 in particular, and the PSD of spinal dorsal horn neurons in general, may be suitable novel targets for successful therapeutic intervention in both prevention and treatment of injury-associated pain.

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References

1. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 1988;33:87–107. [PubMed: 2837713]
2. Boeckers TM, Kreutz MR, Winter C, Zuschratter W, Smalla KH, Sanmarti-Vila L, Wex H, Langnaese K, Bockmann J, Garner CC, Gundelfinger ED. Proline-rich synapse-associated protein-1/cortactin binding protein 1 (ProSAP1/CortBP1) is a PDZ-domain protein highly enriched in the postsynaptic density. J Neurosci 1999;15:6506–6518. [PubMed: 10414979]
3. Boeckers TM, Segger-Junius M, Iglauer P, Bockmann J, Gundelfinger ED, Kreutz MR, Richter D, Kindler S, Kreienkamp HJ. Differential expression and dendritic transcript localization of Shank family members: identification of a dendritic targeting element in the 3′ untranslated region of Shank1 mRNA. Mol Cell Neurosci 2004;26:182–190. [PubMed: 15121189]

4. de Bartolomeis A, Iasevoli F. The homer family and the signal transduction system at glutamatergic postsynaptic density: potential role in behavior and pharmacotherapy. Psychopharmacol Bull 2003;37:51–83. [PubMed: 14608240]
5. Durand CM, Betancur C, Boeckers TM, Bockmann J, Chaste P, Fauchereau F, Nygren G, Rastam M, Gillberg IC, Anckarsater H, Sponheim E, Goubran-Botros H, Delorme R, Chabane N, Mouren-Simeoni MC, de Mas P, Bieth E, Roge B, Heron D, Burglen L, Gillberg C, Leboyer M, Bourgeron T. Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. Nat Genet 2007;39:25–27. [PubMed: 17173049]
6. Guo W, Wei F, Zou S, Robbins MT, Sugiyo S, Ikeda T, Tu JC, Worley PF, Dubner R, Ren K. Group I metabotropic glutamate receptor NMDA receptor coupling and signaling cascade mediate spinal dorsal horn NMDA receptor 2B tyrosine phosphorylation associated with inflammatory hyperalgesia. J Neurosci 2004;24:9161–9173. [PubMed: 15483135]
7. Hargreaves K, Dubner R, Brown F, Flores C, Joris JA. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988;32:77–88. [PubMed: 3340425]
8. Hung AY, Futai K, Sala C, Valtschanoff JG, Ryu J, Woodworth MA, Kidd FL, Sung CC, Miyakawa T, Bear MF, Weinberg RJ, Sheng M. Smaller dendritic spines, weaker synaptic transmission, but enhanced spatial learning in mice lacking Shank1. J Neurosci 2008;28:1697–1708. [PubMed: 18272690]
9. Kreienkamp HJ. Scaffolding proteins at the postsynaptic density: Shank as the architectural framework. Handb Exp Pharmacol 2008;186:365–380. [PubMed: 18491060]
10. Lim S, Naisbitt S, Yoon J, Hwang JI, Suh PG, Sheng M, Kim E. Characterization of the Shank family of synaptic proteins. Multiple genes, alternative splicing, and differential expression in brain and development. J Biol Chem 1999;274:29510–29518. [PubMed: 10506216]
11. Malenka RC, Bear MF. LTP and LTD: an embarrassment of riches. Neuron 2004;44:5–21. [PubMed: 15450156]
12. Mestre C, Pelissier T, Fialip J, Wilcox G, Eschalier AJ. A method to perform direct transcutaneous intrathecal injection in rats. J Pharmacol Toxicol Methods 1994;32:197–200. [PubMed: 7881133]
13. Miletic G, Miyabe T, Gebhardt KJ, Miletic V. Increased levels of Homer1b/c and Shank1a in the post-synaptic density of spinal dorsal horn neurons are associated with neuropathic pain in rats. Neurosci Lett 2005;386:189–193. [PubMed: 16002212]
14. Miletic G, Miletic V. Loose ligation of the sciatic nerve is associated with TrkB receptor-dependent decreases in KCC2 protein levels in the ipsilateral spinal dorsal horn. Pain 2008;137:532–539. [PubMed: 18063479]
15. Millan M. The induction of pain: an integrative review. Prog Neurobiol 1999;57:1–164. [PubMed: 9987804]
16. Miyabe T, Miletic G, Miletic V. Loose ligation of the sciatic nerve in rats elicits transient up-regulation of Homer1a gene expression in the spinal dorsal horn. Neurosci Lett 2006;398:296–299. [PubMed: 16448751]
17. Naisbitt S, Kim E, Tu JC, Xiao B, Sala C, Valtschanoff J, Weinberg RJ, Worley PF, Sheng M. Shank, a novel family of postsynaptic density proteins that binds to the NMDA receptor/PSD-95/GKAP complex and cortactin. Neuron 1999;23:569–582. [PubMed: 10433268]
18. Rosenblum K, Meiri N, Dudai Y. Taste memory: the role of protein synthesis in gustatory cortex. Behav Neural Biol 1993;59:49–56. [PubMed: 8442732]
19. Roussignol G, Ango F, Romorini S, Tu JC, Sala C, Worley PF, Bockaert J, Fagni L. Shank expression is sufficient to induce functional dendritic spine synapses in aspiny neurons. J Neurosci 2005;25:3560–3570. [PubMed: 15814786]
20. Sala C, Piech V, Wilson NR, Passafaro M, Liu G, Sheng M. Regulation of dendritic spine morphology and synaptic function by Shank and Homer. Neuron 2001;31:115–130. [PubMed: 11498055]
21. Sandkuhler J. Models and mechanisms of hyperalgesia and allodynia. Prog Brain Res 2009;89:707–758.
22. Sheng M, Kim E. The Shank family of scaffold proteins. J Cell Science 2000;113:1851–1856. [PubMed: 10806096]
23. Valtschanoff JG, Weinberg RJ. Laminar organization of the NMDA receptor complex within the postsynaptic density. J Neurosci 2001;21:1211–1217. [PubMed: 11160391]

24. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16:109–110. [PubMed: 6877845]

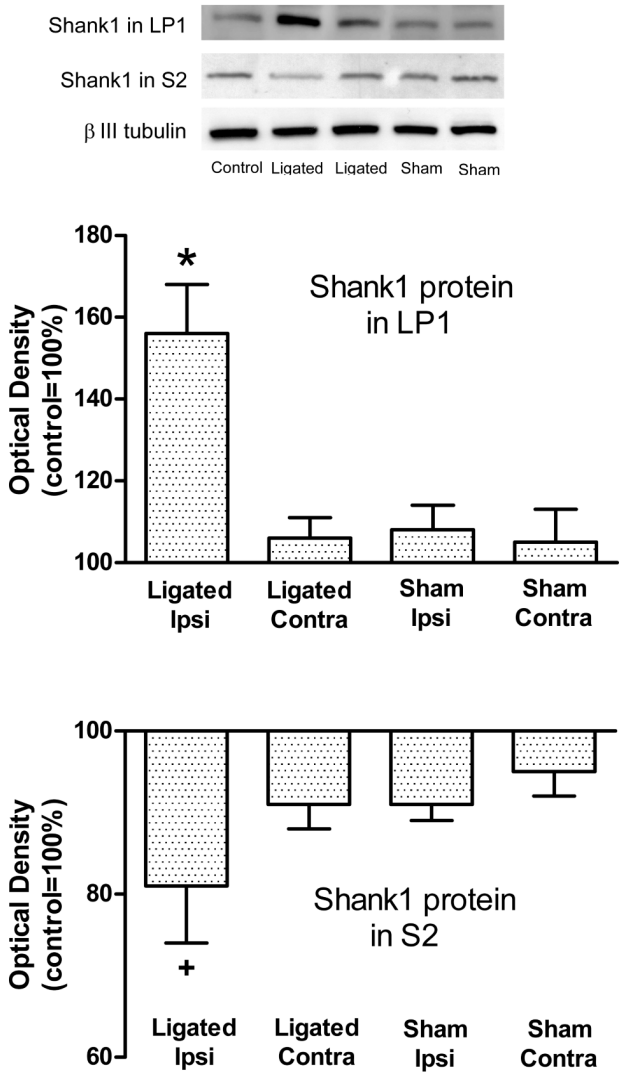

Figure 1. Accumulation of Shank1 protein in the ipsilateral PSD of spinal dorsal horn neurons was associated with loose ligation of the sciatic nerve

A: Immunoblots of Shank1 and beta III-tubulin (loading control) in representative LP1 and S2 fractions of the dorsal horn. **B:** Summary plot of the estimated content of Shank1. Note the increased levels of Shank1 in the ipsilateral PSD of ligated animals. In contrast, Shank1 levels in the contralateral PSD of ligated animals or either side in sham-operated animals were not different from controls. Also note the small but significant decrease in Shank1 protein in the ipsilateral S2 fraction of ligated animals. $^{+}p<0.05$, $*p<0.001$.

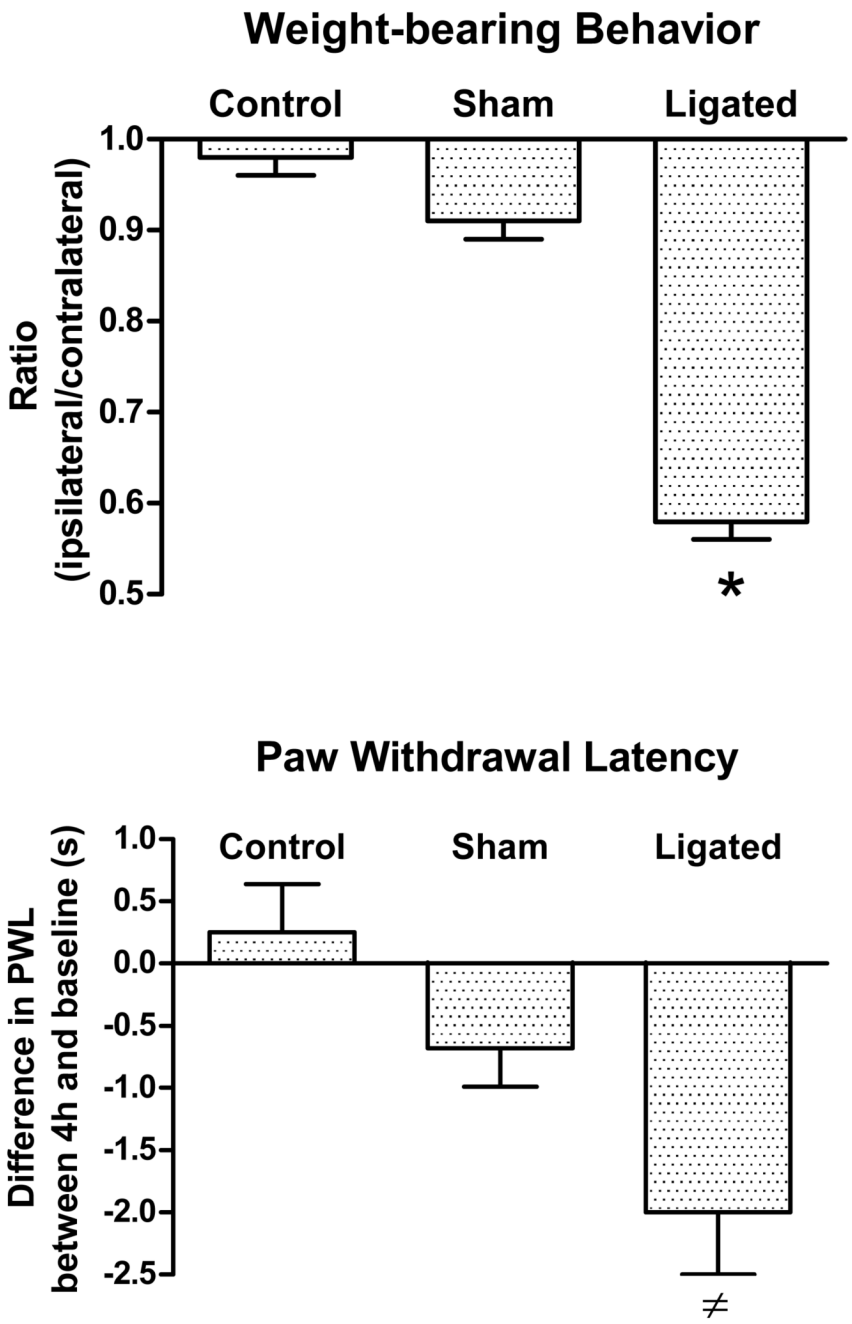

Figure 2. Early signs of pain behavior accompanied loose ligation of the sciatic nerve

Ligated animals exhibited both a shift in weight-bearing behavior and thermal hyperalgesia 4h post-ligation. Neither control, uninjured, nor sham-operated animals exhibited a reduction in the weight distribution ratio or in the second test latency suggesting an absence of pain behavior. ≠$p<0.01$, *$p<0.001$.

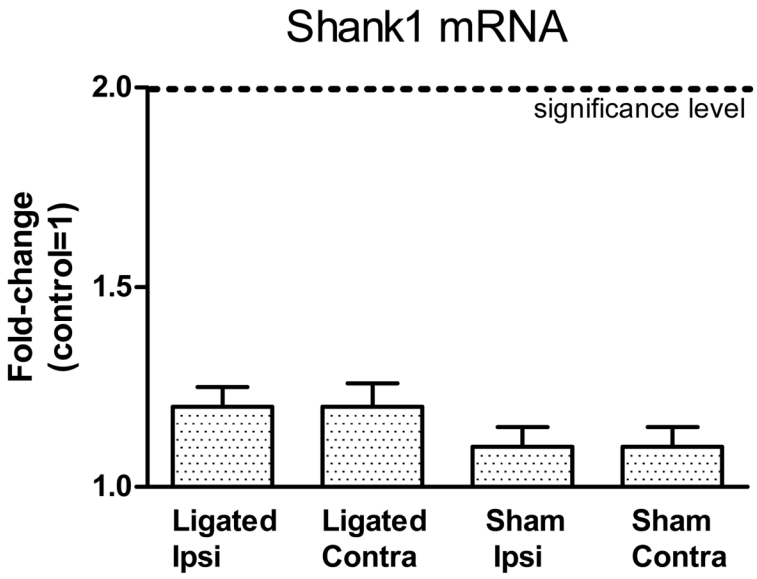

Figure 3. There were no early changes in Shank1 mRNA following loose ligation of the sciatic nerve
Note that there were no significant changes in Shank1 mRNA in either the ipsilateral or contralateral spinal dorsal horn of ligated or sham-operated animals.

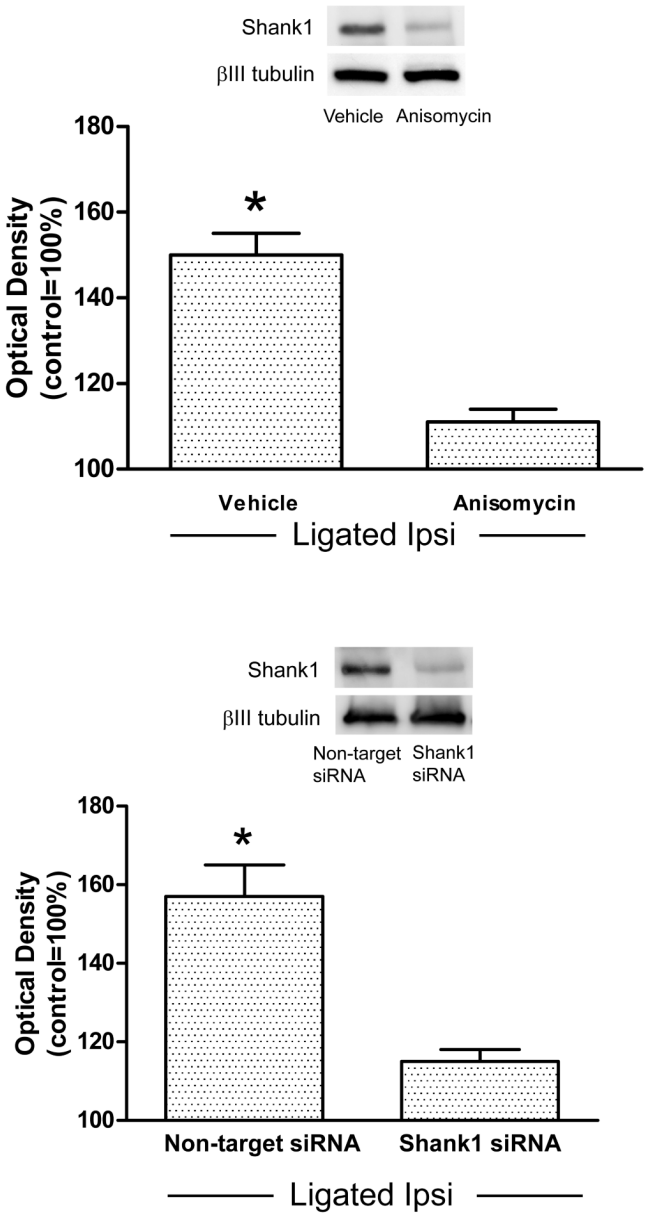

Figure 4. The ligation-associated accumulation of Shank1 protein was abolished by intrathecal pre-treatment with anisomycin and Shank1 siRNA but not non-target siRNA

The typical ligation-associated accumulation of Shank1 in vehicle-treated animals was prevented by pre-treatment with anisomycin (125μg). Similarly, Shank1 siRNA (1μM) but not non-target siRNA (1μM) prevented the Shank1 accumulation in the ipsilateral PSD of dorsal horn neurons. Representative immunoblots accompany each summary plot. *$p<0.001$.

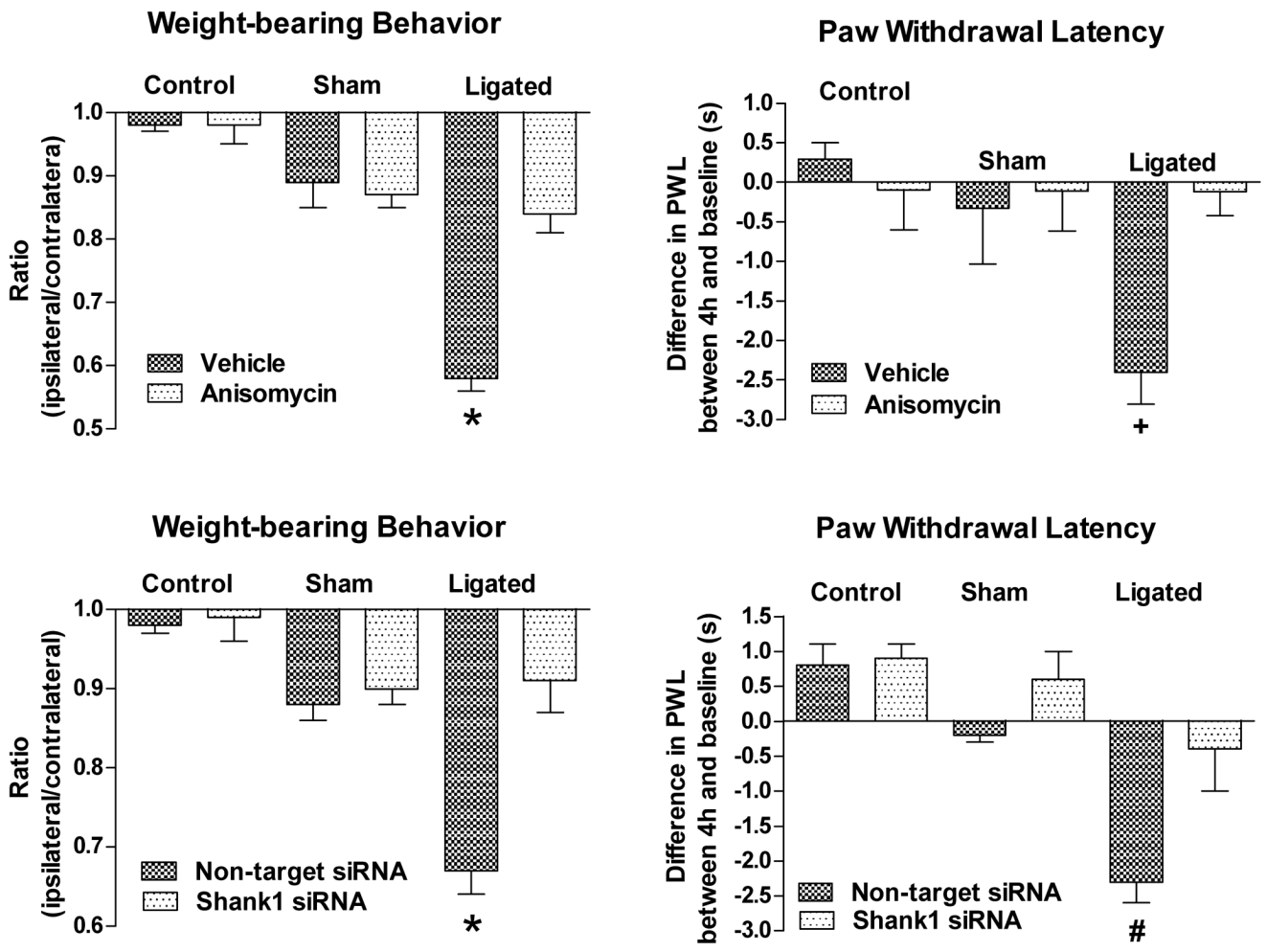

Figure 5. A pre-emptive single intrathecal injection of anisomycin or Shank1 siRNA, but not non-target siRNA, eliminated the early behavioral signs of pain

Pre-treatment with anisomycin or Shank1 siRNA prevented the injury-elicited shift in weight-bearing behavior and the reduction in the second test withdrawal latency 4h after loose ligation of the sciatic nerve. This suggested effective pain relief. Pre-treatment with non-target siRNA failed to prevent either the shift in weight-bearing distribution or the reduction in second test latency. None of the intrathecal injections modified significantly the weight distribution or the second test latency in control (uninjured) or sham-operated animals. $^{+}p<0.05$, #$p<0.005$, *$p<0.001$.

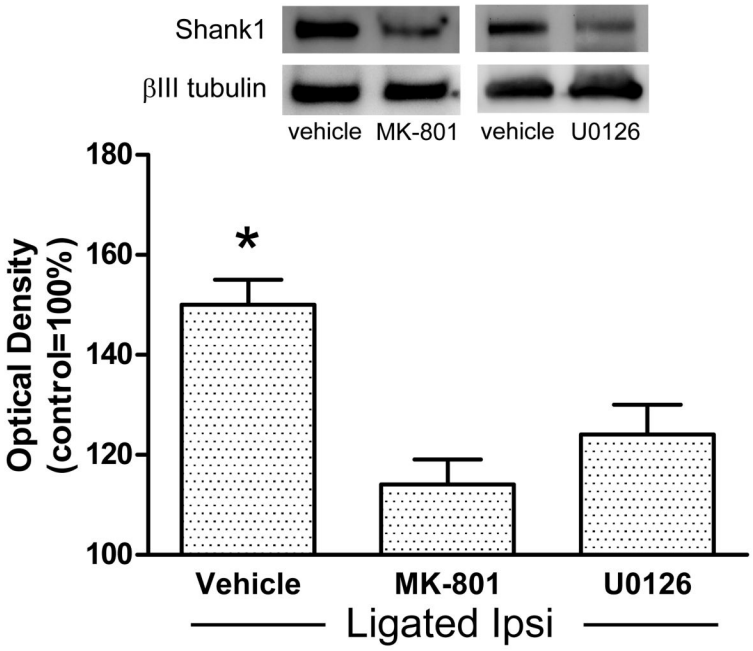

Figure 6. The ligation-associated accumulation of Shank1 was prevented by a block of NMDA receptors or by inhibition of ERK1/2 activation

The ligation-associated accumulation of Shank1 was prevented by pre-treatment with either MK-801 or U0126. Representative immunoblots accompany the summary plot. *p<0.001.

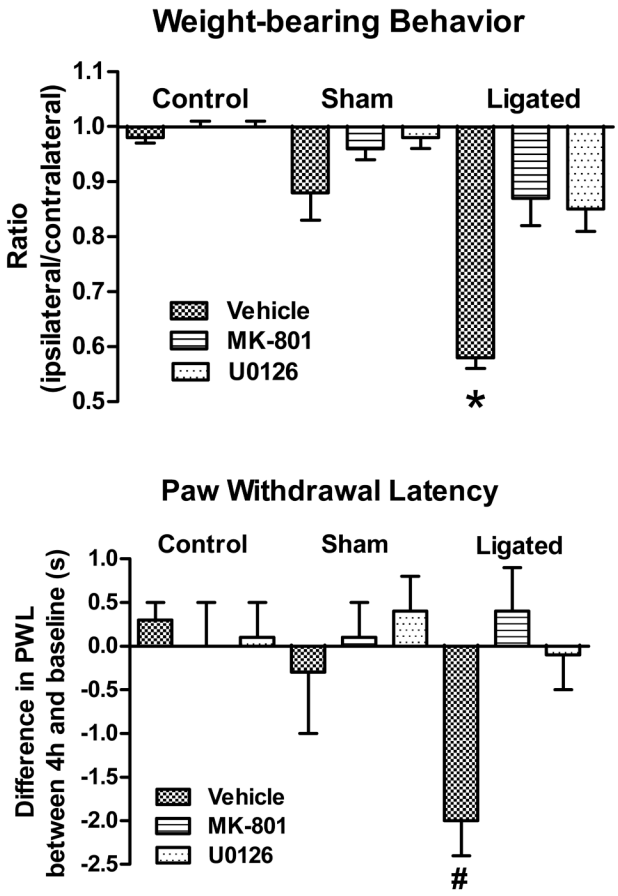

Figure 7. Early signs of pain behavior were alleviated by intrathecal pre-treatment with either MK-801 or U0126

Intrathecal pre-treatment with either MK-801 or U0126 prevented both the ligation-associated significant reduction in weight-bearing ratio and the second test latency. Neither MK-801 nor U0126 significantly modified the weight distribution or paw withdrawal latency in either control or sham-operated animals. #$p<0.005$,*$p<0.001$.

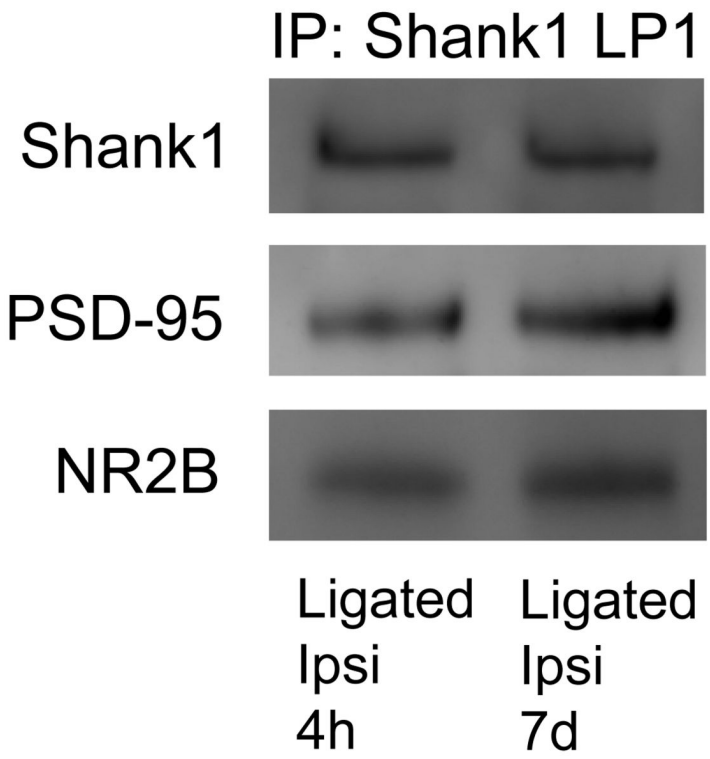

Figure 8. Shank1 protein was linked to PSD-95 and NR2B in the ipsilateral PSD of spinal dorsal horn neurons in ligated animals

A: Representative immunoblots of Shank1 (~218kDa), PSD-95 (~95kDa) and NR2B (~180kDa) in Shank1-immunoprecipitated LP1 fractions. Note the protein-protein link between Shank1 and PSD-95 or NR2B.