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Thrombospondin-4 contributes to spinal sensitization and neuropathic pain states

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Abstract

Neuropathic pain is a common cause of pain after nerve injury, but its molecular basis is poorly understood. In a post-genechip microarray effort to identify new target genes contributing to neuropathic pain development, we report here the characterization of a novel neuropathic pain contributor, thrombospondin-4 (TSP4), using a neuropathic pain model of spinal nerve ligation injury. TSP4 is mainly expressed in astrocytes and significantly upregulated in the injury side of dorsal spinal cord that correlates with the development of neuropathic pain states. TSP4 blockade either by intrathecal antibodies, antisense oligodeoxynucleotides, or inactivation of the TSP4 gene reverses or prevents behavioral hypersensitivities. Intrathecal injection of TSP4 protein into naïve rats is sufficient to enhance the frequency of excitatory postsynaptic currents in spinal dorsal horn neurons, suggesting an increased excitatory pre-synaptic input, and cause similar behavioral hypersensitivities. Together, these findings support that injury-induced spinal TSP4 may contribute to spinal pre-synaptic hypersensitivity and neuropathic pain states. Development of TSP4 antagonists has the therapeutic potential for target-specific neuropathic pain management.

Introduction

Neuropathic pain, or chronic pain derived from nerve injuries, is a multi-mechanism disorder affecting the quality of patients' daily lives adversely. Limited medications for neuropathic pain management are usually associated with unwanted side effects. The development of target specific and safe medications relies on our better understanding of neuropathic pain mechanisms. In an effort to identify novel target genes that play important roles in neuropathic pain processing, our group and others have used the genechip microarray approach to profile dysregulated genes in dorsal root ganglia and spinal cord of

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neuropathic pain models (Costigan et al., 2002; Wang et al., 2002; Valder et al., 2003; Yang et al., 2004; Luo and Figueroa, 2008; Kim et al., 2009). Since thrombospondin proteins have been reported recently to play a critical role in abnormal synaptogenesis (Christopherson et al., 2005; Xu et al., 2010), which may lead to spinal sensitization and pain states (Zeilhofer, 2005), we selected the gene encoding thrombospondin-4 (TSP4), among dysregulated genes, for further characterization to determine whether it is a novel player in neuropathic pain development.

Thrombospondins are a family of large oligomeric, extracellular matrix glycoproteins that mediate cell-cell, and cell-matrix interactions (Adams, 2001; Bornstein, 2001). There are five members in the thrombospondin supperfamily, TSP1–5, which can be subdivided into groups A (TSP1/2) and B (TSP3/4/5) with similar structure and functional domains and secreted as trimers or pentamers, respectively (Adams, 2001). Multiple TSPs may exist in developing tissues, but individual TSP is often expressed by non-overlapping cell populations, suggesting that these proteins may carry out similar functions in different tissues (Adams, 2001). As reviewed recently, TSP1/2 play roles in platelet aggregation, inflammatory response and angiogenesis regulation during tumor growth and wound repair, connective tissue extracellular matrix assembly. TSP5 is mainly expressed in cartilage and certain connective tissues and plays roles in chondrocyte differentiation, attachment and cartilage extracellular matrix assembly. While multiple sites of TSP3 and TSP4 expression are detected, the biology of TSP3 and TSP4 is not well studied (Adams and Lawler, 2004). Notably, TSP4 is expressed and localized to synapses and neuromuscular junction at the adulthood (Arber and Caroni, 1995). Enhanced green fluorescent protein (eGFP) expression in transgenic mice under the control of the TSP4 promoter has confirmed that TSP4 is expressed in spinal dorsal horn glial cells in adult mice (images in NCBI website for the NINDS GENSAT BAC Transgenic Project. http://www.gensat.org/login.jsp).

In this study, we examined the hypothesis that injury-induced TSP4 induces spinal neuron sensitization and neuropathic pain states with an integrative approach involving behavioral pharmacology, biochemistry, and electrophysiology analyses in a rat neuropathic pain model and genetically modified mice.

Experimental Procedures and Materials

Animals

Male young adult Harlan Sprague Dawley rats (< 150 g) were from Harlan Industries (Indianapolis, IN). The eGFP labeled TSP4 expressing transgenic (TSP4-eGFP) mice were from Mutant Mouse Regional Resource Centers (Thbs4-EGFP, MMRRC, supported by NCRR-NIH). TSP4 gene knock-out mice were from The Jackson Laboratory (B6.129P2-Thbs4^{tm1Dgen}/J, Bar Harbor, Maine). These mice were bred internally and only adult male mice were used for the experiments. All the genetically modified mice appeared normal with respect to grooming, social interactions, feeding, and showed no signs of abnormality or any obvious motor defects, tremor, seizure, or ataxia. All animals were exposed to a 12/12 hr day/night cycle with free access to food and water. All animal care and experiments were performed according to protocols approved by the Institutional Animal Care Committees of the University of California, Irvine and the Indiana University.

Intrathecal antisense oligodeoxynucleotides

We designed the antisense oligodeoxynucleotides based on a segment of the rat TSP4 gene (Genbank accession #: X89963) that has been used for producing primers in real-time PCR experiments (TaqMan Gene Expression Assay ID: Rn01494317, Applied Biosystems), and specific to the TSP4 mRNA. Mismatch oligodeoxynucleotides, which contain the same number of nucleotides but in a random order, were used as controls. The nucleotide

sequences for the antisense and mismatch oligodeoxynucleotides were CCATCATTGTTGCTATCTTCC and ACCATCGTTGTTACTTTCTCC, respectively. Modification of the oligodeoxynucleotides was described previously (Li et al., 2004; Boroujerdi et al., 2008; Kim et al., 2009).

Expression and purification of TSP4 proteins

293 EBNA cells (Invitrogen, Carlsbad, CA) stably transfected with the rat TSP4 cDNA (Genbank accession #: X89963) with a six-histidine (His) tag at the N-terminal were used for overexpression of the TSP4 proteins (Dunkle et al., 2007). The TSP4-His proteins were purified using a Ni-NTA column based on the manufacture's instructions (Invitrogene, Carlsbad, CA), concentrated with Amicon Ultra-4 Centrifugal Filter Unit (50K Molecular weight cut off, Millipore, Billerica, MA), aliquoted and stored at −80 °C until use.

Spinal nerve ligation

The surgical procedure of unilateral spinal nerve ligation (SNL) was performed as described (Kim and Chung, 1992). Briefly, under isoflurane anesthesia, the left L5/L6 spinal nerves of Harlan Sprague-Dawley rats or L4 spinal nerve of mice (Rigaud et al., 2008) were exposed and ligated with a silk suture distal to DRGs and proximal to their conjunction to form the sciatic nerve. Sham operations were performed in the same way except that spinal nerves were not ligated. Rats or mice are sacrificed at designated time after behavioral studies, DRG and spinal cord were isolated, and either processed immediately for biochemical studies or kept at −80 °C until use.

Intrathecal injection

Under light isoflurane anesthesia, L5/6 spinal lumbar region was located and saline, TSP4 proteins, His-tag peptides (GenScript, Piscataway, NJ), antisera, antisense or mismatch oligodeoxynucleotides in a total volume of 10 μL per rat were injected intrathecally between lumbar regions L5 and L6 through a 30-gauge needle connected to a microinjector (Tritech Research, Inc., Los Angeles, CA). All the drug treatments in SNL animals started when the animals had developed behavioral hypersensitivities (> one-week post SNL), except for the pre-emptive treatments, which started prior to the SNL procedures as indicated.

Behavioral test

Behavioral tests were performed blindly. For non-SNL experiments, the average values between left and right hindpaws were used for statistical analysis between test groups.

Von Frey filament test—Hindpaw sensitivities to von Frey filament stimulation were tested as described previously (Boroujerdi et al., 2008; Kim et al., 2009). Briefly, animals were allowed to acclimatize for at least 30 min in a clear plastic, wire mesh-bottomed cage. The 50% paw withdrawal thresholds (PWT) to von Frey filament (Stoelting Wood Dale) stimulation were determined using the up-down method of Dixon (Dixon, 1980). A series of filaments, starting with one that has a buckling weight of 2.0 g for rats and 0.41 g for mice, were applied consecutively to the plantar surface of the hindpaw with a force causing the filament to buckle. Paw lifting within 5 seconds indicated a positive response and prompted the use of the next weaker filament. Absence of a paw lifting response after 5 seconds prompted the use of the next filament with increasing weight. This paradigm continued until four more measurements had been made after the initial change in the behavioral response or until five consecutive negative (assigned a score of 15 g for rats, and 3 g for mice) or four consecutive positive (assigned a score of 0.25 g for rats, and 0.01 g for mice) responses had occurred. The resulting scores were used to calculate the 50% response threshold as described previously (Luo et al., 2001).

Hot box (Hargreaves) Test—Thermal hyperalgesia was measured as reduced hindpaw withdrawal latency (PWL) to thermal stimuli using a Hargreaves apparatus (Hargreaves et al., 1988). Free moving animals were acclimated individually for at least 15 minutes on the glass top of a hot box maintained at 30 ± 0.1 °C. A heat stimulus from a high intensity light bulb was applied to the hindpaw plantar surface through a small aperture below the glass surface. Paw withdrawal latency defined as the time between the application of heat stimulus and withdrawal of the targeted hindpaw was measured. A cutoff time of 20 seconds was set to prevent the sensitization and thermal injury of the skin. The averaged value of two readings per paw was calculated and used for statistical analysis.

Paw Pressure (Randall-Selitto) Test—Rats were first acclimated to human touch and holding positions for a week before the experiments. Mechanical hyperalgesia was measured with the Randall-Selitto Test using a Paw Pressure Analgesymeter (Ugo Basil North America) that applies an increasing force with a rate of 16 grams/second to the hindpaw between a flat surface and a blunt pointer (Randall and Selitto, 1957). Paw pressure withdrawal thresholds (PPT) were determined as the amount of force that induced a hindpaw withdrawal.

Motor function tests

To determine if locomotor functions were altered in rats post antisense/antibody treatments or in genetically modified mice, the Basso, Beattie, Bresnahan Locomotor Rating Scale for rats (BBB) (Basso et al., 1995) or the Basso Mouse Scale for Locomotion for mice (BMS) (Basso et al., 2006) were performed blindly. Briefly, animals were acclimated to their testing environment daily by handling and exposing them to the open field test apparatus for a week before testing. The locomotor function tests are based on a scale focused on consistent coordination between the front and hind limbs, such as paw position, toe clearance, trunk stability and tail position (Boroujerdi et al., 2011).

Western blots

Western blot analysis was used to examine levels of proteins of interest in DRG and dorsal spinal cord samples. After electrophoresis, sample proteins were transferred to nitrocellulose membranes electrophoretically. In most cases, membranes were cut into sections for probing with primary antibodies against different target proteins concurrently. After blocking non-specific binding sites with 5% low fat milk in PBS, the membranes were incubated for 1 hr at room temperature with primary antibodies against the proteins of interest followed by secondary antibodies conjugated with horseradish peroxidase. Primary antibodies included that against TSP1 (mouse, Neomarkers Inc., Fremont, CA, 1:400), TSP2 (mouse, BD Transduction Laboratories, Lexington, KY, 1:500), and TSP4 (guinea pig, 1:750). The specificity of the noncommercial guinea pig TSP4 antibody against rat recombinant full-length TSP4 has been characterized previously (Sodersten et al., 2006; Dunkle et al., 2007). Antibodies against β-actin (mouse, Novus Biologicals, LLC, Littleton, CO, 1:10,000) were used for loading control analysis. The antibody-protein complexes were visualized by chemiluminescent reagents. The band densities were quantified by either imaging quantification or densitometry within the linear range of the film sensitivity curve. Ratios of the band density for the protein of interest to that for β-actin was calculated within each sample first before cross-sample comparisons to calculate the percentage changes in the levels of proteins of interest in the experimental groups compared with that from the control groups. Variations in band densities of proteins of interest in the control groups (contralateral side) were determined by comparing each band density with the mean of the band densities from at least two different control samples run in the same Western blot after its ratios to β-actin band densities were calculated.

Fluorescent histochemistry

Spinal cord samples collected from two-week spinal nerve ligated TSP4-eGFP animals were immersion fixed in 4% paraformaldehyde (PFA) overnight followed by sedimentation in 30% sucrose PBS solution. Spinal cord samples frozen in optimal cutting tissue medium were sectioned (10μm). Sections on slides were incubated with primary antibodies diluted in Antibody Diluent Solution (DAKO) for 2 days, followed by incubation with respective secondary antibodies with different color emissions for 2 hrs at room temperature. After washing, slides were mounted with Vectashield DAPI Hardmount (Vector Labs). Primary antibodies against Iba-1 (Wako, 1:1000), myelin basic protein (MBP) (Abcam, 1:500), eGFP (Abcam, 1:500), glial fibrillary acidic protein (GFAP) (BD Biosci 1:500) and NF-70 (Millipore, 1:500) were used. For detecting GFAP or NF-70 immunoreactivities in spinal cord sections, antigen retrieval was determined necessary and performed by incubating the sections in heated sodium citrate buffer (pH 6.0) for five minutes using a pressure cooker before incubation with primary antibodies. Fluorescent images were taken with a Zeiss LSM 780 Two-Photon Laser Scanning Confocal Microscope. Optical sections were line averaged and collected at 0.3μm intervals. Stacks of seven consecutive optical sections with the best quality of immunoreactivities were then merged in Volocity (version 6.0, PerkinElmer) for data analysis. For each cell marker, staining was performed on three slices at least 100 μm apart from each of the four animals used.

For eGFP signal quantification, mirroring regions of interest spanning from the midline to both sides of the dorsal spinal cord were selected. eGFP fluorescence above the background level was selected for analysis using Volocity's "Find object using intensity" function. Data analyses were performed on six slices (minimum 50 μm intervals) per animal from four animals. Surface area and intensity of eGFP-expressing cells from the contralateral (uninjured) or ipsilateral (injured) sides of all four animals were then pooled respectively and compared. For quantifying the number of eGFP-expressing cells, eGFP-expressing cells with nuclei in the same regions of interest as described for surface area and intensity analyses were identified at a higher magnification and counted manually. Data from the injured or uninjured sides were pooled, respectively, and compared. For co-localization data, thresholded Pearson's correlation coefficient values as described by Barlow et al. (Barlow et al., 2010) were obtained using the Volocity colocalization function.

Electrophysiology on spinal cord slices

Spinal cord was collected by hydraulic extrusion from animals anesthetized with ketamine-HCl (1 mg/kg, i.p.). Transverse slices (300–350 μm) were cut from the lumbar enlargement using a vibratome (VT1000S; Leica, Nussloch, Germany) in an ice-cold (4 °C) sucrose solution containing (in mM): 230 sucrose, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 10 $MgSO_4$, 10 glucose, pH 7.4, 290–305 mOsm/L, equilibrated with 95% O_2 and 5% CO_2. The slices were maintained in an artificial cerebrospinal fluid (ACSF) containing (in mM): 130 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose, pH 7.4, 295–305 mOsm/L. The ACSF was continuously equilibrated with 95% O_2 and 5% CO_2, and slices were incubated for > 1 h before recording. Recording electrodes were prepared from borosilicate glass (World Precision Instruments) using a horizontal electrode puller (P-97; Sutter Instruments). Electrodes have resistances of 2–4 MΩ when filled with an intracellular solution containing (in mM): 92 $CsMeSO_4$, 43 CsCl, 1 $MgCl_2$, 2 EGTA, 5 TEA, 10 HEPES and 2 Mg-ATP, pH 7.4, 295–300 mOsm/L. Oxygenated ACSF was used as bath solution, and the flow rate was adjusted to 2–3 ml/min. Neurons in the superficial dorsal horn were visualized with an infrared-differential interference contrast (DIC) microscope (BX50WI; Olympus Optical, Tokyo, Japan) and a CCD camera.

To record α-amino-3-hydroxyl-5-methylisoxazole-4-propionic acid (AMPA) receptor-mediated miniature excitatory post-synaptic currents (mEPSC), bath application of tetrodotoxin (TTX, 1 μM, Sigma), strychnine (1 μM, Sigma), bicuculline (30 μM, Research Biochemicals), and APV (50 mM, Research Biochemicals) were used during recording to block TTX-sensitive Na^+, glycinergic, GABAergic and *N*-methyl-D-aspartate (NMDA) currents, respectively. All recordings were performed at 25 ± 1 °C with an Axopatch 200B amplifier (Molecular Devices). Membrane holding potential was –70 mV for measuring AMPA receptor-mediated mEPSCs. At this holding potential, over 90 percent of the mEPSCs from dorsal spinal cord neurons are mediated by AMPA receptors since they can be blocked by AMPA receptor antagonist, CNQX (Nguyen et al., 2009), and NMDA receptor-mediated currents are not activated due to voltage-dependent Mg^{2+} blockade (Shimoyama et al., 2000). NMDA receptor-mediated mEPSCs were not examined since they only represent less than 10 percent of the mEPSCs even at a depolarizing potential to remove the Mg^{2+} blockade (Nguyen et al., 2009). During whole-cell recordings, series resistance (8–15 MΩ) was monitored periodically, and cells with > 15% changes were excluded from the analysis. Signals were filtered at 2 kHz and digitized at a sampling rate of 5 kHz using a data-acquisition program (Axograph 4.6; Molecular Devices). To compare the frequency of mEPSCs between control and TSP4 treatment groups, the Kolmogorov–Smirnov test was used and percentage cumulative frequency distributions were constructed based on the average frequency of each individual cells.

Statistics

Student's *t* tests were performed for pair-wise comparisons and one-way or two-way ANOVA analyses with post-tests were performed for multi-group comparisons as indicated in figure legends. Significance was indicated by a two-tailed p value < 0.05.

Results

Peripheral nerve injury induced TSP4 upregulation in dorsal spinal cord that correlated with the development of behavioral hypersensitivities

We hypothesized that if TSP4 plays a critical role in neuropathic pain development after nerve injury, the development of neuropathic pain states should have a temporal correlation with injury-induced TSP4 dysregulation. Accordingly, we examined TSP4 expression levels in DRG and spinal cord from a rat neuropathic pain model derived from unilateral L5/6 spinal nerve ligation (SNL) (Kim and Chung, 1992) at designated time points correlated with different stages of behavioral hypersensitivity development. SNL, but not sham (data not shown), rats displayed behavioral hypersensitivities to different stimuli in the injury side compared with basal sensitivities in the contralateral side. The behavioral hypersensitivities included reduced paw withdrawal thresholds to von Frey filament stimulation (tactile allodynia), pressure stimulation (mechanical hyperalgesia), and reduced paw withdrawal latency to thermal stimulation (thermal hyperalgesia), all were followed by a gradual recovery (Fig. 1a–c). Western blot data indicated that TSP4 protein levels were increased in the injury side of L5/6 dorsal spinal cord at day two and 14 post injury, near the onset and correlating with behavioral hypersensitivities, respectively, but were similar to that in the non-injury side at 10 weeks (and 20 weeks, data not shown) post injury when the injured animals recovered from the hypersensitive states (Fig. 1d). In contrast, TSP4 protein levels in L5/6 dorsal root ganglia (DRG) were reduced in the injury side post SNL at all the time points measured, independent from the presence or absence of behavioral hypersensitivities (Fig. 2a). While the role of uninjured L4 DRG in the injury side to neuropathic pain processing is an active topic of discussion (Fukuoka et al., 2012), it has been reported that SNL also induces changes in uninjured L4 DRG at the injury side that could have contributed to pain processing (Schafers et al., 2003; Obata et al., 2004; Katsura et al., 2006;

Xu et al., 2007; Jeon et al., 2009; Shibasaki et al., 2010). Therefore, we also examined TSP4 protein levels in adjacent, uninjured L4 DRG at time points correlating with different developmental stages of neuropathic pain states. Our data indicated that TSP4 protein levels in adjacent, uninjured L4 DRG at the injury side were not altered significantly at all the time points examined (Fig. 2b). Since only TSP4 dysregulation in the injury side of dorsal spinal cord correlated temporarily with behavioral hypersensitivities, which may underlie a central mechanism of pain processing, we focused our subsequent investigation at the dorsal spinal cord level.

Blocking injury-induced spinal TSP4 upregulation reversed behavioral hypersensitivities in the neuropathic pain model

To determine if injury-induced TSP4 upregulation contributed to maintenance of behavioral hypersensitivities, we examined if blocking injury-induced TSP4 upregulation could reverse established neuropathic pain states. Intrathecal bolus injection of TSP4 antibodies (chicken polyclonal) into the lumbar L5/6 region two-weeks post injury could dose-dependently reverse established allodynia in the injury side (Fig. 3a). The effects of TSP4 antisera (80 μg/rat) in allodynia reversal had an onset time about four hours and a duration over six hours (Fig. 3b). Similar treatment also caused a reversal of thermal hyperalgesia (Fig. 3c) and mechanical hyperalgesia (Fig. 3d) in the SNL rats without affecting the behavioral sensitivity at the non-injury side. The specificity of the noncommercial chicken TSP4 antibody against recombinant rat full-length TSP4 has been characterized previously (Dunkle et al., 2007). Intrathecal injection of the guinea pig TSP4 antibody used in the biochemical studies also caused a similar reversal of behavioral hypersensitivity (data not shown). Neither antibodies against another protein, peripherin (chicken polyclonal), chicken IgY, nor heated TSP4 antibodies caused significant changes in behavioral sensitivity over the pre-treatment level (Fig. 3b–d). It is not clear why i.t. antibodies at the peak effect time point caused a complete reversal in thermal hyperalgesia (Fig. 3c) and mechanical hyperalgesia (Fig. 3d), which are mediated by high threshold nociceptors such as myelinated Aδ and non-myelinated C fibers, but a partial reversal in tactile allodynia (Fig. 3a, 3b), which are mediated by low threshold myelinated Aβ fibers. In addition, intrathecal injection for four days with TSP4 antisense (but not mismatch) oligodeoxynucleotides, at a dose (50 μg/rat/day) that is effective in knocking down other target gene mRNAs (Li et al., 2004; Boroujerdi et al., 2008; Kim et al., 2009), caused a time-dependent reversal of established allodynia (Fig. 3e), thermal hyperalgesia (Fig. 3f) and mechanical hyperalgesia (Fig. 3g) at the injury side. The antisense effects had an onset time of one-three days, peaked approximately three-five days after the initiation of the treatments and lasted over two-five days after the last injection depending on the modality tested. It seemed that thermal hyperalgesia was more sensitive to TSP4 antibody and antisense oligodeoxynucleotide treatments with an earlier onset time of reversal than tactile allodynia and mechanical hyperalgesia. Neither the antisense nor mismatch oligodeoxynucleotides altered behavioral thresholds at the non-injury side (Fig. 3e–g). Western blot data indicated that the antisense, but not mismatch, treatment blocked injury-induced dorsal spinal cord TSP4 upregulation (Fig. 3h). Neither the antibody nor the antisense oligodeoxynucleotide treatments affected locomotor functions significantly (Fig. 4a, 4b, respectievly). Together, these data support that injury-induced spinal TSP4 upregulation plays a critical role in the maintenance of neuropathic pain states.

Blocking injury induction of TSP4 prevented the development of behavioral hypersensitivities

To determine if TSP4 induction by SNL was required for initiation of neuropathic pain states, we treated SNL rats pre-emptively with daily intrathecal injection of TSP4 antisera (80 μg/rat/day) for eight days, started immediately before the SNL surgery, followed by

daily behavioral test before the injection. Pre-emptive TSP4 antibody treatment prevented allodynia onset at the injury side without altering the baseline thresholds significantly at the non-injury side. The TSP4 effects lasted for three days after the last injection (Fig. 5a). These data support that injury-induced TSP4 is critical in neuropathic pain initiation. To confirm this, we examined if allodynia was diminished or abolished in SNL TSP4 knockout (KO) mice compared with sex- and age-matched wild-type littermates (WT). We confirmed that TSP4 gene null expression did not affect the appearance and physical activity, including locomotor functions (Fig. 5b insert), of the mice as described (The Jackson Laboratory, B6.129P2-Thbs4^{tm1Dgen}/J), and there was no significant difference in the body weights between the adult WT and KO mice (WT: 27.1 ± 1.9g; KO: 26.9 ± 1.0g. Means ± SEM), nor their basal sensory sensitivity at the time of SNL surgeries. SNL induced allodynia in the injury side of WT, but not the TSP4 null, mice, nor at the non-injury side of WT and TSP4 null mice (Fig. 5b).

To determine if SNL might also cause dysregulation of other TSP proteins in dorsal spinal cord, which could have contributed to behavioral hypersensitivity, and have been affected by TSP4 null expression, we examined TSP1 and TSP2 protein levels in dorsal spinal cord of SNL WT and SNL TSP4 KO mice. Western blot data indicated that TSP1 and TSP2 protein levels in dorsal spinal cord were similar between injury and non-injury sides of WT and TSP4 KO mice (Fig. 6) when allodynia was evident in the injury side of SNL WT, but not TSP4 KO, mice (Fig. 5b). Thus, SNL in the TSP4 KO mice does not seem to induce a compensatory increase of other TSP proteins, nor SNL in WT mice induces dysregulation of other TSP proteins, which could have contributed to the development of abnormal sensations (data not shown). Together, these findings support that injury induction of TSP4 at the spinal level plays a critical role in the initiation of neuropathic pain states.

Dorsal spinal cord location of injury-induced TSP4

To determine the location of injury-induced spinal cord TSP4 upregulation, we take the advantage of a transgenic mouse line in which eGFP are expressed under the control of a TSP4 promoter so that TSP4 expressing cells can be identified by green fluorescence. Data from confocal microscopy indicated that SNL injury (two-weeks) led to increased eGFP fluorescence in the injury side of spinal cord, mainly at the dorsal spinal cord and dorsal column (Fig. 7a, 7b), which correlated with behavioral hypersensitivity in SNL animals (Fig. 5b). Summarized data indicated that SNL injury induced significant increases in the surface area (Fig. 7c) and intensity (Fig. 7d) of eGFP fluorescence in TSP4-eGFP expressing cells, but not the number of TSP4-eGFP expressing cells (Fig. 7e), in the spinal cord, suggesting that peripheral nerve injury causes TSP4 upregulation in TSP4 expressing cells, but not an increase in the number of TSP4 expressing cells. The sum of percentage increases from surface area and intensity of eGFP signals in TSP4-eGFP expressing cells post SNL injury was larger than the amplitude of TSP4 upregulation in dorsal spinal cord of SNL rats detected by Western blots (Fig. 1d), probably reflecting a signal amplification by eGFP fluorescence.

To determine the type(s) of TSP4 expressing cells in dorsal spinal cord, we performed fluorescent immunostaining on injured spinal cord sections with antibodies against different cell type markers, and examined the co-localization of cell-marker immunoreactivity with eGFP. Our data indicated that eGFP signals were detected mainly in cells immunoreactive to antibodies against GFAP, a marker for astrocytes (Fig. 8a), but not in cells immunoreactive to antibodies against NF-70, a marker for neurofilaments (Fig. 8b), Iba-1, a marker for microglia (Fig. 8c), nor myelin basic protein (Fig. 8d), a marker for myelin sheath of myelinated nerve fibers. This was supported by Pearson's Correlation Coefficient analysis (Barlow et al., 2010) for colocolization of cell maker fluorescence with eGFP fluorescence

from pooled data (1, a perfect positive correlation, −1, a perfect inverse correlation, 0, a random distribution) (Fig. 8e).

Increased spinal TSP4 protein alone was sufficient to induce behavioral hypersensitivities that correlated with dorsal horn neuron sensitization

To confirm a contributory role of TSP4 to the development of behavioral hypersensitivities without the influence from other injury factors that are present in the nerve injury model, we examined if increased spinal TSP4 alone is sufficient to induce behavioral hypersensitivity in naive rats. Intrathecal bolus injection of full-length recombinant rat TSP4 fusion proteins with His-tags (for easy purification) into L5/6 spinal level of naive rats, at a dose of 45 μg/rat, but not 20 μg/rat, resulted in the development of reversible tactile allodynia (Fig. 9a), thermal hyperalgesia (Fig. 9b) and mechanical hyperalgesia (Fig. 9c), similar to that seen in the SNL model (Fig. 1a–c). The behavioral hypersensitivities peaked about three-four days, and lasted for four-six days (Fig. 9a–c). Heat-inactivation of TSP4 abolished its pro-nociceptive effect (Fig. 9a). Similarly injected equal molar dose of the His-tag peptide did not cause behavioral hypersensitivities, nor alter baseline behavioral sensitivities (Fig. 9a–c).

Since thrombospondin proteins play a critical role in abnormal synaptogenesis (Christopherson et al., 2005; Xu et al., 2010), which could lead to spinal sensitization and pain states (Zeilhofer, 2005), we studied if increased spinal TSP4 caused behavioral hypersensitivity through a mechanism involving enhanced excitatory synaptic neurotransmission in the dorsal horn. AMPA-receptor mediated miniature excitatory postsynaptic currents (mEPSC) of dorsal horn neurons in spinal cord slices from TSP4 injected rats with peak behavioral hypersensitivities (four days post TSP4 injection) were examined. mEPSC in these neurons are induced by glutamate release from pre-synaptic terminals and changes in its frequency or amplitude reflect a pre-synaptic or post-synaptic mechanism, respectively (Nguyen et al., 2009; Brittain et al., 2011). Our data indicated that the average frequency (Fig. 9d, e_1) as well as cumulative frequency distributions (Fig. 9d, e_2), but not amplitude (Fig. e_3), of mEPSC in dorsal horn neurons from TSP4 treated rats were increased at this time point, compared with that from His-tag peptide injected control rats (Fig. 9d–e). However, acute bath application of recombinant TSP4 proteins to the spinal cord slices for 15 min at a concentration similar to the pro-nociceptive spinal TSP4 concentration at the first day of i.t. injection did not alter mEPSC frequency (nor amplitudes, data not shown) in L5 dorsal horn neurons (Fig. 9f-g). These data support that increased spinal TSP4 is sufficient to induce spinal neuron sensitization and behavioral hypersensitivity highly likely by enhancing pre-synaptic excitatory input through a chronic mechanism.

Discussion

Our results indicate that peripheral nerve injury induces TSP4 upregulation in dorsal spinal cord that correlates with initiation/maintenance of behavioral hypersensitivities mediated by different sensory fibers. Blocking injury-induced TSP4 with intrathecal antisense oligodeoxynucleotides or TSP4 gene knockout as well as blocking its activity with intrathecal antibodies prevent the onset and/or reverse established neuropathic pain states. Intrathecal injection of TSP4 proteins alone is sufficient to induce behavioral hypersensitivities that correlate with dorsal horn neuron sensitization through enhanced frequency of mEPSCs. Together, these findings support our hypothesis that injury-induced spinal TSP4 contributes to the initiation and maintenance of neuropathic pain states through a yet identified mechanism involving most likely enhanced pre-synaptic excitatory neurotransmitter release.

The detail mechanism underlying injury-induced spinal TSP4 in neuropathic pain processing is under active investigation. Our data support that TSP4 protein levels are not altered in uninjured, adjacent L4 DRG from the injury side of SNL rats with neuropathic pain states, which is consistent with the latest evaluation that L4 DRG is less likely contributing to neuropathic pain processing in this model (Fukuoka et al., 2012). Therefore, it is likely that dysregulated TSP4 in the injury segment plays a critical role in the development of neuropathic pain states. It is interesting that injury leads to an increase of TSP4 protein levels in the injured spinal cord segment, but a reduction in associated DRG, suggesting that peripheral nerve injury induces differential TSP4 regulations in the peripheral and central nervous systems, respectively, which may underlie distinct mechanisms in neuropathic pain processing.

Our data confirm that peripheral nerve injury induces TSP4 expression mainly in GFAP immunoreactive cells in the spinal cord. The profiles of eGFP/GFAP positive cells in the injury side closely resemble the morphology of immature astrocytes in dorsal spinal cord (Fig. 2G in Edwards et al., 1990). However, we cannot exclude the possibility that some of them are radial glial cells, an embryonic precursor of astrocytes (Edwards et al., 1990), which can also be detected by GFAP antibodies (Choi, 1988) (also see Pixley and de Vellis, 1984; Edwards et al., 1990). It is possible that peripheral nerve injury reactivates these cells that otherwise are mostly silent in adult stage of development. The presence of these embryonic astrocytes is known to correlate with the peak periods of neurogenesis (Edwards et al., 1990), suggesting a potential role of these cells in nerve regeneration after peripheral nerve injury.

TSP is considered a member of the endoneurial extracellular matrix molecules and TSP expression is increased dramatically along the path of regenerating axons post injury, it has been hypothesized that TSP may play a role in cell signaling during nerve regeneration post injury (Hoffman and O'Shea, 1999; Dubovy et al., 2002). Our data support that SNL-induced TSP4 expression is mainly distributed along fiber profiles in dorsal spinal cord and dorsal column of the injury side (Fig. 7a, b). Since both high threshold nociceptive fibers (Aδ and C fibers), which transmit painful signals, and low threshold sensory fibers (Aβ fibers), which transmit activity induced by innocuous stimuli, are sending their axons to the dorsal spinal cord at the segment of entry (Yaksh and Luo, 2007), enhanced TSP4 expression in immature astrocytes surrounding these fibers in dorsal spinal cord may play a critical role in mediating behavioral hypersensitivities such as thermal/mechanical hyperalgesia (by Aδ and C fibers), and tactile allodynia (by Aβ fibers). In addition, myelinated Aβ fibers are also sending axons collaterally into the dorsal column (Yaksh and Luo, 2007) and enhanced TSP4 expression in immature astrocytes surrounding these axons in the dorsal column may play a critical role in mediating tactile allodynia. The detail mechanism underlying peripheral nerve injury-induced TSP4 overexpression in mediating sensory information processing is under active investigation.

Recently, it has been reported that astrocyte-secreted TSP induces excitatory synaptogenesis in the central nervous system (Christopherson et al., 2005) through interactions with its neuronal receptor, the calcium channel alpha-2-delta-1 subunit proteins (Eroglu et al., 2009). In addition, trigeminal nerve injury is shown to induce astrocyte activation in the brain stem sensory trigeminal nuclei that leads to reactive synaptogenesis presumably involving thrombospondins (Lo et al., 2011). TSP1 is shown to increase the speed of synaptogenesis in young, but not mature, hippocampal neurons (Xu et al., 2010). Even though intrathecal injection of TSP1 proteins also induces behavioral hypersensitivity in rats (data not shown), our data indicated that TSP1/2 protein levels in dorsal spinal cord remain similar between SNL WT mice with allodynia and SNL TSP4 KO mice without allodynia (Fig. 5b),

suggesting that peripheral nerve injury induced behavioral hypersensitivity is not mediated by dysregulation of other TSP proteins.

It is possible that injury-induced TSP4 overexpression in immature astrocytes surrounding the injured sensory fibers provides a TSP4-rich perisynaptic environment, which may lead to abnormal excitatory synaptogenesis in the spinal cord. This plasticity can contribute to enhanced dorsal horn neuron sensitization, wider spreading of sensory signals, and evoked behavioral hypersensitivities. This is supported by our data showing that intrathecal TSP4 injection into naive rats can lead to sensitization of dorsal horn neurons through a mechanism most likely involving enhanced excitatory neurotransmitter release from pre-synaptic terminals, and behavioral hypersensitivities similar to that observed post peripheral nerve injury (Fig. 9a–e). Since acute bath application of TSP4 failed to elicit dorsal horn neuron sensitization (Fig. 9f–g), our data support that the pronociceptive effects of TSP4 in the injury model is likely mediated through a chronic mechanism, which may include, but not limited to, de novo TSP4 synthesis and/or altered excitatory synaptogenesis that requires days to complete (Christopherson et al., 2005). Our data supporting this conclusion include the slow onset of behavioral hypersensitivity after pre-emptive TSP4 antibody treatment in SNL rats (Fig. 5a), the slow onset and long-lasting effects of TSP4-induced behavioral hypersensitivity in naive animals (Fig. 9a–c), of TSP4 antisense reversal in behavioral hypersensitivities in SNL animals (Fig. 3e–f). Neutralizing TSP4 secreted from astrocytes in dorsal spinal cord post injury by antibody treatment (Fig. 3a–d, Fig. 5a) may present an alternative intervention that is faster in onset (hrs), but shorter in duration (hrs) in reversing neuropathic pain states.

In summary, our findings provide a large body of evidence to support that injury-induced TSP4 expression in presumably immature astrocytes in spinal cord plays a critical role in the initiation and maintenance of neuropathic pain states. Blocking the initiation of this pathological pathway may serve as a new approach in developing the next generation of target specific medications for neuropathic pain management.

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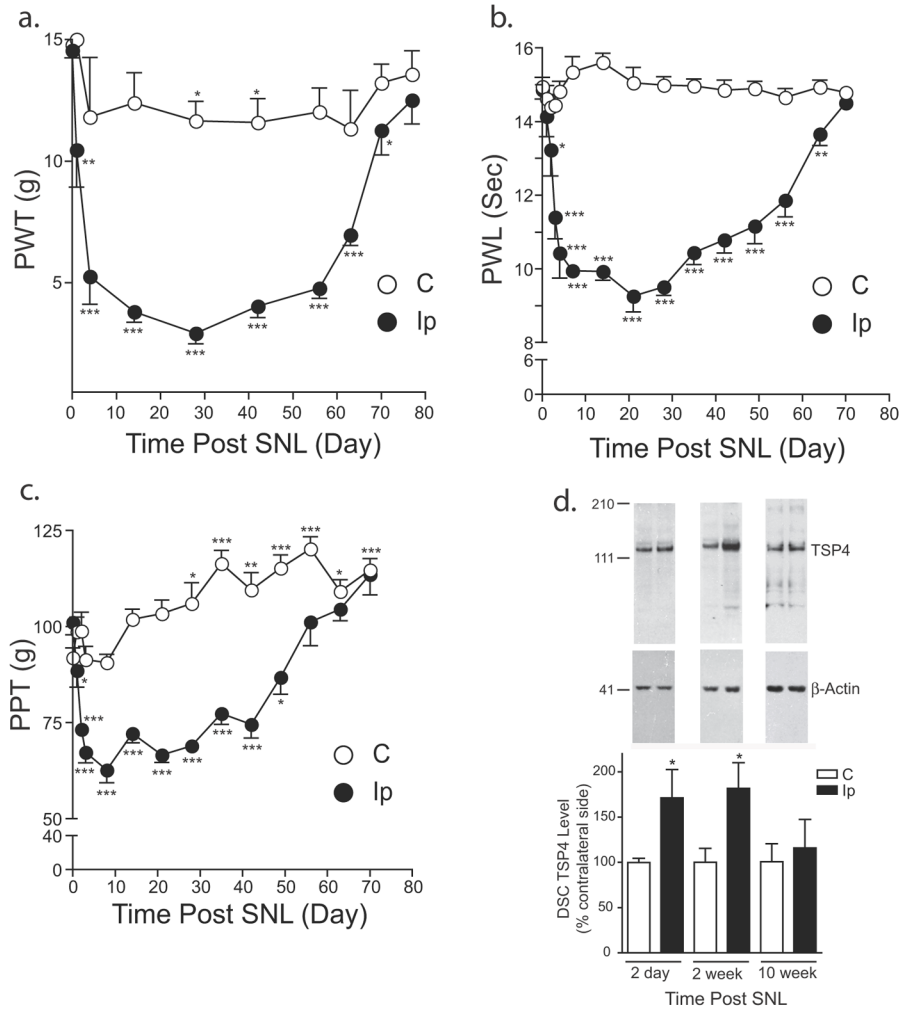

Figure 1. Nerve injury-induced TSP4 upregulation correlated temporally with the development of behavioral hypersensitivities

a–c. Unilateral L5/6 SNL injury caused a reduction in **(a)** hindpaw withdrawal thresholds (PWT) to light touch (tactile allodynia), **(b)** hindpaw withdrawal latency (PWL) to thermal stimulation (thermal hyperalgesia), and **(c)** hindpaw pressure withdrawal thresholds (PPT) (mechanical hyperalgesia) in the injury side compared to that in the non-injury side. In the paw pressure testing, there was a time-dependent conditioning adaptive increase in PPT in both groups. All behavioral hypersensitive states recovered gradually after approximately 7–10 weeks post ligation injury. Data presented are the means ± SEM from at least five rats in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the pre-treatment level as determined by Two-Way ANOVA analysis with Bonferroni Post-Tests. **d.** Western blot data indicated that L5/6 SNL injury caused TSP4 protein upregulation in dorsal spinal cord of the injury side at time points that correlated with the initiation and maintenance of behavioral hypersensitivities. The injury-induced TSP4 upregulation returned to a level similar to that in the uninjury side when injured animals recovered from the behavioral hypersensitive states. Representative Western blot bands are shown on top of each bar group and estimated molecular weights (Kd) are shown on the left. Data shown are the means ± SEM from five to six rats in each group.

*$p < 0.05$ compared with the contralateral side by Students' *t* test. C - contralateral, Ip - ipsilateral to SNL

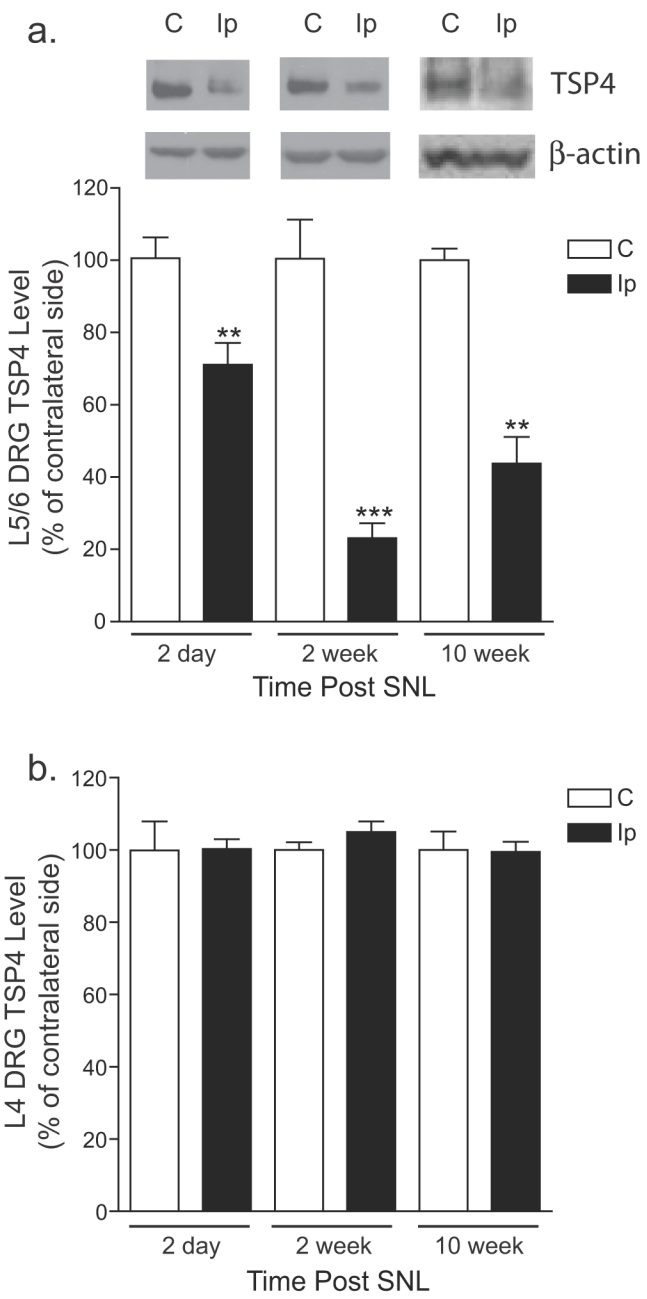

Fig. 2. Spinal nerve injury decreased TSP4 protein expression in injured, but not in adjacent uninjured, dorsal root ganglia

a. Western blot data indicated that L5/6 spinal nerve ligation injury caused down-regulation of TSP4 proteins in L5/6 DRG of the injury side at all the time points examined. Representative Western blot bands are shown on top of each bar group in the same order as the summarized bar graph data, which are the means ± SEM from at least nine rats in each group. **p < 0.01, ***p < 0.001 compared with contralateral side by Students' *t* test. **b.** In contrast, L5/6 spinal nerve ligation injury did not change TSP4 protein levels in uninjured, adjacent L4 DRG from the injury side. Data presented are the means ± SEM from nine rats in each group. C - contralateral, Ip - ipsilateral to SNL.

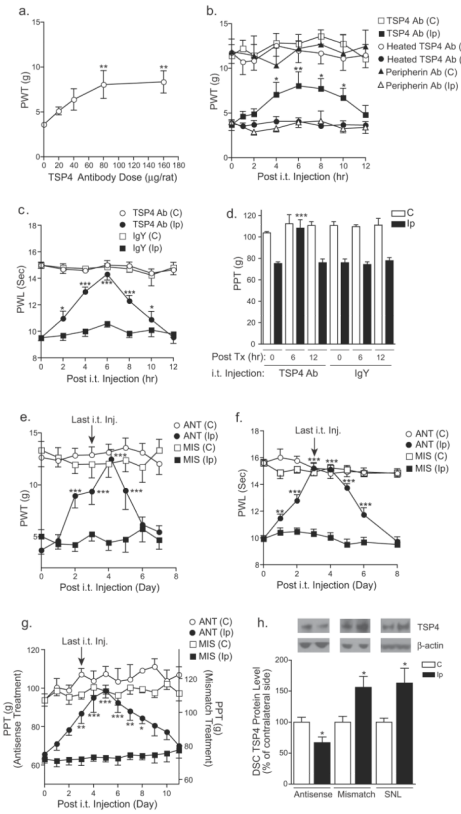

Figure 3. Blocking injury-induced spinal TSP4 resulted in reversal of behavioral hypersensitivities

a. Bolus i.t. injection of TSP4 antibodies dose-dependently reversed injury-induced tactile allodynia in SNL rats. Behavioral test on the injury side started six hrs after the bolus injection of TSP4 antibodies. Data shown are the means ± SEM from three to six rats in each group. ** $p < 0.01$ compared with the pre-treatment level as determined by One-Way ANOVA analysis with Dunnett's Multiple Comparison Tests.

b. Bolus i.t. injection of TSP4 antibodies (80 μg/rat) reversed injury-induced tactile allodynia in SNL rats without affecting the behavioral thresholds on the non-injury side. The anti-allodynic effect of TSP4 antibodies had an onset time of four hrs, peaked at six hrs, and a duration over six hrs. Neither heated TSP4 antibodies nor antibodies against another protein, peripherin, showed anti-allodynia effects. Data shown are the means ± SEM from six rats in each group. *$p < 0.05$, **$p < 0.01$ compared with the pre-treatment level as determined by Two-Way ANOVA analysis with Bonferroni Post-Tests. C –contralateral, Ip - ipsilateral to SNL.

c, d. Bolus i.t. injection of TSP4 antibodies (80 μg/rat) reversed injury-induced thermal hyperalgesia (**c**) and mechanical hyperalgesia (**d**) in SNL rats without affecting the behavioral thresholds on the non-injury side. The same dose of chicken IgY was used as control. To avoid sensitization of the hindpaw in paw pressure testing, mechanical hyperalgesia was tested in a six-hour interval as shown (**d**). Data shown are the means ± SEM from five to six rats in each group. *$p < 0.05$, ***$p < 0.001$ compared with the pre-treatment level as determined by Two-Way ANOVA analysis with Bonferroni Post-Tests. C - contralateral, Ip - ipsilateral to SNL.

e–g. Daily i.t. injection of TSP4 antisense (ANT), but not mismatch (MIS), oligodeoxynucleotides (50 μg/rat/day) for four days reversed injury-induced tactile allodynia **(e)**, thermal hyperalgesia **(f)** and mechanical hyperalgesia **(g)** in SNL rats.

Behavioral tests were performed before each daily injection, and daily after the last injection. Depending on the modality tested, the anti-nociceptive effect of TSP4 antisense oligodeoxynucleotides had an onset time from one to three days, peaked at three to five days, and lasted for over two to five days after the last injection. The baseline PPT value for the mismatch treatment in panel **g** (right Y axis) was higher than that in the antisense treatment due to the conditioning adaptive increase in PPT (as shown in Fig. 1c) in the same group of rats that was used for the antisense treatment first, followed by the mismatch treatment at least 48 hrs apart. Data shown are the means ± SEM from at least five rats in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the pre-treatment level as determined by Two-Way ANOVA analysis with Bonferroni Post-Tests. C – contralateral, Ip –ipsilateral to SNL.

h. Intrathecal TSP4 antisense treatment (50 μg/rat/day for 4 days) blocked injury-induced TSP4 expression in dorsal spinal cord. L5/6 dorsal spinal cord samples were collected one-day after the last oligodeoxynucleotide treatment from SNL rats, and subjected to Western blot analyses. Representative Western blot bands are shown on top of each bar group. Data shown are the means ± SEM from at least seven rats in each group. SNL –six-week SNL rats without treatment. C - contralateral, Ip - ipsilateral to SNL.

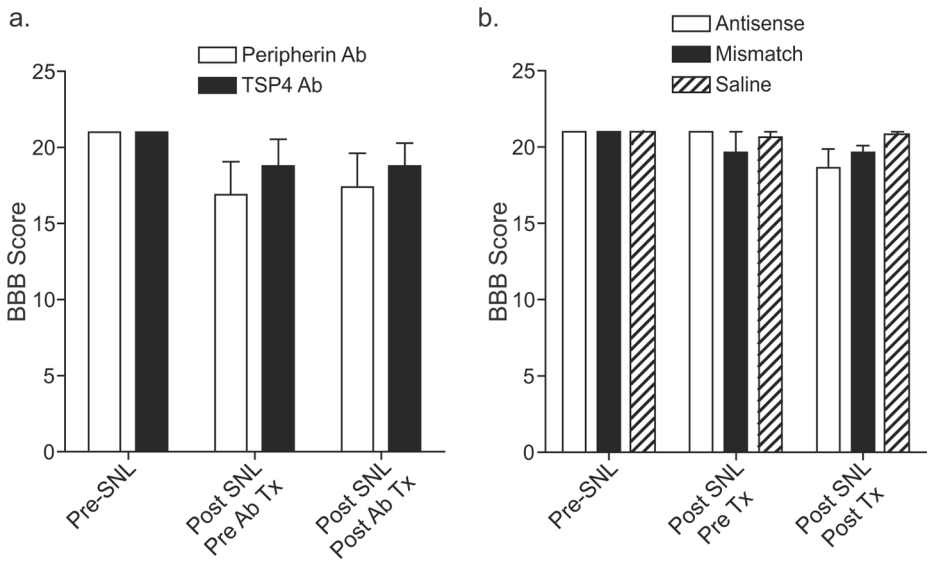

Fig. 4. Locomotor functions were not impaired by intrathecal treatments with TSP4 antibody or antisense oligodeoxyneucleotides

Locomotor functions were analyzed with the Basso, Beattie, Bresnahan (BBB) locomotor rating scale in SNL rats before and after SNL surgery and treatments with antibodies (**a**) or oligodeoxyneucleotides (**b**) as described. The post treatment time points were six hrs post bolus antibody injections (**a**) or overnight after the last i.t. injection of the four-day antisense treatments (**b**), both were correlated with reversals of behavioral hypersensitivities shown in Fig. 3b–d and 3e–g, respectively. Data presented are the means ± SEM from at least five rats in each group.

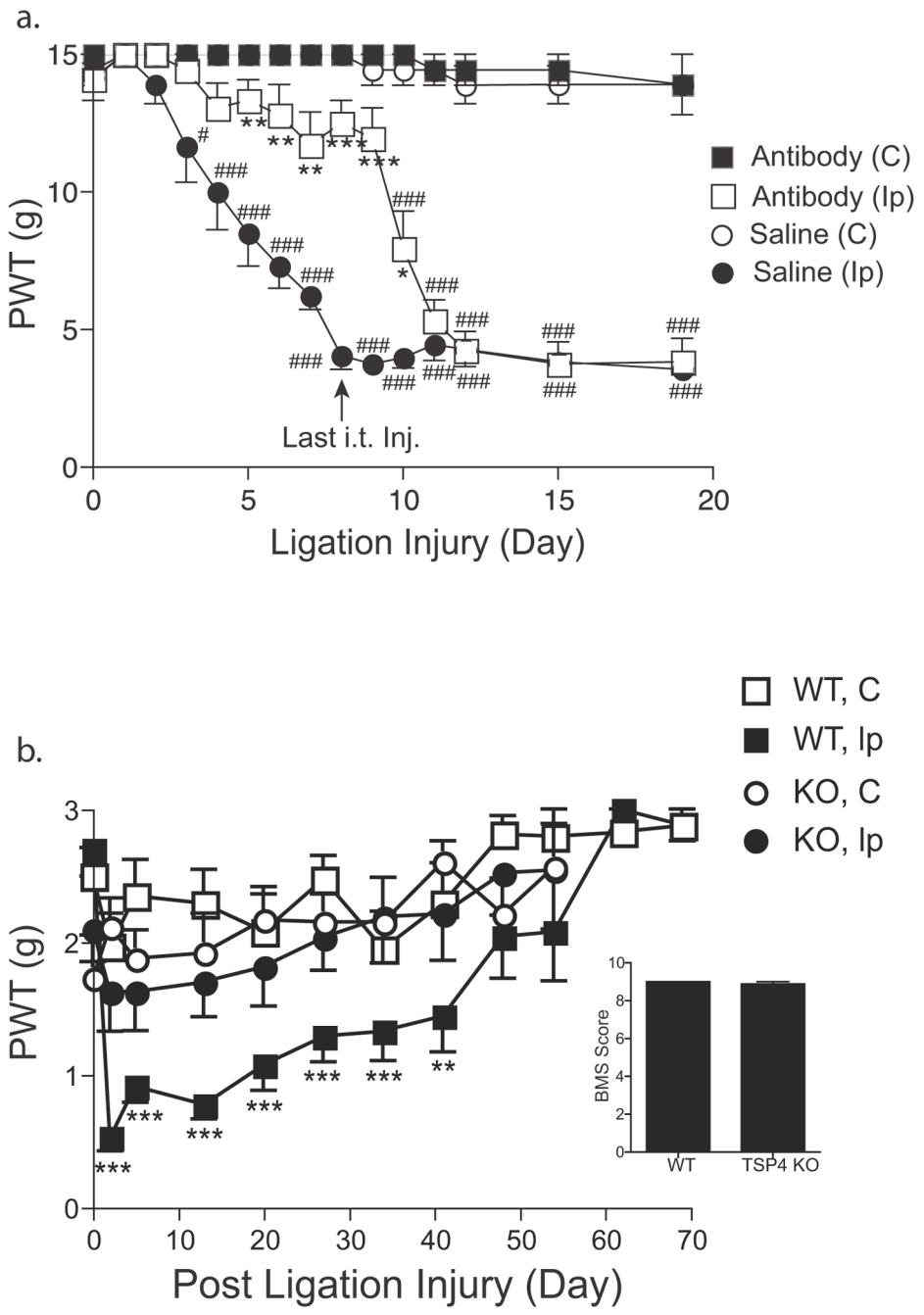

Figure 5. Blocking injury induction of spinal TSP4 prevented neuropathic allodynia initiation

a. Intrathecal pre-emptive treatment with TSP4 antibodies prevented allodynia onset in spinal nerve ligated rats in a reversible manner. TSP4 antibodies (80 μg/rat/day) were injected directly into L5/6 spinal region immediately before, and daily after SNL for 8 days. Behavioral tests were performed before and daily after SNL and prior to each daily injection. Data presented are the means ± SEM from 6 rats in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the saline injected group. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with the pre-treatment level using Two-way ANOVA analysis with Bonferroni Post-Tests. C - contralateral, Ip - ipsilateral to SNL.

b. TSP4 null mice had diminished tactile allodynia post SNL. Unilateral L5 SNL was performed in adult male TSP4 null mice (KO) and their age-, sex-matched wild type littermates (WT). Behavioral tests were performed blindly before and after SNL. Data presented are the means ± SEM from at least 7–12 mice in each group. TSP4 null expression did not impair the locomotor functions of the TSP4 KO mice compared with their WT littermates as analyzed with the Basso Mouse Scale for locomotion (BMS) from at least eight mice in each group (insert, means ± SEM). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with pre-SNL level using Two-Way ANOVA analysis with Bonferroni Post-Tests.

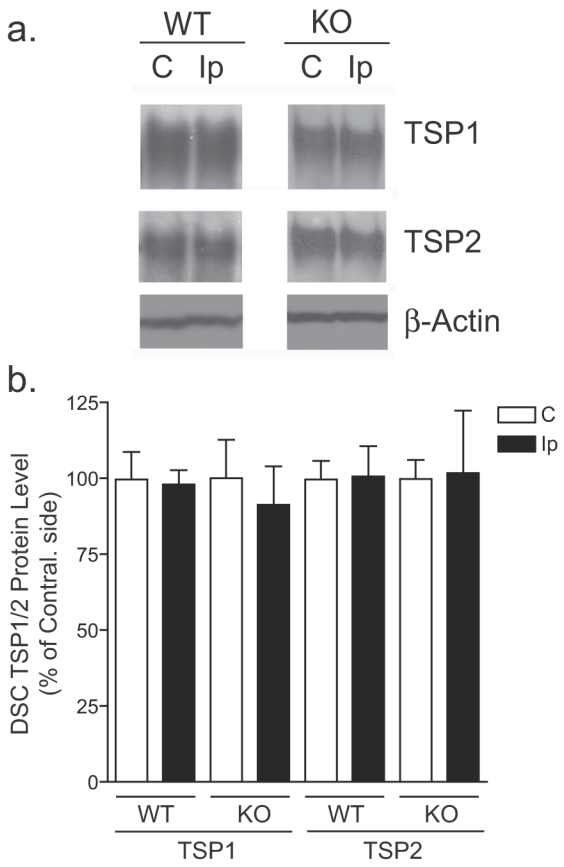

Fig. 6. SNL did not alter TSP1 and TSP2 protein levels in dorsal spinal cord of WT and TSP4 KO mice

Western blot data indicated that L5 SNL injury (four weeks) did not alter dorsal spinal cord TSP1/2 protein levels significantly in WT and TSP4 KO mice when allodynia was observed in SNL WT, but not SNL TSP4 KO mice as shown in Fig. 5b. **a.** Representative Western blot data. **b.** Summarized Western blot data presented as the means ± SEM from three to four independent experiments in each group.

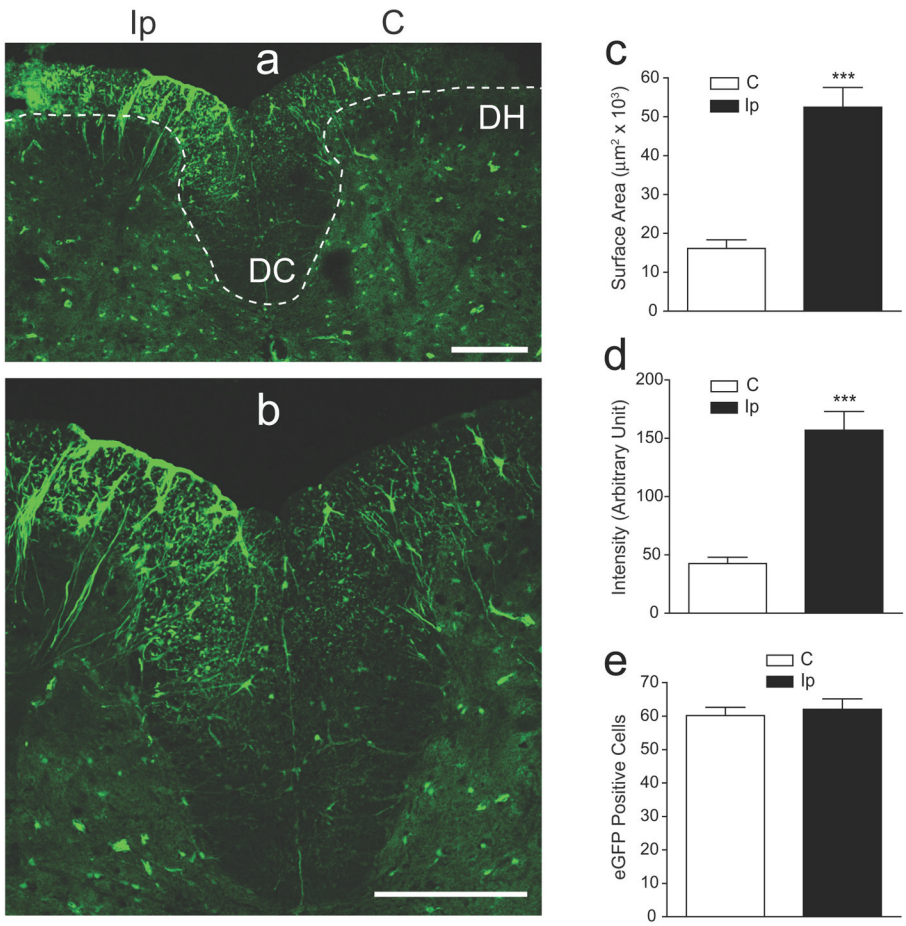

Figure 7. eGFP immunoreactivity in dorsal spinal cord of SNL TSP4-eGFP mice

a. Over view of a L5 dorsal spinal cord section from a two-week SNL TSP4-eGFP mouse (an representative image from four mice). The dot line outlines the dorsal horn (DH) and dorsal column (DC) areas in both sides. C - contralateral, Ip - ipsilateral to SNL. Scale bar: 200μm.

b. Enlarged view of dorsal spinal cord presented in panel (**a)** showing the structure of eGFP positive cells. Scale bar: 200 μm.

c–e. SNL injury induced significant increases in the surface area (**c**), and intensity (**d**) of eGFP signals in TSP4-eGFP expressing cells in dorsal spinal cord without altering the total number of eGFP expressing cells (**e**). Data presented are the means ± SEM from a total of 24 sections, six from each of the four SNL mice. ***$p < 0.001$ compared with contralateral side by Students' *t* test. C - contralateral, Ip - ipsilateral to SNL.

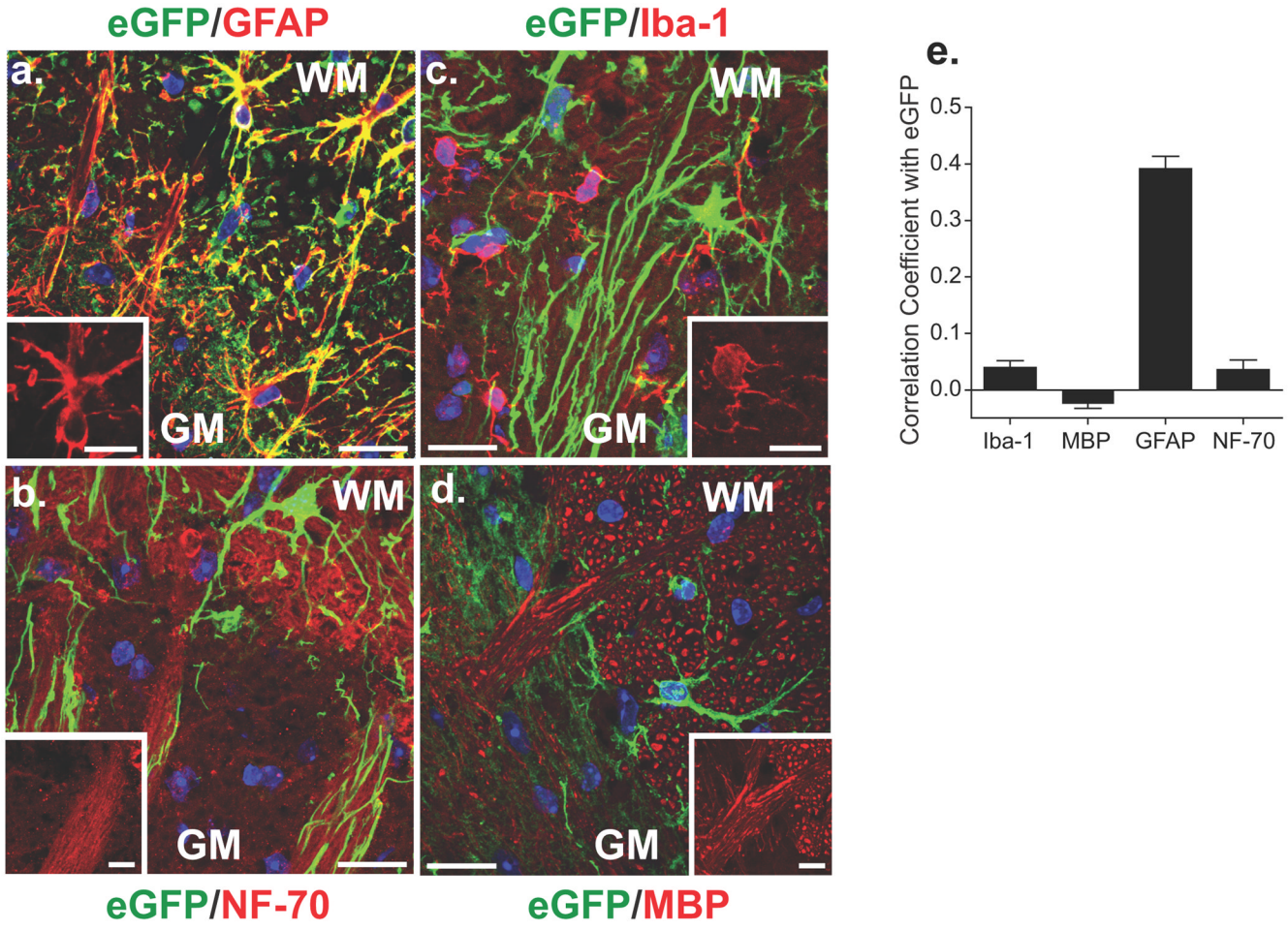

Figure 8. TSP4-eGFP signals in dorsal spinal cord were mainly in astrocytes

a–d. Fluorescent immunoreactivity to antibodies of commonly used cell-type specific markers was superimposed with TSP4-eGFP signals in ipsilateral dorsal spinal cord sections from two-week SNL TSP4-eGFP mice. Representative images from four mice are shown. TSP4 promoter-driven eGFP expression signals (green) mainly co-localized with immnoreactivity to antibodies against GFAP, a marker for activated astrocytes (red, **a**), but not with immunoreactivities to antibodies against NF-70, a marker for neurofilaments (red, **b**), Iba-1, a marker for microglial cells (red, **c**), nor myelin basic protein (MBP, red, **d**). WM – white matter. GM – grey matter. Scale bars: 20μm. Blue – nuclei. **Insert**: Enlarged images in each panel showing morphology of each type of immunoreactive cells, respectively. Scale bars: 10μm. **e.** Possibility of colocalization of fluorescent signals from eGFP and immunoreactivity to each cell marker antibody was analyzed as described (Barlow et al., 2010). Data are presented as Pearson's correlation coefficient values from the means ± SEM derived from pooled data of 12 sections from four mice.

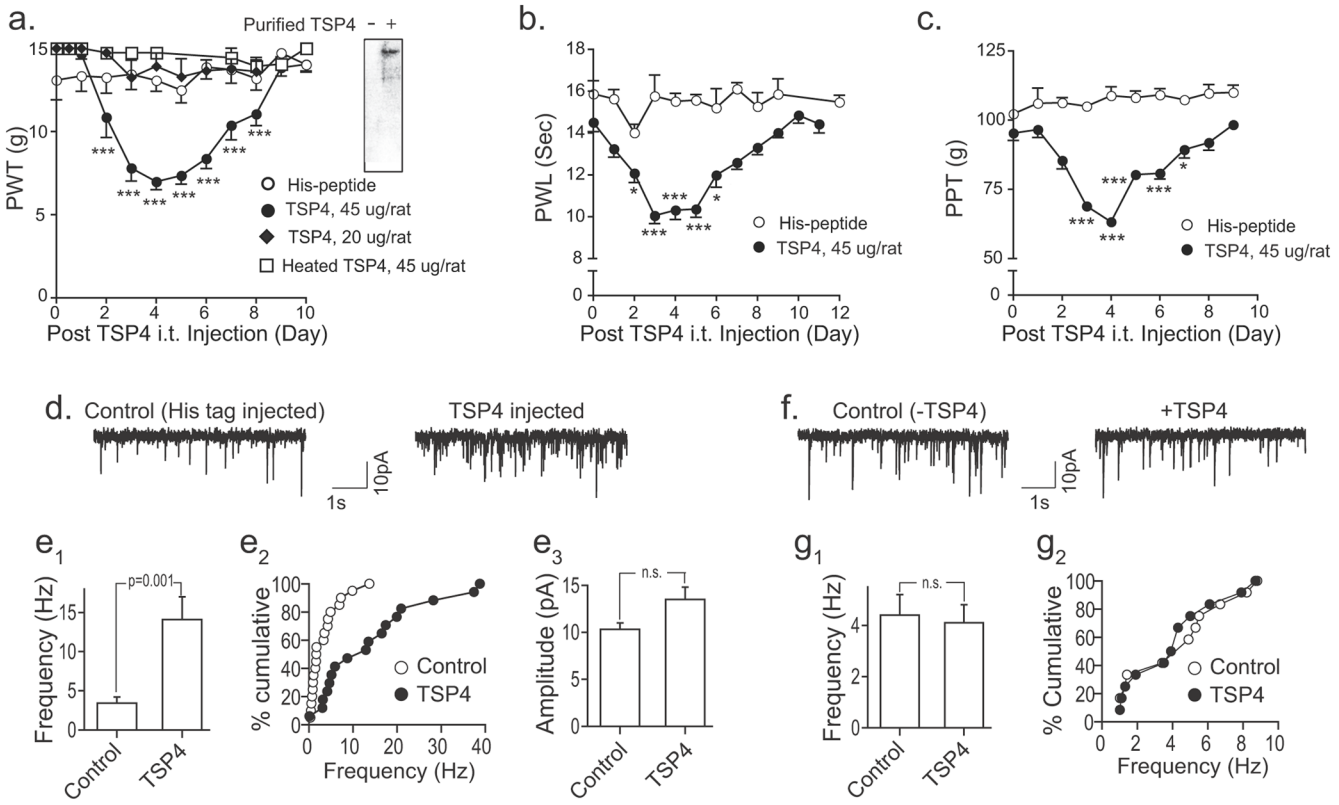

Figure 9. Increased spinal TSP4 is sufficient to induce dorsal horn neuron sensitization and behavioral hypersensitivities through a chronic machanism

a–c. Bolus intrathecal injection of 45 μg/rat TSP4 into L5/6 spinal region of naive rats at time 0 led to long-lasting, and reversible hindpaw hypersensitivities (averaged from both sides) assessed by blind daily behavioral test, including tactile allodynia (**a**), thermal hyperalgesia (**b**), and mechanical hyperalgesia (**c**). Intrathecal injection of 20 μg/rat TSP4 proteins, heated (45 μg/rat) TSP4 proteins (**a**), equal molar dose of His-tag peptides (**a–c**) did not cause behavioral hypersensitivities nor alter baseline behavioral thresholds. **Up right insert in (a):** A Western blot showing purified TSP4-His fusion proteins detected by TSP4 antibodies. Data presented are the means ± SEM from five to six rats in each group. *p < 0.05, **p < 0.01, ***p < 0.001 compared with pre-treatment level by Two-Way ANOVA analysis with Bonferroni Post-Tests.

d–e. Intrathecal injection of TSP4 proteins into L5/6 spinal region induced spinal neuron sensitization in naive rats that correlated with behavioral hypersensitivities. (**d**) Representative currents of AMPA receptor-mediated mEPSC from superficial dorsal horn neuron whole-cell patch recording in L5 spinal cord slices of rats injected intrathecally with equal molar dose of His-tag peptides (Control) or TSP4-His fusion proteins (TSP4, 45 μg/rat) four-days ago. Intrathecal TSP4 proteins induced an increase in the average frequency (e_1) as well as cumulative frequency distributions (e_2), but not amplitude (e_3), of mEPSC. Data presented are summarized means ± SEM from a total of 17–20 dorsal horn neurons recorded from spinal cord slices of five rats in each group. n.s. – not significant. **f–g.** Acute application of TSP4 proteins to spinal cord slices did not alter mEPSCs in dorsal horn neurons. mEPSCs from naive rat L5 superficial dorsal horn neurons were recorded under identical conditions for spinal cord slice recording shown in panels (**d**) and (**e**) except that TSP4 proteins were added into the recording bath for 15 min. **f.** Representative traces of mEPSC before and after bath application of 15 μg/mL TSP4, a concentration that is similar

to the spinal TSP4 concentration in vivo at the first injection day that caused behavioral and dorsal horn neuron sensitization later as shown in **a–e** (assuming the rate of CSF formation is 2.2 μL/min, about 3 mL/day/rat, (Cserr, 1965) without considering the turnover of CSF and TSP4 proteins). **g.** Summarized graphs showing the lack of acute TSP4 effects on mEPSC average frequency (g_1) and cumulative frequency distribution (g_2) from rat superficial dorsal horn neurons. Data presented are the means ± SEM from a total of 12 dorsal horn neurons recorded from spinal cord slices of three rats in each group. n.s. – not significant.