Title
Mechanisms of Thrombospondin-4 in Pain Modulation

Author
Park, John

Publication Date
2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA,
IRVINE

Mechanisms of Thrombospindin-4 in Pain Modulation

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In Pharmacology and Toxicology

by

John Francisco Park

Dissertation Committee:
Professor Zhigang David Luo, Chair
Professor Frances Leslie
Professor Frederick Ehlert

2014

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 3

CHAPTER 4

LIST OF TABLES

ACKNOWLEDGMENTS

I would like to express the deepest appreciation to my committee chair, Dr. Z. David Luo for his patience, mentorship, and faith in me. Dr. Luo has provided me with intellectual freedom to devise this dissertation project and opportunities to improve all aspects of my scholarly career. Without Dr. Luo's utmost faith and trust in my abilities and intellect, this dissertation would not have been possible.

I would like to thank my committee members Dr. Frances Leslie and Dr. Frederick Ehlert. Dr. Leslie provided unparalleled guidance in overcoming all obstacles that lay before me. I could not have achieved this level of success without Dr. Leslie's support. I am thankful for Dr. Ehlert who provided continuous support and mentorship throughout my graduate studies.

I would like to express my sincere gratitude and appreciation to my colleagues Dr. Amin Boroujerdi, Dr. Kang-wu Li, Dr. Mi-Kyeong Kim, and Dr. Chunyi Zhou for their overall guidance and knowledge regarding pain mechanisms. I would like to thank Dr. Eric Chang for his words of wisdom and career development advice. I am thankful for my friend Peter Yu, who has provided insightful discussions about this dissertation. I thank Nancy Trinh for all her hard-work, devotion, and help with my dissertation project. I could not have achieved this quality of work without her. Thank you Ben Vo for your overall assistance. I would like to thank all the lab members for making work enjoyable and fun.

I would like to thank Dr. Oswald Steward and Ilse Sears-Kraxberger for their contribution, assistance, and technical support on my electron microscopy studies.

I also would like to thank all my family and friends. I truly thank Hyuna Lee for her unconditional love and support. I know that I can always rely on her when times are difficult. I would like to thank Revival Church and Spring of Spirit for their continuous encouragement.

Financial support was partially provided by Pharmaceuticals Research and Manufacturers of America Foundation (PhRMA) Pre-Doctoral fellowship award.

CURRICULUM VITAE

EDUCATION

University of California, Irvine
Doctor of Philosophy Candidate, Pharmacology & Toxicology, December 2014

University of California, San Diego
Bachelor of Science, Animal Physiology and Neuroscience, March 2008

ACADEMIC HONORS

- Henry Wood Elliot Award for the most outstanding graduate student, UC Irvine, 2014
- The Pharmaceutical Research and Manufacturers of America Foundation Pre-Doctoral Fellowship Award, Washington DC, 2012 - 2014

RELATED RESEARCH EXPERIENCE

Dissertation Laboratory, Dr. Z. David Luo, UC Irvine
September 2009 – December 2014

- Identified target genes and underlying pathways that contributes to the development and maintenance of chronic pain using multidisciplinary approach.
- Studied protein-protein interactions and designing target specific drugs by utilizing surface plasmon resonance, peptide arrays with SPOT synthesis, and receptor binding assays.
- Examined how changes in synapse morphology affect sensory information processing at the ultrastructural level using electron microscopy in collaboration with Dr. Oswald Steward, the Director of the Reeves-Irvine Research Center.
- Used Mass Spectrometry-based proteomics in identifying protein networks during chronic pain states in collaboration with Dr. Lan Huang in Dept. of Biophysics at UC Irvine.

Staff Research Associate, Dr. Palmer Taylor, UC San Diego
September 2005 - July 2009

- Made modifications using molecular biology on acetylcholine binding protein structure, a soluble surrogate to nicotinic receptors, to study the selectivity for ligands that mimics certain human neuronal receptors.
- Analyzed the efficacy of neonicotinoids, which are a class of insecticides that are nicotine like, on mammalian nicotinic acetylcholine receptors (nAChR) to insect nAChR using receptor-binding assays by collaborating with Dr. John E. Cassida at UC Berkeley.
- Examined the binding protein structure and it's selectivity of the ligand binding sites by collecting and analyzing x-ray diffraction data from crystalized drug-receptor complexes.
- Monitored laboratory equipment. Contacted and negotiated with sales representatives.

Research Associate, UC San Diego Hillcrest Hospital
September 2006 - May 2008

- Analyzed and collected data for ongoing clinical research projects such as headaches and alternative medicine treatments, abdominal pain preventions, and chronic obstructive pulmonary disease.
- Assisted alongside faculty physicians, medical students, and with patients in collecting and analyzing clinical data.

PUBLICATIONS

Research Papers:

- **Park, J.,** Trinh, VT., Zauke, F., Luo, ZD. Molecular determinant of TSP4 in pain processing. (manuscript in preparation)
- **Park, J**., Zhou, CY., Li, KW., Wang, D., Chang, E., Deng, P., Kim, DK., Zhang, X., Vu, J., Kim, MK., Sharp, K., Steward, O., Vitko, I., Perez-Reyes, E., Eroglu, C., Barres, B., Zauke, F., Xu, ZC., Feng, G., Luo, ZD. (2014). Interactions of voltage-gated calcium channel alpha-2-delta-1 subunit with thrombospondin-4 proteins mediate sensory-neuron-type and modality-specific nociception. (submitted, in revision)
- **Park, J**., Trinh, VT., Sears-Kraxberger, I., Li, KW., Steward, O., Luo, ZD. (2014) Synaptic ultrastructure changes in trigeminal-spinal-complex post trigeminal nerve injury. Journal of Comparative Neurology. (submitted)
- Pan, B., Yu, H., **Park, J.**, Yu, Y.P., Luo, Z.D., and Hogan, Q.H. (2014). Painful nerve injury upregulates thrombospondin-4 expression in dorsal root ganglia. J Neurosci Res.

- Kim, D.S., Li, K.W., Boroujerdi, A., Peter Yu, Y., Zhou, C.Y., Deng, P., **Park, J.**, Zhang, X., Lee, J., Corpe, M., et al. (2012). Thrombospondin-4 contributes to spinal sensitization and neuropathic pain states. J Neurosci 32, 8977-8987.
- **Park, J.,** and Luo, Z.D. (2010). Calcium channel functions in pain processing. Channels 4, 510-517.
- Tomizawa M*, Talley TT*, **Park JF**, Maltby D, Medzihradszky KF, Durkin KA, Cornejo-Bravo JM, Burlingame AL, Casida JE, Taylor P (2009). Nicotinic agonist binding site mapped by methionine- and tyrosine-scanning coupled with azidochloropyridinyl photoaffinity labeling. J. Med. Chem., 52 (12), 3735-3741.
  *First Co-authors

Abstracts:

- Ultrastructural Changes in Spinal Dorsal Horn Synaptic Types in an Orofacial Neuropathic Pain Model. **John F Park**, Ilse Spears-Kraxberger, Kang-Wu Li, Nancy Trinh, Oswald Steward, Z. David Luo. International Association for the Study of Pain 2013.
- Epidermal growth factor-like domain of thrombospondin-4 proteins plays a crucial role in mediating behavioral hypersensitivities. **John F. Park**, Joshua Lee, Kang-Wu Li, Z. David Luo. Society of Neuroscience 2011.
- Structural determinants of TSP4 mediated pain processing. **John F. Park**, Kang-Wu Li, Z. David Luo. Society of Neuroscience 2010.

- Crystallographic comparison of nicotinic ligands in complex with the acetylcholine binding protein. Todd T. Talley**, John F. Park**, Joshua Wu, Kwok-Yiu Ho, Banumathi Sankaran and Palmer Taylor Experimental Biology 2010.
- A structure-guided design strategy to develop ligands with subtype selectivity for human nicotinic acetylcholine receptors. John G. Yamauchi, Ákos Nemecz, Kwok-Yiu Ho, Joseph R. Fotsing, Timo Weide, Neil Grimster, Bernhard Stump, **John F. Park**, Deepika Nayyar, Todd T. Talley, Valery V. Fokin, K. Barry Sharpless, Palmer Taylor. Experimental Biology 2009.
- Analysis of nicotinoid and neonicotinoid ligand binding to acetylcholine binding proteins (AChBPs) by crystallographic and affinity labeling procedures. Jose M. Cornejo-Bravo, Todd T. Talley, **John F. Park**, Moto Tomizawa, John E. Casida, Palmer Taylor. Society of Neuroscience 2008.
- Understanding receptor selectivity and ligand stereochemistry of insecticides and therapeutic agents for nicotinic acetylcholine receptor subtypes. John Yamauchi, Todd T. Talley, **John F. Park**, Motohiro Tomizawa, John E. Casida, Michal Harel, Palmer Taylor. Experimental Biology 2008

TECHNICAL SKILLS

- Proteomics (SPOT Peptide assay, Mass-spectrometry based immunoprecipitation).
- Behavioral Pharmacology (Handling animals, surgeries, injections, perfusions, behavioral nociception assays, etc).
- Biochemical Techniques (Protein Purification, Western blots, Tissue Cell Culture, etc).
- Pharmacology (radio ligand binding, scintillation proximity assay, solid-phase binding).
- Trained in Biacore 2000 by GE Life Sciences.
- Molecular Biology Techniques (PCR, Cloning, Electrophoresis, etc).
- Microscopy (Light, Fluorescent, and transmission electron).
- X-Ray Crystallography
- Proficient in Microsoft Office (Word, Excel, PowerPoint).
- Proficient in Adobe Creative Suite 5.0 (Photoshop, Illustrator, InDesign).

TEACHING EXPERIENCE

Teaching Assistant, UC Irvine
January 2011 - March 2011

- Instructed an undergraduate laboratory course on cell and developmental biology.
- Created lesson-plans, handouts, and experiments to clearly communicate basic science knowledge and simplify complex materials.

Teaching Assistant, UC San Diego
January 2007 - January 2008

- Instructed discussion classes for multi-cellular biology, system neurobiology, and human physiology courses.
- Promoted student's learning of complex materials and topics.

ABSTRACT OF THE DISSERTATION

Mechanisms of Thrombospondin-4 in Pain Modulation

By

John Park

Doctor of Philosophy in Pharmacology and Toxicology

University of California, Irvine, 2014

Professor Zhigang David Luo, Chair

Upregulation of the thrombospondin-4 (TSP4) or calcium channel α2δ-1 subunit ($Ca_v\alpha_2\delta_1$) in the dorsal spinal cord and dorsal root ganglia plays a causal role in neuropathic pain development through an unidentified mechanism. TSP4 blockade either by antibodies or inactivation of the TSP4 gene prevents development of chronic pain states. Intrathecal injection of TSP4 proteins into naive rats can cause dorsal horn neuron hyperexcitability and allodynia, which are blocked by the $Ca_v\alpha_2\delta_1$ ligand gabapentin. These findings suggest that TSP4 and $Ca_v\alpha_2\delta_1$ may interact together in mediating pain processing. Here, I show that TSP4 binding to $Ca_v\alpha_2\delta_1$ is detectable in the same immunocomplexes from rodent spinal cords and in solid-phase binding. SPOT Peptide array analysis and in vitro binding of TSP4 recombinant truncation proteins to $Ca_v\alpha_2\delta_1$ reveal multiple binding sites within the TSP4 Epidermal Growth Factor-like domains (EGF-like) and coil-coil domain. Functionally, lumbar intrathecal injection of EGF-like domain proteins is sufficient to induce behavioral hypersensitivity. Selective ablation of $Ca_v\alpha_2\delta_1$ from $Na_v1.8$-positive sensory neurons abolishes EGF-like domain induced thermal hyperalgesia, but not tactile allodynia. A 15-mer TSP4 peptide from the EGF-like domains can block both $Ca_v\alpha_2\delta_1$- and TSP4-induced behavioral hypersensitivity, presumably by disrupting the interaction between TSP4 and $Ca_v\alpha_2\delta_1$. My data suggest that the EGF-like domains of TSP4 interact with $Ca_v\alpha_2\delta_1$ to induce chronic pain states in a modality specific manner. Emerging data also suggest that $Ca_v\alpha_2\delta_1$ and TSP4 interact to induce synapse formation. To determine

if maladaptive changes in synapses correlate with neuropathic pain states in a nerve injury model with injury-induced upregulation of TSP4 and $Ca_v\alpha_2\delta_1$, I examined synapse numbers at ultrastructure level in superficial dorsal horn of rats after trigeminal nerve injury. I found a significant increase in both excitatory and inhibitory synapses within lamina I and II of the superficial dorsal horn, respectively, and a decrease in mean synaptic length, a marker for synapse strength, of inhibitory interneurons mainly in lamina I of the injury side. Together, it is likely that injury-induced TSP4/$Ca_v\alpha_2\delta_1$ contribute to the development of chronic pain states after nerve injury through a mechanism involving dysregulation of excitatory and inhibitory synapses in the superficial dorsal horn.

INTRODUCTION

Pain is the most common symptom for which patients seek medical attention. A recent survey has indicated that chronic pain, including neuropathic pain caused by injuries in the peripheral or central nervous systems (CNS), is one of the leading causes of disability in the United States. The prevalence of chronic pain in the United States is estimated to be 35.5% or 105 million people (Harstall, 2003; Mitka, 2003). This disorder costs more than $100 billion per year in direct health-care expenditure and lost work time. Neuropathic pain syndromes include spontaneous pain and evoked pain such as allodynia (exaggerated response to otherwise innocuous tactile stimuli) and hyperalgesia (exaggerated pain sensations as a result of exposure to mildly noxious stimuli) (Bridges et al., 2001; Woolf and Mannion, 1999). The use of conventional analgesics such as opioids and non-steroidal anti-inflammatory drugs is less effective for treating neuropathic pain and often associated with poor tolerability, unfavorable side effects, and concerns over long-term safety and abuse potential (Woodcock, 2009). The lack of specific therapeutics for neuropathic pain is mainly due to our insufficient knowledge in understanding the molecular mechanism of neuropathic pain.

Pain Processing

Pain information processing starts from activation of peripheral nociceptors, causing action potentials to propagate along the primary afferent nerve fibers into sensory neurons in dorsal root ganglia (DRG). They are further relayed to the spinal dorsal horn through the central axons of sensory neurons. The action potentials reaching the central terminals of sensory afferents can cause membrane depolarization, activation of voltage-gated calcium channels (VGCCs), and calcium influx, which triggers synaptic vesicle exocytosis. This then leads to the release of excitatory neurotransmitters including glutamate, pain-inducing peptides such as substance P, and calcitonin gene-related peptide (CGRP) into

the synaptic cleft. These neurotransmitters can then cause activation of post-synaptic dorsal horn projection neurons and interneurons, leading to spinal modulation of sensory signals (Park and Luo, 2010).

Noxious stimuli of various modalities are processed by a set of sensory nerve fibers, including unmyelinated C fibers, thinly myelinated Aδ fibers, and myelinated Aβ fibers. Aδ and C primary fibers, also known as nociceptors because they are activated mainly by high intensity noxious stimuli, originate from mainly small to medium size neurons in DRG and form synaptic connections with distinct spinal dorsal horn neurons primarily in spinal laminae I and II (Todd, 2010). Nociceptors can be further defined as peptidergic and non-peptidergic expressing neurons. Many peptidergic nociceptors typically express neuropeptides substance P and CGRP, and can be further defined by the expression of nerve growth factor recetor trkA (Snider and McMahon, 1998). In contrast, non-peptidergic nociceptors are defined by its binding of the plant isolectin B4 and its expression of glial cell-derived neuroptrophic factor receptors and purinergic P2X3 receptor (Molliver and Snider, 1997; Snider and McMahon, 1998). Aβ fibers are mainly from larger DRG neurons that transmit non-painful tactile stimuli and terminate in laminae III-V (Light and Perl, 1979; Rethelyi et al., 1982; Shortland and Woolf, 1993; Shortland et al., 1989).

The physiological changes that underlie chronic pain processing could occur at multiple levels, including changes in sensory afferent activity, alterations in neurotransmitter release, spinal reorganization, and alterations in descending pain facilitation and inhibition (Kuner, 2010). An important aspect of this neuroplasticity is the altered expression of a wide range of receptors and channels. For instance, our lab has reported data from gene-chip microarray analysis demonstrating that nerve injury causes an upregulation of the VGCC subunit α2δ-1 ($Ca_v\alpha_2\delta_1$) and thrombospondin-4 mRNA in DRG, both are important for neuropathic pain information processing (Kim et al., 2009; Kim et al., 2012; Valder et al., 2003; Wang et al., 2002).

Voltage-Gated Calcium Channel Subunits

The VGCC complex typically consists of channel-forming α1-subunit (~170kDa) along with auxiliary β- (~52kDa), α2- (~15-kDa), δ- (~17-25kDa) and γ-subunits (~32kDa) (Catterall et al., 2005; Dolphin, 2013). So far, ten α1-subunits have been identified in mammals and are encoded by distinct genes (Catterall et al., 2005). This subunit is also subjected to alternative splicing (Altier et al., 2007; Bourinet et al., 1999; Castiglioni et al., 2006; Lin et al., 2004; Lin et al., 1997; Lin et al., 1999; Lipscombe and Raingo, 2007; Rajapaksha et al., 2008; Tsunemi et al., 2002). The α1-subunit consists of four homologous domains (I-IV), each having six transmembrane helices (S1 through S6), which together form the calcium conduction pore, voltage sensors, and gating apparatus (Yu et al., 2005). The S4 transmembrane domain contains positive charged amino acids for voltage sensing. There are four known β-subunits (β-1 through β-4), which are intracellular proteins that enhance cell surface expression of the α1-subunits and modulate the gating properties through their interactions with the channel-forming α1-subunit and intracellular signaling molecules (Flynn and Zamponi, 2010; Hidalgo and Neely, 2007; Karunasekara et al., 2009). Four α2δ-subunits have been identified (α2δ-1 through α2δ-4), each consisting of two disulfide-linked peptides (α2 and δ) that are encoded by the same gene (De Jongh et al., 1990a; Ellis et al., 1988). Similar to the β-subunit, α2δ subunits promote and stabilize cell surface expression of VGCCs (Davies et al., 2007; Klugbauer et al., 2003). Eight γ-subunits have been identified and appear to act as glycoproteins with four transmembrane segments, but the exact function of the γ-subunit is not well defined (Dolphin, 2009; Iftinca and Zamponi, 2009). Together, the auxiliary subunits modulate the functional properties of the α1-subunit (Fig. 1).

$Ca_v\alpha_2\delta$ in Pain Processing

$Ca_v\alpha_2\delta$ subunit has been implicated to play a role in neuropathic pain. It has been shown that injury-induced increase of $Ca_v\alpha_2\delta_1$ subunit in sensory neurons and spinal

dorsal horn correlates with neuropathic pain states (Li et al., 2006; Luo et al., 2001). Blocking nerve injury-induced spinal $Ca_v\alpha_2\delta_1$ protein upregulation with dorsal rhizotomy or intrathecal $Ca_v\alpha_2\delta_1$ anti-sense oligodeoxynucleotide treatment either prevented (Boroujerdi et al., 2008) or reversed (Li et al., 2004) tactile allodynia. This is further supported by evidence indicating that upregulated $Ca_v\alpha_2\delta_1$ in transgenic mice over-expressing $Ca_v\alpha_2\delta_1$ in neuronal cells results in behavioral hypersensitivities in the absence of nerve damage (Li et al., 2006). In contrast, $Ca_v\alpha_2\delta_1$ genetically ablated mice show a defect in mechanical and thermal sensitivity, and a delayed response to nerve injury compared to wild-type mice (Patel et al., 2013). Most importantly, $Ca_v\alpha_2\delta_1$ subunit has been identified as the binding site for gabapentin (Neurontin) and pregabalin (Lyrica), which are anti-convulsant and anti-neuropathic pain medications. $Ca_v\alpha_2\delta_1$ ligands pregabalin and gabapentin are effective in alleviating neuropathic pain (Brown et al., 1998; Field et al., 2006), but their anti-neuropathic pain properties are abolished in knock-in mice expressing a mutant $Ca_v\alpha_2\delta_1$ that cannot bind gabapentin and pregabalin (Field et al., 2006). However, the cellular mechanism underlying the mode of action of these drugs is unclear.

At the spinal level, elevated $Ca_v\alpha_2\delta_1$ mediates behavioral hypersensitivities through enhanced excitatory pre-synaptic input that activates glutamate receptors at post-synaptic dorsal horn neurons (Nguyen et al., 2009). Recent studies suggest that injury-induced increase of $Ca_v\alpha_2\delta_1$ in DRG leads to increase trafficking of $Ca_v\alpha_2\delta_1$ proteins to the pre-synaptic terminals, which can be reduced by pregabalin (Bauer et al., 2009; Hendrich et al., 2008; Li et al., 2004). In fact, it has been shown that $Ca_v\alpha_2\delta_1$ proteins promote trafficking of α1-pore forming VGCC subunit to presynaptic terminals and increase synaptic vesicle release probability (Hoppa et al., 2012). Together, these findings suggest that injury-induced $Ca_v\alpha_2\delta_1$ subunit may contribute to the initiation and maintenance of neuropathic pain by altering VGCC cell surface expression and/or neurotransmitter release at their afferent presynaptic terminals, thus modulating the excitability and/or synaptic neuroplasticity in the dorsal horn.

Thrombospondin-4 and its role in chronic pain states

A recent study has shown that $Ca_v\alpha_2\delta_1$ is the neuronal receptor for astrocyte-secreted thrombospondins (TSPs) in mediating excitatory CNS synaptogenesis (Eroglu et al., 2009). TSPs are large oligomeric, multidomain, extracellular matrix proteins that mediate cell-cell and cell-matrix interactions through binding to other extracellular matrix proteins, membrane proteins, and cytokines (Adams and Lawler, 2004; Bornstein, 2001). TSP consists of five isoforms in mammals, TSP1-5, which can be broken down into two subfamilies, subgroup A and B (Fig. 2). TSP1 and TSP2 are part of the subgroup A family that shares the same structure and functional domains, and are assembled as trimers for secretion. These proteins are shown to be highly expressed during early postnatal ages, and remarkably reduced during adulthood when the amount of excitatory synaptogenesis is significantly reduced (Christopherson et al., 2005). Subgroup B consists of TSP3-5 that forms a pentameric structure with functional domains different from that in Subgroup A.

TSPs are found to be localized to different tissues in a non-overlapping manner, suggesting that these proteins may carry out similar functions in different tissues (Adams et al., 2001). TSP1 and 2 seem to play a role in platelet aggregation, inflammatory response, and angiogenesis regulation during tumor growth and wound repair, and connective tissue extracellular matrix assembly. The biology of TSP3 is not well studied (Adams and Lawler, 2004). TSP5 is mainly expressed in cartilage and certain connective tissues and plays a role in chondrocyte differentiation, attachment and cartilage extracellular matrix assembly (Adams and Lawler, 2004). TSP4 has been recently demonstrated to be involved in the following: 1) intracellular functions in the secretory pathways by interacting with transcriptional factor Atf6α (Lynch et al., 2012); 2) regulation of vascular inflammation and adaptive responses of cardiomyocytes to pressure overload and in human hypertrophied hearts (Cingolani et al., 2011; Frolova et al., 2012; Gabrielsen et al., 2007; Mustonen et al., 2008; Mustonen et al., 2010); 3) promotion of atherosclerotic processes by attracting and retaining macrophages in atherosclerotic lesions (Frolova et al., 2010); 4) regulation

of protective astrogenesis following ischemic injury to the brain cortex in a NOTCH1-dependent manner (Benner et al., 2013).

Furthermore, TSP4 has recently been identified by our lab to play a role in neuropathic pain development. We have shown that TSP4 is upregulated in spinal cord post peripheral (Kim et al., 2012) or trigeminal nerve injury (Li et al., 2014). Upregulated TSP4 is involved in mediating behavioral hypersensitivities because injury-induced pain states can be prevented or reversed by blocking TSP4 with intrathecal TSP4 anti-sense oligodeoxynucleotides or antibodies, and genetic ablation of TSP4 gene in spinal nerve ligated mice (Kim et al., 2012; Li et al., 2014). In addition, intrathecal injection of TSP4 proteins leads to spinal neuron hyperexcitability and behavioral hypersensitivity (Kim et al., 2012). Interestingly, TSP4 has been shown to bind to the von Willebrand factor A (VWF-A) domain of integrin αM and $Ca_v\alpha_2\delta_1$ that plays a role in inflammatory responses underlying atherosclerosis (Pluskota et al., 2005) and excitatory CNS synaptogenesis (Eroglu et al., 2009), respectively. However, it remains unclear if TSP4 and $Ca_v\alpha_2\delta_1$ interaction is important in mediating chronic pain states. We hypothesized that spinal TSP4 induces abnormal synapse modulation and pain states by interacting with $Ca_v\alpha_2\delta_1$.

References

Adams JC, Kureishy N, Taylor AL. 2001. A role for syndecan-1 in coupling fascin spike formation by thrombospondin-1. J Cell Biol 152(6):1169-1182.

Adams JC, Lawler J. 2004. The thrombospondins. Int J Biochem Cell Biol 36(6):961-968.

Altier C, Dale CS, Kisilevsky AE, Chapman K, Castiglioni AJ, Matthews EA, Evans RM, Dickenson AH, Lipscombe D, Vergnolle N, Zamponi GW. 2007. Differential role of N-type calcium channel splice isoforms in pain. J Neurosci 27(24):6363-6373.

Bauer CS, Nieto-Rostro M, Rahman W, Tran-Van-Minh A, Ferron L, Douglas L, Kadurin I, Sri Ranjan Y, Fernandez-Alacid L, Millar NS, Dickenson AH, Lujan R, Dolphin AC. 2009. The increased trafficking of the calcium channel subunit alpha2delta-1 to presynaptic terminals in neuropathic pain is inhibited by the alpha2delta ligand pregabalin. J Neurosci 29(13):4076-4088.

Benner EJ, Luciano D, Jo R, Abdi K, Paez-Gonzalez P, Sheng H, Warner DS, Liu C, Eroglu C, Kuo CT. 2013. Protective astrogenesis from the SVZ niche after injury is controlled by Notch modulator Thbs4. Nature 497(7449):369-373.

Bornstein P. 2001. Thrombospondins as matricellular modulators of cell function. J Clin Invest 107(8):929-934.

Boroujerdi A, Kim HK, Lyu YS, Kim DS, Figueroa KW, Chung JM, Luo ZD. 2008. Injury discharges regulate calcium channel alpha-2-delta-1 subunit upregulation in the dorsal horn that contributes to initiation of neuropathic pain. Pain 139(2):358-366.

Bourinet E, Soong TW, Sutton K, Slaymaker S, Mathews E, Monteil A, Zamponi GW, Nargeot J, Snutch TP. 1999. Splicing of alpha 1A subunit gene generates phenotypic variants of P- and Q-type calcium channels. Nat Neurosci 2(5):407-415.

Bridges D, Thompson SW, Rice AS. 2001. Mechanisms of neuropathic pain. Br J Anaesth 87(1):12-26.

Brown JP, Dissanayake VU, Briggs AR, Milic MR, Gee NS. 1998. Isolation of the [3H] gabapentin-binding protein/alpha 2 delta Ca2+ channel subunit from porcine brain: development of a radioligand binding assay for alpha 2 delta subunits using [3H] leucine. Anal Biochem 255(2):236-243.

Castiglioni AJ, Raingo J, Lipscombe D. 2006. Alternative splicing in the C-terminus of CaV2.2 controls expression and gating of N-type calcium channels. Journal of Physiology (Cambridge) 576(Pt 1):119-134.

Catterall WA, Perez-Reyes E, Snutch TP, Striessnig J. 2005. International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. Pharmacol Rev 57(4):411-425.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA. 2005. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120(3):421-433.

Cingolani OH, Kirk JA, Seo K, Koitabashi N, Lee DI, Ramirez-Correa G, Bedja D, Barth AS, Moens AL, Kass DA. 2011. Thrombospondin-4 is required for stretch-mediated contractility augmentation in cardiac muscle. Circ Res 109(12):1410-1414.

Davies A, Hendrich J, Van Minh AT, Wratten J, Douglas L, Dolphin AC. 2007. Functional biology of the alpha(2)delta subunits of voltage-gated calcium channels. Trends Pharmacol Sci 28(5):220-228.

De Jongh KS, Warner C, Catterall WA. 1990. Subunits of purified calcium channels. Alpha 2 and delta are encoded by the same gene. J Biol Chem 265(25):14738-14741.

Dolphin AC. 2009. Calcium channel diversity: multiple roles of calcium channel subunits. Curr Opin Neurobiol 19(3):237-244.

Dolphin AC. 2013. The alpha2delta subunits of voltage-gated calcium channels. Biochim Biophys Acta 1828(7):1541-1549.

Ellis SB, Williams ME, Ways NR, Brenner R, Sharp AH, Leung AT, Campbell KP, McKenna E, Koch WJ, Hui A, et al. 1988. Sequence and expression of mRNAs encoding the alpha 1 and alpha 2 subunits of a DHP-sensitive calcium channel. Science 241(4873):1661-1664.

Eroglu C, Allen NJ, Susman MW, O'Rourke NA, Park CY, Ozkan E, Chakraborty C, Mulinyawe SB, Annis DS, Huberman AD, Green EM, Lawler J, Dolmetsch R, Garcia KC, Smith SJ, Luo ZD, Rosenthal A, Mosher DF, Barres BA. 2009. Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell 139(2):380-392.

Field MJ, Cox PJ, Stott E, Melrose H, Offord J, Su TZ, Bramwell S, Corradini L, England S, Winks J, Kinloch RA, Hendrich J, Dolphin AC, Webb T, Williams D. 2006. Identification of the alpha2-delta-1 subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin. Proc Natl Acad Sci U S A 103(46):17537-17542.

Flynn R, Zamponi G. 2010. Calcium channel regulation by RGK proteins. Channels (Austin) 4(6).

Frolova EG, Pluskota E, Krukovets I, Burke T, Drumm C, Smith JD, Blech L, Febbraio M, Bornstein P, Plow EF, Stenina OI. 2010. Thrombospondin-4 regulates vascular inflammation and atherogenesis. Circ Res 107(11):1313-1325.

Frolova EG, Sopko N, Blech L, Popovic ZB, Li J, Vasanji A, Drumm C, Krukovets I, Jain MK, Penn MS, Plow EF, Stenina OI. 2012. Thrombospondin-4 regulates fibrosis and remodeling of the myocardium in response to pressure overload. FASEB J 26(6):2363-2373.

Gabrielsen A, Lawler PR, Yongzhong W, Steinbruchel D, Blagoja D, Paulsson-Berne G, Kastrup J, Hansson GK. 2007. Gene expression signals involved in ischemic injury, extracellular matrix composition and fibrosis defined by global mRNA profiling of the human left ventricular myocardium. J Mol Cell Cardiol 42(4):870-883.

Harstall C. 2003. How Prevalent Is Chronic Pain? . Pain Clinical Updates XI(2):4.

Hendrich J, Van Minh AT, Heblich F, Nieto-Rostro M, Watschinger K, Striessnig J, Wratten J, Davies A, Dolphin AC. 2008. Pharmacological disruption of calcium channel trafficking by the alpha2delta ligand gabapentin. Proc Natl Acad Sci U S A 105(9):3628-3633.

Hidalgo P, Neely A. 2007. Multiplicity of protein interactions and functions of the voltage-gated calcium channel beta-subunit. Cell Calcium 42(4-5):389-396.

Hoppa MB, Lana B, Margas W, Dolphin AC, Ryan TA. 2012. alpha2delta expression sets presynaptic calcium channel abundance and release probability. Nature 486(7401):122-125.

Iftinca MC, Zamponi GW. 2009. Regulation of neuronal T-type calcium channels. Trends Pharmacol Sci 30(1):32-40.

Karunasekara Y, Dulhunty AF, Casarotto MG. 2009. The voltage-gated calcium-channel beta subunit: more than just an accessory. Eur Biophys J 39(1):75-81.

Kim DS, Figueroa KW, Li KW, Boroujerdi A, Yolo T, Luo ZD. 2009. Profiling of dynamically changed gene expression in dorsal root ganglia post peripheral nerve injury and a critical role of injury-induced glial fibrillary acidic protein in maintenance of pain behaviors [corrected]. Pain 143(1-2):114-122.

Kim DS, Li KW, Boroujerdi A, Peter Yu Y, Zhou CY, Deng P, Park J, Zhang X, Lee J, Corpe M, Sharp K, Steward O, Eroglu C, Barres B, Zaucke F, Xu ZC, Luo ZD. 2012. Thrombospondin-4 contributes to spinal sensitization and neuropathic pain states. J Neurosci 32(26):8977-8987.

Klugbauer N, Marais E, Hofmann F. 2003. Calcium channel alpha2delta subunits: differential expression, function, and drug binding. J Bioenerg Biomembr 35(6):639-647.

Kuner R. 2010. Central mechanisms of pathological pain. Nat Med 16(11):1258-1266.

Li CY, Song YH, Higuera ES, Luo ZD. 2004. Spinal dorsal horn calcium channel alpha2delta-1 subunit upregulation contributes to peripheral nerve injury-induced tactile allodynia. J Neurosci 24(39):8494-8499.

Li CY, Zhang XL, Matthews EA, Li KW, Kurwa A, Boroujerdi A, Gross J, Gold MS, Dickenson AH, Feng G, Luo ZD. 2006. Calcium channel alpha2delta1 subunit mediates spinal hyperexcitability in pain modulation. Pain 125(1-2):20-34.

Li KW, Kim DS, Zaucke F, Luo ZD. 2014. Trigeminal nerve injury-induced thrombospondin-4 up-regulation contributes to orofacial neuropathic pain states in a rat model. Eur J Pain 18(4):489-495.

Light AR, Perl ER. 1979. Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. J Comp Neurol 186(2):133-150.

Lin Y, McDonough SI, Lipscombe D. 2004. Alternative splicing in the voltage-sensing region of N-Type CaV2.2 channels modulates channel kinetics. J Neurophysiol 92(5):2820-2830.

Lin Z, Haus S, Edgerton J, Lipscombe D. 1997. Identification of functionally distinct isoforms of the N-type Ca2+ channel in rat sympathetic ganglia and brain. Neuron 18(1):153-166.

Lin Z, Lin Y, Schorge S, Pan JQ, Beierlein M, Lipscombe D. 1999. Alternative splicing of a short cassette exon in alpha1B generates functionally distinct N-type calcium channels in central and peripheral neurons. J Neurosci 19(13):5322-5331.

Lipscombe D, Raingo J. 2007. Alternative splicing matters: N-type calcium channels in nociceptors. Channels (Austin) 1(4):225-227.

Luo ZD, Chaplan SR, Higuera ES, Sorkin LS, Stauderman KA, Williams ME, Yaksh TL. 2001. Upregulation of dorsal root ganglion (alpha)2(delta) calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. J Neurosci 21(6):1868-1875.

Lynch JM, Maillet M, Vanhoutte D, Schloemer A, Sargent MA, Blair NS, Lynch KA, Okada T, Aronow BJ, Osinska H, Prywes R, Lorenz JN, Mori K, Lawler J, Robbins J, Molkentin JD. 2012. A thrombospondin-dependent pathway for a protective ER stress response. Cell 149(6):1257-1268.

Mitka M. 2003. “Virtual textbook” on pain developed: effort seeks to remedy gap in medical education. JAMA 290(18):2395.

Molliver DC, Snider WD. 1997. Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurons. J Comp Neurol 381(4):428-438.

Mustonen E, Aro J, Puhakka J, Ilves M, Soini Y, Leskinen H, Ruskoaho H, Rysa J. 2008. Thrombospondin-4 expression is rapidly upregulated by cardiac overload. Biochem Biophys Res Commun 373(2):186-191.

Mustonen E, Leskinen H, Aro J, Luodonpaa M, Vuolteenaho O, Ruskoaho H, Rysa J. 2010. Metoprolol treatment lowers thrombospondin-4 expression in rats with myocardial infarction and left ventricular hypertrophy. Basic Clin Pharmacol Toxicol 107(3):709-717.

Nguyen D, Deng P, Matthews EA, Kim DS, Feng G, Dickenson AH, Xu ZC, Luo ZD. 2009. Enhanced pre-synaptic glutamate release in deep-dorsal horn contributes to calcium channel alpha-2-delta-1 protein-mediated spinal sensitization and behavioral hypersensitivity. Mol Pain 5:6.

Park J, Luo ZD. 2010. Calcium channel functions in pain processing. Channels (Austin) 4(6):510-517.

Patel R, Bauer CS, Nieto-Rostro M, Margas W, Ferron L, Chaggar K, Crews K, Ramirez JD, Bennett DL, Schwartz A, Dickenson AH, Dolphin AC. 2013. alpha2delta-1 gene deletion affects somatosensory neuron function and delays mechanical hypersensitivity in response to peripheral nerve damage. J Neurosci 33(42):16412-16426.

Pluskota E, Stenina OI, Krukovets I, Szpak D, Topol EJ, Plow EF. 2005. Mechanism and effect of thrombospondin-4 polymorphisms on neutrophil function. Blood 106(12):3970-3978.

Rajapaksha WR, Wang D, Davies JN, Chen L, Zamponi GW, Fisher TE. 2008. Novel splice variants of rat CaV2.1 that lack much of the synaptic protein interaction site are expressed in neuroendocrine cells. J Biol Chem 283(23):15997-16003.

Rethelyi M, Light AR, Perl ER. 1982. Synaptic complexes formed by functionally defined primary afferent units with fine myelinated fibers. J Comp Neurol 207(4):381-393.

Shortland P, Woolf CJ. 1993. Chronic peripheral nerve section results in a rearrangement of the central axonal arborizations of axotomized A beta primary afferent neurons in the rat spinal cord. J Comp Neurol 330(1):65-82.

Shortland P, Woolf CJ, Fitzgerald M. 1989. Morphology and somatotopic organization of the central terminals of hindlimb hair follicle afferents in the rat lumbar spinal cord. J Comp Neurol 289(3):416-433.

Snider WD, McMahon SB. 1998. Tackling pain at the source: new ideas about nociceptors. Neuron 20(4):629-632.

Todd AJ. 2010. Neuronal circuitry for pain processing in the dorsal horn. Nature reviews Neuroscience 11(12):823-836.

Tsunemi T, Saegusa H, Ishikawa K, Nagayama S, Murakoshi T, Mizusawa H, Tanabe T. 2002. Novel Cav2.1 splice variants isolated from Purkinje cells do not generate P-type Ca2+ current. J Biol Chem 277(9):7214-7221.

Valder CR, Liu JJ, Song YH, Luo ZD. 2003. Coupling gene chip analyses and rat genetic variances in identifying potential target genes that may contribute to neuropathic allodynia development. J Neurochem 87(3):560-573.

Wang H, Sun H, Della Penna K, Benz RJ, Xu J, Gerhold DL, Holder DJ, Koblan KS. 2002. Chronic neuropathic pain is accompanied by global changes in gene expression and shares pathobiology with neurodegenerative diseases. Neuroscience 114(3):529-546.

Woodcock J. 2009. A difficult balance--pain management, drug safety, and the FDA. N Engl J Med 361(22):2105-2107.

Woolf CJ, Mannion RJ. 1999. Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet 353(9168):1959-1964.

Yu FH, Yarov-Yarovoy V, Gutman GA, Catterall WA. 2005. Overview of molecular relationships in the voltage-gated ion channel superfamily. Pharmacol Rev 57(4):387-395.

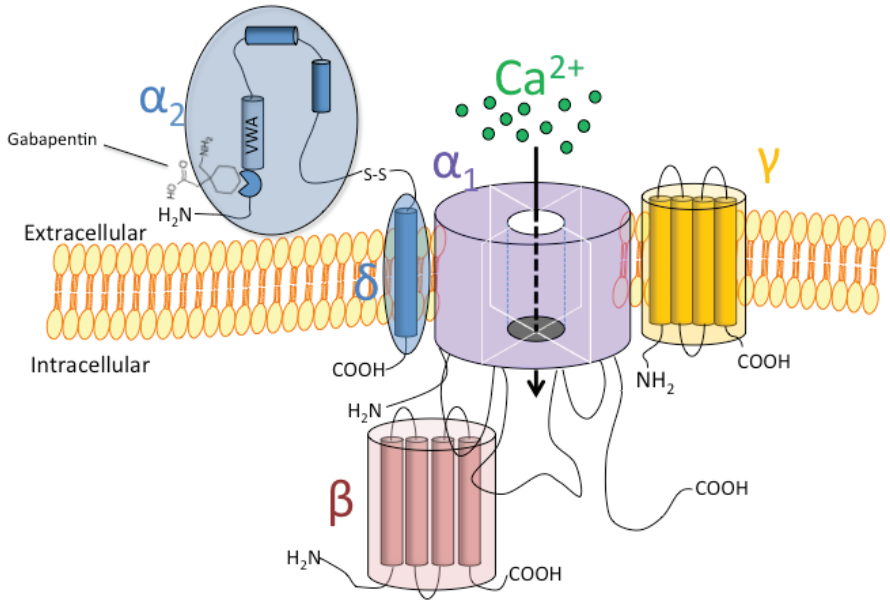

Figure 1. Structural organization of voltage-gated calcium channel subunits (VGCC). The VGCC is composed of the α1- (purple), α2δ- (blue), β- (red), and γ- (yellow) subunits. The predicted α-helices are depicted as cylinders. The α2δ subunit is encoded by the same gene, but post-translationally cleaved into α2 and δ peptides that are linked by disulfide bonds (S-S). Sequence analysis of α2δ-subunit reveals the presence of the von Willebrand Factor type A (VWA) domain. The gabapentin binding site is upstream of the VWA domain.

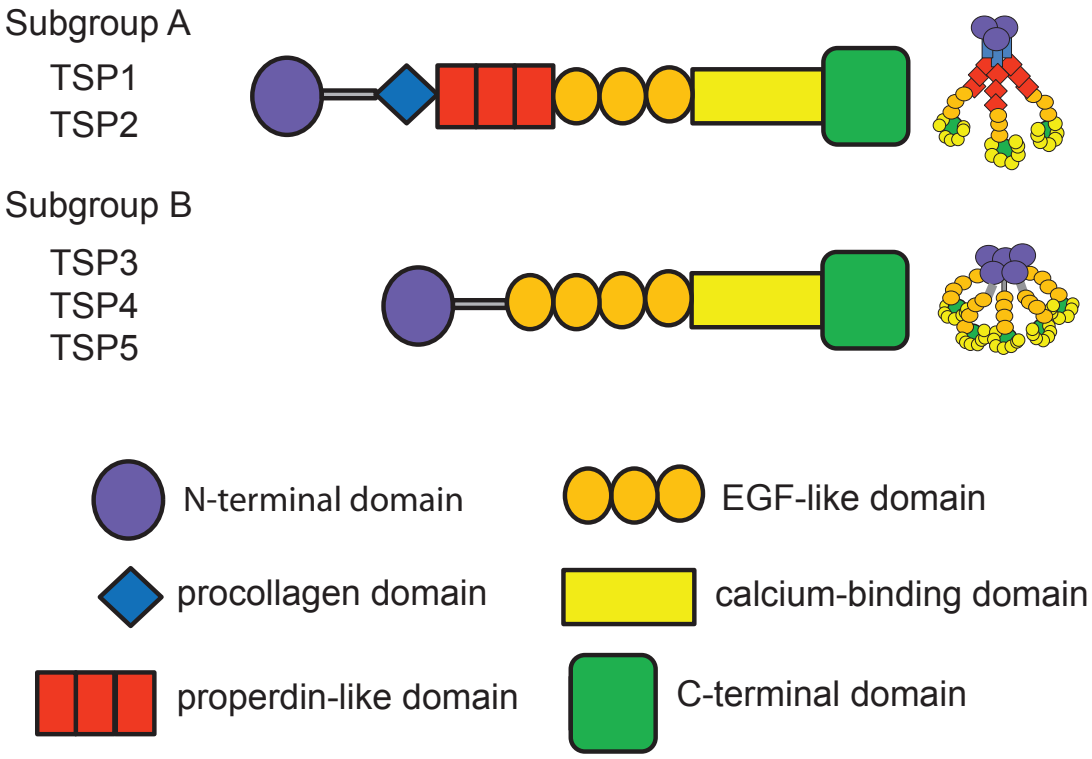

Figure 2. Schematic diagram of the domain structures of thrombospondin family members. N-terminal laminin G-like domain (purple), oligomerization domain (grey), procollagen repeat (blue), properdin-like type 1 domain (red), epidermal growth factor-like type 2 domain (orange), calcium binding type 3 domain (yellow), and C-terminal L-type lectin-like domain (green).

CHAPTER 1

Interactions of voltage-gated calcium channel alpha-2-delta-1 subunit with thrombospondin-4 proteins

Abstract

Dysregulation of voltage-gated calcium channel $\alpha_2\delta_1$ subunit ($Ca_v\alpha_2\delta_1$) and thrombospondin-4 (TSP4) proteins at the spinal level after peripheral nerve injury contributes to neuropathic pain states through an unknown mechanism. A recent finding showed that $Ca_v\alpha_2\delta_1$ is the neuronal TSP receptor in synapse formation. Based on these findings, I hypothesized a novel mechanism in which TSP4 and $Ca_v\alpha_2\delta_1$ proteins interact to cause neuropathic pain states. To determine if TSP4 and $Ca_v\alpha_2\delta_1$ indeed interact, I examined whether TSP4 and $Ca_v\alpha_2\delta_1$ interact in vivo and in vitro. Immunoprecipitation data indicate that both proteins are detectable in the same immunocomplexes from rodent spinal cords. In addition, $Ca_v\alpha_2\delta_1$ recombinant proteins bind to immobilized TSP4 proteins dose-dependently in vitro. These findings support that $Ca_v\alpha_2\delta_1$ proteins indeed interact directly with TSP4 in vitro and in vivo. To investigate the binding domains of TSP4 to $Ca_v\alpha_2\delta_1$, I made TSP4 recombinant truncation proteins for in vitro binding and found that the epidermal growth factor-like (EGF-like) domains and coil-coil domain of TSP4 have similar binding affinity to recombinant $Ca_v\alpha_2\delta_1$ proteins. This is confirmed by mapping the $Ca_v\alpha_2\delta_1$-binding domains of TSP4 with SPOT Peptide Array analysis that indicates multiple high affinity $Ca_v\alpha_2\delta_1$-binding peptides within these domains and the C-terminal domain. Thus, the interaction between $Ca_v\alpha_2\delta_1$ and TSP4 is likely mediated by multiple sites in the coil-coil and EGF-like domains of TSP4.

Introduction

$Ca_v\alpha_2\delta_1$ is a structural subunit of high voltage-gated calcium channels (Dolphin, 2009; Kim and Chung, 1992). So far, four $Ca_v\alpha_2\delta$-subunits have been identified ($Ca_v\alpha_2\delta_1$ through $Ca_v\alpha_2\delta_4$), each consisting of two disulfide-linked peptides (α_2 and δ) that are encoded by the same gene (De Jongh et al., 1990a; De Jongh et al., 1990b; Ellis et al., 1988). $Ca_v\alpha_2\delta_1$ subunit is ubiquitously expressed in many tissues and is highly expressed by CNS neurons (Cole et al., 2005). Functions of $Ca_v\alpha_2\delta_1$ include regulation and stabilization of VGCC trafficking to the plasma membrane and presynaptic terminals (Davies et al., 2007; Elmariah et al., 2004; Elmariah et al., 2005; Hoppa et al., 2012), fine-tuning channel functions and gating properties (Davies et al., 2006; Davies et al., 2010; Klugbauer et al., 1999; Klugbauer et al., 2003; Mori et al., 1991; Singer et al., 1991) and modulation of synaptogenesis and synaptic functions (Eroglu et al., 2009; Hoppa et al., 2012). Gabapentinoids, such as gabapentin and pregabalin, bind to $Ca_v\alpha_2\delta_1$ and $Ca_v\alpha_2\delta_2$ subunits with high affinity (Gee et al., 1996; Marais et al., 2001), and exhibit anti-neuropathic pain properties in patients (Backonja et al., 1998; Backonja and Glanzman, 2003; Dworkin and Kirkpatrick, 2005; Guay, 2005; Levendoglu et al., 2004; Rosner et al., 1996; Stacey et al., 2008; To et al., 2002; Zareba, 2005) and animal models (Hwang and Yaksh, 1997; Luo et al., 2002; Luo et al., 2001). Interestingly, only the $Ca_v\alpha_2\delta_1$, but not $Ca_v\alpha_2\delta_2$, gene is upregulated in neuropathic pain models after nerve injuries (Bauer et al., 2009; Li et al., 2014), and anti-neuropathic pain properties of gabapentinoids are abolished in knock-in mice expressing a mutant $Ca_v\alpha_2\delta_1$ that cannot bind gabapentin and pregabalin (Field et al., 2006).

Thrombospondins (TSPs) are secreted multi-domain extracellular glycoproteins involved in cell to cell and cell to matrix interactions. TSP consists of five isoforms in mammals, TSP1-5, which can be broken down into two subfamilies, subgroup A and B. TSP1 and TSP2 are part of the subgroup A family that shares the same structure and functional domains, and are assembled as trimers for secretion. Subgroup B consists

of TSP3-5 that forms a pentameric structure with functional domains different from that in Subgroup A. This subfamily lacks the procollagen domain and type 1 repeats, and they are less than 50% homologous to TSP1/2 (Adams and Lawler, 2004). TSP1 and TSP2 are involved in modulation of platelet aggregation, angiogenesis, inflammation, extracellular matrix assembly and wound repair. TSP5 is mainly expressed in cartilage and connective tissues, involved in regulation of differentiation, attachment of chondrocyte, as well as assembly of cartilage extracellular matrix. While TSP3/4 are expressed in multiple sites, their functional roles are not well defined (Adams and Lawler, 2004). It has been suggested that TSP4 may be involved in local signaling in the nervous system because it is a preferred substrate for neurons in promoting neurite outgrowth (Arber and Caroni, 1995). Recent studies have suggested that TSP4 proteins interact with $Ca_v\alpha_2\delta_1$ in mediating pathophysiological conditions (Eroglu et al., 2009; Xu et al., 2009). In this study, I investigated if interaction between TSP4 and $Ca_v\alpha_2\delta_1$ proteins occur, and what TSP4 domains are involved in binding to $Ca_v\alpha_2\delta_1$.

Methods and Materials

Construction of the Recombinant TSP4 Truncated cDNA: The recombinant truncated TSP4 proteins were prepared by the PCR-driven overlap extension technique (Heckman and Pease, 2007) using full-length rat TSP4 cDNA (Genbank accession #: X89963) in pCEP-Pu vector as template. Segments of the TSP4 cDNA were amplified using two flanking master primers and internal primers that introduced the mutation for truncation and created an overlapping nucleotide sequence. The construct with the N-terminal domain of TSP4 deletion was prepared with the internal primers 5'- CACGCGCTAGTCTCTCTGTTCCAG-3' (forward), 5'- CTGGAACAGAGAGACTAGCGCGTG-3' (reverse), and master primers 5'-CGCAGAACTGGTAGGTATGG-3' (forward), 5'-CTTATCATGTCTGGATCCGGC-3' (reverse). The construct containing only the EGF-like domain of TSP4 was obtained with internal primers 5'-CGCGCTAGTCCCAACACGTC-3' (forward) and 5'- GCTTCTTGGTTTATCCACAGACATAG-3' (reverse), 5'-CTATGTCTGTGGATAAACCAAGAAGC-3' (forward) and 5'-GACGTGTTGGGACTAGCGCG-3' (reverse), and master primers 5'-CGGGACTTTCCTACTTGGCAG-3' (forward), 5'-CCCGACACCCGCCAACACC-3' (reverse). The construct containing the type-3 calcium binding domain and C-terminal domain of TSP4 was created with the internal primer 5'- CACGCGCTAGTCAAGGATGTGGAC-3' (forward), 5'-GTCCACATCCTTGACTAGCGCGTG-3' (reverse), and master primers 5'-CGCAGAACTGGTAGGTATGG-3' (forward), 5'-CTTATCATGTCTGGATCCGGC-3' (reverse). The construct containing the N-terminal domain only construct was prepared with the internal primers 5'-GTGGTGAGGGGCTAAACCAAGAAG-3' (forward), 5'-CTTCTTGGTTTAGCCCCTCACCAC-3' (reverse), and master primers 5'-CTAGAAGCTGGGTACCTTAAGGC-3' (forward), and 5'-CAAGCTGTGACCGTCTCCG-3' (reverse). The construct containing

the N-terminal domain and coil-coil domain of TSP4 was obtained with the internal primers 5'-CTCCTCCAGCATAAACCAAGAAGC-3' (forward) and 5'-GCTTCTTGGTTTATGCTGGAGGAG-3' (reverse). The same master primers for the N-terminal domain only construct was used. The constructs were inserted back into pCEP-pu vector containing the BM40 signal peptide (Kohfeldt et al., 1997) (Narouz-Ott et al., 2000) and N-terminal 6x-Histdine tag using restriction sites KpnI and BamHI. The constructs were confirmed by sequencing (Eton Biosciences, San Diego, CA).

Expression and Purification of Recombinant TSP4 proteins: The recombinant rat TSP4 cDNA (Genbank accession #: X89963) with a six-histidine tag at the N-terminal was transfected into human embryonic kidney cell line 293-EBNA cells (Invitrogen, Carlsbad, CA) using the calcium phosphate transfection method. The transfected cells were selected with 0.5µg/ml puromycin and grown to confluency. Secretion of the full-length TSP4-His and truncated TSP4-His proteins into DMEM/F-12 medium (Mediatech, Manassas, VA) was confirmed by Western blot using anti-penta His monoclonal antibody (Qiagen, Valencia, CA). The TSP4-His proteins were purified using a Ni-NTA column based on the manufacturer's instructions (Invitrogen, 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. The recombinant truncated TSP4-His proteins were expressed and purified in a similar manner.

Purification of FLAG-$Ca_V\alpha_2\delta_1$: FLAG-$Ca_V\alpha_2\delta_1$ cDNA was transiently transfected into human embryonic kidney cells-293 (HEK 293) using lipofecatime 2000 (Invitrogen). The transfected cells were washed twice with PBS buffer, then extracted in protein extraction buffer (50mM Tris, 150mM NaCl, 1mM EDTA, 0.1% Triton-X, pH7.4) in two-three days. The cell lysate was incubated on ice for 15 min followed by centrifugation at 13,000 g for 20 minutes at 4 °C. The supernatant was incubated with anti-FLAG M2

agarose affinity resin (Sigma-Aldridge, St. Louis, MO) by rotating for 2 hr at 4 °C, and then washed three times with protein extraction buffer. FLAG-$Ca_V\alpha_2\delta_1$ was eluted in elution buffer (0.1M glycine, pH 3.5) and stored at -20 °C for future use.

Solid-Phase Binding: The reagents for solid-phase binding were from Invitrogen (Cat # CNB0011). Recombinant truncated TSP4 proteins were immobilized onto a 96-well polystyrene plates (Thermo) overnight at 4 °C in coating buffer A. All further incubations were carried out at RT for 1 hr, and proteins or antibodies were diluted in assay buffer containing bovine serum albumin (BSA). After blocking, the plates were incubated with affinity purified FLAG-$Ca_v\alpha_2\delta_1$ for 1 hr, washed, then incubated with mouse monoclonal anti-FLAG antibody (1:1000; Sigma-Aldridge), followed by horse radish peroxidase (HRP)-conjugated secondary antibody. The bound FLAG-$Ca_v\alpha_2\delta_1$ complexes were detected by measuring a color reaction (yellow product) at 450 nm after adding tetramethylbenzidine (TMB) for 15 min followed by adding sulfuric acid to stop the reaction.

Membrane Preparation and Radioligand Binding: TsA-201 cells stably expressing $Ca_V2.2e$ [Δ24a, 31a], $Ca_V\beta_3$, and $Ca_V\alpha_2\delta_1$ (Lin et al., 2004) were harvested in Tris/EDTA buffer (5mM Tris/HCL, 5mM EDTA, pH 7.4 containing PMSF, leupeptin, and pepstatin A), incubated on ice for 15 min, sonicated, and then centrifuged at 1000 g for 10 min. The supernatant was collected and centrifuged at 50,000 g for 30 min at 4 °C. The pellet was re-suspended in the same buffer. Cell membranes (50μg) were incubated in the presence of 20nM of [^{3}H]gabapentin (PerkinElmer, Waltham, MA) with or without purified recombinant TSP4 proteins in 10mM HEPES (pH 7.4) for 90 min at RT, and filtered onto pre-wetted GF/C filters under vacuum. The filters containing the cell membranes were washed three times with 3ml of ice-cold Tris buffer (pH 7.4), and used for liquid-scintillation counting. For nonspecific binding, the binding assay was performed in the presence of 10μM gabapentin. Specific binding was obtained by subtracting nonspecific binding from total binding. All experiments were carried out in duplicates and plotted using

Graphpad Prism (Graphpad Software, San Diego, CA)

Surface Plasmon Resonance Binding: All experiments were carried out using BIAcore 3000 and CM5 Sensor Chip (GE Healthcare Sciences, Piscataway, NJ), and at 25°C. $Ca_V\alpha_2\delta_1$ antibody (mouse, Sigma-Aldrich St. Louis, MO) was coupled to the dextran matrix of a CM5 sensor chip using Amine Coupling Kit as described by (Johnsson et al., 1991). Excess reactive esters were quenched by injection of 1.0M ethanolamine-hydrochloride, pH 8.5. The binding assays were performed using HBS-P buffer (0.01M HEPES, pH 7.4, 0.15M NaCl, 0.005% and surfactant P20) as running buffer. Purified TSP4 proteins and $Ca_V\alpha_2\delta_1$ protein extracts from tsA-201 cells stably expressing $Ca_V2.2e$ [Δ24a, 31a], $Ca_V\beta_3$, and $Ca_V\alpha_2\delta_1$ (Lin et al., 2004) as described earlier were diluted in HBS-P buffer (GE Healthcare). $Ca_V\alpha_2\delta_1$ protein extracts were injected at a flow rate of 10µl/min over the immobilized $Ca_V\alpha_2\delta_1$ antibody flow cells, followed by injection of purified TSP4 proteins at a flow rate of 20µl/min. Non-specific binding of TSP4 to the flow cell without the presence of immobilized $Ca_V\alpha_2\delta_1$ antibody was subtracted from all binding curves using BIAevaluation software version 3.0 (GE Healthcare) and plotted using Graphpad Prism (Graphpad Software).

Results

TSP4 binds to $Ca_v\alpha_2\delta_1$

To examine potential interactions between TSP4 and $Ca_v\alpha_2\delta_1$, I examined if both proteins were detectable in immunoprecipitation complexes from in vivo and in vitro experiments. My data showed that $Ca_v\alpha_2\delta_1$ proteins were detectable in TSP4 immunoprecipitates from rat and mouse spinal cord extracts (Fig. 1A). In addition, TSP4 proteins were detectable from $Ca_v\alpha_2\delta_1$-immunoprecipitates from cells overexpressing $Ca_v\alpha_2\delta_1$ and TSP4 recombinant proteins in vitro (Fig. 1B). These data support that $Ca_v\alpha_2\delta_1$/TSP4 proteins are at least in the same immunocomplexes. To determine if a direct interaction between $Ca_v\alpha_2\delta_1$/TSP4 proteins occur, I performed an ELISA-based ligand binding of recombinant $Ca_v\alpha_2\delta_1$ proteins to immobilized TSP4 in transient expression experiments in vitro. My data indicated that recombinant $Ca_v\alpha_2\delta_1$ proteins bound to TSP4 proteins dose-dependently (Fig. 2A), supporting a direct interaction of these proteins. Conversely, I used surface plasmon resonance spectroscopy (SPR) to measure the real-time binding of TSP4 proteins to immobilized $Ca_v\alpha_2\delta_1$ on a BIAcore CM5 sensor chip. $Ca_v\alpha_2\delta_1$ proteins from tsA-201 cell lysates overexpressing VGCC α_{1b}, β_3, and $Ca_v\alpha_2\delta_1$ subunits were immobilized on the chip surface by covalently bound $Ca_v\alpha_2\delta_1$ antibodies. Injected TSP4 proteins bound to immobilized $Ca_v\alpha_2\delta_1$ in a dose-dependent manner, which was in agreement with the ELISA-based ligand binding assay (Fig. 2B).

To examine whether the interaction between TSP4 and $Ca_v\alpha_2\delta_1$ can be blocked by $Ca_v\alpha_2\delta_1$-ligand gabapentin, I measured [^{3}H]gabapentin binding to tsA-201 cell lysates overexpressing VGCC α_{1b}, β_3, and $Ca_v\alpha_2\delta_1$ subunits in the presence of purified TSP4 proteins. Binding analysis showed that TSP4 could dose-dependently decrease bound [^{3}H]gabapentin binding to $Ca_v\alpha_2\delta_1$, further supporting an interaction between TSP4 and $Ca_v\alpha_2\delta_1$ proteins.

Mapping of the TSP4 binding motifs to $Ca_v\alpha_2\delta_1$

To identify the TSP4 binding domain to $Ca_v\alpha_2\delta_1$, I created the following five recombinant truncation constructs of TSP4: N-terminal domain deletion construct that encodes the EGF-like domain, calcium binding domain, and C-terminal domain [ND]; EGF-like domains alone construct (EGF-like); calcium binding domain and C-terminal domain (CD); N-terminal domain alone construct (NT); and N-terminal domain together with coil-coil domain (NT+CC) (Fig. 3A). The TSP4 mutant proteins from these constructs were expressed in vitro and purified through His-tag columns (Fig. 3B). The recombinant truncated TSP4 proteins were tested in an ELISA-based ligand-binding assay for binding to recombinant $Ca_v\alpha_2\delta_1$ proteins. The EGF-like domain containing proteins (ND and EGF-like) and coil-coil domain proteins of TSP4 bound to $Ca_v\alpha_2\delta_1$ with a higher affinity than proteins containing the N-terminal domain, calcium-binding domain, and C-terminal domain (Fig. 4A). My data indicated that the coil-coil and EGF-like domains of TSP4 contained the major binding sites to $Ca_v\alpha_2\delta_1$ proteins.

I also synthesized a series of overlapping 15-mer peptides that cover the entire length of TSP4 to further delineate the TSP4 binding motif to $Ca_v\alpha_2\delta_1$. Similarly, specific TSP4 linear peptides encoding the coil-coil and EGF-like domains, but not calcium-binding domain peptides bound to $Ca_v\alpha_2\delta_1$ over-expressing lysates (Fig. 4B). A few specific TSP4 peptides from the N-terminal and C-terminal domain also bound to $Ca_v\alpha_2\delta_1$ (Fig. 4B), which may contribute to the low binding affinity to $Ca_v\alpha_2\delta_1$ observed from the NT and CD truncated proteins (Fig. 4A).

Discussion

TSPs are secreted multidomain glycoproteins, and are involved in diverse biological processes given their ability to bind numerous proteins in the extracellular matrix. For instance, a recent study has identified $Ca_v\alpha_2\delta_1$ as the neuronal TSP receptor involved in promoting excitatory synaptogenesis (Christopherson et al., 2005; Eroglu et al., 2009). Interestingly, TSP4 and $Ca_v\alpha_2\delta_1$ proteins are known to play a causal role in neuropathic pain through an unknown mechanism (Kim et al., 2012; Li et al., 2004). It is likely that injury-induced TSP4 causes spinal sensitization and neuropathic pain through interactions with the $Ca_v\alpha_2\delta_1$. Here, I show that TSP4 proteins directly bind to $Ca_v\alpha_2\delta_1$ in vitro and in the spinal cord. Through the use of an array of linear peptides that cover the whole amino acid sequence of TSP4, I determined the core $Ca_v\alpha_2\delta_1$-binding motifs to the coil-coil domain, EGF-like domain, and C-terminal domain of TSP4. Deletion of the $Ca_v\alpha_2\delta_1$-binding motifs from TSP4 significantly diminished $Ca_v\alpha_2\delta_1$ binding to immobilized TSP4 truncated proteins in an ELISA-based binding assay. Thus, the interaction of these proteins may be critical in mediating spinal dorsal horn neuron sensitization and regulating behavioral hypersensitivity during chronic pain states.

Binding sites can be typically divided as: 1) linear sites that follow the primary amino acid sequences; 2) nonlinear binding sites that are made up of short fragments and are adjacent in spatial proximity. To better understand the binding sites in TSP4 to $Ca_v\alpha_2\delta_1$, I used SPOT peptide synthesis method and ELISA-based binding assay for identification of linear binding sites and discontinuous binding sites. Specific TSP4 linear peptides from the coil-coil domain or EGF-like domains of TSP4, but not the calcium binding type-3 domains bind to $Ca_v\alpha_2\delta_1$ with considerable affinity; thereby suggesting that the coil-coil domain and EGF-like domains of TSP4, when presented as unstructured parts of TSP4, may represent a linear binding site for $Ca_v\alpha_2\delta_1$. However, a few discontinuous TSP4 peptides from the N-terminal and C-terminal domain of TSP4 do bind to $Ca_v\alpha_2\delta_1$ with high affinity. In fact, truncated TSP4 proteins containing the N-terminal or C-terminal domain

exhibit low affinity binding to $Ca_v\alpha_2\delta_1$. Since $Ca_v\alpha_2\delta_1$ binds to multiple non-continuous TSP4 peptides, this suggests that the interaction between TSP4 and $Ca_v\alpha_2\delta_1$ is mediated through multi-domains (N-terminal, coil-coil domain, EGF-like domain, and C-terminal domain of TSP4) and may be conformationally defined.

The functional effect of the coil-coil domain and EGF-like domains of TSP4 in pain processing is unclear. TSPs are characterized by containing the coil-coil domain, EGF-like domains, calcium-binding type 3 repeats, and a highly conserved C-terminal domain. The coil-coil domain of TSPs is important for the assembly of TSP proteins into trimers (TSP1/2) or pentamers (TSP3/4/5) (Adams and Lawler, 2004; Lawler et al., 1985; Lawler et al., 1995; Sottile et al., 1991). In contrast, the EGF-like domains of TSP4 have been shown to bind to the von Willebrand Factor A (VWF-A) domain of integrin proteins that interact with extracellular matrix proteins (Pluskota et al., 2005; Whittaker and Hynes, 2002). In addition, the EGF-like domains of TSPs bind to the VWF-A domain of $Ca_v\alpha_2\delta_1$ proteins and cause excitatory synapse formation (Eroglu et al., 2009). Interestingly, injury-induced upregulation of $Ca_v\alpha_2\delta_1$ proteins in the superficial dorsal spinal cord may cause abnormal excitatory synaptogenesis and neuronal hyperexcitability (Li et al., 2014). Thus, I propose that the binding of the EGF-like domains of TSP4 to $Ca_v\alpha_2\delta_1$ may trigger a structural conformational change since it is known that VWF-A domains can switch a protein's structure from an inactive to an activate state upon ligand binding (Whittaker and Hynes, 2002). The TSP4/$Ca_v\alpha_2\delta_1$ complex may then induce an intracellular signaling cascade that ultimately leads abnormal excitatory synaptogenesis and chronic pain states. Identification of proteins associated with TSP4/$Ca_v\alpha_2\delta_1$ complexes could provide critical insight on downstream mechanisms of TSP4/$Ca_v\alpha_2\delta_1$ and development of chronic pain states.

The $Ca_v\alpha_2\delta_1$ subunits were identified as the molecular targets for gabapentin. However, it is unclear whether gabapentin has any consistent drug effect on VGCC function, despite the high affinity of gabapentin to $Ca_v\alpha_2\delta_1$ subunits. It has been reported

that the anti-neuropathic pain effects of gabapentin correlate with the expression level of its binding protein $Ca_v\alpha_2\delta_1$ in animal models (Luo et al., 2002). Mutation of the binding site for gabapentinoids within $Ca_v\alpha_2\delta_1$ abolishes the anti-hyperalgesic properties of the drugs (Field et al., 2006), establishing that binding of gabapentinoids to $Ca_v\alpha_2\delta_1$ is responsible for their anti-hyperalgesic actions. Gabapentin can also inhibit TSP4-induced dorsal horn neuron sensitization and behavioral hypersensitivity (Park et al., submitted; in revision), suggesting that elevated spinal TSP4 mediates spinal neuron sensitization and pain states through its interaction with $Ca_v\alpha_2\delta_1$. Since gabapentin binding site is located upstream of the VWF-A domain of $Ca_v\alpha_2\delta_1$ (Wang et al., 1999), it is less likely that the inhibitory effects of gabapentin on synaptogenesis, dorsal horn neuron sensitization, and hyperalgesia derive directly from binding of TSP4 to $Ca_v\alpha_2\delta_1$. In support of this notion, competitive binding between TSP4 and [^{3}H]gabapentin showed only partially diminished binding of gabapentin to $Ca_v\alpha_2\delta_1$ at high doses of TSP4. In addition, gabapentin did not affect the binding between TSP4 and $Ca_v\alpha_2\delta_1$ using the ELISA-based binding assay and SPR (data not shown). Instead, gabapentin binding to $Ca_v\alpha_2\delta_1$ may cause conformational changes to the protein and therefore interfere with TSP4/$Ca_v\alpha_2\delta_1$ interactions indirectly.

In summary, my findings provide evidences to support that TSP4 directly interacts with $Ca_v\alpha_2\delta_1$ proteins through its EGF-like domains and coil-coil domain. Blocking the interaction of these proteins could lead to long-term downstream effects in modulating chronic pain states.

References

Adams JC, Lawler J. 2004. The thrombospondins. Int J Biochem Cell Biol 36(6):961-968.

Arber S, Caroni P. 1995. Thrombospondin-4, an extracellular matrix protein expressed in the developing and adult nervous system promotes neurite outgrowth. J Cell Biol 131(4):1083-1094.

Backonja M, Beydoun A, Edwards KR, Schwartz SL, Fonseca V, Hes M, LaMoreaux L, Garofalo E. 1998. Gabapentin for the symptomatic treatment of painful neuropathy in patients with diabetes mellitus: a randomized controlled trial [see comments]. Jama 280(21):1831-1836.

Backonja M, Glanzman RL. 2003. Gabapentin dosing for neuropathic pain: evidence from randomized, placebo-controlled clinical trials. Clin Ther 25(1):81-104.

Bauer CS, Nieto-Rostro M, Rahman W, Tran-Van-Minh A, Ferron L, Douglas L, Kadurin I, Sri Ranjan Y, Fernandez-Alacid L, Millar NS, Dickenson AH, Lujan R, Dolphin AC. 2009. The increased trafficking of the calcium channel subunit alpha2delta-1 to presynaptic terminals in neuropathic pain is inhibited by the alpha2delta ligand pregabalin. J Neurosci 29(13):4076-4088.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA. 2005. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120(3):421-433.

Cole RL, Lechner SM, Williams ME, Prodanovich P, Bleicher L, Varney MA, Gu G. 2005. Differential distribution of voltage-gated calcium channel alpha-2 delta (alpha2delta) subunit mRNA-containing cells in the rat central nervous system and the dorsal root ganglia. J Comp Neurol 491(3):246-269.

Davies A, Douglas L, Hendrich J, Wratten J, Tran Van Minh A, Foucault I, Koch D, Pratt WS, Saibil HR, Dolphin AC. 2006. The calcium channel alpha2delta-2 subunit partitions with CaV2.1 into lipid rafts in cerebellum: implications for localization and function. J Neurosci 26(34):8748-8757.

Davies A, Hendrich J, Van Minh AT, Wratten J, Douglas L, Dolphin AC. 2007. Functional biology of the alpha(2)delta subunits of voltage-gated calcium channels. Trends Pharmacol Sci 28(5):220-228.

Davies A, Kadurin I, Alvarez-Laviada A, Douglas L, Nieto-Rostro M, Bauer CS, Pratt WS, Dolphin AC. 2010. The alpha2delta subunits of voltage-gated calcium channels form GPI-anchored proteins, a posttranslational modification essential for function. Proc Natl Acad Sci U S A 107(4):1654-1659.

De Jongh KS, Warner C, Catterall WA. 1990a. Subunits of purified calcium channels. Alpha 2 and delta are encoded by the same gene. J Biol Chem 265(25):14738-14741.

Dolphin AC. 2009. Calcium channel diversity: multiple roles of calcium channel subunits. Curr Opin Neurobiol 19(3):237-244.

Dworkin RH, Kirkpatrick P. 2005. Pregabalin. Nat Rev Drug Discov 4(6):455-456.

Ellis SB, Williams ME, Ways NR, Brenner R, Sharp AH, Leung AT, Campbell KP, McKenna E, Koch WJ, Hui A, et al. 1988. Sequence and expression of mRNAs encoding the alpha 1 and alpha 2 subunits of a DHP-sensitive calcium channel. Science 241(4873):1661-1664.

Elmariah SB, Hughes EG, Oh EJ, Balice-Gordon RJ. 2004. Neurotrophin signaling among neurons and glia during formation of tripartite synapses. Neuron glia biology 1(4):1-11.

Elmariah SB, Oh EJ, Hughes EG, Balice-Gordon RJ. 2005. Astrocytes regulate inhibitory synapse formation via Trk-mediated modulation of postsynaptic GABAA receptors. J Neurosci 25(14):3638-3650.

Eroglu C, Allen NJ, Susman MW, O'Rourke NA, Park CY, Ozkan E, Chakraborty C, Mulinyawe SB, Annis DS, Huberman AD, Green EM, Lawler J, Dolmetsch R, Garcia KC, Smith SJ, Luo ZD, Rosenthal A, Mosher DF, Barres BA. 2009. Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for

excitatory CNS synaptogenesis. Cell 139(2):380-392.

Field MJ, Cox PJ, Stott E, Melrose H, Offord J, Su TZ, Bramwell S, Corradini L, England S, Winks J, Kinloch RA, Hendrich J, Dolphin AC, Webb T, Williams D. 2006. Identification of the {alpha}2-{delta}-1 subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin. Proc Natl Acad Sci U S A 103(46):17537-17542. Epub 12006 Nov 17536.

Gee NS, Brown JP, Dissanayake VU, Offord J, Thurlow R, Woodruff GN. 1996. The novel anticonvulsant drug, gabapentin (Neurontin), binds to the alpha2delta subunit of a calcium channel. J Biol Chem 271(10):5768-5776.

Guay DR. 2005. Pregabalin in neuropathic pain: a more “pharmaceutically elegant” gabapentin? Am J Geriatr Pharmacother 3(4):274-287.

Heckman KL, Pease LR. 2007. Gene splicing and mutagenesis by PCR-driven overlap extension. Nat Protoc 2(4):924-932.

Hoppa MB, Lana B, Margas W, Dolphin AC, Ryan TA. 2012. alpha2delta expression sets presynaptic calcium channel abundance and release probability. Nature 486(7401):122-125.

Hwang JH, Yaksh TL. 1997. Effect of subarachnoid gabapentin on tactile-evoked allodynia in a surgically induced neuropathic pain model in the rat. Reg Anesth 22(3):249-256.

Johnsson B, Lofas S, Lindquist G. 1991. Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. Anal Biochem 198(2):268-277.

Kim DS, Li KW, Boroujerdi A, Peter Yu Y, Zhou CY, Deng P, Park J, Zhang X, Lee J, Corpe M, Sharp K, Steward O, Eroglu C, Barres B, Zaucke F, Xu ZC, Luo ZD. 2012. Thrombospondin-4 contributes to spinal sensitization and neuropathic pain states. J Neurosci 32(26):8977-8987.

Kim SH, Chung JM. 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50(3):355-363.

Klugbauer N, Lacinova L, Marais E, Hobom M, Hofmann F. 1999. Molecular diversity of the calcium channel alpha2delta subunit. J Neurosci 19(2):684-691.

Klugbauer N, Marais E, Hofmann F. 2003. Calcium channel alpha2delta subunits: differential expression, function, and drug binding. J Bioenerg Biomembr 35(6):639-647.

Kohfeldt E, Maurer P, Vannahme C, Timpl R. 1997. Properties of the extracellular calcium binding module of the proteoglycan testican. FEBS letters 414(3):557-561.

Lawler J, Derick LH, Connolly JE, Chen JH, Chao FC. 1985. The structure of human platelet thrombospondin. J Biol Chem 260(6):3762-3772.

Lawler J, McHenry K, Duquette M, Derick L. 1995. Characterization of human thrombospondin-4. J Biol Chem 270(6):2809-2814.

Levendoglu F, Ogun CO, Ozerbil O, Ogun TC, Ugurlu H. 2004. Gabapentin is a first line drug for the treatment of neuropathic pain in spinal cord injury. Spine 29(7):743-751.

Li CY, Song YH, Higuera ES, Luo ZD. 2004. Spinal dorsal horn calcium channel alpha2delta-1 subunit upregulation contributes to peripheral nerve injury-induced tactile allodynia. J Neurosci 24(39):8494-8499.

Li KW, Yu YP, Zhou C, Kim DS, Lin B, Sharp K, Steward O, Luo ZD. 2014. Calcium channel alpha2delta1 proteins mediate trigeminal neuropathic pain states associated with aberrant excitatory synaptogenesis. J Biol Chem 289(10):7025-7037.

Lin Y, McDonough SI, Lipscombe D. 2004. Alternative splicing in the voltage-sensing region of N-Type CaV2.2 channels modulates channel kinetics. J Neurophysiol 92(5):2820-2830.

Luo ZD, Calcutt NA, Higuera ES, Valder CR, Song YH, Svensson CI, Myers RR. 2002. Injury type-specific calcium channel alpha 2 delta-1 subunit up-regulation in rat

neuropathic pain models correlates with antiallodynic effects of gabapentin. J Pharmacol Exp Ther 303(3):1199-1205.

Luo ZD, Chaplan SR, Higuera ES, Sorkin LS, Stauderman KA, Williams ME, Yaksh TL. 2001. Upregulation of dorsal root ganglion (alpha)2(delta) calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. J Neurosci 21(6):1868-1875.

Marais E, Klugbauer N, Hofmann F. 2001. Calcium channel alpha2delta subunits-structure and gabapentin binding. Molecular Pharmacology 59(5):1243-1248.

Mori Y, Friedrich T, Kim MS, Mikami A, Nakai J, Ruth P, Bosse E, Hofmann F, Flockerzi V, Furuichi T, et al. 1991. Primary structure and functional expression from complementary DNA of a brain calcium channel. Nature 350(6317):398-402.

Narouz-Ott L, Maurer P, Nitsche DP, Smyth N, Paulsson M. 2000. Thrombospondin-4 binds specifically to both collagenous and non-collagenous extracellular matrix proteins via its C-terminal domains. J Biol Chem 275(47):37110-37117.

Pluskota E, Stenina OI, Krukovets I, Szpak D, Topol EJ, Plow EF. 2005. Mechanism and effect of thrombospondin-4 polymorphisms on neutrophil function. Blood 106(12):3970-3978.

Rosner H, Rubin L, Kestenbaum A. 1996. Gabapentin adjunctive therapy in neuropathic pain states. Clin J Pain 12(1):56-58.

Singer D, Biel M, Lotan I, Flockerzi V, Hofmann F, Dascal N. 1991. The roles of the subunits in the function of the calcium channel. Science 253(5027):1553-1557.

Sottile J, Selegue J, Mosher DF. 1991. Synthesis of truncated amino-terminal trimers of thrombospondin. Biochemistry 30(26):6556-6562.

Stacey BR, Dworkin RH, Murphy K, Sharma U, Emir B, Griesing T. 2008. Pregabalin in the treatment of refractory neuropathic pain: results of a 15-month open-label trial. Pain Med 9(8):1202-1208.

To TP, Lim TC, Hill ST, Frauman AG, Cooper N, Kirsa SW, Brown DJ. 2002. Gabapentin for neuropathic pain following spinal cord injury. Spinal Cord 40(6):282-285.

Wang M, Offord J, Oxender DL, Su TZ. 1999. Structural requirement of the calcium-channel subunit alpha2delta for gabapentin binding. Biochem J 342 (Pt 2):313-320.

Whittaker CA, Hynes RO. 2002. Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Mol Biol Cell 13(10):3369-3387.

Xu J, Xiao N, Xia J. 2009. Thrombospondin 1 accelerates synaptogenesis in hippocampal neurons through neuroligin 1. Nat Neurosci 13(1):22-24.

Zareba G. 2005. Pregabalin: a new agent for the treatment of neuropathic pain. Drugs Today (Barc) 41(8):509-516.

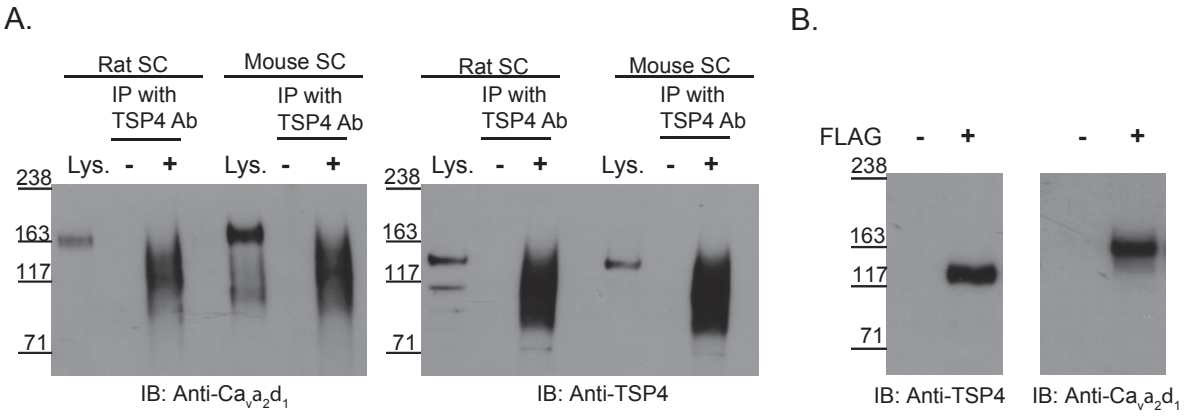

Figure 1. TSP4 interacts with $Ca_v\alpha_2\delta_1$.

A. Representative Western blots showing co-immunoprecipitation (IP) of $Ca_v\alpha_2\delta_1$ with TSP4 proteins by anti-TSP4 antibody. IP from rat and mouse spinal cord samples. Lys.: spinal cord lysate as a positive control. -: no IP anti-TSP4 antibody. +: with IP anti-TSP4 antibody. Estimated molecular weights (kDA) are shown on the left.

B. Representative Western blot showing IP of FLAG-tagged $Ca_v\alpha_2\delta_1$ with TSP4 proteins by anti-FLAG antibodies. IP from 293-EBNA cell line co-transfected with TSP4 and FLAG-tagged $Ca_v\alpha_2\delta_1$ cDNA. +: with transfection of FLAG-tagged $Ca_v\alpha_2\delta_1$. -: without transfection of the FLAG-tagged $Ca_v\alpha_2\delta_1$ construct. Estimated molecular weights (kDA) are shown on the left.

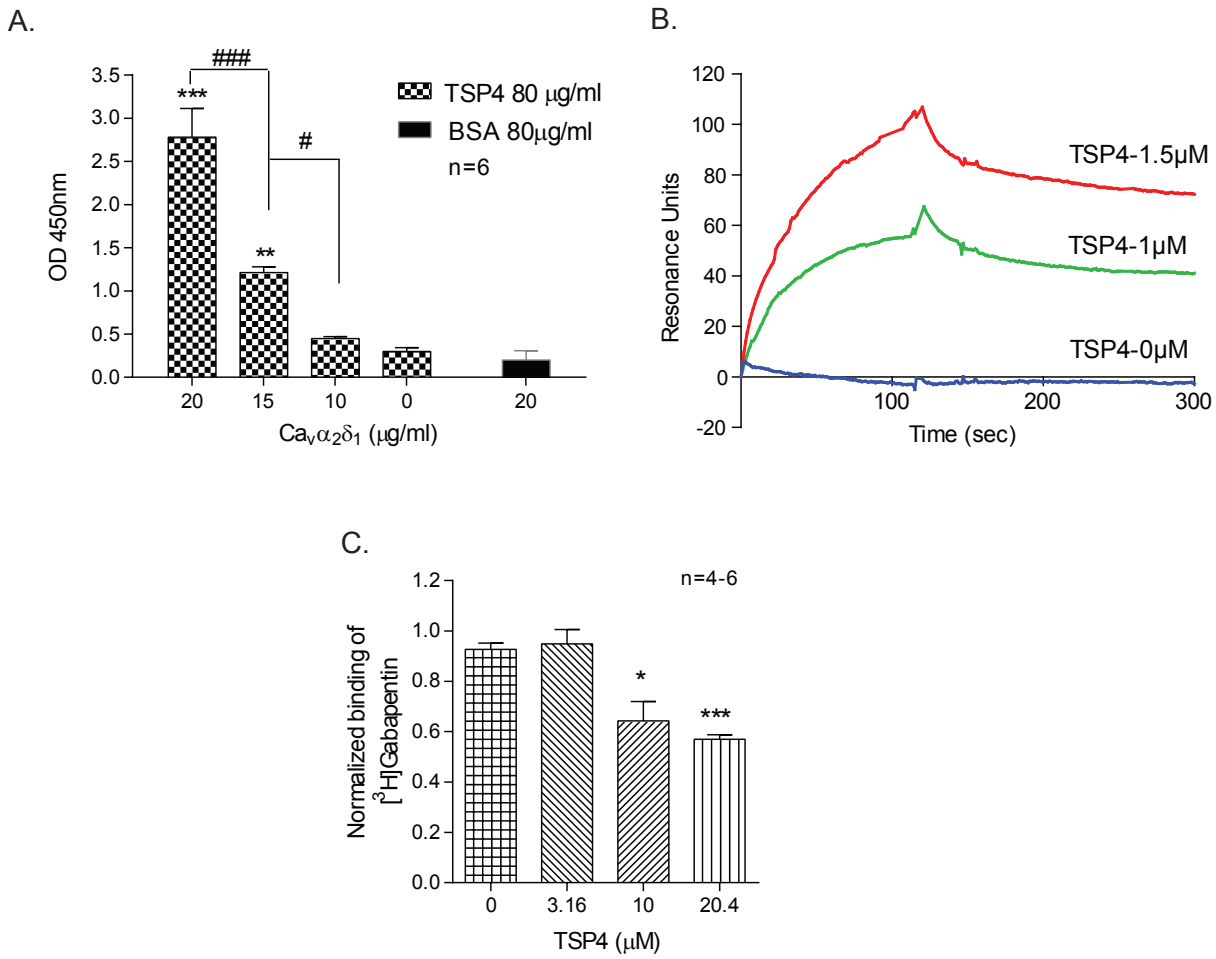

Figure 2. Direct binding of TSP4 to $Ca_v\alpha_2\delta_1$.

A. Summarized data (means ± SEM; n=6) showing that FLAG-$Ca_v\alpha_2\delta_1$, but not bovine serum albumin (BSA), dose-dependently binds to immobilized TSP4. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the background level (without adding FLAG- $Ca_v\alpha_2\delta_1$), $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared between adjacent doses by one-way ANOVA analysis with Bonferroni post-tests.

B. Sensogram of TSP4 dose-dependently binding to captured $Ca_v\alpha_2\delta_1$. $Ca_v\alpha_2\delta_1$ antibody was immobilized to a CM5 sensor chip to capture $Ca_v\alpha_2\delta_1$ proteins from $Ca_v\alpha_2\delta_1$ overexpressing protein extracts at a flow rate of 10μl/min. HBS-P buffer or purified TSP4 proteins were injected over a captured $Ca_v\alpha_2\delta_1$ flow cell at a flow rate of 20μl/min.

C. Radioligand binding of [^{3}H]gabapentin to $Ca_v\alpha_2\delta_1$ over-expressing lysates in the presence of purified TSP4 proteins. The final concentration of [^{3}H]gabapentin was 20nM. Values are the means ± SEM; n=4-6. $^{*}p < 0.05$, $^{***}p < 0.001$ compared with the background level (without adding TSP4) by one-way ANOVA analysis with Bonferroni post-tests.

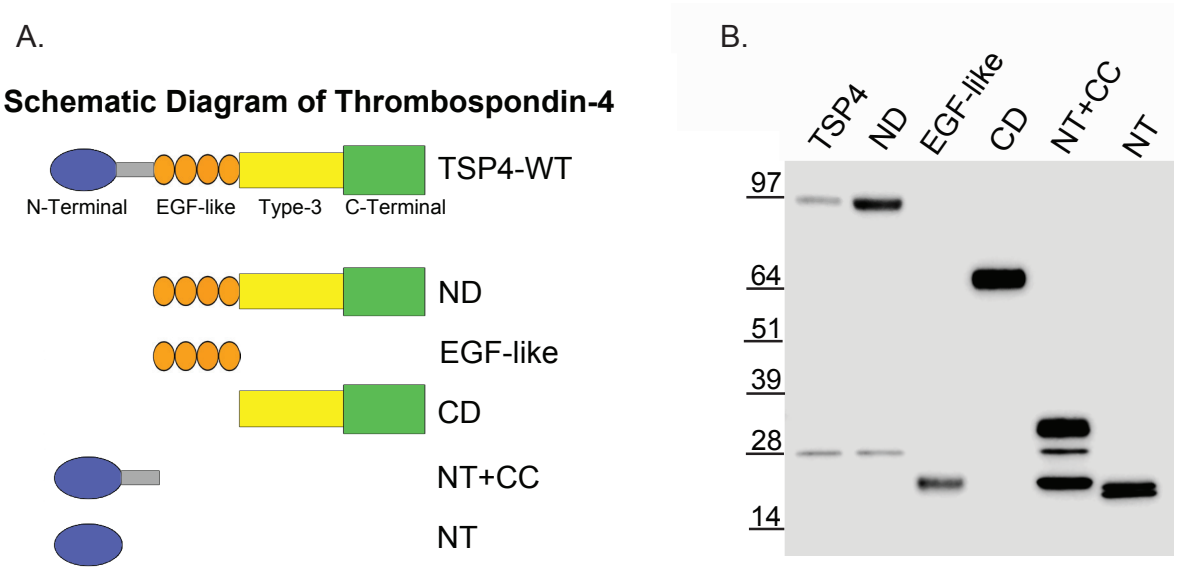

Figure 3. Creation and expression of recombinant TSP4 proteins.
A. Domain structure of full-length TSP4 and recombinant TSP4 truncation proteins. Top: N-terminal domain (blue), coiled-coil oligomerization domain (grey), four type II EGF-like domain repeats (orange), seven type III calcium binding repeats (yellow), and the C-terminal domain (green).
B. A representative Western blot of the purified recombinant TSP4 proteins detected by anti-His antibodies under reducing conditions. The approximate molecular weight (kDa) is show on the left.

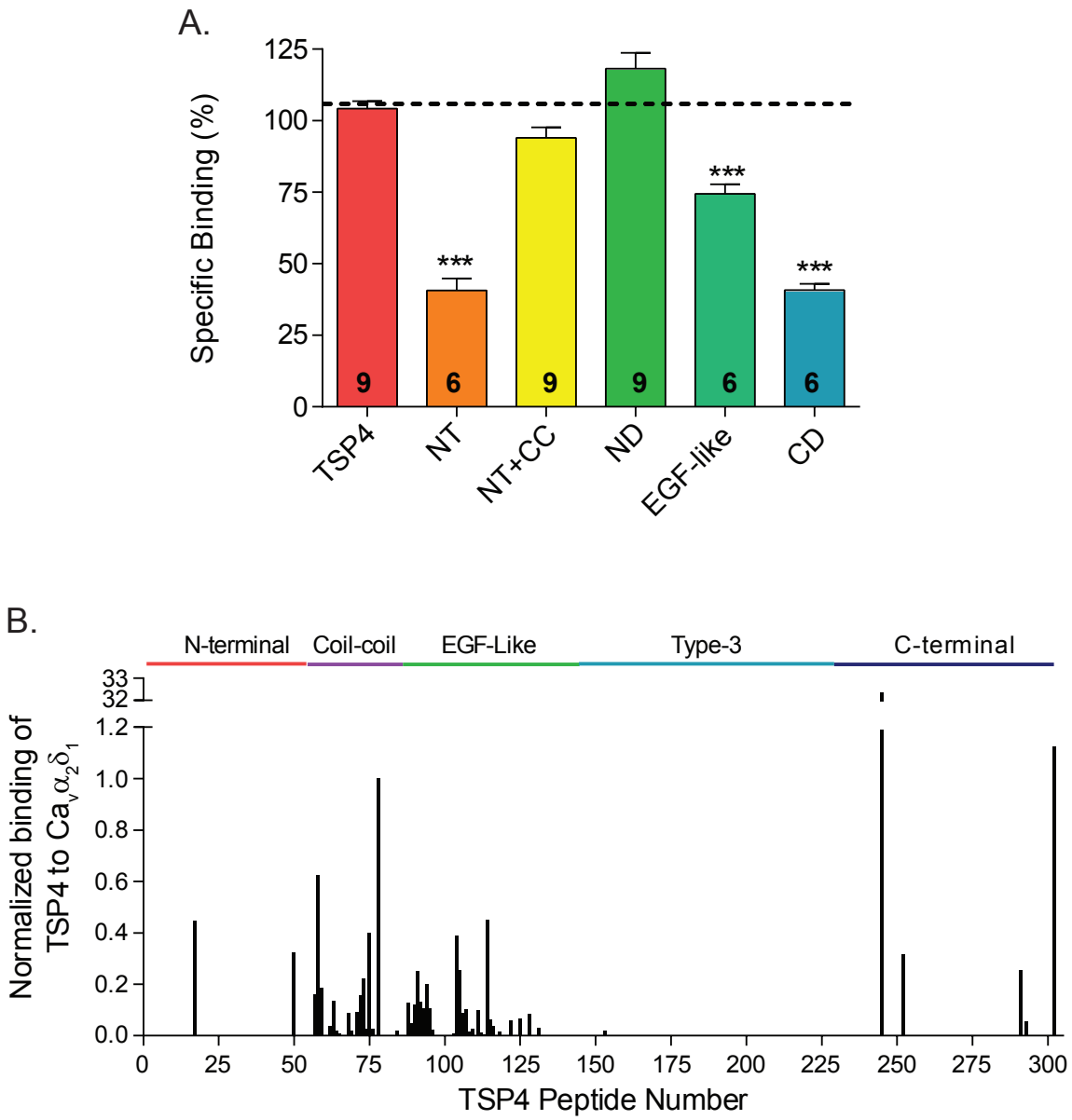

Figure 4. Identification of $Ca_v\alpha_2\delta_1$ binding sites in TSP4.

A. Binding of FLAG-$Ca_v\alpha_2\delta_1$ to immobilized TSP4 or truncated TSP4 proteins (n=6-9) at equal molar concentration to TSP4 (80µg/ml) in an ELISA-based ligand binding assay. Values are the Mean±SEM; ***p <0.001 compared with full-length TSP4 by one-way ANOVA analysis with Bonferroni post-hoc test.

B. Summary of normalized binding of $Ca_v\alpha_2\delta_1$ lysates to 15-mer peptides (overlapping by 12 amino acids) encompassing the entire TSP4 amino acid sequence. Summarized data normalized to TSP4 peptide number 78.

CHAPTER 2

Epidermal growth factor-like domain of thrombospondin-4 proteins plays a crucial role in mediating behavioral hypersensitivities

Abstract

Upregulation of thrombospondin-4 (TSP4) or voltage-gated calcium channel subunit alpha-2-delta-1 ($Ca_v\alpha_2\delta_1$) proteins in the dorsal spinal cord contributes to neuropathic pain development through an unidentified mechanism. Intrathecal injection of TSP4 proteins into naive rats can cause dorsal horn neuron hyperexcitability and dose-dependent, slow-onset and long-lasting allodynia, which can be blocked by the $Ca_v\alpha_2\delta_1$ ligand gabapentin. These findings suggest that TSP4 and $Ca_v\alpha_2\delta_1$ may interact together in mediating pain processing. Here, I used TSP4 recombinant truncations proteins to identify the molecular determinant(s) of TSP4 in mediating behavioral hypersensitivity. I examined what TSP4 domains are pro-nociceptive, and whether blocking/deleting $Ca_v\alpha_2\delta_1$ could abolish its nociceptive effects. Lumbar intrathecal injection of the TSP4 EGF-like domains is sufficient to induce gabapentin-sensitive tactile allodynia, mechanical and thermal hyperalgesia. Selective ablation of $Ca_v\alpha_2\delta_1$ from $Na_v1.8$-positive sensory neurons abolishes TSP4 EGF-like domain-induced thermal hyperalgesia, but not tactile allodynia. A $Ca_v\alpha_2\delta_1$–binding TSP4 peptide within the EGF-like domains can block both $Ca_v\alpha_2\delta_1$- and TSP4-induced behavioral hypersensitivity, presumably by disrupting the interaction between TSP4 and $Ca_v\alpha_2\delta_1$. Together, these findings indicate that the interactions of TSP4 with $Ca_v\alpha_2\delta_1$ through its EGF-like domains are important in mediating chronic pain in a modality-specific manner.

Introduction

Neuropathic pain is caused by injuries in the peripheral or central nervous systems (CNS) and can lead to pain syndromes that include spontaneous pain and evoked pain such as allodynia (exaggerated response to otherwise innocuous tactile stimuli) and hyperalgesia (exaggerated pain sensations as a result of exposure to mildly noxious stimuli) (Bridges et al., 2001; Woolf and Mannion, 1999). Upon peripheral nerve injury, there is a parallel upregulation of the voltage gated calcium channel auxiliary subunit alpha-2-delta-1 ($Ca_v\alpha_2\delta_1$) and astrocyte-secreted thrombospondin-4 (TSP4) mRNA in the dorsal root ganglia (DRG), and protein in the spinal cord that correlates with the onset of allodynia (Kim et al., 2009; Kim et al., 2012; Li et al., 2004; Luo et al., 2002; Luo et al., 2001; Pan et al., 2014; Valder et al., 2003; Wang et al., 2002). Studies have shown that increased $Ca_v\alpha_2\delta_1$ proteins contribute to the neuropathic pain development because tactile allodynia was either prevented (Boroujerdi et al., 2008) or reversed (Li et al., 2004) by blocking nerve injury-induced spinal $Ca_v\alpha_2\delta_1$ protein upregulation with dorsal rhizotomy or intrathecal $Ca_v\alpha_2\delta_1$ anti-sense oligodeoxynucleotide treatment, respectively. This is further supported by evidence indicating that upregulated $Ca_v\alpha_2\delta_1$ plays a significant role in the development of behavioral hypersensitivities in the absence of nerve damage from mice over-expressing $Ca_v\alpha_2\delta_1$ in neuronal cells (Li et al., 2006). In addition, $Ca_v\alpha_2\delta_1$ ligands pregabalin and gabapentin are effective in alleviating neuropathic pain (Brown et al., 1998; Field et al., 2006), but their anti-neuropathic pain properties are abolished in $Ca_v\alpha_2\delta_1$ genetically ablated mice (Patel et al., 2013) and knock-in mice expressing a mutant $Ca_v\alpha_2\delta_1$ that cannot bind gabapentin and pregabalin (Field et al., 2006b).

Currently, the mechanisms underlying $Ca_v\alpha_2\delta_1$-mediated neuropathic pain states are not clear. Gabapentin has been shown to inhibit synaptogenesis by blocking the interaction of $Ca_v\alpha_2\delta_1$ with thrombospondin (TSP) proteins (Eroglu et al., 2009). TSPs are large oligomeric, multidomain, extracellular matrix proteins that mediate cell-cell and cell-matrix interactions through binding to other extracellular matrix proteins, membrane

proteins, and cytokines (Adams and Lawler, 2004; Bornstein, 2001). The TSP family consists of two subfamilies, subgroup A (TSP1-2) and subgroup B (TSP3-5), which is organized by oligomerization state and domain structure. TSP4 plays an important role in the development of neuropathic pain states (Kim et al., 2012). TSP4 blockade by intrathecal anti-sense oligodeoxynucleotides, antibodies, or genetic ablation of the TSP4 gene reverses or prevents behavioral hypersensitivities (Kim et al., 2012). Also, I showed that TSP4 directly binds to $Ca_v\alpha_2\delta_1$ through its coil-coil domain and EGF-like domains of TSP4 (see Chapter 1). Together, these observations support my hypothesis that spinal TSP4 interacts with $Ca_v\alpha_2\delta_1$ to promote chronic pain states. In this study, I investigated the interaction between TSP4 and $Ca_v\alpha_2\delta_1$ and its role in development of behavioral hypersensitivity, and identified the functional determinant of TSP4's pro-nociceptive effects.

Methods and Materials

Animals: Male adult Harlan Sprague Dawley rats (<150g) were from Harlan Sprague Dawley. The $Ca_V\alpha_2\delta_1$ over-expressing transgenic mice were generated as described previously (Li et al., 2006). The $Ca_V\alpha_2\delta_1^{fl/fl}$ mice were generated by floxing the exon 6 of the $Ca_V\alpha_2\delta_1$ gene (MGI ID: 88295) with loxP sites (collaboration with Dr. G. Feng at MIT). Homozygous $Ca_V\alpha_2\delta_1^{fl/fl}$ mice were crossed with the $Na_v1.8$-Cre mice with Cre recombinase expression only in $Na_v1.8$-positive sensory neurons (Minett et al., 2012; Nassar et al., 2004; Stirling et al., 2005) to generate heterozygous $Ca_V\alpha_2\delta_1^{Nav1.8\text{-}Cre+/-}$ mice, which were used to generate homozygous $Ca_V\alpha_2\delta_1^{Nav1.8\text{-}Cre+/-}$ mice for experiments. Mouse genotyping was performed by TransnetYX, Inc. (Cordova, TN). All animals were housed in separate cages and exposed to a 12hr light/dark cycle with food and water *ad libitum*. All animal care and experiments were performed according to protocols approved by the Institutional Animal Care Committees of the University of California, Irvine.

Peptides: The following peptides were synthesized and verified by mass spectrometry and high-performance liquid chromatography analysis by Genscript (Piscataway, NJ): $Ca_v\alpha_2\delta_1$-binding peptide 23 (QACGPLSFQSPTPN) and its scramble peptide (TNPTPSQFSLPGCAQ); $Ca_v\alpha_2\delta_1$-binding peptide 49 (PGVRCTNLAPGFRCD) and its scramble peptide (GLAFVNCPRRDTGCP); $Ca_v\alpha_2\delta_1$-binding peptide 143 (YTAFNGVDFEGTFHV) and its scramble peptide (DAVFFTNEVGGYFHT). The lyophilized peptides were dissolved in sterile water.

Tactile Allodynia: The animals were placed in Plexiglass chambers on a wire-mesh bottomed cage for acclimatization (at least 60 minutes). Von Frey filaments (Stoelting, Wood Dale, IL) were used to determine the 50% paw withdrawal threshold (PWT) using the up-down method of Dixon (Dixon, 1980). A series of von Frey filaments were applied to both plantar surfaces of the hind paws, starting with a buckling weight of 2.0g or 0.41g

for rat or mice, respectively, in ascending order of strength with sufficient force to cause the filament to buckle. A positive response was defined as a rapid withdrawal and/or licking of the paw upon application of the stimulus, which prompted the use of the next weaker filament. Absence of a paw withdrawal response after five seconds prompted the use of the next filament of increasing weight. This paradigm was continued until four more measurements have been made after the initial change of the behavioral response or until five consecutive negative (assigned a score of 15 g for rats or 3g for mice) or four consecutive positive (assigned a score of 0.25 g for rats or 0.01g for mice) responses have occurred. The resulting scores were used to calculate the 50% response threshold as described previously (Luo et al., 2001b).

Thermal Hyperalgesia (Hargreaves Method): The animals were placed in a Plexiglass chambers on a glass panel that was maintained at 30°C for acclimatization (at least 60 min). A heat stimulus was applied to both the plantar surface of the hind paw using a high intensity light bulb projecting through a small aperture below the glass panel. When the animals move their paw away from the thermal stimulus, the light beam automatically shuts off. The length of time between the start of the light beam and the withdrawal of the hind paw was defined as the paw withdrawal latency (PWL). A cut off time of 20 seconds was used to avoid tissue damage to the hind paw.

Mechanical Hyperalgesia (Randall-Selitto test): The rats were first acclimated to human touch and holding positions for a week ahead of the experiments. Mechanical hyperalgesia was measured with the Randall-Selitto Test using a Paw Pressure Analgesymeter (Ugo Basile North America, Collegeville, PA) 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, measured through a scale located on the Analgesymeter, which

induced a hind paw withdrawal. Rats were acclimated to holding posture for additional five minutes before each reading and constantly watched for conditioned false responses that were not included in data analysis.

Results

EGF-like domains of TSP4 are pro-nociceptive

Previous findings have shown that increased spinal TSP4 protein alone is sufficient to induce tactile allodynia, thermal and mechanical hyperalgesia (Kim et al., 2012) through interaction of TSP4 with $Ca_v\alpha_2\delta_1$ (Park et al., submitted, in revision). To confirm that a direct interaction between TSP4 and $Ca_v\alpha_2\delta_1$ in vivo is required for TSP4's pro-nociceptive effects, I examined whether deletion of the $Ca_v\alpha_2\delta_1$ binding motif from TSP4 could abolish its pro-nociceptive effects in vivo. Accordingly, I injected the recombinant truncated TSP4 proteins into L5/6 spinal region of naïve adult male rats and tested their hind paw sensitivity to mechanical and thermal stimuli. The design of the TSP4 recombinant truncation constructs is shown in Figure 1. These constructs were expressed in vitro and purified through His-tag columns (Fig. 1B) as described in Chapter 1. Intrathecal (i.t.) injection of the EGF-like domain containing constructs (EGF-like and ND) at equal molar dose to full-length TSP4 (45μg/rat), which was shown to be pro-nociceptive (Kim et al., 2012), induced a reduction in paw withdrawal threshold to von Frey filaments (tactile allodynia), withdrawal latency (thermal hyperalgesia), and withdrawal threshold (mechanical hyperalgesia) with an onset time and duration similar to that induced by full-length TSP4 proteins (Kim et al., 2012) (Fig. 2A-C). In contrast, lowering the dose of EGF-like proteins to an equal molar dose of the sub-threshold dose of full-length TSP4 (20μg/rat) protein or i.t. injection of recombinant truncated proteins in the absence of the EGF-like domains of TSP4 (CD, NT, and NT+CC) did not cause behavioral hypersensitivities (Fig. 2A-C and Fig. 3A). Similar to that of full-length TSP4, i.t. injection of $Ca_v\alpha_2\delta_1$ ligand gabapentin can dose-dependently reverse tactile allodynia induced by EGF-like domains of TSP4 within an hour (Fig. 3A, B). Together, these findings suggest that the EGF-like domains of TSP4 are the functional determinant of TSP4's pro-nociceptive effects through an interaction with the $Ca_v\alpha_2\delta_1$ proteins.

Next, to examine whether EGF-like's pro-nociceptive effect requires $Ca_v\alpha_2\delta_1$, I used a Cre-Lox strategy to delete $Ca_v\alpha_2\delta_1$ from primary afferent voltage-gated sodium channel 1.8 ($Na_v1.8$) expressing neurons. Our lab generated a mouse line harboring a floxed exon 6 of $Ca_v\alpha_2\delta_1$ gene ($Ca_v\alpha_2\delta_1^{fl/fl}$) (collaboration with Dr. G. Feng at MIT) and then crossed it with a well-characterized mouse line expressing Cre recombinase in $Na_v1.8$ positive neurons ($Na_v1.8^{Cre}$) (Fig. 4A, B). The Cre recombinase in $Na_v1.8^{Cre}$ mice is expressed by up to 75% of DRG neurons, including unmyelinated C, thinly myelinated Aδ, low-threshold mechanoreceptors and some Aβ fibers (Abrahamsen et al., 2008; Shields et al., 2012; Stirling et al., 2005). Previously, i.t. injection of TSP4 (5μg/mouse) into mice lacking $Ca_v\alpha_2\delta_1$ in $Na_v1.8$ Cre-expressing neurons ($Ca_v\alpha_2\delta_1^{Nav1.8}$) resulted in tactile allodynia with a similar onset time and duration as that observed in similarly treated control $Ca_v\alpha_2\delta_1^{fl/fl}$ mice, but TSP4-induced thermal and cold hyperalgesia was abolished in $Ca_v\alpha_2\delta_1^{Nav1.8}$ mice (Park et al., submitted, in revision). Similarly, i.t. injection of EGF-like domain proteins at equal molar dose (1μg/mouse) to full-length TSP4 (5μg/mouse) caused tactile allodynia with a similar onset time and duration as that induced by similar EGF-like domain protein injections in rats (Fig. 2 and Fig. 3) and control $Ca_v\alpha_2\delta_1^{fl/fl}$ mice (Fig. 4C). However, i.t. injection of EGF-like domain protein-induced thermal hyperalgesia seen in control $Ca_v\alpha_2\delta_1^{fl/fl}$ mice and naïve rats was abolished in $Ca_v\alpha_2\delta_1^{Nav1.8}$ mice (Fig. 4D). Taken together, these findings highly suggest that interaction between the EGF-like domains of TSP4 and $Ca_v\alpha_2\delta_1$ in $Na_v1.8$-positive sensory fiber terminals is required for EGF-like domain induced thermal hyperalgesia in a modality specific manner.

Effects of intrathecal $Ca_v\alpha_2\delta_1$-binding TSP4 peptides on behavioral hypersensitivity

To determine if a direct interaction between TSP4 and $Ca_v\alpha_2\delta_1$ in vivo is required for pain processing, I examined if $Ca_v\alpha_2\delta_1$-binding peptides of TSP4 from the SPOT peptide array analysis (see Chapter 1) could block TSP4-induced tactile allodynia. I synthesized

three specific high-affinity $Ca_v\alpha_2\delta_1$-binding peptides from different domains of TSP4 (coil-coil domain, Peptide 23; EGF-like domain, Peptide 49; C-terminal domain, Peptide 143) and injected the $Ca_v\alpha_2\delta_1$-binding peptides into the L5/6 spinal region of post-TSP4 injected animals with peak allodynia (Day 3 post-bolus TSP4 injection). $Ca_v\alpha_2\delta_1$-binding peptide 49, but not its scramble peptide blocked TSP4-induced tactile allodynia dose-dependently at 4 hr post-peptide injection (Fig. 5A). This effect lasted for <6 hrs. $Ca_v\alpha_2\delta_1$-binding peptide 143 partially attenuated TSP4-induced tactile allodynia, but this effect is not significantly different from its respective scramble peptide (Fig. 5B). Also, i.t. injection of peptide 23 had no effect on TSP4-induced tactile allodynia (Fig. 5C). Bolus injection of peptide 23, 49, or 143 into naïve rats did not have a pro-nociceptive effect (Fig. 5D). Together, my data suggest that the $Ca_v\alpha_2\delta_1$-binding peptide within the EGF-like domain (peptide 49) of TSP4 has an anti-nociceptive effect in TSP4-induced tactile allodynia.

Previously, our lab has reported that transgenic mice with $Ca_v\alpha_2\delta_1$ overexpression in neuronal cells (TG) cause an enhanced excitatory synaptic input in the superficial dorsal horn and behavioral hypersensitivity in the absence of nerve injury (Li et al., 2006; Zhou and Luo, 2014). $Ca_v\alpha_2\delta_1$-induced spinal neuron sensitization and pain states can be blocked by gabapentin (Li et al., 2006; Zhou and Luo, 2014), TSP4 antibodies, and genetic ablation of TSP4 from TG mice (Park et al., submitted, in revision). Using this TG model, I examined if $Ca_v\alpha_2\delta_1$-binding TSP4 peptide 49 and 143 that were found effective in blocking TSP4-induced tactile allodynia could also attenuate $Ca_v\alpha_2\delta_1$-induced behavioral hypersensitivity. My data indicated that i.t. injection of $Ca_v\alpha_2\delta_1$-binding peptide 49, but not its respective scramble peptides, could reverse allodynia in the $Ca_v\alpha_2\delta_1$ TG mice with an onset time of 2- to 4-hr and a duration of <6-hr (Fig 6A). $Ca_v\alpha_2\delta_1$-binding peptide 143 could partially attenuate allodynia in the $Ca_v\alpha_2\delta_1$ TG mice, but this anti-nociceptive effect is not significantly different from its respective scramble peptide (Fig. 6B). These results indicate that the $Ca_v\alpha_2\delta_1$-binding peptide from the EGF-like domains of TSP4 can attenuate $Ca_v\alpha_2\delta_1$-induced tactile allodynia by possibly interfering with the TSP4 and

$Ca_v\alpha_2\delta_1$ interaction.

I next examined the effect of the $Ca_v\alpha_2\delta_1$-binding peptides on a unilateral L5/6 spinal nerve ligation (SNL) neuropathic pain model. We have previously shown that TSP4 and $Ca_v\alpha_2\delta_1$ protein levels are increased in L5/6 dorsal spinal cord at day 2 and 14 post injury (Kim et al., 2012; Li et al., 2004; Luo et al., 2001). These time points correlated respectively with the onset and established behavioral hypersensitivity, thereby suggesting that injury-induced TSP4 and $Ca_v\alpha_2\delta_1$ proteins in the dorsal spinal cord may play a role in neuropathic pain development. Intrathecal injection of $Ca_v\alpha_2\delta_1$-binding peptide 49, but not peptide 143 or respective scramble peptide, into the lumbar L5/6 region two-weeks post injury caused a dose-dependent increase in paw withdrawal threshold after nerve injury with an onset time of 4 hr and a duration of <6 hr (Fig. 7A, B). Thus, these findings suggest that spinal TSP4 interacts with $Ca_v\alpha_2\delta_1$ proteins to cause tactile allodynia, whereby blocking this interaction with $Ca_v\alpha_2\delta_1$-binding peptide 49 can attenuate nerve-injury induced allodynia.

Discussion

We have previously shown that peripheral nerve injury induces upregulation of TSP4 and $Ca_v\alpha_2\delta_1$ at the spinal cord level (Kim et al., 2012; Li et al., 2004), which contributes to chronic pain states through possible interactions between TSP4 and $Ca_v\alpha_2\delta_1$. My findings here identified the EGF-like domains of TSP4 as the pro-nociceptive domain that interacts with $Ca_v\alpha_2\delta_1$. Administration of $Ca_v\alpha_2\delta_1$-binding TSP4 peptide from the EGF-like domains can partially attenuate nerve-injury induced tactile allodynia, and block TSP4- and $Ca_v\alpha_2\delta_1$-induced tactile allodynia. Together, these results support that the EGF-like domains of TSP4 are the functional determinants in mediating behavioral hypersensitivity through its interactions with $Ca_v\alpha_2\delta_1$ proteins.

Here, I showed that all truncated TSP4 proteins containing the EGF-like domains induce similar behavioral hypersensitivities as full-length TSP4 proteins, but those containing the N-terminal, coil-coil, calcium-binding, and C-terminal domains do not. Conversely, a study has shown that the EGF-like domains of TSP interact with the von Willebrand Factor A (VWF-A) domain of $Ca_v\alpha_2\delta_1$ and mediates its synapse-inducing effects (Eroglu et al., 2009). The VWF-A domain is conserved within the α_2 moiety of all $Ca_v\alpha_2\delta_1$ subunits and is also found in integrins that have been shown to interact with extracellular matrix proteins (Whittaker and Hynes, 2002). Thus, the EGF-like domains of TSP4 may directly interact with the VWF-A domain of $Ca_v\alpha_2\delta_1$ in mediating behavioral hypersensitivity. Since gabapentin binds to a site upstream of the VWF domain of $Ca_v\alpha_2\delta_1$ (Wang et al., 1999), it is less likely that the inhibitory effects of gabapentin on EGF-like-domain-induced behavioral hypersensitivities are mediated through a direct blockage of TSP4 binding to $Ca_v\alpha_2\delta_1$ (Fig. 3A, B). Instead, gabapentin binding to $Ca_v\alpha_2\delta_1$ may cause conformational changes to the protein that may interfere with TSP4/$Ca_v\alpha_2\delta_1$ interactions.

Since $Ca_v\alpha_2\delta_1$ is expressed in the central terminals of DRG neurons that project to the superficial laminae of dorsal spinal cord (Bauer et al., 2009), I examined whether genetic ablation of $Ca_v\alpha_2\delta_1$ from a subpopulation of primary afferent terminals affect

TSP4 EGF-like domain induced behavioral hypersensitivity. Genetic ablation of $Ca_v\alpha_2\delta_1$ from $Na_v1.8$-positive neurons had no affect on basal behavioral responses to innocuous mechanical stimuli and noxious thermal stimuli. This is consistent with studies showing that behavioral responses to noxious thermal stimuli and innocuous mechanical stimuli remain intact in mice in which all $Na_v1.8$-positive neurons have been ablated with diphtheria toxin (Abrahamsen et al., 2008). However, genetic ablation of $Ca_v\alpha_2\delta_1$ from $Na_v1.8$-positive neurons resulted in a significantly reduced response to EGF-like induced thermal hyperalgesia, but had no effect on EGF-like induced tactile allodynia (Fig. 4C, D). These data suggest that $Ca_v\alpha_2\delta_1$ presumably at the central afferent terminals of $Na_v1.8$-positive neurons, expressed by up to 75% of DRG neurons (Shields et al., 2012), are required for EGF-like domain induced thermal hyperalgesia, but that at central terminals of $Na_v1.8$-negative sensory neurons are responsible for EGF-like domain induced mechanical allodynia. Interestingly, global knockout of $Ca_v\alpha_2\delta_1$ showed no effect to noxious thermal stimuli, but a significantly reduced behavioral hypersensitivity to innocuous mechanical and noxious cold stimuli (Patel et al., 2013). These discrepancies between the $Ca_v\alpha_2\delta_1^{Nav1.8}$ and global $Ca_v\alpha_2\delta_1$ knockout may result from compensatory factors or pathways as a result of general deletion of $Ca_v\alpha_2\delta_1$. Together, these findings indicate that the EGF-like domains of TSP4 and its interaction with $Ca_v\alpha_2\delta_1$ mediate behavioral hypersensitivity in a modality-specific manner.

To determine if $Ca_v\alpha_2\delta_1$ binding peptides of TSP4 can block interactions between TSP4 and $Ca_v\alpha_2\delta_1$ in nociception, I designed $Ca_v\alpha_2\delta_1$-binding peptides from the coil-coil domain (Peptide 23; QACGPLSFQSPTPN), C-terminal domain (Peptide 143; YTAFNGVDFEGTFHV) and EGF-like domain (Peptide 49; PGVRCTNLAPGFRCD) of TSP4. I chose high affinity $Ca_v\alpha_2\delta_1$-binding peptides from the coil-coil domain and EGF-like domains of TSP4 since direct in vitro binding studies revealed these domains to be the major binding site for $Ca_v\alpha_2\delta_1$. The C-terminal domain peptide 143 bound to $Ca_v\alpha_2\delta_1$ with the highest affinity amongst other TSP4 peptides. The amino acid sequence

of peptide 143 is highly conserved among the TSP family (TSP1-5) and fully conserved among subgroup B (TSP3-5) in rodents and humans. In contrast, the coil-coil and EGF-like domain peptide sequences are highly conserved to TSP4 in rodents.

Since the EGF-like domain is the pro-nociceptive domain of TSP4 that directly binds to $Ca_v\alpha_2\delta_1$, I hypothesized that a TSP4 peptide from the EGF-like domain could block the interaction between TSP4 and $Ca_v\alpha_2\delta_1$ and thereby attenuate behavioral hypersensitivity. To determine the effects of $Ca_v\alpha_2\delta_1$-binding peptides from the EGF-like domain, coil-coil domain, and C-terminal domain on nociception, I used a rat neuropathic pain model. Intrathecal injection of $Ca_v\alpha_2\delta_1$-binding peptide 49, but not peptide 143, can partially attenuate nerve-injury induced tactile allodynia, thereby providing further support for interaction of TSP4 to $Ca_v\alpha_2\delta_1$ proteins that contribute to neuropathic pain states. To confirm the anti-nociceptive effect of the $Ca_v\alpha_2\delta_1$-binding peptide that may block TSP4/$Ca_v\alpha_2\delta_1$ interaction without the influence from other injury factors, I also examined if behavioral hypersensitivity induced by an increased spinal TSP4 or $Ca_v\alpha_2\delta_1$ proteins alone can be attenuated by $Ca_v\alpha_2\delta_1$-binding peptide 49. I found that $Ca_v\alpha_2\delta_1$-binding peptides 49, but not peptide 23 and 143, could block TSP4-induced tactile allodynia. Also, $Ca_v\alpha_2\delta_1$-induced tactile allodynia from transgenic mice overexpressing $Ca_v\alpha_2\delta_1$ in neuronal cells can be attenuated by $Ca_v\alpha_2\delta_1$-binding peptide 49, but not peptide 143 after i.t. injection. Therefore, $Ca_v\alpha_2\delta_1$-binding peptide from the EGF-like domain, but not coil-coil domain or C-terminal domain can diminish behavioral hypersensitivity by blocking the interaction between TSP4 and $Ca_v\alpha_2\delta_1$.

Furthermore, i.t. injection of $Ca_v\alpha_2\delta_1$-binding peptides alone does not have any acute effect or pro-nociceptive effect. Even though i.t. injection of EGF-like domain containing proteins is pro-nociceptive (ND and EGF-like), the EGF-like domain containing linear peptide (Peptide 49) alone cannot induce behavioral hypersensitivity in naïve animals. The results suggest that protein-protein, but not peptide-protein interaction is critical for TSP4's pro-nociceptive effect. Thus, the functional effect of TSP4 may require multiple

binding regions and a protein interface upon folding, which is supported by my SPOT peptide data (see Chapter 1).

Recent studies reveal that gabapentin can inhibit $Ca_v\alpha_2\delta_1$ and VGCC trafficking, thereby reducing the increase of $Ca_v\alpha_2\delta_1$ to the pre-synaptic terminals of nerve-injured DRGs in the dorsal horn in vivo (Bauer et al., 2009; Hendrich et al., 2008). Moreover, transgenic overexpression of $Ca_v\alpha_2\delta_1$ in neuronal cells causes an enhanced excitatory transmission and behavioral hypersensitivity that can be attenuated by gabapentin (Li et al., 2004; Li et al., 2006). Similarly, bolus injection of $Ca_v\alpha_2\delta_1$-binding TSP4 peptide 49 can also block $Ca_v\alpha_2\delta_1$-induced tactile allodynia. Future studies will need to explore whether the $Ca_v\alpha_2\delta_1$-binding peptides could modulate excitatory transmission and reduce $Ca_v\alpha_2\delta_1$ trafficking to presynaptic sites in the dorsal spinal cord.

The ability of gabapentin to inhibit TSP4-induced dorsal horn sensitization and behavioral hypersensitivity highlights the critical role between TSP4 and $Ca_v\alpha_2\delta_1$ interaction in mediating chronic pain states. We have previously shown that TSP4 and $Ca_v\alpha_2\delta_1$ blockade either by intrathecal antibodies, antisense oligodeoxynucleotides, genetic ablation of TSP4 gene, or pharmacological treatment of gabapentin can prevent behavioral hypersensitivity and spinal neuron hyperexcitability (Park et al., submitted, in revision). Also, gabapentin can block TSP-induced synapse formation by interfering with TSP and $Ca_v\alpha_2\delta_1$ interaction (Eroglu et al., 2009), indicating that the interactions of $Ca_v\alpha_2\delta_1$ with TSP4 may contribute to aberrant excitatory synaptogenesis in neuropathic pain states. This is further supported by recent findings showing that trigeminal nerve-injury causes an increase in $Ca_v\alpha_2\delta_1$ in the superficial dorsal horn and correlates with aberrant excitatory synaptogenesis and enhances presynaptic excitatory input (Li et al., 2014). However, it is unclear how synaptogenesis is involved in the rapid reduction of behavioral hypersensitivity by gabapentin. Even though I cannot rule out the possibility that gabapentin mediates its effect independent of binding to $Ca_v\alpha_2\delta_1$, it is reasonable to suspect that TSP4 and $Ca_v\alpha_2\delta_1$ interaction may be important in maintaining TSP4-induced

dorsal horn sensitization and behavioral hypersensitivity. The fast action of gabapentin may derive from interfering with TSP4/$Ca_v\alpha_2\delta_1$ interactions that could increase recycling of VGCC at the cell surface/synapse, subsequently leading to decrease synaptic activity. Likewise, it will be interesting to explore whether $Ca_v\alpha_2\delta_1$-binding peptides can block excitatory synapse formation and surface expression of VGCC in a similar manner as gabapentin.

In summary, my findings demonstrate that the EGF-like domains of TSP4 is the molecular determinant in mediating hypersensitivities to different modalities that requires its interaction with $Ca_v\alpha_2\delta_1$. Blocking the interaction with $Ca_v\alpha_2\delta_1$-binding peptides can attenuate pain hypersensitivity, which can be used as a future tool to study the mechanism underlying TSP4 and $Ca_v\alpha_2\delta_1$ interaction in chronic pain states. Extensive studies examining $Ca_v\alpha_2\delta_1$-binding peptides as a potential treatment for chronic pain are warranted. Together, my findings indicate a new mechanism of neuropathic pain and point to a new direction for managing chronic pain.

References

Abrahamsen B, Zhao J, Asante CO, Cendan CM, Marsh S, Martinez-Barbera JP, Nassar MA, Dickenson AH, Wood JN. 2008. The cell and molecular basis of mechanical, cold, and inflammatory pain. Science 321(5889):702-705.

Adams JC, Lawler J. 2004. The thrombospondins. Int J Biochem Cell Biol 36(6):961-968.

Bauer CS, Nieto-Rostro M, Rahman W, Tran-Van-Minh A, Ferron L, Douglas L, Kadurin I, Sri Ranjan Y, Fernandez-Alacid L, Millar NS, Dickenson AH, Lujan R, Dolphin AC. 2009. The increased trafficking of the calcium channel subunit alpha2delta-1 to presynaptic terminals in neuropathic pain is inhibited by the alpha2delta ligand pregabalin. J Neurosci 29(13):4076-4088.

Bornstein P. 2001. Thrombospondins as matricellular modulators of cell function. J Clin Invest 107(8):929-934.

Boroujerdi A, Kim HK, Lyu YS, Kim DS, Figueroa KW, Chung JM, Luo ZD. 2008. Injury discharges regulate calcium channel alpha-2-delta-1 subunit upregulation in the dorsal horn that contributes to initiation of neuropathic pain. Pain 139(2):358-366.

Bridges D, Thompson SW, Rice AS. 2001. Mechanisms of neuropathic pain. Br J Anaesth 87(1):12-26.

Brown JP, Dissanayake VU, Briggs AR, Milic MR, Gee NS. 1998. Isolation of the [3H] gabapentin-binding protein/alpha 2 delta Ca2+ channel subunit from porcine brain: development of a radioligand binding assay for alpha 2 delta subunits using [3H] leucine. Anal Biochem 255(2):236-243.

Eroglu C, Allen NJ, Susman MW, O'Rourke NA, Park CY, Ozkan E, Chakraborty C, Mulinyawe SB, Annis DS, Huberman AD, Green EM, Lawler J, Dolmetsch R, Garcia KC, Smith SJ, Luo ZD, Rosenthal A, Mosher DF, Barres BA. 2009. Gabapentin

receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell 139(2):380-392.

Field MJ, Cox PJ, Stott E, Melrose H, Offord J, Su TZ, Bramwell S, Corradini L, England S, Winks J, Kinloch RA, Hendrich J, Dolphin AC, Webb T, Williams D. 2006. Identification of the alpha2-delta-1 subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin. Proc Natl Acad Sci U S A 103(46):17537-17542.

Hendrich J, Van Minh AT, Heblich F, Nieto-Rostro M, Watschinger K, Striessnig J, Wratten J, Davies A, Dolphin AC. 2008. Pharmacological disruption of calcium channel trafficking by the alpha2delta ligand gabapentin. Proc Natl Acad Sci U S A 105(9):3628-3633.

Kim DS, Figueroa KW, Li KW, Boroujerdi A, Yolo T, Luo ZD. 2009. Profiling of dynamically changed gene expression in dorsal root ganglia post peripheral nerve injury and a critical role of injury-induced glial fibrillary acidic protein in maintenance of pain behaviors [corrected]. Pain 143(1-2):114-122.

Kim DS, Li KW, Boroujerdi A, Peter Yu Y, Zhou CY, Deng P, Park J, Zhang X, Lee J, Corpe M, Sharp K, Steward O, Eroglu C, Barres B, Zaucke F, Xu ZC, Luo ZD. 2012. Thrombospondin-4 contributes to spinal sensitization and neuropathic pain states. J Neurosci 32(26):8977-8987.

Li CY, Song YH, Higuera ES, Luo ZD. 2004. Spinal dorsal horn calcium channel alpha2delta-1 subunit upregulation contributes to peripheral nerve injury-induced tactile allodynia. J Neurosci 24(39):8494-8499.

Li CY, Zhang XL, Matthews EA, Li KW, Kurwa A, Boroujerdi A, Gross J, Gold MS, Dickenson AH, Feng G, Luo ZD. 2006. Calcium channel alpha2delta1 subunit mediates spinal

hyperexcitability in pain modulation. Pain 125(1-2):20-34.

Li KW, Yu YP, Zhou C, Kim DS, Lin B, Sharp K, Steward O, Luo ZD. 2014. Calcium channel alpha2delta1 proteins mediate trigeminal neuropathic pain states associated with aberrant excitatory synaptogenesis. J Biol Chem 289(10):7025-7037.

Luo ZD, Calcutt NA, Higuera ES, Valder CR, Song YH, Svensson CI, Myers RR. 2002. Injury type-specific calcium channel alpha 2 delta-1 subunit up-regulation in rat neuropathic pain models correlates with antiallodynic effects of gabapentin. J Pharmacol Exp Ther 303(3):1199-1205.

Luo ZD, Chaplan SR, Higuera ES, Sorkin LS, Stauderman KA, Williams ME, Yaksh TL. 2001. Upregulation of dorsal root ganglion (alpha)2(delta) calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. J Neurosci 21(6):1868-1875.

Minett MS, Nassar MA, Clark AK, Passmore G, Dickenson AH, Wang F, Malcangio M, Wood JN. 2012. Distinct Nav1.7-dependent pain sensations require different sets of sensory and sympathetic neurons. Nat Commun 3:791.

Nassar MA, Stirling LC, Forlani G, Baker MD, Matthews EA, Dickenson AH, Wood JN. 2004. Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. Proc Natl Acad Sci U S A 101(34):12706-12711.

Pan B, Yu H, Park J, Yu YP, Luo ZD, Hogan QH. 2014. Painful nerve injury upregulates thrombospondin-4 expression in dorsal root ganglia. J Neurosci Res.

Patel R, Bauer CS, Nieto-Rostro M, Margas W, Ferron L, Chaggar K, Crews K, Ramirez JD, Bennett DL, Schwartz A, Dickenson AH, Dolphin AC. 2013. alpha2delta-1 gene deletion affects somatosensory neuron function and delays mechanical hypersensitivity in response to peripheral nerve damage. J Neurosci 33(42):16412-

16426.

Randall LO, Selitto JJ. 1957. A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther 111(4):409-419.

Shields SD, Ahn HS, Yang Y, Han C, Seal RP, Wood JN, Waxman SG, Dib-Hajj SD. 2012. Nav1.8 expression is not restricted to nociceptors in mouse peripheral nervous system. Pain 153(10):2017-2030.

Stirling LC, Forlani G, Baker MD, Wood JN, Matthews EA, Dickenson AH, Nassar MA. 2005. Nociceptor-specific gene deletion using heterozygous NaV1.8-Cre recombinase mice. Pain 113(1-2):27-36.

Valder CR, Liu JJ, Song YH, Luo ZD. 2003. Coupling gene chip analyses and rat genetic variances in identifying potential target genes that may contribute to neuropathic allodynia development. J Neurochem 87(3):560-573.

Wang H, Sun H, Della Penna K, Benz RJ, Xu J, Gerhold DL, Holder DJ, Koblan KS. 2002. Chronic neuropathic pain is accompanied by global changes in gene expression and shares pathobiology with neurodegenerative diseases. Neuroscience 114(3):529-546.

Wang M, Offord J, Oxender DL, Su TZ. 1999. Structural requirement of the calcium-channel subunit alpha2delta for gabapentin binding. Biochem J 342 (Pt 2):313-320.

Whittaker CA, Hynes RO. 2002. Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Mol Biol Cell 13(10):3369-3387.

Woolf CJ, Mannion RJ. 1999. Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet 353(9168):1959-1964.

Zhou C, Luo ZD. 2014. Electrophysiological characterization of spinal neuron sensitization by elevated calcium channel alpha-2-delta-1 subunit protein. Eur J Pain 18(5):649-658.

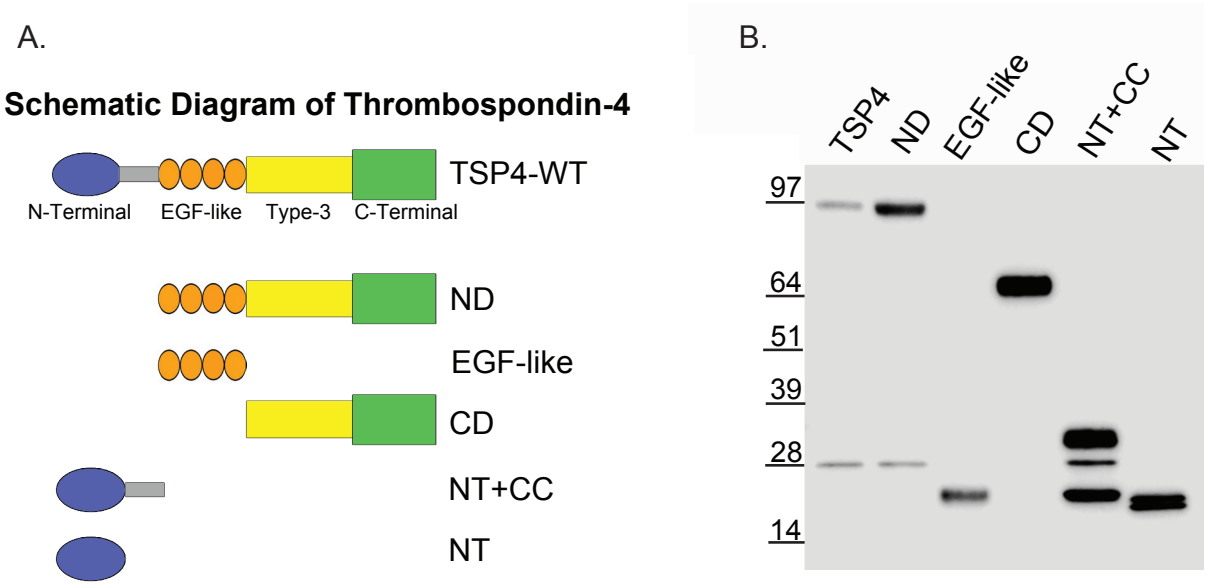

Figure 1. Design and purification of recombinant TSP4 proteins.
A. Domain structure of full-length TSP4 and recombinant TSP4 truncation proteins. Top: N-terminal domain (blue), coiled-coil oligomerization domain (grey), four type II EGF-like domain repeats (orange), seven type III calcium binding repeats (yellow), and the C-terminal domain (green). **B.** A representative Western blot of the purified recombinant TSP4 proteins detected by anti-His antibodies under reducing conditions. The approximate molecular weight (kDa) is show on the left.

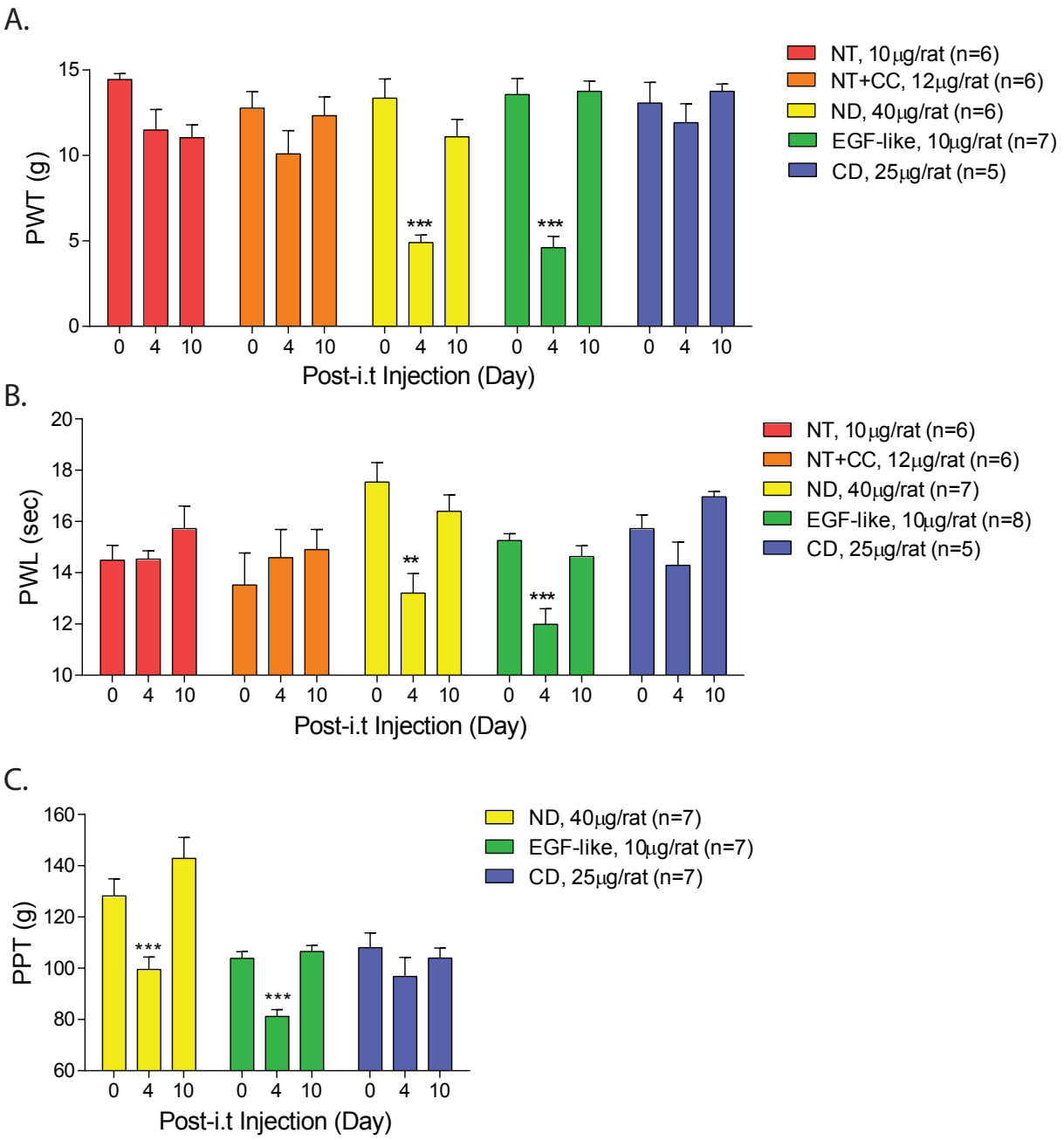

Figure 2. Direct intrathecal (i.t.) injection of TSP4 truncation proteins containing the EGF-like domains into L5/6 spinal region of naïve rats induced behavioral hypersensitivities. Paw withdrawal threshold (PWT) (**A.**), paw withdrawal latency (PWL) (**B.**), and paw pressure threshold (**C.**) were tested before, at day-four (peak behavioral hypersensitivity), and day-ten (recovery) post-i.t. injection of recombinant TSP4 truncated proteins using von Frey filaments, Hargreaves thermal stimulation, and Randall-Selitto paw pressure stimulation, respectively. Data presented are the means ± SEM from 5-8 rats in each group. **p < 0.01, ***p < 0.001 compared with pre-treatment level by one-way ANOVA analysis with Bonferroni post-hoc test.

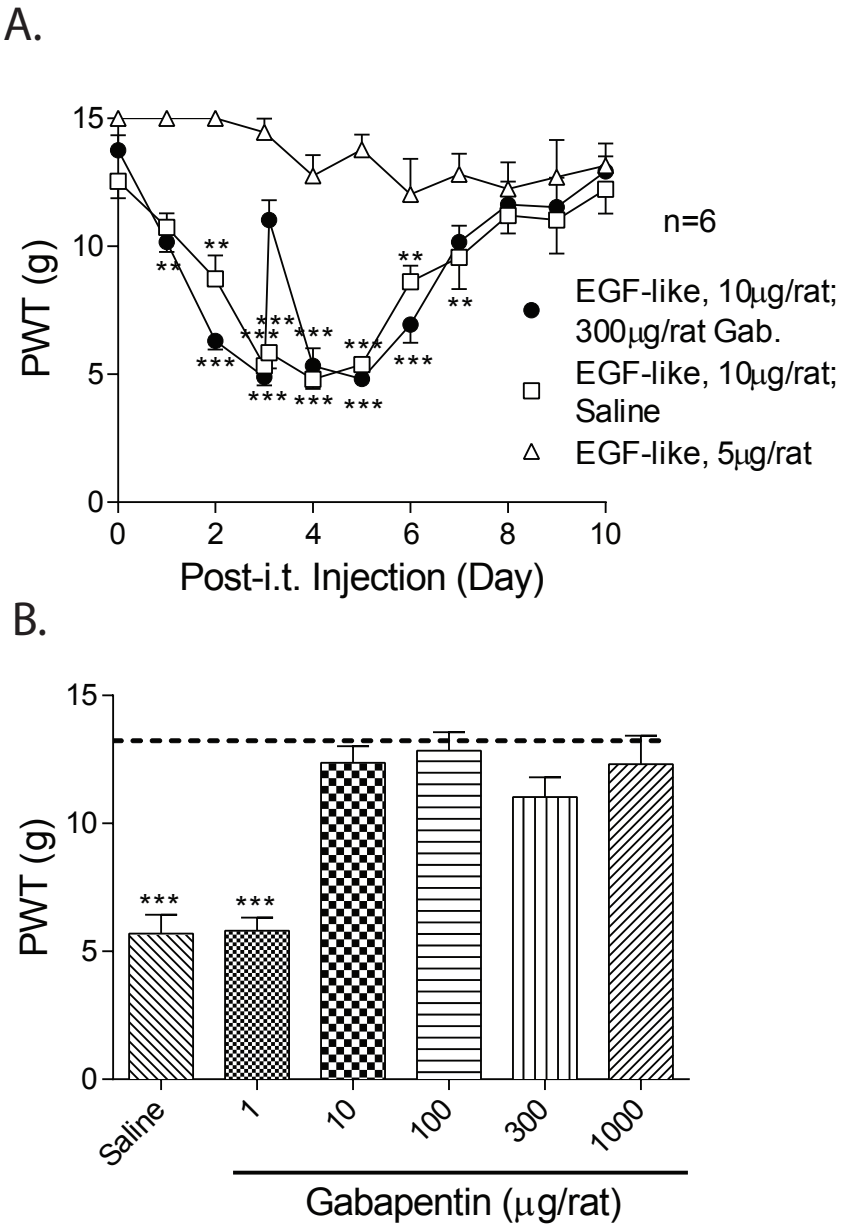

Figure 3. Intrathecal injection (i.t.) of EGF-like domains of TSP4 into naïve rats induced dose-dependent and reversible allodynia that could be blocked by gabapentin. A. Bolus EGF-like construct injection started at time 0 followed by blind daily behavioral test using von Frey filaments. Bolus injection of gabapentin (300μg/rat) or saline was administered at day-three post TSP4 injection, followed by behavioral tests after 1 hr, and then continued daily.

B. Dose-dependent effect of gabapentin/rat in reversal of tactile allodynia. The dotted line represents the PWT before i.t. injections. Data presented are the Means ± SEM from 6 rats in each group. **p <0.01, ***p <0.001 compared with pre-treatment level by one-way ANOVA analysis with Bonferroni post-hoc tests.

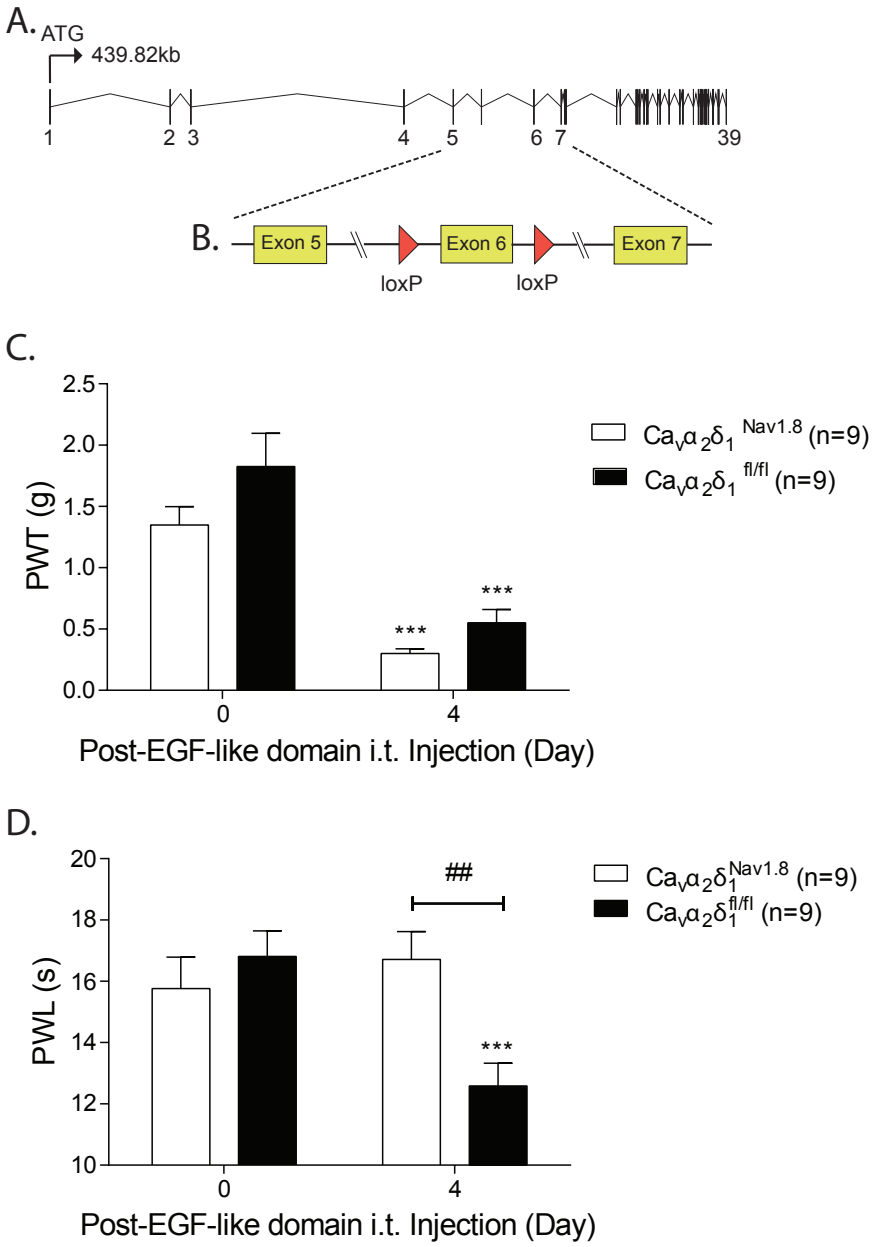

Figure 4. Intrathecal injection (i.t.) of EGF-like domains of TSP4 into $Ca_v\alpha_2\delta_1^{Nav1.8}$ mice induced tactile allodynia, but not thermal hyperalgesia.

A. Gene structure of $Ca_v\alpha_2\delta_1$ gene. **B.** A diagram showing the strategy of generating the $Ca_v\alpha_2\delta_1$ conditional knockout (KO) mice by floxing exon 6 of the $Ca_v\alpha_2\delta_1$ gene with loxP sites. **C, D.** Bolus EGF-like injection (1μg/mouse) into the L4/5 region of $Ca_v\alpha_2\delta_1^{Nav1.8}$ or control $Ca_v\alpha_2\delta_1^{fl/fl}$ mice were tested before and at day-four post-i.t. injection using von Frey filaments (**C.**) or Hargreaves thermal stimuli (**D.**). Data presented are the Means ± SEM from 9 mice in each group. ***p <0.001compared with pre-treatment level, $^{\#\#}$p<0.01 compared between groups by one-way ANOVA analysis with Bonferroni post-hoc tests.

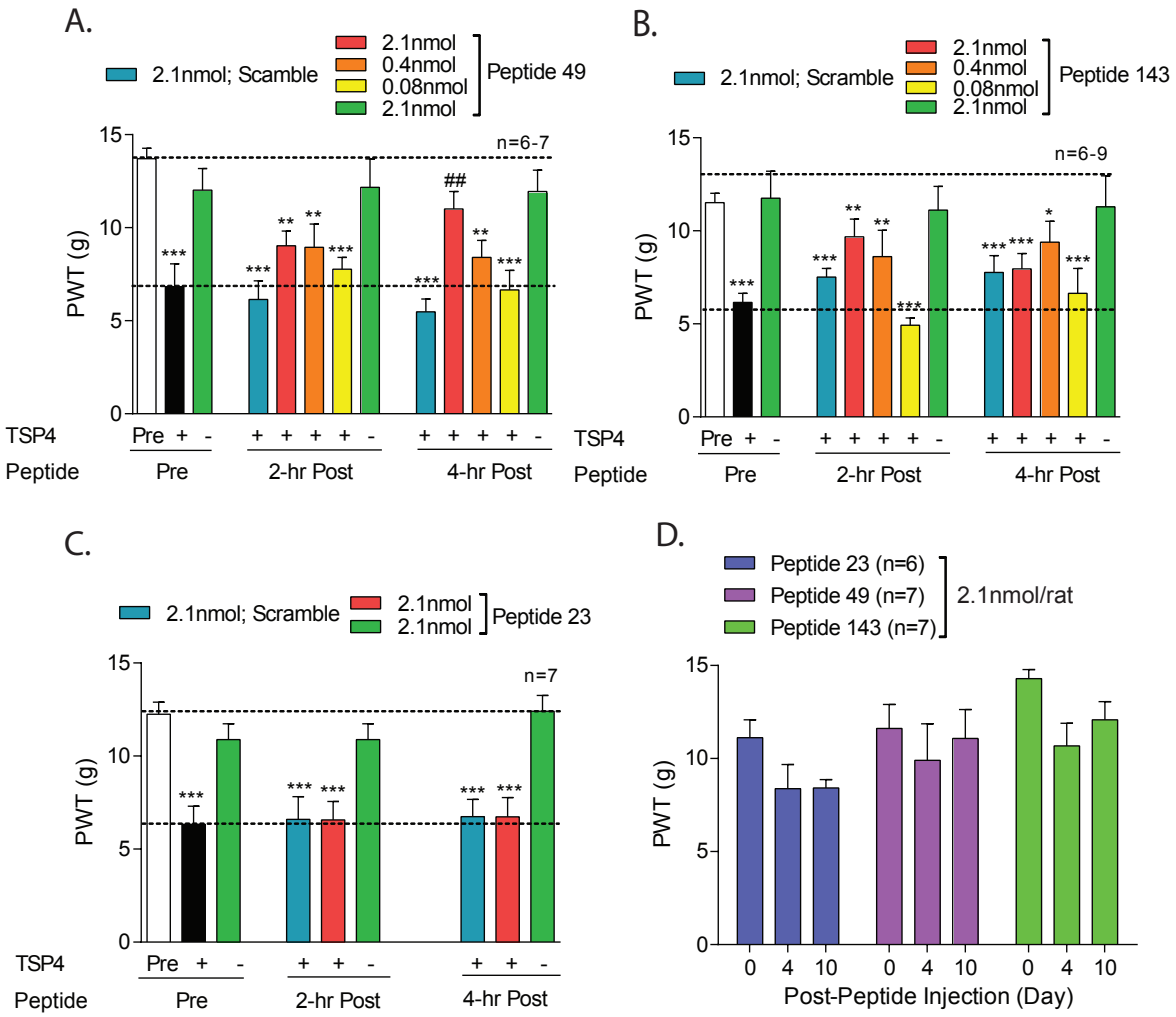

Figure 5. $Ca_v\alpha_2\delta_1$-binding TSP4 peptide 49 has an anti-nociceptive effect in TSP4-induced tactile allodynia. **A-C.** TSP4 (45µg/rat) proteins or PBS buffer were injected into L5/6 spinal region of naïve rats at day 0 after baseline testing, followed by daily behavioral tests using von Frey filaments. +: rats treated with TSP4, -: rats treated with PBS. Tactile allodynia peaked between 3-4 days post TSP4 injection. The top and bottom dotted line represents the mean PWT before and three days after i.t. injection of TSP4, respectively. Bolus i.t. injection of $Ca_v\alpha_2\delta_1$-binding TSP4 peptide 49 (**A.**), 143 (**B.**), 23 (**C.**), or respective scramble peptides was administered at day-three post injection, followed by behavioral tests at 2 hr and 4 hr post-peptide treatment. The peptide was administered at 5X Molar excess (2.1nmol/rat), molar equivalent (0.4nmol/rat), or 5X less molar (0.08nmol/rat) dose to the pro-nociceptive dose of TSP4 (45µg/rat). Data presented are the Means ± SEM from 6-9 rats in each group. *p<0.05, **p<0.01, ***p <0.001 compared with pre-treatment level by one-way ANOVA analysis with Bonferroni post-hoc tests. $^{\#\#}$p<0.01 compared between $Ca_v\alpha_2\delta_1$-binding peptide and scramble peptide by one-way ANOVA analysis with Bonferroni post-hoc test.

D. Bolus i.t. injection of $Ca_v\alpha_2\delta_1$-binding TSP4 peptide 23, 49, or 143 into naïve rats was tested before, at day-four, and day-ten post-peptide injection using von Frey filaments. Data presented are the Means ± SEM from 6-7 rats in each group.

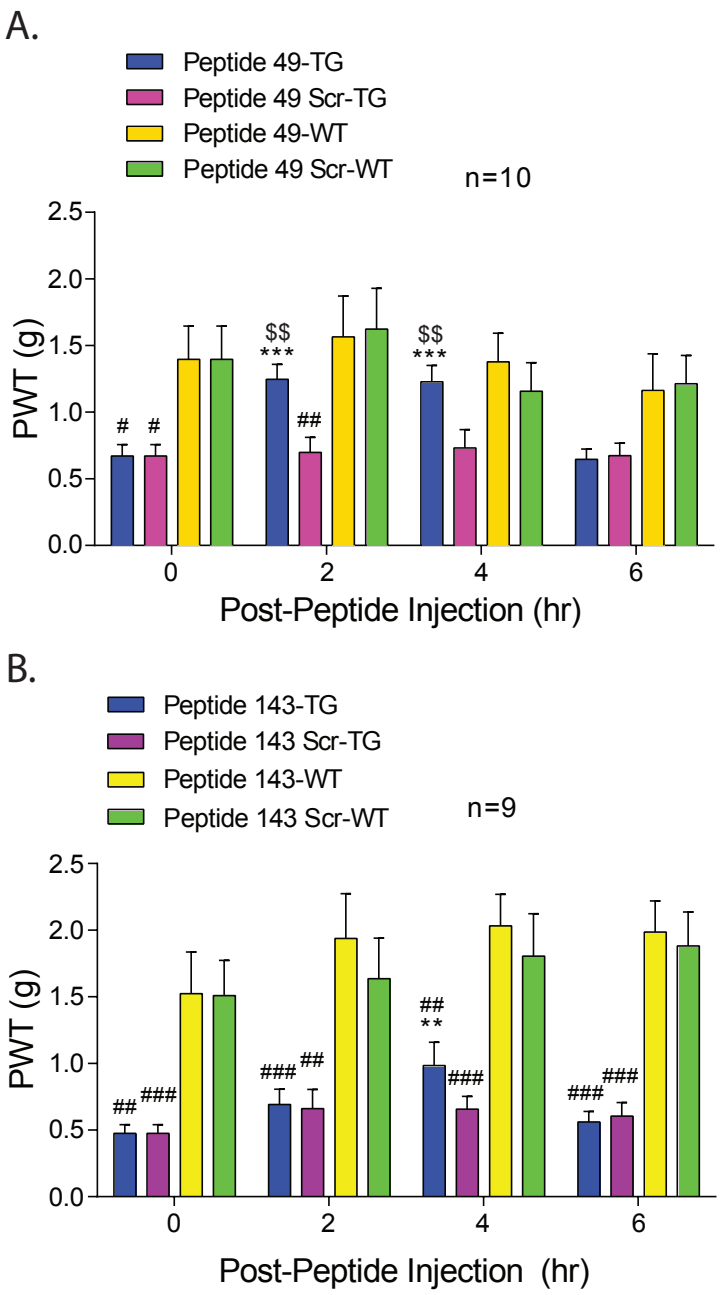

Figure 6. $Ca_v\alpha_2\delta_1$-binding TSP4 peptide 49 attenuates $Ca_v\alpha_2\delta_1$-induced tactile allodynia. Bolus i.t. injection of $Ca_v\alpha_2\delta_1$-binding peptide 49 (**A.**), 143 (**B.**), or respective scramble peptides (0.047nmol/mouse) at equal molar dose to TSP4 (5µg/mouse) was administered to $Ca_v\alpha_2\delta_1$-overexpressing transgenic mice (TG) and their age- and sex-matched wild-type littermates (WT) at time 0, followed by behavioral test for PWT at 2-hr, 4-hr, and 6-hr post peptide treatment. Data presented are the Means ± SEM from 9-10 mice in each group. **p<0.01, ***p <0.001 compared with pre-treatment level by one-way ANOVA analysis with Bonferroni post-hoc tests. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared between TG and WT mice by two-way ANOVA analysis with Bonferroni post-hoc test. $^{\$\$}p<0.01$ compared between $Ca_v\alpha_2\delta_1$-binding peptide and scramble peptide treated TG mice by two-way ANOVA analysis with Bonferroni post-hoc test.

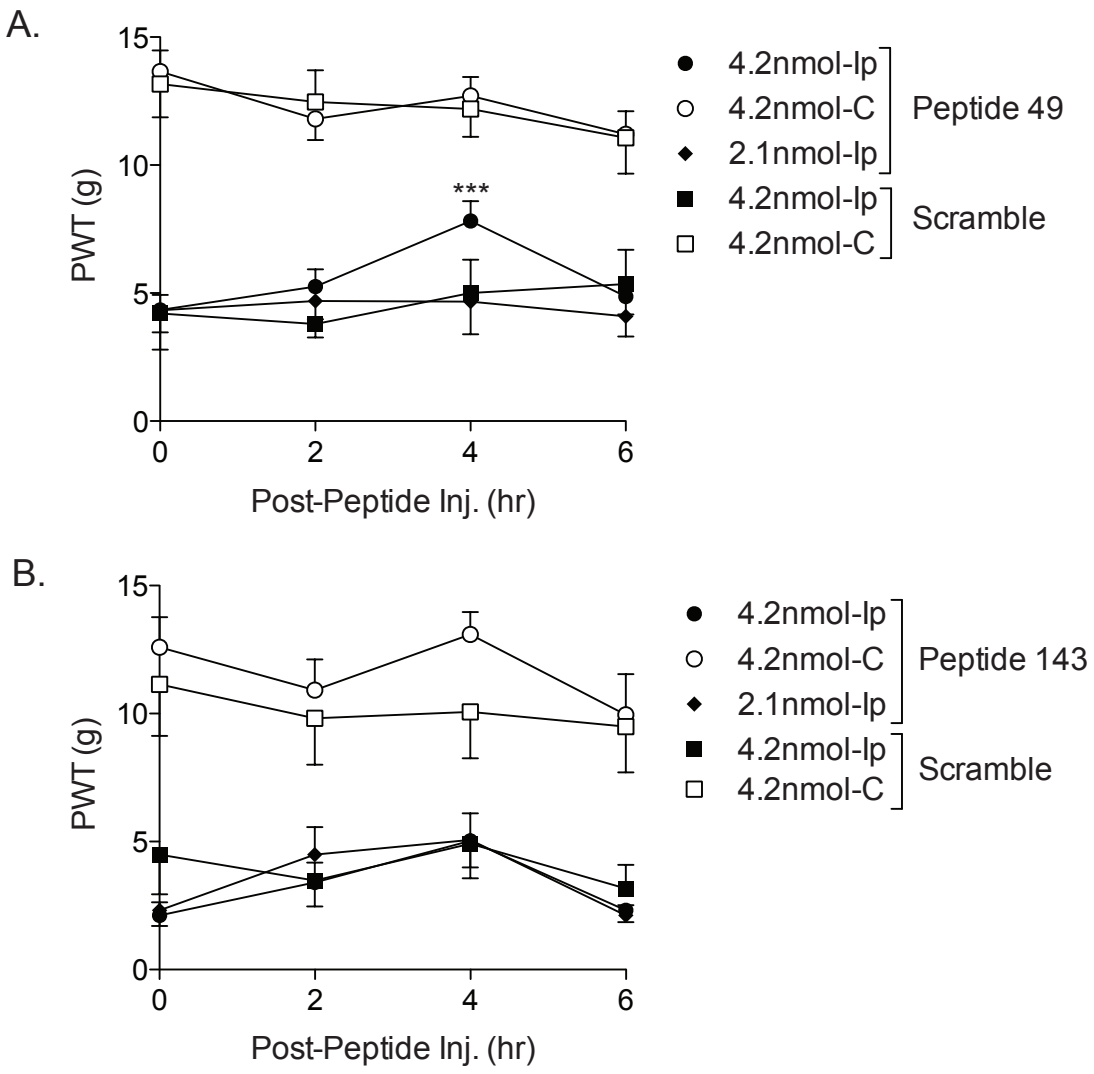

Figure 7. $Ca_v\alpha_2\delta_1$-binding TSP4 peptide 49 causes partial reversal of injury-induced tactile allodynia in two-week SNL rats. Bolus i.t. injection of $Ca_v\alpha_2\delta_1$-binding peptide 49 (**A.**), 143 (**B.**), or respective scramble peptides at 10X molar excess (4.2nmol/rat) or 5X molar excess (2.1nmol/rat) of TSP4 (45µg/rat) was administered to post-two week SNL rats at time 0, followed by behavioral test for PWT at 2-hr, 4-hr, and 6-hr post peptide treatment. Injection of peptides did not affect the behavioral threshold on the non-injury side. Ip, ipsilateral to injury; C, contralateral to injury. Data presented are the Means ± SEM from 6-12 rats in each group. ***p <0.001 compared with pre-treatment level by one-way ANOVA analysis with Bonferroni post-hoc tests.

CHAPTER 3

Synaptic ultrastructure changes in trigeminal-spinal-complex post trigeminal nerve injury

Abstract

Trigeminal nerves collecting sensory information from the orofacial area synapse on second order neurons in the superficial dorsal horn of subnucleus caudalis and cervical C1/C2 spinal cord (Vc/C2), which is a critical site for sensory information processing. Injury to the trigeminal nerves may cause maladaptive changes in synaptic connectivity that plays an important role in chronic pain development. Here, I examined whether injury to the infraorbital nerve, a branch of the trigeminal nerves, led to synaptic ultrastructural changes when the injured animals have developed neuropathic pain states. Transmission electron microscopy was used to examine synaptic profiles in Vc/C2 at three-weeks post-injury, corresponding to the time of peak behavioral hypersensitivity following chronic constriction injury to the infraorbital nerve (CCI-ION). Using established criteria, synaptic profiles were classified as associated with excitatory (R-), inhibitory (F-), and primary afferent (C-) terminals. Counts were made of each type within the superficial dorsal horn of the Vc/C2 and compared between sham and injured animals, and synaptic contact length was also measured. Compared with data from sham controls, rats with trigeminal nerve injury had increased numbers of R- (laminar I) and F- (lamina II) profiles and decreased mean synaptic length of F-profiles (lamina II) within the Vc/C2 superficial dorsal horn three-weeks post injury. However, the synapse numbers and mean synaptic length of C-type profiles are similar between sham and CCI-ION rats. Together, these changes in excitatory and inhibitory synaptic profiles in the superficial dorsal horn of Vc/C2 may contribute to the development of orofacial pain states.

Introduction

Trigeminal nerve injuries can lead to the development of neuropathic pain, which is debilitating and difficult to treat (Bennetto et al., 2007; Zakrzewska and McMillan, 2011). This disorder can lead to pain sensations with innocuous stimuli (allodynia) or exaggerated pain sensations with supra-threshold stimuli (hyperalgesia) (Iwata et al., 2011; Zakrzewska and McMillan, 2011). Mechanisms underlying neuropathic pain are not well understood. Recently, our group and others have reported that neuropathic pain has been attributed, at least in part, to synaptic changes in the neuronal circuitry of the spinal cord that may mediate injury-induced pain sensations (Li et al., 2014a; Li et al., 2014b; Sandkuhler, 2009).

The trigeminal spinal subnucleus caudalis and cervical C1/C2 spinal cord (Vc/C2) is the major projection site for trigeminal nerves transmitting both noxious and non-noxious information (Sessle, 2000). The Vc/C2 receives nociceptive information through glutamatergic peripheral inputs (Bae et al., 2000; Hu, 1990). Vc/C2 neuron excitability is significantly enhanced after trigeminal nerve injury due to increased receptive field sizes, basal level and evoked hyperexcitability that reflect central sensitization (Chiang et al., 2005; Iwata et al., 2001; Iwata et al., 2004; Li et al., 2014b; Tsuboi et al., 2004). Also, local inhibitory interneurons involving γ-aminobutyric acid (GABAergic) and/or glycinergic interneurons have been shown to modulate Vc/C2 neuron activities (Avendano et al., 2005; Bae et al., 2005; Jacquin et al., 1989) and pre-synaptic inhibition of primary afferent terminals (Todd, 1996). After nerve injury, there appears to be a disinhibition in the superficial spinal dorsal horn and spinal trigeminal nucleus through a reduction in GABAergic inhibitory transmission (Martin et al., 2010; Moore et al., 2002) and loss in GABA-immunoreactive neurons (Castro-Lopes et al., 1993; Eaton et al., 1998; Ibuki et al., 1997; Martin et al., 2010; Moore et al., 2002; Somers and Clemente, 2002). Together, synaptic alterations of excitatory or inhibitory neurons in the Vc/C2 may contribute to chronic orofacial pain states. However, it is not clear if these synaptic changes are

mediated by increased spinal synapses and/or enhanced synaptic activity of existing synapses.

In this study, I examined ultrastructural changes of synapses in a rat model of chronic constriction injury of the infraorbital nerve (CCI-ION) in relation to orofacial neuropathic pain development. Since the ION branch of the trigeminal nerve is exclusively sensory and nociceptive fibers terminating mainly in lamina I-III of the Vc/C2 (Morris et al., 2004; Todd, 2010), and CCI-ION injury leads to peak orofacial hypersensitivity three-weeks after nerve injury (Li et al., 2014a; Vos et al., 1994), I examined changes in synapse profiles in the superficial dorsal horn of Vc/C2 from sham and CCI-ION rats three-weeks post surgery with electron microscopy.

Materials and Methods

Experimental Animals: Six adult male Harlan Sprague-Dawley rats weighing approximately 200g were used for this study. The animals were exposed to 12 h light/12 h dark cycle with food and water available *ab libitum*. The animal usage and experimental protocols were approved by Institutional Animal Care and Use Committee of the University of California Irvine.

Surgery: The unilateral chronic constriction injury to the infraorbital nerve was performed as described (Kernisant et al., 2008). Briefly, isoflurane anesthetized rats with hair shaved above the left eye were put in a stereotaxic frame. An anterior-posterior skin incision following the curve of the frontal bone was made above the left eye. The ION lying on the maxillary bone was exposed and dissected free from surrounding connective tissue within the orbit. Fine forceps and a silk suture (5-0) loaded needle with a bended tip were used to place two loose ligations around the IoN, 3-4 mm apart. Sham operated animals were similarly prepared with no ION ligation. The muscle layers and skin incisions were closed with silk sutures (5-0) and rats were recovered on a warm heating pad.

Behavioral Testing: Blinded orofacial behavioral testing was performed as described (Li et al., 2014a; Vos et al., 1994). Briefly, the rat vibrissal pad was shaved under light isoflurane anesthesia one day before behavioral test. Rats were habituated at least one hr prior to behavioral testing. Orofacial mechanical sensitivity to von Frey filament stimulation was performed with a series of von Frey filaments (numbers 3.61, 3.84, 4.08, 4.31, 4.56, 4.74, 4.93 and 5.18; equivalent to 0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0 and 15 g of force, respectively) with Dixon's Up-Down method (Dixon, 1980) on both sides of the vibrissal pad. The following behaviors were considered a positive response to von Frey filaments: 1) a brisk withdrawal reaction; 2) an escape/attack reaction in which a rat avoided the filament either by moving away from the filament to assume a crouching

position against a cage wall or burying its head under the body, or attacked the filament by biting and grabbing; 3) asymmetric face grooming shown as at least three uninterrupted face-wash strokes directed to the facial area being stimulated, often preceded by a brisk withdrawal reaction. Presence or absence of a positive response prompted the use of the next lower or higher stimulating force, respectively, which was repeated for a total of six responses to von Frey stimuli. The 50% withdrawal threshold values were calculated using the formula: $10^{(X+kd)}/10^4$, where X is the value of the final von Frey filament used in log units, k is the tabular value for the positive/negative response patterns from Chaplan et al. (Chaplan et al., 1994) and d is the mean differences between stimuli in log units. Scores of 0.25 or 15 g were assigned, respectively, when consecutive positive or negative responses were observed.

Electron Microscopy: CCI-ION and sham rats were perfused with 2% paraformaldehyde/2% glutaraldehyde three-weeks post CCI-ION that correlates with orofacial hypersensitivity in the injury side of CCI-ION rats (Li et al., 2014). The Vc/C2 was isolated, rinsed in 0.1M sodium cacodylate buffer, and post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 1 hour, dehydrated in increasing serial dilutions of ethanol (70%, 85%, 95%, 100%) for 10 min each, incubated in propylene oxide for 1 hour, incubated in propylene oxide/Spurr's resin (1:1) for 1 hour, and then embedded in Spurr's resin overnight. Ultrathin sections (~60nm thickness) were cut using a Leica Ultracut UCT ultramicrotome (Leica, Vienna, Austria), mounted on copper grids, stained with uranyl acetate and lead citrate. Images of the Vc/C2 were viewed on a JEOL 1400 transmission electron microscope (JEOL, Tokyo, Japan) and images were captured using a Gatan Digital camera (Gatan, Pleasanton, CA, USA).

Image Processing: The sampling procedure was performed as described by Darian-Smith et al. (Darian-Smith et al., 2010). Briefly, synapses in electron micrographs

(5000X) were counted along tracks (4μm wide, with 10μm between tracks) taken at different depths and running parallel to the dorsal surface of the spinal cord. Data from three tracks were combined for analysis to identify any laminar specificity. In each non-overlapping image, the following synapse types were identified: R = synapses with round vesicles, asymmetric synapse, and a single large synaptic contact (presumed to be excitatory); C = large presynaptic terminal with round vesicles and often form multiple synaptic contacts and contain dense-cored vesicles (presumed to be primary afferent axons); F = presynaptic terminals with pleomorphic-flattened vesicles and symmetric synapses (presumed to be inhibitory synapses). An observer who was blinded for animal groups identified these synaptic profiles from each image.

To identify changes in the proportion of a particular type of synaptic profile after CCI-ION, counts of each synaptic profile were divided by the total of all synaptic profile counts across all tracks per dorsal horn section and expressed as a percentage of total synaptic counts. Synaptic profile numbers for each track were also normalized to area of 100μm^2. To determine whether any changes in synaptic profile numbers were actual changes in particular synapse types, but not changes in size of the profiles, I estimated their numerical density by using the size-frequency method (DeFelipe et al., 1999). The number of synapse profile per unit volume was estimated using the formula $N_v = N_A/d$, in which "N_A" is the number of a particular type of synapses per unit area and "d" is the average length of corresponding synaptic densities (μm). The synapse length provides a normalization factor for sampling bias introduced by tissue thickness. For all synapses identified, the post-synaptic density length was measured using ImageJ software.

To determine if unilateral CCI-ION injury caused bilateral effects on synaptic profiles, sham rats were also included as controls for comparisons using univariate analysis of variance (ANOVA). For changes in total synaptic profile counts, one-way ANOVA with *post hoc* Bonferroni tests was used to compare sections between the ipsilateral and contralateral Vc/C2 of both CCI-ION and sham rats. For laminar specificity,

sections from the ipsilateral and contralateral Vc/C2 of CCI-ION rats were compared with that from the ipsilateral and contralateral Vc/C2 of sham rats using two-way ANOVA with repeated measures, followed by Bonferroni *post hoc* test between tracks (ipsilateral vs. contralateral Vc/C2 of CCI-ION; ipsilateral Vc/C2 of CCI-ION vs. ipsilateral Vc/C2 of sham). All statistical analysis were performed by using PRISM 6.0 (Graphpad, San Diego, CA).

Results

I examined whether there were changes in the number of different types of synapses during the development of chronic orofacial pain after CCI-ION. As shown in Figure 1, unilateral CCI-ION injury caused a reduction in orofacial threshold to von Frey filament stimuli, or tactile allodynia in the injury side three weeks post-surgery compared with that from the non-injury side and sham operated rats. This is consistent with previous reports from this model (Li et al., 2014a; Vos et al., 1994). Thin sections of the Vc/C2 spinal cord were collected from CCI-ION and sham rats three weeks post-surgery and examined for synaptic changes. Profiles were identified and counted from 12 most superficial tracks (4μm wide spaced at 10μm intervals) running parallel to the dorsal surface of the spinal cord and covering lamina I/II (Darian-Smith et al., 2010). I combined every three tracks for analysis to identify any laminar specificity. Figure 2 shows typical examples of each type of profile quantified in my analysis. The R-profiles were defined as having a single asymmetric synapse (large post-synaptic density) with round vesicles (Figure 2A). In contrast, C-profiles were large, contain round and dense core vesicles, and often form multiple asymmetric synapses (Figure 2C). The F-profiles contained pleomorphic vesicles and form symmetrical synapses (Figure 2B).

Table 1 shows the total number of each synaptic profile (R-, F-, and C-profile) counted and the total area of Vc/C2 dorsal horn analyzed across all tracks. A total of 4,516 and 5,041 profiles were quantified from three CCI-ION and sham-operated animals, respectively. Each profile type was normalized with respect to the tissue area examined (per 100μm^2) or expressed as a proportion of total profiles summed across all tracks per animal. There was no significant difference in either the counts per unit area or the percentage of synapse profiles between the nerve-injured and sham dorsal horn sections (Table 1; Figure 3A and B). These data suggest that CCI-ION injury did not affect total synaptic profile counts or proportion of each synapse type within the superficial dorsal horn. Since the total areas of Vc/C2 dorsal horn sections examined were not significantly

different between CCI-ION and sham rats (Figure 3C), potential sampling bias of synaptic profile counts is less likely.

CCI-ION injury led to increased R-profiles in superficial dorsal horn

Area

To examine whether there was a laminar difference in the synaptic profile numbers after CCI-ION, I quantified each synapse profile type per 100μm^2 at the superficial laminae of the dorsal horn, and compared data from the ipsilateral CCI-ION to that from contralateral CCI-ION and ipsilateral sham rats using two-way ANOVA with Bonferroni *post hoc* test. As shown in Table 2, there was no significant "group" (CCI-ION ipsilateral, CCI-ION contralateral, sham ipsilateral, sham contralateral) effect on R-, F-, or C-synaptic profile counts per 100μm^2, which is in agreement with the results shown in Table 1 and Figure 3. However, there was a significant interaction between "group" effects and laminar specificity for the R-profiles, but not the C- and F-profiles (Table 2; Figure 4), which corresponds to a significant increase in R-profile types within 45μm of the superficial dorsal horn three-weeks after CCI-ION injury (Figure 4A).

Volumetric Density

To ensure that changes in profile types observed in Figure 4 were not due to sampling bias introduced in ultra thin sections, I used the size-frequency method (Darian-Smith et al., 2010; DeFelipe et al., 1999) to estimate the numbers of profiles per unit volume and compared data from the CCI-ION ipsilateral dorsal horn with that from the CCI-ION contralateral and sham ipsilateral dorsal horn using two-way ANOVA with Bonferroni *post hoc* test. My data indicated that the overall group effect was not significant, but there was a significant interaction between the effects of injury and laminar specificity for the R- and F-synaptic profiles, but not the C-synaptic profiles (Table 2; Figure 5), which corresponds to a significant increase in the R-profiles within 45μm of the superficial dorsal horn and

F-profiles within 45μm-90μm of the superficial dorsal horn three-weeks after CCI-ION injury (Figure 5).

Synapse Length

To provide a measure of the strength of synaptic contacts associated with different synaptic profile types, I measured the post-synaptic length for each profile type, a parameter that can be affected by changes in synaptic efficacy and plasticity (Desmond and Levy, 1986; Pierce and Lewin, 1994). For F-profiles, there was a significant reduction in post-synaptic length post CCI-ION injury, particularly within 45μm of the superficial dorsal horn (Figure 6B). In contrast, CCI-ION injury did not cause a significant change on post-synaptic length of R- and C-synaptic profiles.

Discussion

Findings from our previous studies have indicated that CCI-ION trigeminal nerve injury leads to aberrant excitatory synaptogenesis in the superficial dorsal horn of trigeminal spinal complex (Vc/C2) that correlates with dorsal horn neuron sensitization and orofacial neuropathic pain states (Li et al., 2014b). This suggests that nerve injury-induced aberrant synaptogenesis is likely a form of neuroplasticity underlying the development of orofacial neuropathic pain post trigeminal nerve injury. However, it is not clear if this nerve injury-induced maladaptive change affects the number and/or strength of different synapse types. In this study, I examined synapse ultrastructure in superficial dorsal horn of Vc/C2 from sham and CCI-ION rats three-weeks post injury when the injured rats exhibit severe orofacial pain states (Figure 1) (Li et al., 2014a). My data indicate that trigeminal nerve injury leads to an increase in mainly R-synaptic profiles (presumably excitatory synapses) and a decrease in F-synaptic length (presumably inhibitory synapses) in lamina I of Vc/C2, as well as an increase in F-synapse profiles in lamina II of Vc/C2 (Figure 7). In contrast, there is no significant change in C-synaptic profiles, presumably primary afferents, on the injury side. Together, my data support that maladaptive synaptic changes in dorsal spinal cord post trigeminal nerve injury may contribute to the development of orofacial neuropathic pain states.

To ensure that the increase in R-synaptic profiles was not due to an increase in size of R-profiles that could increase the likelihood of detecting the same profile more than once in individual micrographs, I applied the size-frequency method to correct for sampling biases introduced by the fact that the synapses are several times larger than the tissue section thickness. This sampling method has been shown to be efficient in analyzing large areas in spinal dorsal horn and reducing variations across samples (Benes and Lange, 2001; Darian-Smith et al., 2010; DeFelipe et al., 1999). My data indicate that findings from the size-frequency method correlate well with results of synaptic profile counts per unit area, confirming an increase in the number of R-profiles, presumably

excitatory synapses, in superficial dorsal horn of the injury side three-weeks after CCI-ION nerve injury compared to that from non-injury side of CCI-ION and sham control rats. A potential limitation in this synapse-profiling scheme is that an identified “R-profile” could be a “C-Profile” since both profiles share similar structural morphology. For instance, if a synapse profile consists of round vesicles, one asymmetrical synapse, and at least one dense-core vesicle, it would be considered a C-profile since at the ultrastructural level, dense core vesicles are associated with primary afferent fibers (Bailey and Ribeiro-da-Silva, 2006; Ribeiro-da-Silva et al., 1989). However, if tissue sectioning leads to loss of the dense core vesicle, the profile could be misidentified as an R-profile instead. While I cannot conclusively determine whether the significant increase in R-profiles is from excitatory interneurons or primary afferent fibers, our previous data indicate that almost all increased synapses in superficial dorsal horn of Vc/C2 contain VGLUT2 vesicular glutamate transporter (Li et al., 2014b), which is expressed in many of the excitatory interneurons (Braz et al., 2014; Maxwell et al., 2007; Oliveira et al., 2003; Santos et al., 2009; Todd et al., 2003; Yasaka et al., 2010), and myelinated, but not unmyelinated, afferents in lamina I (Todd et al., 2003). This suggests that increased excitatory synapses in superficial dorsal horn post nerve injury may have direct effects on at least orofacial tactile allodynia (Li et al., 2014b), which is mediated mainly by myelinated sensory fibers (Julius and Basbaum, 2001). Interestingly, genetic ablation of VGLUT2, but not VGLUT1, attenuates nerve-injury induced tactile allodynia, cold and thermal hyperalgesia (Leo et al., 2009; Rogoz et al., 2012). Since cold and thermal hyperalgesia are mainly transmitted by unmyelinated nociceptive afferents (Julius and Basbaum, 2001), it is possible that increased synapses in excitatory interneurons contribute to hypersensitivities to these modalities indirectly. Further functional studies are warranted to further delineate the functionality of nerve injury-induced R-synaptic profiles.

Interestingly, I also show an increase of F-synaptic profile counts, presumably inhibitory, on the injury side of CCI-ION compared to sham rats within lamina II of the

Vc/C2 superficial dorsal horn after corrections for sampling bias by the size-frequency method (DeFelipe et al., 1999). In addition, a significant decrease in mean synaptic length of F-profiles was observed within lamina I of Vc/C2 superficial dorsal horn from the injury side of CCI-ION rats compared with that from non-injury side of CCI-ION and sham rats. Synaptic length, the length of post-synaptic density along the parallel pre- and post-synaptic membranes of the profiles, is a structural correlate of synaptic strength (Pierce and Lewin, 1994). There was no significant change in mean synaptic length of R- and C-synaptic profiles in the injury side of superficial dorsal horn post CCI-ION injury, supporting that synaptic strength of these profiles were not likely changed post injury. Together, these results suggest that an increase in inhibitory circuitry in lamina II combined with a decrease in inhibitory synaptic strength in lamina I of Vc/C2 may contribute to abnormal sensation processing.

The contributory role of inhibitory interneurons to neuropathic pain states processing remains controversial due to confounding findings from neuropathic pain models. Findings from some studies have shown a reduction of GABAergic inhibitory transmission in lamina II neurons following chronic constriction injury (CCI) or spared nerve injury (SNI) of the sciatic nerve (Moore et al., 2002) and a substantial loss of GABA-immunoreactive neurons following CCI (Eaton et al., 1998; Ibuki et al., 1997; Martin et al., 2010). These changes could attribute, at least in part, to neuronal cell death following nerve injury that could result in the loss of inhibitory input onto excitatory neurons, or disinhibition, in spinal superficial dorsal horn, leading to chronic pain states after nerve injury (Castro-Lopes et al., 1993; Eaton et al., 1998; Ibuki et al., 1997; Moore et al., 2002; Somers and Clemente, 2002). However, findings from other stereological studies have shown no loss in neuronal population, or GABA-immunoreactivity in the superficial dorsal horn following CCI (Polgar et al., 2004; Polgar et al., 2003). These discrepancies may be attributed in part to variations in examining specific populations of inhibitory interneurons by selective immunoreactive markers (such as GABA, glycine, and glutamate decarboxylase) and/

or global measurements of inhibitory interneurons without assessing for synapses and laminar specificity at the ultrastructural level. My findings showed no overall injury effect on total F-synaptic profile counts in the Vc/C2 superficial dorsal horn that complements previous findings using stereological methods (Polgar et al., 2004; Polgar et al., 2003), but an increase in F-synaptic profile counts only within lamina II of the Vc/C2 after CCI-ION injury compared to sham animals. It is possible that this injury-induced plasticity leads to increased inhibitory synaptic input (from other interneurons) into lamina II inhibitory interneurons, resulting in disinhibition that may contribute to neuropathic pain states. This is supported by findings that the numbers of F-synaptic profiles and GABA-immunoreactive inhibitory synapses are significantly increased within superficial dorsal horn of a primate model following cervical dorsal rhizotomy (Darian-Smith et al., 2010). In addition, inhibitory interneurons within lamina II of the superficial dorsal horn can receive inhibitory synaptic input from glycinergic and/or GABAergic interneurons (Labrakakis et al., 2009; Lu et al., 2013; Miraucourt et al., 2007; Takazawa and MacDermott, 2010), forming a feed-forward inhibitory control within lamina II/III of the superficial dorsal horn to polysynaptic low-threshold Aβ-fiber input onto nociceptive neurons (Labrakakis et al., 2009; Lu et al., 2013; Miraucourt et al., 2007; Miraucourt et al., 2009). Thus, disrupting this inhibitory circuit by increased inhibitory synapses may elicit CCI-ION injury-induced allodynia, similar to that resulted from suppression of this feed-forward inhibitory circuit following spinal nerve ligation injury (Lu et al., 2013) or strychnine-induced disinhibition (Labrakakis et al., 2009). Furthermore, my findings suggest that a reduction in inhibitory synaptic strength and/or connectivity in lamina I of superficial dorsal horn, presumably through reduced inhibitory control to nociceptors at this location, may contribute to the development of neuropathic pain states.

Bilateral behavioral hypersensitivity has been reported in this model, even though that in the contralateral side is much less server (Li et al., 2014a; Martin et al., 2010; Vos et al., 1994). This bilateral effect may be due to trigeminal primary afferents projecting

to the contralateral Vc/C2 region (Jacquin et al., 1990; Panneton et al., 1991) or other systemic effects such as bilateral activation of microglial cells and over production of cytokines post injury. Since these bilateral effects post CCI-ION injury are not specifically correlated with the onset and duration of orofacial pain states (Li et al., 2014a; Martin et al., 2010; Vos et al., 1994), and my data show injury-side specific synaptic plasticity, but similar synaptic ultrastructures between non-injury (contralateral) side of CCI-ION and injury side of sham rats, it is likely that changes in synapse ultrastructures in the Vc/C2 region of the injury side play a dominant role in orofacial hypersensitivity.

Spinal neurons are heterogeneous and include, but are not limit to, projection neurons, excitatory and inhibitory interneurons. Sensory afferents projecting to Vc/C2 dorsal horn can form direct synaptic connections with these neurons, and excitatory or inhibitory interneurons can directly or indirectly modulate the activity of projection neurons, and/or other interneurons through specific synaptic connections (Braz et al., 2014). This well-organized spinal circuitry, in conjunction with descending modulation, forms the molecular basis for sensory information processing at the level of trigeminal-spinal complex. In order to understand the role of nerve injury-induced aberrant synaptic neurotransmission in orofacial pain processing, it is critical to understand the nature of injury-induced changes in synaptic ultrastructures. In combination with other recent findings, my findings presented here allow us to propose the following molecular mechanism underlying the CCI-ION induced aberrant synaptic transmission. 1) CCI-ION injury leads to overexpression of the calcium channel alpha-2-delta-1 proteins in trigeminal neurons and pre-synaptic afferent terminals in superficial Vc/C2 that contributes to orofacial neuropathic pain states (Li et al., 2014b). 2) CCI-ION injury also induces upregulation of thrombospondin-4 (TSP4) in Vc/C2 regions that contributes to orofacial neuropathic pain states (Li et al., 2014a). 3) Elevated alpha-2-delta-1 proteins are synaptogenic and promote aberrant excitatory synaptogenesis in superficial dorsal horn by interacting with thrombospondin proteins (Chou et al., 2002; Eroglu et al., 2009; Kim et al., 2012; Li et al., 2014a; Lo et al., 2011). 4)

CCI-ION may also cause disinhibition of inhibitory interneurons in lamina II and projection neurons in lamina I (Figure 7). In conclusion, my findings support that trigeminal nerve injury leads to maladaptive excitatory/inhibitory synaptic plasticity that likely contributes to central sensitization and orofacial neuropathic pain states (Li et al., 2014a; Li et al., 2014b). Identification of affected subpopulations of neurons as well as their synaptic connectivity in future studies will shed some light on the detail mechanism of orofacial neuropathic pain processing post trigeminal nerve injury.

References

Avendano C, Machin R, Bermejo PE, Lagares A. 2005. Neuron numbers in the sensory trigeminal nuclei of the rat: A GABA- and glycine-immunocytochemical and stereological analysis. J Comp Neurol 493(4):538-553.

Bae YC, Ihn HJ, Park MJ, Ottersen OP, Moritani M, Yoshida A, Shigenaga Y. 2000. Identification of signal substances in synapses made between primary afferents and their associated axon terminals in the rat trigeminal sensory nuclei. J Comp Neurol 418(3):299-309.

Bae YC, Park KS, Bae JY, Paik SK, Ahn DK, Moritani M, Yoshida A, Shigenaga Y. 2005. GABA and glycine in synaptic microcircuits associated with physiologically characterized primary afferents of cat trigeminal principal nucleus. Exp Brain Res 162(4):449-457.

Bailey AL, Ribeiro-da-Silva A. 2006. Transient loss of terminals from non-peptidergic nociceptive fibers in the substantia gelatinosa of spinal cord following chronic constriction injury of the sciatic nerve. Neuroscience 138(2):675-690.

Benes FM, Lange N. 2001. Two-dimensional versus three-dimensional cell counting: a practical perspective. Trends in neurosciences 24(1):11-17.

Bennetto L, Patel NK, Fuller G. 2007. Trigeminal neuralgia and its management. BMJ 334(7586):201-205.

Braz J, Solorzano C, Wang X, Basbaum AI. 2014. Transmitting pain and itch messages: a contemporary view of the spinal cord circuits that generate gate control. Neuron 82(3):522-536.

Castro-Lopes JM, Tavares I, Coimbra A. 1993. GABA decreases in the spinal cord dorsal horn after peripheral neurectomy. Brain Res 620(2):287-291.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. 1994. Quantitative assessment of tactile allodynia in the rat paw. Journal of neuroscience methods 53(1):55-63.

Chiang CY, Zhang S, Xie YF, Hu JW, Dostrovsky JO, Salter MW, Sessle BJ. 2005. Endogenous ATP involvement in mustard-oil-induced central sensitization in trigeminal subnucleus caudalis (medullary dorsal horn). J Neurophysiol 94(3):1751-1760.

Chou AK, Muhammad R, Huang SM, Chen JT, Wu CL, Lin CR, Lee TH, Lin SH, Lu CY, Yang LC. 2002. Altered synaptophysin expression in the rat spinal cord after chronic constriction injury of sciatic nerve. Neurosci Lett 333(3):155-158.

Darian-Smith C, Hopkins S, Ralston HJ, 3rd. 2010. Changes in synaptic populations in the spinal dorsal horn following a dorsal rhizotomy in the monkey. J Comp Neurol 518(1):103-117.

DeFelipe J, Marco P, Busturia I, Merchan-Perez A. 1999. Estimation of the number of synapses in the cerebral cortex: methodological considerations. Cereb Cortex 9(7):722-732.

Desmond NL, Levy WB. 1986. Changes in the postsynaptic density with long-term potentiation in the dentate gyrus. J Comp Neurol 253(4):476-482.

Dixon WJ. 1980. Efficient analysis of experimental observations. Annual review of pharmacology and toxicology 20:441-462.

Eaton MJ, Plunkett JA, Karmally S, Martinez MA, Montanez K. 1998. Changes in GAD- and GABA- immunoreactivity in the spinal dorsal horn after peripheral nerve injury and promotion of recovery by lumbar transplant of immortalized serotonergic precursors. Journal of chemical neuroanatomy 16(1):57-72.

Eroglu C, Allen NJ, Susman MW, O'Rourke NA, Park CY, Ozkan E, Chakraborty C, Mulinyawe SB, Annis DS, Huberman AD, Green EM, Lawler J, Dolmetsch R, Garcia KC, Smith SJ, Luo ZD, Rosenthal A, Mosher DF, Barres BA. 2009. Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell 139(2):380-392.

Hu JW. 1990. Response properties of nociceptive and non-nociceptive neurons in the rat's trigeminal subnucleus caudalis (medullary dorsal horn) related to cutaneous and deep craniofacial afferent stimulation and modulation by diffuse noxious inhibitory controls. Pain 41(3):331-345.

Ibuki T, Hama AT, Wang XT, Pappas GD, Sagen J. 1997. Loss of GABA-immunoreactivity in the spinal dorsal horn of rats with peripheral nerve injury and promotion of recovery by adrenal medullary grafts. Neuroscience 76(3):845-858.

Iwata K, Imai T, Tsuboi Y, Tashiro A, Ogawa A, Morimoto T, Masuda Y, Tachibana Y, Hu J. 2001. Alteration of medullary dorsal horn neuronal activity following inferior alveolar nerve transection in rats. J Neurophysiol 86(6):2868-2877.

Iwata K, Imamura Y, Honda K, Shinoda M. 2011. Physiological mechanisms of neuropathic pain: the orofacial region. International review of neurobiology 97:227-250.

Iwata K, Masuda Y, Ren K. 2004. Trigeminal neuronal recording in animal models of orofacial pain. Methods Mol Med 99:123-137.

Jacquin MF, Chiaia NL, Rhoades RW. 1990. Trigeminal projections to contralateral dorsal horn: central extent, peripheral origins, and plasticity. Somatosens Mot Res 7(2):153-183.

Jacquin MF, Golden J, Rhoades RW. 1989. Structure-function relationships in rat brainstem subnucleus interpolaris: III. Local circuit neurons. J Comp Neurol 282(1):24-44.

Julius D, Basbaum AI. 2001. Molecular mechanisms of nociception. Nature 413(6852):203-210.

Kernisant M, Gear RW, Jasmin L, Vit JP, Ohara PT. 2008. Chronic constriction injury of the infraorbital nerve in the rat using modified syringe needle. Journal of neuroscience methods 172(1):43-47.

Kim DS, Li KW, Boroujerdi A, Peter Yu Y, Zhou CY, Deng P, Park J, Zhang X, Lee J, Corpe M, Sharp K, Steward O, Eroglu C, Barres B, Zaucke F, Xu ZC, Luo ZD. 2012. Thrombospondin-4 contributes to spinal sensitization and neuropathic pain states.

J Neurosci 32(26):8977-8987.

Labrakakis C, Lorenzo LE, Bories C, Ribeiro-da-Silva A, De Koninck Y. 2009. Inhibitory coupling between inhibitory interneurons in the spinal cord dorsal horn. Mol Pain 5:24.

Leo S, Moechars D, Callaerts-Vegh Z, D'Hooge R, Meert T. 2009. Impairment of VGLUT2 but not VGLUT1 signaling reduces neuropathy-induced hypersensitivity. Eur J Pain 13(10):1008-1017.

Li KW, Kim DS, Zaucke F, Luo ZD. 2014a. Trigeminal nerve injury-induced thrombospondin-4 up-regulation contributes to orofacial neuropathic pain states in a rat model. Eur J Pain 18(4):489-495.

Li KW, Yu YP, Zhou C, Kim DS, Lin B, Sharp K, Steward O, Luo ZD. 2014b. Calcium channel alpha2delta1 proteins mediate trigeminal neuropathic pain states associated with aberrant excitatory synaptogenesis. J Biol Chem 289(10):7025-7037.

Lo FS, Zhao S, Erzurumlu RS. 2011. Astrocytes promote peripheral nerve injury-induced reactive synaptogenesis in the neonatal CNS. J Neurophysiol 106(6):2876-2887.

Lu Y, Dong H, Gao Y, Gong Y, Ren Y, Gu N, Zhou S, Xia N, Sun YY, Ji RR, Xiong L. 2013. A feed-forward spinal cord glycinergic neural circuit gates mechanical allodynia. J Clin Invest 123(9):4050-4062.

Martin YB, Malmierca E, Avendano C, Nunez A. 2010. Neuronal disinhibition in the trigeminal nucleus caudalis in a model of chronic neuropathic pain. Eur J Neurosci 32(3):399-408.

Maxwell DJ, Belle MD, Cheunsuang O, Stewart A, Morris R. 2007. Morphology of inhibitory and excitatory interneurons in superficial laminae of the rat dorsal horn. The Journal of physiology 584(Pt 2):521-533.

Miraucourt LS, Dallel R, Voisin DL. 2007. Glycine inhibitory dysfunction turns touch into pain through PKCgamma interneurons. PLoS One 2(11):e1116.

Miraucourt LS, Moisset X, Dallel R, Voisin DL. 2009. Glycine inhibitory dysfunction induces a selectively dynamic, morphine-resistant, and neurokinin 1 receptor- independent mechanical allodynia. J Neurosci 29(8):2519-2527.

Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ. 2002. Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. J Neurosci 22(15):6724-6731.

Morris R, Cheunsuang O, Stewart A, Maxwell D. 2004. Spinal dorsal horn neurone targets for nociceptive primary afferents: do single neurone morphological characteristics suggest how nociceptive information is processed at the spinal level. Brain research Brain research reviews 46(2):173-190.

Oliveira AL, Hydling F, Olsson E, Shi T, Edwards RH, Fujiyama F, Kaneko T, Hokfelt T, Cullheim S, Meister B. 2003. Cellular localization of three vesicular glutamate transporter mRNAs and proteins in rat spinal cord and dorsal root ganglia. Synapse 50(2):117-129.

Panneton WM, Klein BG, Jacquin MF. 1991. Trigeminal projections to contralateral dorsal horn originate in midline hairy skin. Somatosens Mot Res 8(2):165-173.

Pierce JP, Lewin GR. 1994. An ultrastructural size principle. Neuroscience 58(3):441-446.

Polgar E, Gray S, Riddell JS, Todd AJ. 2004. Lack of evidence for significant neuronal loss in laminae I-III of the spinal dorsal horn of the rat in the chronic constriction injury model. Pain 111(1-2):144-150.

Polgar E, Hughes DI, Riddell JS, Maxwell DJ, Puskar Z, Todd AJ. 2003. Selective loss of spinal GABAergic or glycinergic neurons is not necessary for development of thermal hyperalgesia in the chronic constriction injury model of neuropathic pain. Pain 104(1-2):229-239.

Ribeiro-da-Silva A, Tagari P, Cuello AC. 1989. Morphological characterization of substance P-like immunoreactive glomeruli in the superficial dorsal horn of the rat spinal cord and trigeminal subnucleus caudalis: a quantitative study. J Comp Neurol

281(4):497-415.

Rogoz K, Lagerstrom MC, Dufour S, Kullander K. 2012. VGLUT2-dependent glutamatergic transmission in primary afferents is required for intact nociception in both acute and persistent pain modalities. Pain 153(7):1525-1536.

Sandkuhler J. 2009. Models and mechanisms of hyperalgesia and allodynia. Physiol Rev 89(2):707-758.

Santos SF, Luz LL, Szucs P, Lima D, Derkach VA, Safronov BV. 2009. Transmission efficacy and plasticity in glutamatergic synapses formed by excitatory interneurons of the substantia gelatinosa in the rat spinal cord. PLoS One 4(11):e8047.

Sessle BJ. 2000. Acute and chronic craniofacial pain: brainstem mechanisms of nociceptive transmission and neuroplasticity, and their clinical correlates. Crit Rev Oral Biol Med 11(1):57-91.

Somers DL, Clemente FR. 2002. Dorsal horn synaptosomal content of aspartate, glutamate, glycine and GABA are differentially altered following chronic constriction injury to the rat sciatic nerve. Neurosci Lett 323(3):171-174.

Takazawa T, MacDermott AB. 2010. Glycinergic and GABAergic tonic inhibition fine tune inhibitory control in regionally distinct subpopulations of dorsal horn neurons. The Journal of physiology 588(Pt 14):2571-2587.

Todd AJ. 1996. GABA and glycine in synaptic glomeruli of the rat spinal dorsal horn. Eur J Neurosci 8(12):2492-2498.

Todd AJ. 2010. Neuronal circuitry for pain processing in the dorsal horn. Nature reviews Neuroscience 11(12):823-836.

Todd AJ, Hughes DI, Polgar E, Nagy GG, Mackie M, Ottersen OP, Maxwell DJ. 2003. The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. Eur J Neurosci 17(1):13-27.

Tsuboi Y, Takeda M, Tanimoto T, Ikeda M, Matsumoto S, Kitagawa J, Teramoto K, Simizu K, Yamazaki Y, Shima A, Ren K, Iwata K. 2004. Alteration of the second branch of the trigeminal nerve activity following inferior alveolar nerve transection in rats. Pain 111(3):323-334.

Vos BP, Strassman AM, Maciewicz RJ. 1994. Behavioral evidence of trigeminal neuropathic pain following chronic constriction injury to the rat's infraorbital nerve. J Neurosci 14(5 Pt 1):2708-2723.

Yasaka T, Tiong SY, Hughes DI, Riddell JS, Todd AJ. 2010. Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. Pain 151(2):475-488.

Zakrzewska JM, McMillan R. 2011. Trigeminal neuralgia: the diagnosis and management of this excruciating and poorly understood facial pain. Postgraduate medical journal 87(1028):410-416.

Table 1. Profile counts within Vc/C2 superficial dorsal horn of 3-week CCI-ION rats

Contralateral (non-injured side)												
			R-Profiles			F-Profiles			C-Profiles			
Animal ID	Total Counts	Total Area (μm^2)	Counts	% Total	per 100μm^2	Counts	% Total	per 100μm^2	Counts	% Total	per 100μm^2
CCI-ION												
1	813	8600	103	12.7	1.2	398	49.0	4.6	312	38.4	3.6
2	841	7938	126	15.0	1.6	308	36.6	3.9	407	48.4	5.1
3	711	9049	180	25.3	2.0	179	25.2	2.0	352	49.5	3.9
Sham												
1	904	8652	167	18.5	1.9	281	31.1	3.2	456	50.4	5.3
2	803	8070	153	19.1	1.9	258	32.1	3.2	392	48.8	4.9
3	1041	10108	186	17.9	1.8	333	32.0	3.3	522	50.1	5.2

Ipsilateral (injured side)												
			R-Profiles			F-Profiles			C-Profiles			
Animal ID	Total Counts	Total Area (μm^2)	Counts	% Total	per 100μm^2	Counts	% Total	per 100μm^2	Counts	% Total	per 100μm^2
CCI-ION												
1	779	6615	122	15.7	1.8	382	49.0	5.8	275	35.3	4.2
2	846	7144	123	14.5	1.7	360	42.6	5.0	363	42.9	5.1
3	526	7118	119	22.6	1.7	206	39.2	2.9	260	49.4	3.7
Sham												
1	752	8229	138	18.4	1.7	260	34.6	3.2	354	47.1	4.3
2	656	7382	136	20.7	1.8	192	29.3	2.6	328	50.0	4.4
3	885	7594	102	11.5	1.3	269	30.4	3.5	514	58.1	6.8

Total numbers of synaptic profile counts for 12 tracks within the superficial laminae of Vc/C2 dorsal horn in each of the sections sampled were summed. Profile counts are expressed per 100μm^2 or as proportion of synaptic profile counts/total profile counts.

TABLE 2

Univariate Analyis of Variance for Different Profile Types

	Counts/100µm²			Counts/100µm³			Synapse Length		
	R	**F**	**C**	**R**	**F**	**C**	**R**	**F**	**C**
Interaction	F=3.072	F=1.896	F=0.356	F=3.933	F=2.353	F=0.361	F=2.224	F=1.681	F=1.71
	p=0.013	p=0.102	p=0.945	p=0.003	p=0.046	p=0.942	p=0.057	p=0.149	p=0.13
Group Effect	F=0.746	F=1.067	F=0.887	F=0.683	F=1.575	F=0.196	F=0.463	F=6.440	F=1.63
	p=0.554	p=0.416	p=0.488	p=0.586	p=0.269	p=0.896	p=0.716	p=0.016	p=0.25
Distance	F=22.27	F=35.44	F=35.88	F=19.40	F=32.44	F=25.10	F=2.928	F=11.17	F=0.43
	p=0.000	p=0.000	p=0.000	p=0.000	p=0.000	p=0.000	p=0.054	p=0.000	p=0.73

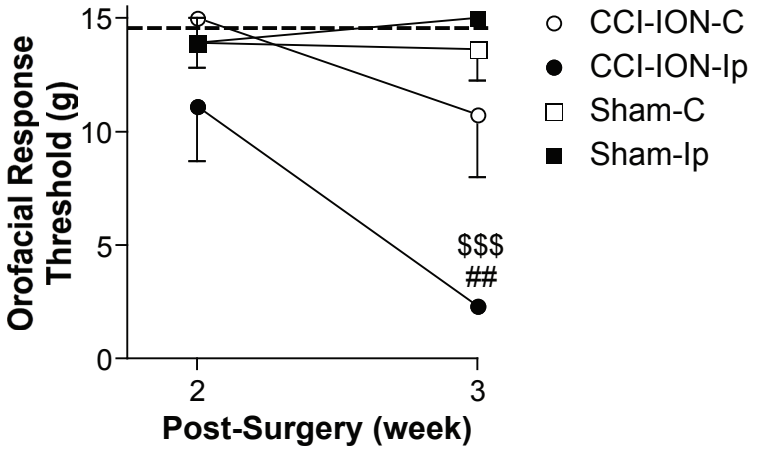

Figure 1. Unilateral chronic constriction injury to the infraorbital nerve (CCI-ION) caused orofacial hypersensitivity to mechanical stimuli three-weeks post-surgery in rats. Sensitivity to von Frey filament stimulation on the whisker pad was tested two- or three-weeks post CCI-ION or sham operations. Dashed line represents averaged orofacial response thresholds to von Frey filaments without injury in naïve rats. Data presented are the means ± SEM from 3 rats for each group. Ip, ipsilateral to injury; C, contralateral to injury. $^{\#\#}p < 0.01$ for CCI-ION-Ip vs. CCI-ION-C; $^{\$\$\$}p < 0.001$ for CCI-ION-Ip vs. sham-Ip by two-way ANOVA followed by Bonferroni post hoc test.

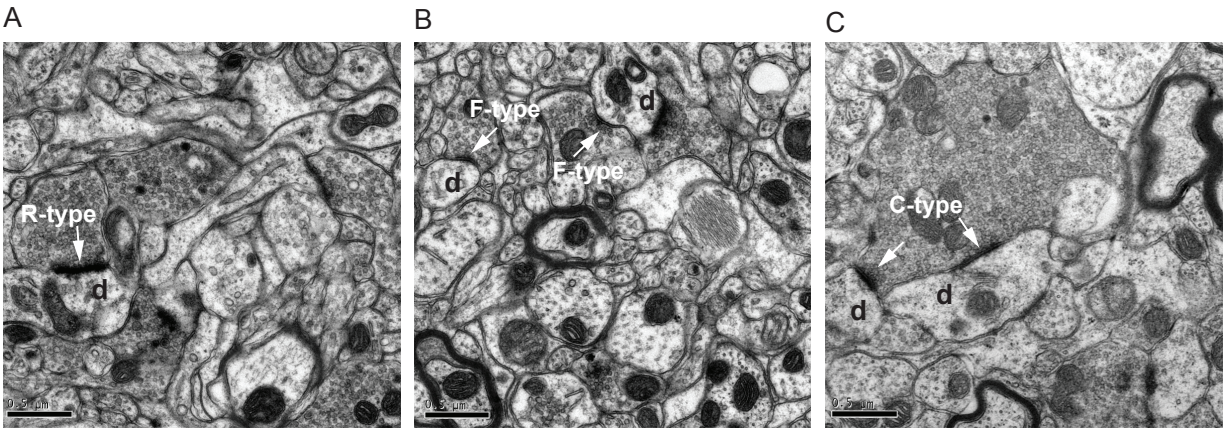

Figure 2. Example of different synaptic profiles in electron micrographs of Vc/C2 superficial dorsal horn from CCI-ION rats. A. a R-type terminal (arrow) with a large synaptic profile and uniform round vesicles typical of an excitatory synapse. **B.** F-type terminals (arrows) with pleomorphic vesicles and synapse typical of an inhibitory synapse. **C.** a C-type primary afferent terminal synapsing with multiple dendrites (arrows). d. dendrite. Scale bars = 0.5µm.

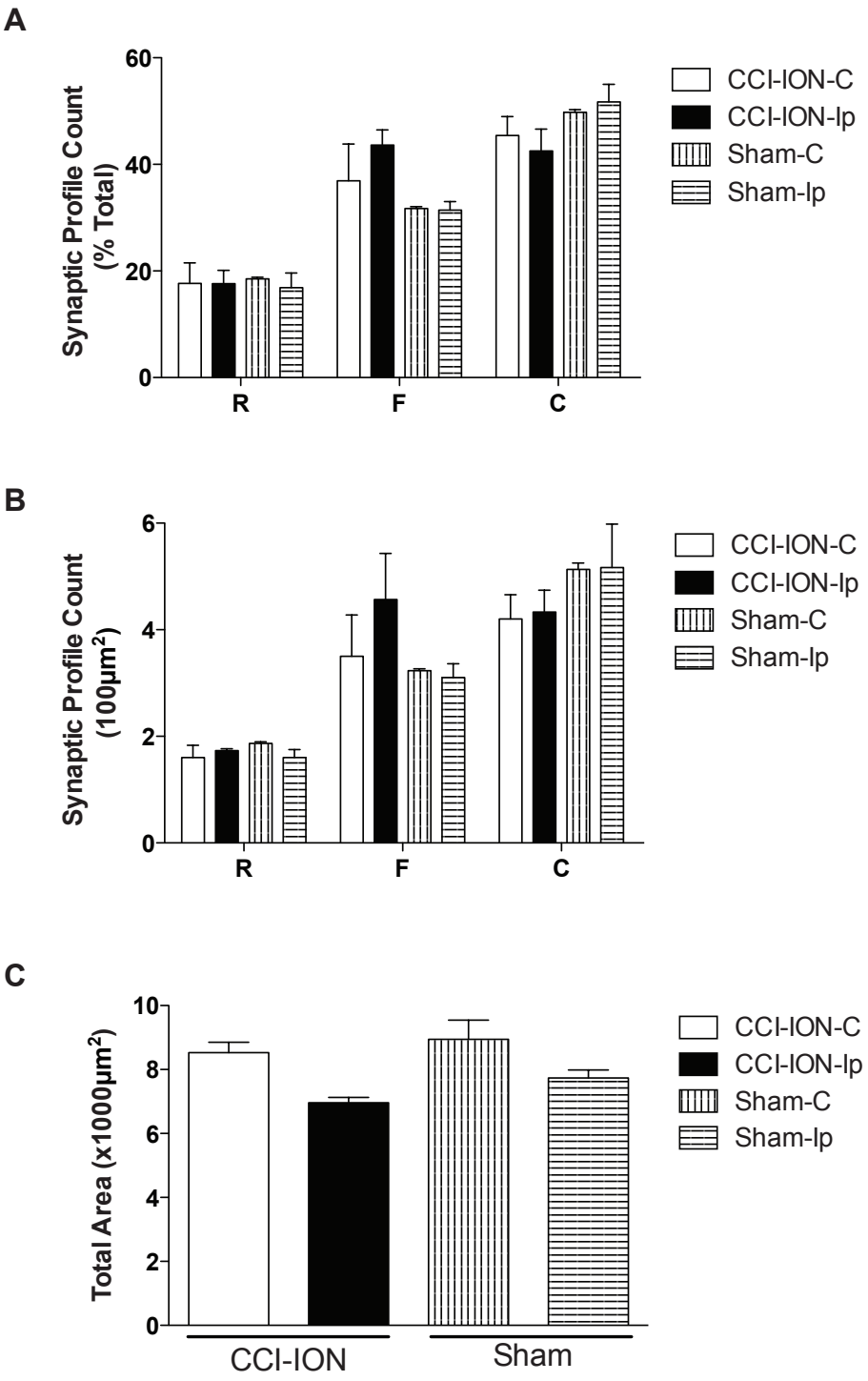

Figure 3. Quantification of total synaptic profile counts in Vc/C2 superficial dorsal horn of CCI-ION and sham rats. A, B: Summarized data of the proportion of each synaptic profile (R, F, and C) per total profile count (**A**) and per 100µm^2 (**B**) through dorsal horn superficial laminae. **C:** Summarized data of the total area of tissue examined. Data presented are the means ± SEM from 3 rats for each group. Ip, ipsilateral to injury; C, contralateral to injury.

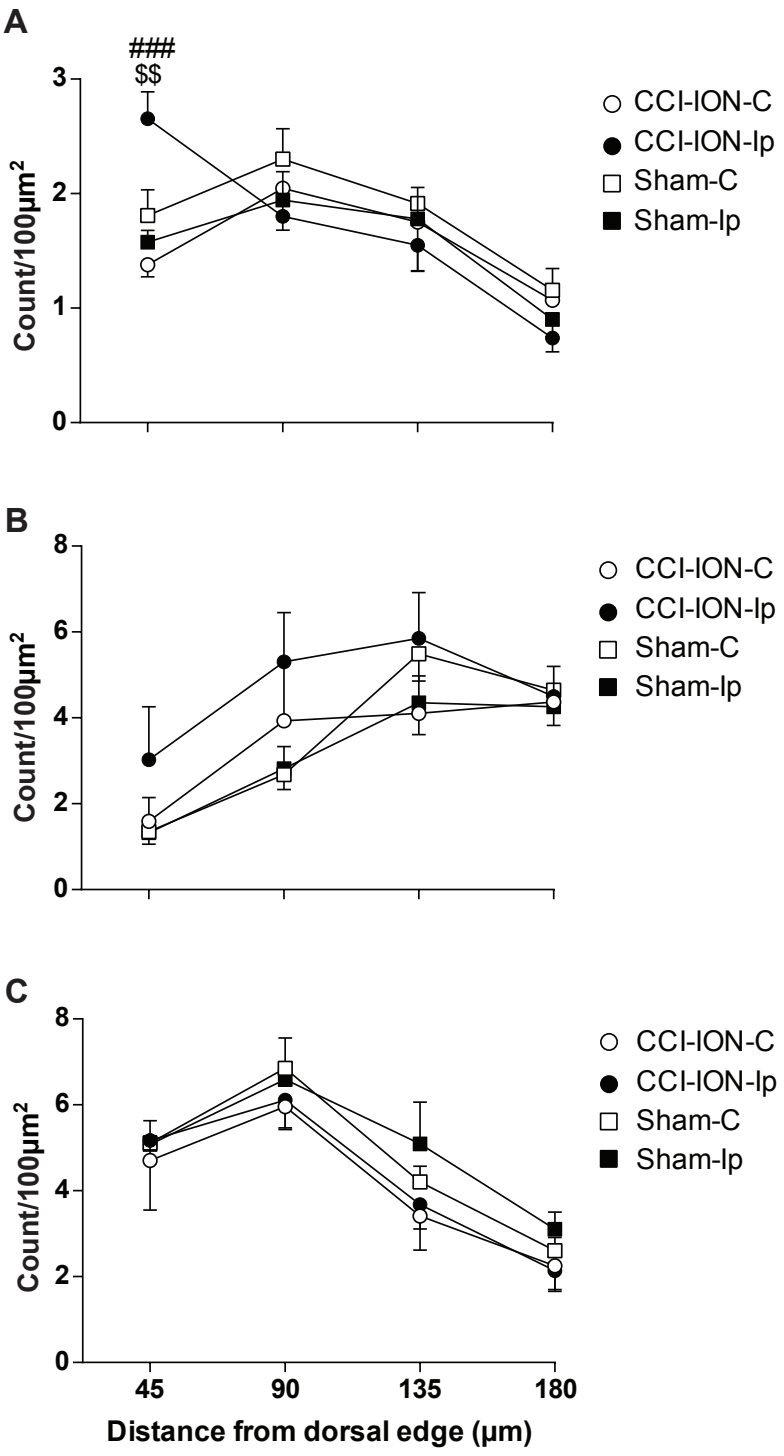

Figure 4. R- (**A**), F- (**B**), and C- (**C**) profile counts expressed per 100µm^2 through the superficial laminae (I-II) of Vc/C2 dorsal spinal cord. Data presented are the means ± SEM from 3 rats for each group. $^{###}p < 0.001$ for CCI-ION-Ip vs. CCI-ION-C; $^{\$\$}p < 0.01$ for CCI-ION-Ip vs. sham-Ip by two-way ANOVA followed by Bonferroni post hoc test. Ip, ipsilateral to injury; C, contralateral to injury.

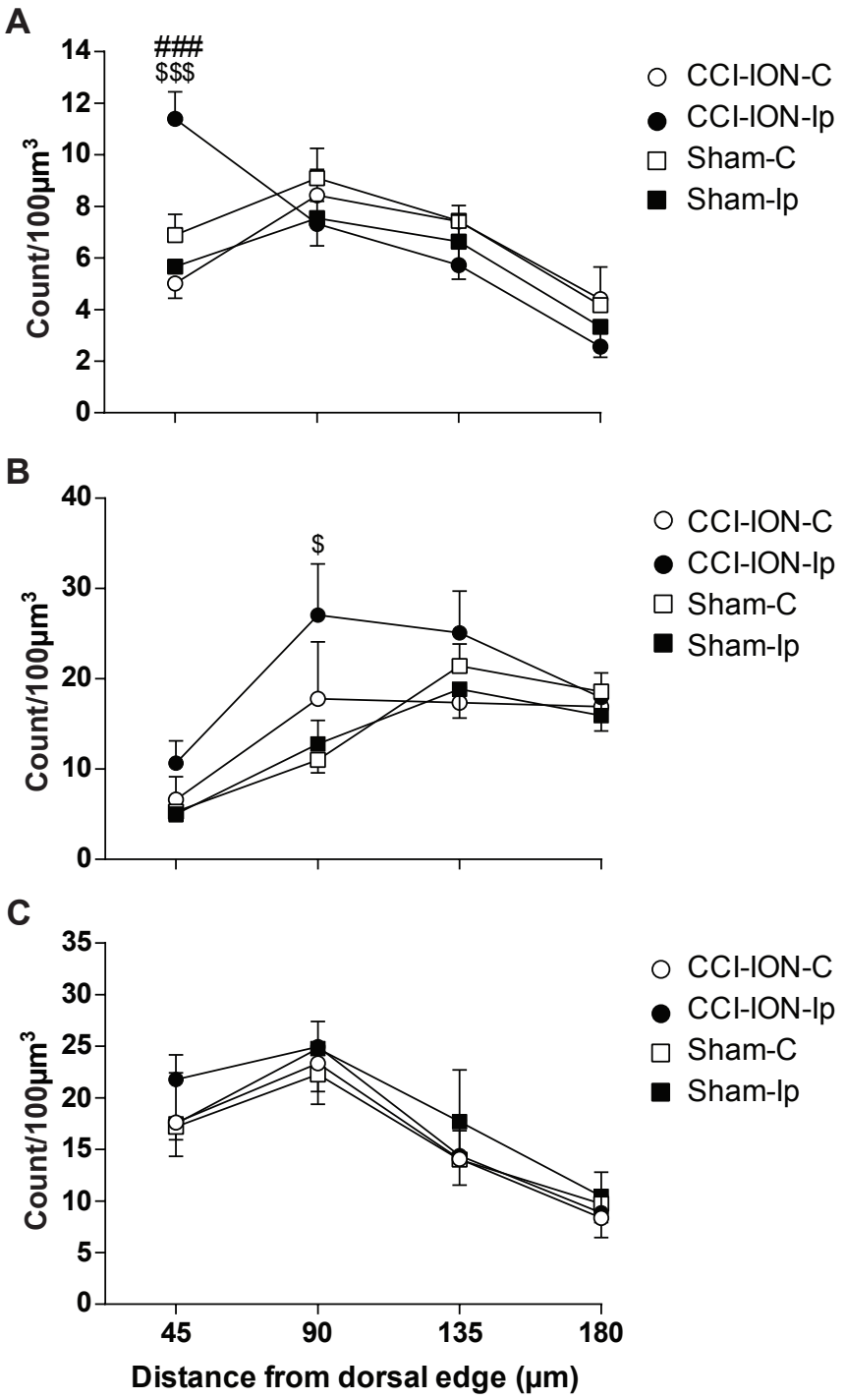

Figure 5. R- (**A**), F- (**B**), and C- (**C**) profile counts using the size-frequency method (profile/100µm^3) through the superficial laminae (I-II) of Vc/C2 dorsal spinal cord. Data presented are the means ± SEM from 3 rats for each group. $^{###}p < 0.001$ for CCI-ION-Ip vs. CCI-ION-C; $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ for CCI-ION-Ip vs. sham-Ip by two-way ANOVA followed by Bonferroni post hoc test. Ip, ipsilateral to injury; C, contralateral to injury.

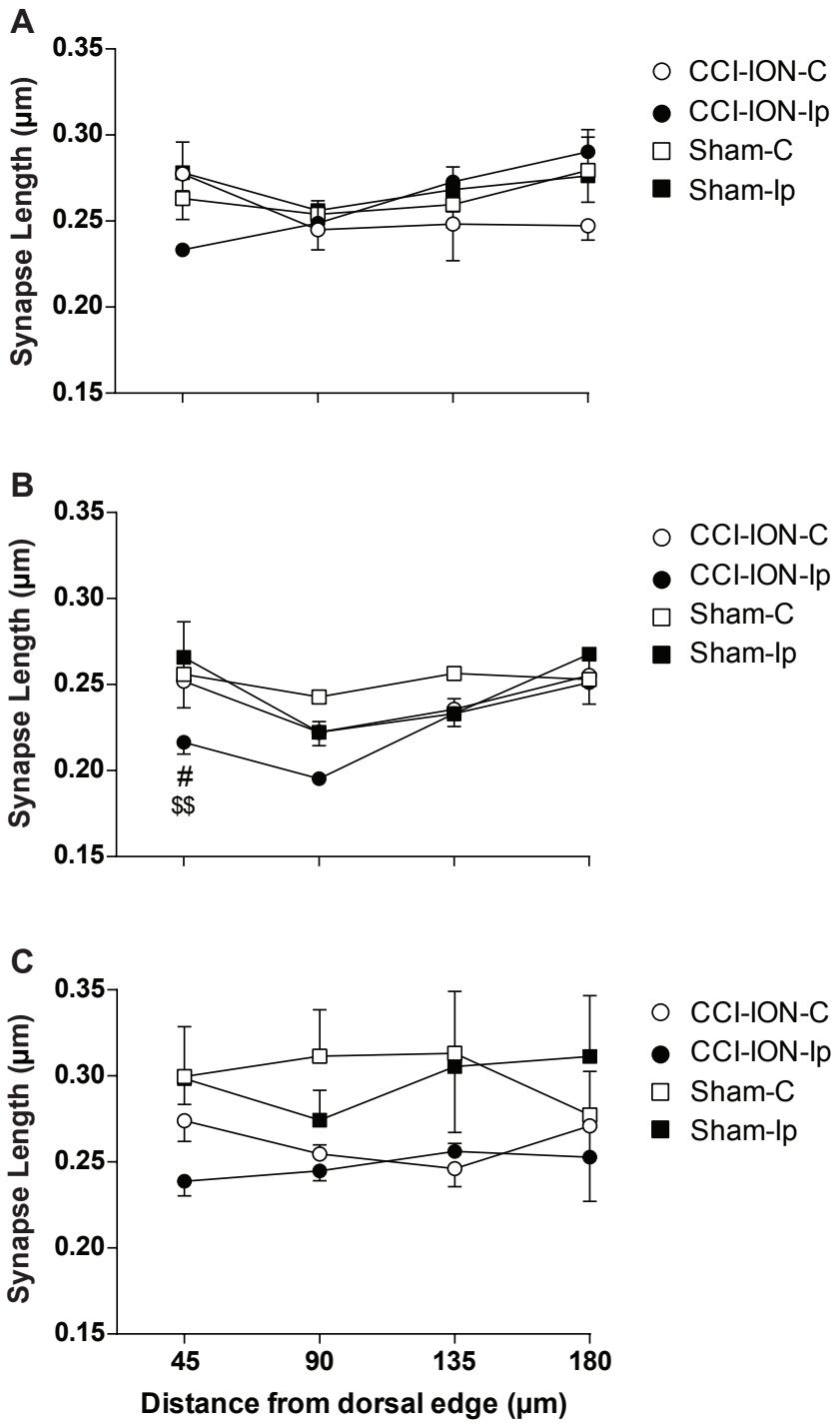

Figure 6. Mean synaptic length for R- (**A**), F- (**B**), and C- (**C**) profiles through the superficial laminae (I-II) of Vc/C2 dorsal spinal cord. Data presented are the means ± SEM from 3 rats for each group. $^{\#}p < 0.05$ for CCI-ION-Ip vs. CCI-ION-C; $^{\$\$}p < 0.01$ for CCI-ION-Ip vs. sham-Ip by two-way ANOVA followed by Bonferroni post hoc test. Ip, ipsilateral to injury; C, contralateral to injury.

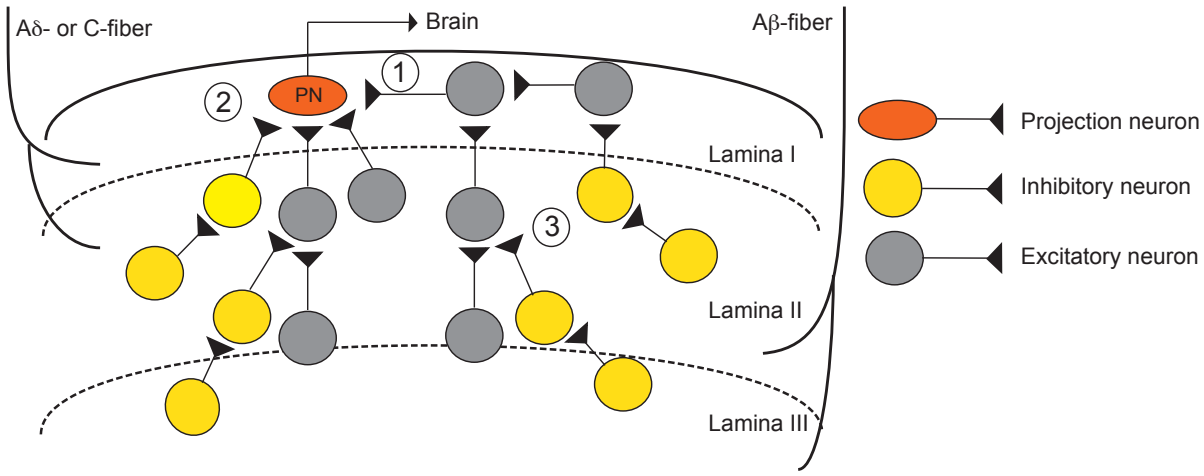

Figure 7. Schematic representation of proposed synaptic changes in the superficial dorsal horn after trigeminal nerve injury. 1) After nerve-injury, there is an increase of excitatory synapses by presumably excitatory interneurons (grey) onto other interneurons or projection neurons (orange) in lamina I. This enhance excitatory input can increase the excitability of projection neurons and facilitate the transmission of pain signals to the brain. **2)** Under non-injured conditions, there is a preexisting inhibitory tone by inhibitory interneurons (yellow) on lamina I projection neurons that result in decrease excitability of lamina I neurons and thereby modulate pain transmission. After nerve-injury, there is a decrease of inhibitory synaptic strength in lamina I, which may result in disinhibition of lamina I projection neurons. **3)** Inhibitory interneurons at lamina II receive increase inhibitory input after nerve-injury that may result in disinhibition of nociceptive pathways under inhibitory control by local inhibitory neurons, thereby allowing non-nociceptive signals to activate nociceptive pathways and consequently increase excitability of projection neurons.

CHAPTER 4

Conclusions and Perspectives

Chronic pain is a rising health problem that is predicted to affect up to 30% of the adult population in the United States alone (Johannes et al., 2010). Few patients with chronic pain obtain complete relief from drugs that are currently available such as opioids and non-steroidal anti-inflammatory drugs (NSAIDS), while more than half report inadequate pain relief (Woodcock, 2009). The underlying challenge of developing better drugs to manage chronic pain relies on better understanding of the mechanisms that contribute to altered sensory information processing, including sensitization of peripheral primary sensory neurons and nociceptive neurons in the spinal cord.

The $Ca_v\alpha_2\delta_1$ ligand gabapentin and pregablin is widely used as an anticonvulsant and for treating chronic pain, but has a 40-50% success rate in achieving adequate pain relief in clinical trials (Bockbrader et al., 2010; Perret and Luo, 2009). Despite the success of these gabapentinoid drugs, the underlying mechanism to its anti-hyperalgesic effects is still unknown. A number of studies have demonstrated upregulation of $Ca_v\alpha_2\delta_1$ gene and protein expression in the dorsal root ganglia (DRG) from nerve injury models that contribute to neuropathic pain states (Li et al., 2004; Li et al., 2006; Luo et al., 2002; Luo et al., 2001; Valder et al., 2003; Wang et al., 2002), which may be a key target for the action of gabapentin. Mice expressing a mutated $Ca_v\alpha_2\delta_1$ gabapentin-binding site abolish gabapentin's anti-hyperalgesic effects, lending further support for $Ca_v\alpha_2\delta_1$ as the target for gabapentinoid drugs (Field et al., 2006). Another action of gabapentin is shown to inhibit synaptogenesis via blocking the interaction of $Ca_v\alpha_2\delta_1$ with thrombospondin (TSP) proteins. This function of $Ca_v\alpha_2\delta_1$ is independent of voltage-gated calcium channel function (Eroglu et al., 2009). Interestingly, our lab has demonstrated that TSP4 protein is upregulated in the dorsal spinal cord after nerve-injury and contributes to spinal

presynaptic hypersensitivity and neuropathic pain states (Kim et al., 2012). Thus, it is highly likely that TSP4 interacts with $Ca_v\alpha_2\delta_1$ in mediating abnormal synapse modulation and chronic pain states, which may be attenuated by gabapentin.

Our studies provide a large body of evidence indicating that TSP4 and $Ca_v\alpha_2\delta_1$ indeed interact to mediate behavioral hypersensitivity in a modality-specific manner, which can be blocked by gabapentin or a specific TSP4 peptide that binds to $Ca_v\alpha_2\delta_1$. We mapped the nociceptive function of TSP4 to the EGF-like domains; however, the down-stream mechanism of TSP4/$Ca_v\alpha_2\delta_1$ interaction in modulating pain state processing remains elusive. Interestingly, the EGF-like domains of TSP4 have been shown to bind to the von Willebrand Factor A (VWF-A) domain of integrins (Pluskota et al., 2005), and $Ca_v\alpha_2\delta_1$ (Eroglu et al., 2009). The primary role of VWF-A domain of $Ca_v\alpha_2\delta_1$ is intracellular trafficking, especially for $Ca_v\alpha_1$ subunits to the plasma membrane (Canti et al., 2005). Also, gabapentinoids can inhibit $Ca_v\alpha_2\delta_1$ trafficking to the superficial and deep dorsal horn in primary afferents without affecting the upregulation of $Ca_v\alpha_2\delta_1$ gene or protein expression after nerve injury (Bauer et al., 2009). Since TSP4 is an extracellular matrix protein secreted by astrocytes (Christopherson et al., 2005; Eroglu et al., 2009), it is unlikely that TSP4 has an effect on $Ca_v\alpha_2\delta_1$ intracellular trafficking. Instead, an extracellular interaction between TSP4 and $Ca_v\alpha_2\delta_1$ could modulate voltage-gated calcium channel activity, retain or recruit channels at the cell surface of pre-synaptic terminals such that recycling is reduced, or triggers intracellular signaling cascades that subsequently lead to increased synaptic activity and chronic pain (Figure 1). This enhanced synaptic activity is supported by our electrophysiology data showing that either upregulation of TSP4 (Kim et al., 2012) or $Ca_v\alpha_2\delta_1$ (Zhou and Luo, 2014) leads to an increase in frequency, but not amplitude, of AMPA-receptor mediated spontaneous miniature excitatory postsynaptic currents (mEPSC) in dorsal horn neurons, which can be normalized by gabapentin (Zhou and Luo, 2014). Gabapentin's effects in normalizing abnormal pre-synaptic neurontransmission induced by TSP4/$Ca_v\alpha_2\delta_1$ interactions may represent a yet identified mechanism for the

anti-hyperalgesic actions of gabapentin. Future studies on how TSP4/$Ca_v\alpha_2\delta_1$ regulate synaptic neurotransmission, affect surface expression of calcium channels or anterograde trafficking of $Ca_v\alpha_2\delta_1$ will show new insights on downstream mechanisms of TSP4/$Ca_v\alpha_2\delta_1$ interactions.

Our findings at the ultrastructural level support that trigeminal nerve injury leads to maladaptive changes in excitatory synaptogenesis and inhibitory interneuron synaptic plasticity that likely contribute to orofacial neuropathic pain states through abnormal sensitization of dorsal horn neurons and synaptic circuitry. This is supported by the following findings: 1) Both peripheral and orofacial neuropathic pain models demonstrate an increase in $Ca_v\alpha_2\delta_1$ proteins in DRG and trigeminal neurons, and pre-synaptic afferent terminals that results in spinal neuron sensitization (Li et al., 2004; Li et al., 2006; Li et al., 2014b; Zhou and Luo, 2014). 2) Similarly, peripheral and orofacial neuropathic pain models also show an upregulation of TSP4 proteins in the DRG/trigeminal ganglia and dorsal spinal cord that contributes to chronic pain states (Kim et al., 2012; Li et al., 2014a; Pan et al., 2014). 3) Elevated $Ca_v\alpha_2\delta_1$ proteins are synaptogenic and promote excitatory synaptogenesis by interacting with TSP proteins (Eroglu et al., 2009; Kim et al., 2012; Lo et al., 2011). In addition, TSP4 proteins are known to regulate actin cytoskeleton that plays a pivotal role in synapse formation and remodeling (Arber and Caroni, 1995; Narouz-Ott et al., 2000; Tan and Lawler, 2009). Together, these observations suggest that aberrant excitatory synaptogenesis induced by TSP4/$Ca_v\alpha_2\delta_1$ interactions may contribute to the pathophysiology of neuropathic pain.

However, it is unclear whether TSP4/$Ca_v\alpha_2\delta_1$ complex plays a direct role in inhibitory synapse formation after nerve injury. Previous findings showed that TSPs promote excitatory, but not inhibitory synaptogenesis (Christopherson et al., 2005; Eroglu et al., 2009). Instead, non-TSP secreted proteins from astrocytes are involved in the formation of inhibitory synapses that use gamma amino butyric acid (GABA) as the neurotransmitter (Elmariah et al., 2004; Elmariah et al., 2005). Interestingly, high

concentrations of inhibitory neurotransmitter GABA can inhibit TSP-induced excitatory synapse formation, thereby suggesting that GABA inhibitory neurotransmission may control excitatory synapse connections (Eroglu et al., 2009). This concerted balance between excitatory and inhibitory synapses in the spinal dorsal horn may be lost during chronic pain states. This is supported by our electron microscopy data that show a reduction in inhibitory synaptic strength and/or connectivity, presumably through reduced inhibitory control to nociceptors at this location, and aberrant excitatory synaptogenesis in the superficial dorsal horn. Thus, maladaptive excitatory/inhibitory synaptic plasticity most likely contributes to the development of chronic pain states. Identification of affected subpopulations of neurons as well as their synaptic connectivity in future studies will shed some light on the detail mechanism of chronic pain mechanisms.

Gabapentin can prevent the formation of new synapses by blocking the interaction between TSP and $Ca_v\alpha_2\delta_1$ proteins (Eroglu et al., 2009). However, how synaptogenesis is involved in the rapid reduction of behavioral hypersensitivity by gabapentin (within 15min of drug administration) remains unclear. It is more plausible to hypothesize that the effect of gabapentin on synaptogenesis and/or trafficking could have more clinical implications for chronic uses and instead, the rapid action of gabapentin may influence recycling endosomes and membrane stability of calcium channels at the cell surface. It is presently unclear whether the rapid action of gabapentin is mediated by a direct drug interaction between $Ca_v\alpha_2\delta_1$- and $Ca_v\alpha_1$-subunits or requires protein-protein interaction between TSP4 and $Ca_v\alpha_2\delta_1$. Future studies characterizing how TSP4/$Ca_v\alpha_2\delta_1$ interactions regulate synapse formation during development and maintenance of chronic pain states will need to be conducted.

We hypothesize that directly blocking the interaction between TSP4 and $Ca_v\alpha_2\delta_1$ could serve as a novel therapeutic target in the treatment of chronic pain conditions. We developed a 15-amino acid peptide from the EGF-like domain of TSP4 that bind to $Ca_v\alpha_2\delta_1$ as a tool to study TSP4/$Ca_v\alpha_2\delta_1$ interactions. Intrathecal injection of this TSP4 peptide

can attenuate behavioral hypersensitivity induced by TSP4 proteins or overexpression of $Ca_v\alpha_2\delta_1$ in mouse neuronal cells. In addition, we found that the $Ca_v\alpha_2\delta_1$-binding TSP4 peptide suppressed tactile allodynia in a rat neuropathic pain model derived from unilateral L5/6 spinal nerve ligation (Kim and Chung, 1992). This model causes an increase in TSP4 and $Ca_v\alpha_2\delta_1$ protein levels in the dorsal spinal cord that contributes to the initiation and development of behavioral hypersensitivity (Kim et al., 2012; Li et al., 2004), which can be blocked by gabapentin (Luo et al., 2001; Zhou and Luo, 2014). Even though we cannot exclude the possibility that $Ca_v\alpha_2\delta_1$-binding peptide effects are mediated by other mechanisms independent from its binding to $Ca_v\alpha_2\delta_1$ proteins, these observations suggest that the $Ca_v\alpha_2\delta_1$-binding peptide can attenuate behavioral hypersensitivity and that the interaction between TSP4 and $Ca_v\alpha_2\delta_1$ are important in mediating chronic pain states. Although, how the $Ca_v\alpha_2\delta_1$-binding peptide leads to its anti-nociceptive effect is unclear. Since peptides typically have poor plasma membrane permeability, the anti-nociceptive effect of $Ca_v\alpha_2\delta_1$-binding peptide through $Ca_v\alpha_2\delta_1$-mediated intracellular trafficking is less plausible. Instead, the $Ca_v\alpha_2\delta_1$-binding peptide is most likely binding to extracellular $Ca_v\alpha_2\delta_1$ in the dorsal spinal cord that leads to its anti-nociceptive effect. Further experiments regarding whether the $Ca_v\alpha_2\delta_1$-binding peptides have similar effect as gabapentin on dorsal horn sensitization and synaptogenesis may shed light on how this peptide leads to its anti-nociceptive effect.

The clinical implications of TSP4 peptides that block the interaction between TSP4 and $Ca_v\alpha_2\delta_1$ is promising. Our $Ca_v\alpha_2\delta_1$-binding TSP4 peptide is anti-nociceptive and does not alter the basal behavioral responses to innocuous mechanical stimuli. We will need to further access the effect of the $Ca_v\alpha_2\delta_1$-binding TSP4 peptide on nociception with different pain modalities and animal pain models such as inflammatory pain. Also, gabapentinoid drugs are known to cause somnolence, but it is unclear whether interfering with TSP4/$Ca_v\alpha_2\delta_1$ interactions contributes to the sedative effects of these drugs. If not, TSP4 peptides may potentially lead to better side effect profiles. Clearly, more work is needed to

examine and resolve the tolerability and efficacy of TSP4 peptides in chronic pain states.

Together, these findings lead us to the following proposed mechanism. The EGF-like domains of TSP4 bind to the VWF-A domain of $Ca_v\alpha_2\delta_1$ to trigger structural rearrangement since VWF-A domains can cause structural conformational changes from an “inactive” to an “active” state upon ligand-binding (Whittaker and Hynes, 2002). The activated TSP4/$Ca_v\alpha_2\delta_1$ complex can then induce an intracellular signaling cascade that results in abnormal formation of excitatory synapses, dorsal horn neuronal sensitization, and behavioral hypersensitivity (Fig. 1 and Fig. 2). Further studies unraveling the mechanisms underlying the contribution of $Ca_v\alpha_2\delta_1$ and TSP4 to neuropathic pain states will help to reveal the detail downstream mechanisms. Nonetheless, blocking interactions of these proteins may serve as a novel strategy for improving therapeutic efficacy and specificity for pain management interventions.

References

Arber S, Caroni P. 1995. Thrombospondin-4, an extracellular matrix protein expressed in the developing and adult nervous system promotes neurite outgrowth. J Cell Biol 131(4):1083-1094.

Bauer CS, Nieto-Rostro M, Rahman W, Tran-Van-Minh A, Ferron L, Douglas L, Kadurin I, Sri Ranjan Y, Fernandez-Alacid L, Millar NS, Dickenson AH, Lujan R, Dolphin AC. 2009. The increased trafficking of the calcium channel subunit alpha2delta-1 to presynaptic terminals in neuropathic pain is inhibited by the alpha2delta ligand pregabalin. J Neurosci 29(13):4076-4088.

Bockbrader HN, Wesche D, Miller R, Chapel S, Janiczek N, Burger P. 2010. A comparison of the pharmacokinetics and pharmacodynamics of pregabalin and gabapentin. Clin Pharmacokinet 49(10):661-669.

Canti C, Nieto-Rostro M, Foucault I, Heblich F, Wratten J, Richards MW, Hendrich J, Douglas L, Page KM, Davies A, Dolphin AC. 2005. The metal-ion-dependent adhesion site in the Von Willebrand factor-A domain of alpha2delta subunits is key to trafficking voltage-gated Ca2+ channels. Proc Natl Acad Sci U S A 102(32):11230-11235.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA. 2005. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120(3):421-433.

Elmariah SB, Hughes EG, Oh EJ, Balice-Gordon RJ. 2004. Neurotrophin signaling among neurons and glia during formation of tripartite synapses. Neuron glia biology 1(4):1-11.

Elmariah SB, Oh EJ, Hughes EG, Balice-Gordon RJ. 2005. Astrocytes regulate inhibitory synapse formation via Trk-mediated modulation of postsynaptic GABAA receptors. J Neurosci 25(14):3638-3650.

Eroglu C, Allen NJ, Susman MW, O'Rourke NA, Park CY, Ozkan E, Chakraborty C, Mulinyawe SB, Annis DS, Huberman AD, Green EM, Lawler J, Dolmetsch R, Garcia

KC, Smith SJ, Luo ZD, Rosenthal A, Mosher DF, Barres BA. 2009. Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell 139(2):380-392.

Field MJ, Cox PJ, Stott E, Melrose H, Offord J, Su TZ, Bramwell S, Corradini L, England S, Winks J, Kinloch RA, Hendrich J, Dolphin AC, Webb T, Williams D. 2006. Identification of the alpha2-delta-1 subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin. Proc Natl Acad Sci U S A 103(46):17537-17542.

Johannes CB, Le TK, Zhou X, Johnston JA, Dworkin RH. 2010. The prevalence of chronic pain in United States adults: results of an Internet-based survey. J Pain 11(11):1230-1239.

Kim DS, Li KW, Boroujerdi A, Peter Yu Y, Zhou CY, Deng P, Park J, Zhang X, Lee J, Corpe M, Sharp K, Steward O, Eroglu C, Barres B, Zaucke F, Xu ZC, Luo ZD. 2012. Thrombospondin-4 contributes to spinal sensitization and neuropathic pain states. J Neurosci 32(26):8977-8987.

Kim SH, Chung JM. 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50(3):355-363.

Li CY, Song YH, Higuera ES, Luo ZD. 2004. Spinal dorsal horn calcium channel alpha2delta-1 subunit upregulation contributes to peripheral nerve injury-induced tactile allodynia. J Neurosci 24(39):8494-8499.

Li CY, Zhang XL, Matthews EA, Li KW, Kurwa A, Boroujerdi A, Gross J, Gold MS, Dickenson AH, Feng G, Luo ZD. 2006. Calcium channel alpha2delta1 subunit mediates spinal hyperexcitability in pain modulation. Pain 125(1-2):20-34.

Li KW, Kim DS, Zaucke F, Luo ZD. 2014a. Trigeminal nerve injury-induced thrombospondin-4 up-regulation contributes to orofacial neuropathic pain states in a rat model. Eur J Pain 18(4):489-495.

Li KW, Yu YP, Zhou C, Kim DS, Lin B, Sharp K, Steward O, Luo ZD. 2014b. Calcium channel alpha2delta1 proteins mediate trigeminal neuropathic pain states associated with aberrant excitatory synaptogenesis. J Biol Chem 289(10):7025-7037.

Lo FS, Zhao S, Erzurumlu RS. 2011. Astrocytes promote peripheral nerve injury-induced reactive synaptogenesis in the neonatal CNS. J Neurophysiol 106(6):2876-2887.

Luo ZD, Calcutt NA, Higuera ES, Valder CR, Song YH, Svensson CI, Myers RR. 2002. Injury type-specific calcium channel alpha 2 delta-1 subunit up-regulation in rat neuropathic pain models correlates with antiallodynic effects of gabapentin. J Pharmacol Exp Ther 303(3):1199-1205.

Luo ZD, Chaplan SR, Higuera ES, Sorkin LS, Stauderman KA, Williams ME, Yaksh TL. 2001. Upregulation of dorsal root ganglion (alpha)2(delta) calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. J Neurosci 21(6):1868-1875.

Narouz-Ott L, Maurer P, Nitsche DP, Smyth N, Paulsson M. 2000. Thrombospondin-4 binds specifically to both collagenous and non-collagenous extracellular matrix proteins via its C-terminal domains. J Biol Chem 275(47):37110-37117.

Pan B, Yu H, Park J, Yu YP, Luo ZD, Hogan QH. 2014. Painful nerve injury upregulates thrombospondin-4 expression in dorsal root ganglia. J Neurosci Res.

Perret D, Luo ZD. 2009. Targeting voltage-gated calcium channels for neuropathic pain management. Neurotherapeutics 6(4):679-692.

Pluskota E, Stenina OI, Krukovets I, Szpak D, Topol EJ, Plow EF. 2005. Mechanism and effect of thrombospondin-4 polymorphisms on neutrophil function. Blood 106(12):3970-3978.

Tan K, Lawler J. 2009. The interaction of Thrombospondins with extracellular matrix proteins. Journal of cell communication and signaling 3(3-4):177-187.

Valder CR, Liu JJ, Song YH, Luo ZD. 2003. Coupling gene chip analyses and rat genetic variances in identifying potential target genes that may contribute to neuropathic

allodynia development. J Neurochem 87(3):560-573.

Wang H, Sun H, Della Penna K, Benz RJ, Xu J, Gerhold DL, Holder DJ, Koblan KS. 2002. Chronic neuropathic pain is accompanied by global changes in gene expression and shares pathobiology with neurodegenerative diseases. Neuroscience 114(3):529-546.

Whittaker CA, Hynes RO. 2002. Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Mol Biol Cell 13(10):3369-3387.

Woodcock J. 2009. A difficult balance--pain management, drug safety, and the FDA. N Engl J Med 361(22):2105-2107.

Zhou C, Luo ZD. 2014. Electrophysiological characterization of spinal neuron sensitization by elevated calcium channel alpha-2-delta-1 subunit protein. Eur J Pain 18(5):649-658.

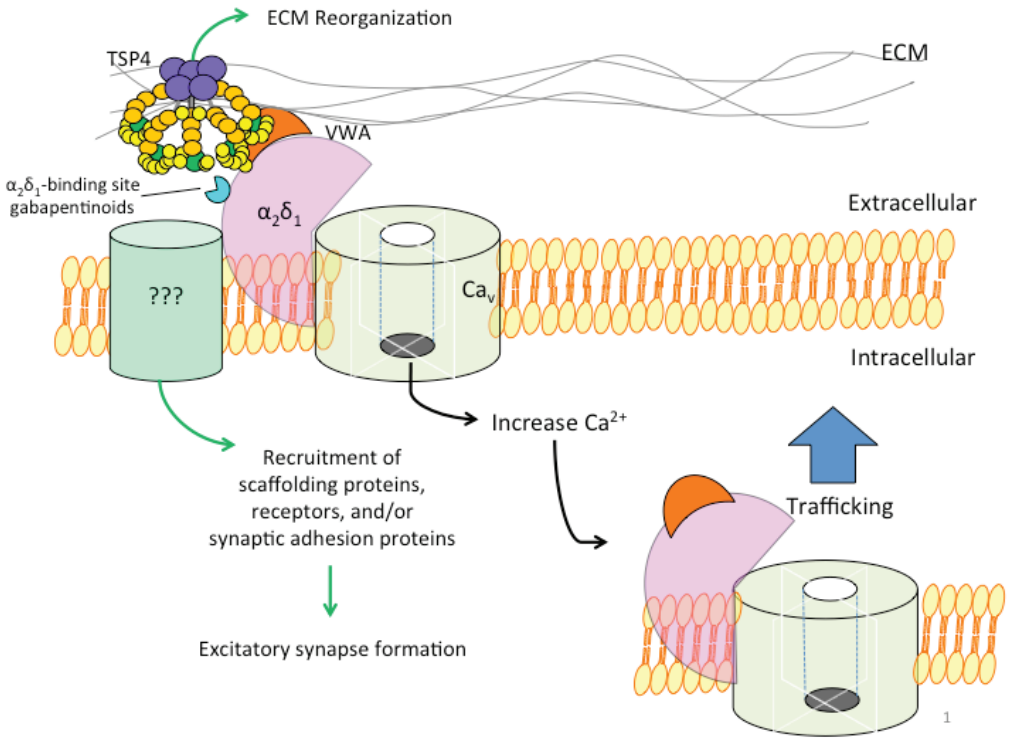

Figure 1. Predicted mechanisms of molecular interaction between TSP4 and $Ca_v\alpha_2\delta_1$. Astrocyte secreted TSP4 proteins bind to the von Willebrand factor-A (VWF-A) domain of the $Ca_v\alpha_2\delta_1$ subunits through its EGF-like domains to cause a conformational change in the protein from an inactive to an active state. This activated complex may trigger the following events: 1) the activated $Ca_v\alpha_2\delta_1$ subunit may bind to and modulate the pore-forming α1-subunit function. The α1-subunit is important for calcium entry that trigger presynaptic neurotransmitter release in response to depolarization. Through an unidentified mechanism, activated $Ca_v\alpha_2\delta_1$ subunit may stabilize calcium channel localization to the plasma membrane. The TSP4/$Ca_v\alpha_2\delta_1$ complex may also interact directly with extracellular matrix proteins (i.e. integrins, collagens, laminin, fibronectin) that bind to other structural proteins and cytoskeleton that may contribute to the stabilization of calcium channel localization. 2) The activated $Ca_v\alpha_2\delta_1$ proteins may modulate unidentified interacting proteins independent of calcium channels and trigger a cascade of molecular events, which include but are not limited to recruitment of receptors, scaffolding proteins, and/or reorganizing extracellular matrix proteins that promote synaptic adhesion between presynaptic and post-synaptic terminals.

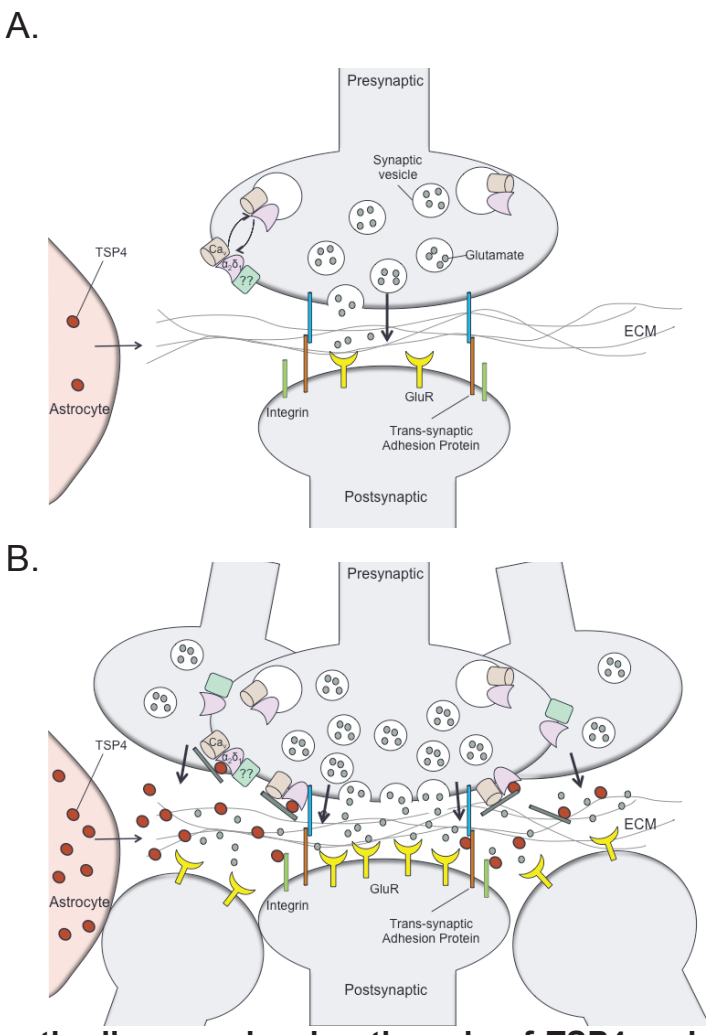

Figure 2. A schematic diagram showing the role of TSP4 and $Ca_v\alpha_2\delta_1$ in chronic pain states. A. Under non-injured states, activation of nociceptors by a mild stimuli leads to excitatory neurotransmitter glutamate release from the presynaptic afferent nerve terminals in the spinal dorsal horn. This signaling to dorsal horn neurons can transmit information about the intensity of the stimuli from the periphery to the brain. Astrocytes remain unchanged during this non-injured state. **B.** Under injury-induced chronic pain states, there is an increase of TSP4 and $Ca_v\alpha_2\delta_1$ proteins in the spinal dorsal horn. Upregulated TSP4 proteins are secreted by activated astrocytes and it can bind to $Ca_v\alpha_2\delta_1$ proteins transported from DRG to presynaptic terminals, extracellular matrix proteins (i.e. collagen, laminin, fibronectin), cell adhesion proteins (i.e. neuroligin), and/or other unidentified proteins in the spinal cord. So far, the TSP4/$Ca_v\alpha_2\delta_1$ complex can induce an increase in excitatory neurotransmitter release through an unidentified mechanism and an increase in excitatory synapse formation by interacting with unidentified proteins involved in synaptic organization and function. These feed-forward mechanisms cause amplification of postsynaptic neuron activation and excitatory synaptic connections, which together contribute to the development and maintenance of chronic pain states.