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IRVINE

Maladaptive synaptogenesis induced by interaction of voltage gated calcium channel subunit α2δ-1 and thrombospondin-4 represents a new mechanism underlying neuropathic pain processing in nerve injury models

DISSERTATION

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by

Peter Yanhui Yu

Dissertation Committee:

Professor David Z Luo, Chair

Professor Geoffrey Abbott

Professor Catherine Cahill

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DEDICATION

To

Mom, dad and my friends:

Without you, I would not be who I am today,

Or where I am today.

Thank you for your support.

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CURRICULUM VITAE

Peter Yanhui Yu

2006	Intern, Protein Crystallography, The Scripps Research Institute
2006-08	Lab Assistant I, Torrey Pines Institute of Molecular Studies
2008	Teaching Assistant, School of Biology, University of California, San Diego
2008	B.S. in Neuroscience and Physiology, University of California, San Diego
2011	Teaching Assistant, School of Developmental Biology, University of California, Irvine
2015	Ph.D. in Pharmacology University of California, Irvine

FIELD OF STUDY

Molecular mechanism of neuropathic pain

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ABSTRACT OF THE DISSERTATION

Maladaptive synaptogenesis induced by interaction of voltage gated calcium channel subunit α2δ-1 and thrombospondin-4 represents a new mechanism underlying neuropathic pain processing in nerve injury models

By

Peter Yanhui Yu

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Professor David Z. Luo, Chair

The molecular basis of neuropathic pain is poorly understood. We have previously shown that nerve injury induces increased production of thrombospondin-4 and voltage-gated calcium channel subunit α2δ-1 and that these proteins play a critical role in mediating behavioral hypersensitivity in animal models. However, the mechanism of action has not been elucidated. I use immunohistochemical staining in nerve injured animal model spinal cords to show that interaction of thrombospondin-4 with α2δ-1 promote excitatory synaptogenesis. Furthermore, I use immunocytochemistry staining in spinal cord and dorsal root ganglia neuron co-culture to show that thrombospondins-4/α2δ-1 interaction mediated synaptogenesis depends on pre-synaptic T-type voltage-gated calcium channel function.

Introduction

The American Academy of Pain Medicine estimates that there may be up to 100 million people in the US suffering from chronic pain. This in turn costs $500 billion dollars per year in medical care and loss of productivity [1]. To meet the demand for chronic pain management, 59 drugs were introduced from 1960 to 2009 that remain in use. However only 7 (Opiods, NSAIDS, Triptans, Anticonvulsants, Antidepressants, Channel blockers, Capsaicin) of the 59 drugs are directed towards novel targets [2]. Part of the difficulty of finding drug targets for chronic pain management is due to the diverse etiology of chronic pain that can derive from different disorders such as cancer, diabetes, arthritis or neuropathy. Pharmaceutical companies are facing complex pain-inducing diseases with poorly understood pathophysiology and a broad patient heterogeneity [3]. A good example is neuropathic pain, or pain states resulting from injuries to the peripheral or central nervous systems. Opiates remain a treatment option even though they exhibit limited efficacy and significant side effects in treating neuropathic pain. Gabapentinoids (gabapentin and pregabalin) are relatively new anti-neuropathic pain medications with milder side effect profiles, but only approximately 30% of neuropathic pain patients have at least moderate pain relief after gabapentin treatment [4]. The development of novel effective analgesics, thus, heavily relies on better understanding of neuropathic pain mechanisms.

Background and Significance

One of the main difficulties in studying neuropathic pain mechanisms is that while neuropathic pain develops from a direct nerve injury, the resulting pathophysiology is diverse. Nerve injury can result in loss of injured sensory neurons leading to denervation and hypersensitivity [5, 6]. Injury to the primary afferents may also lead to changes in neuronal ion channel expression, resulting in increased excitatory tone in the spinal cord circuitry [7-9]. In addition to changes in electrophysiological properties, nerve injury can also lead to phenotypic switching in sensory neurons including expression of neurotransmitters associated with pain in non-nociceptors [10-12].

Furthermore, nerve injury-related discharges can create increased neuronal excitability that leads to spinal sensitization [9, 13, 14]. Non-neuronal cells also play a role in induction of neuropathic pain states. It has been shown in animal models that inflammatory mediators such as InterLeukin-1β and Tumor Necrosis Factor α secreted by activated microglia also play a role in the initiation of pain by affecting channel expression and changing neuronal excitability [15-18]. Furthermore, microglia secreted Bone Derived Neurotrophic Factor has been shown to reverse chloride gradient in GABA neurons, leading to disregulation of inhibitory GABAergic neuron currents that contributes to disinhibition and enhanced circuit excitability [19-22]. Supraspinal inputs modulating pain sensation come in the form of descending modulation from the periaqueductal gray, locus coeruleus, anterior cingulate gyrus [23], amygdala [24] and hypothalamus [25].

Due to the complexity involved in performing a comprehensive study of the mechanisms underlying neuropathic pain, I chose to focus my investigation to studying the role of alpha2delta-1 (α2δ) in pain processing. This is because α2δ-1 is the binding site for gabapentin [26], which has shown efficacy in managing neuropathic pain in clinical trials [27-31]. The pain-relief property of gabapentin in human patients suggests that α2δ-1 plays an important role in pain processing. Furthermore, the Luo laboratory has shown that spinal nerve ligation (SNL), an animal model of nerve injury, causes an increase in α2δ-1 expression in sensory neurons and spinal cord that is correlated to the development of behavioral hypersensitivity [8, 14].

α2δ-1 is an accessory subunit of Voltage Gated Calcium Channels (VGCC). VGCC are channels that respond to depolarization of cell membranes by opening a pore that allows the influx of calcium ions into the cell. Once inside the cell, calcium ions can act as a second messenger by binding to signaling molecules such as PhosphoLipase C (PLC), Protein Kinase C (PKC) and calcium CalModulin dependent protein Kinase (CaMK) to transduce signal cascades or they can bind to other proteins, such as the Soluble N-ethylmaleimide-sensitive fusion protein Attachment Protein REceptor (SNARE) complex, leading to vesicle fusion and neurotransmitter release. VGCCs are divided into L-, N-, P/Q, R- and T-type based on their electrophysiological

properties as well as their sensitivity to certain antagonists. VGCC can also be divided as high-voltage (HVA: L-, N-, P/Q, and R-type) and low-voltage (LVA: T-type) activated channels based on their membrane voltage activation thresholds. The L-type channels have long lasting currents and are sensitive to dihydropyridines (DHP) and are found predominantly in skeletal muscles, smooth muscles, heart and neurons [32]. The N-type VGCC are mainly present in neurons and pre-synaptic terminals and is blocked by ω-conotoxin [33]. The P/Q-type VGCC are mainly present in Purkinje cells and can be blocked by ω-agatoxin [34, 35]. The R-type VGCC are present in the brain as well as cerebellar granule cells and are resistant to DHP, ω-conotoxin and ω-agatoxin [35]. The T-type VGCC found mainly in sinoatria nodes, thalamus, sensory neurons and heart are blocked by ethosuximide [36, 37].

In general, VGCCs are composed of one each of the α1, β, α2δ and γ subunits with the α1 subunit being the pore-forming subunit allowing calcium ions to flow through when opened by membrane depolarization [38].The entirely cytoplasmic β subunit plays a role in chaperoning the VGCC complex from the endoplasmic reticulum to the cell membrane and also modulates gating by interacting with regulatory proteins such as G-proteins [39]. The role of the γ subunit is currently unclear but has been suggested to be involved in α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor recruitment and also reducing VGCC activity by causing a hyperpolarizing shift in the inactivation curve [40]. The α2δ subunit can modulate trafficking of the VGCC complex as well as the current properties of the VGCC [39]. There are 4 members in the α2δ family: α2δ-1, α2δ-2, α2δ-3, α2δ-4 of which α2δ-1 and α2δ-2 are binding sites for gabapentinoids [26, 41]. Both the α2 (~150kDa) and δ (~25kDa) proteins are encoded by the same gene, post-translationally cleaved and subsequently linked via disulfide bonds. The α2 subunit is entirely extracellular while the δ subunit is membrane bound via a glycosylphosphatidylinositol anchor (GPI) [42]. α2δ-1 is expressed in many tissues including skeletal muscles, cardiac and smooth muscles as well as in the central and peripheral nervous system [43]. α2δ-2 and α2δ-3 are expressed in neurons [43]. α2δ-4 expression is mainly non-neuronal and located in specialized cells such as pituitary, adrenal gland, colon and fetal liver [44], as well as retinal neurons [43].

Mutations in α2δ-3 has been implicated in thermal pain processing in drosophila, mice and humans and in reduced sensitivity to chronic back pain in humans [45]. There has been no reported role of α2δ-2 or α2δ-4 in the field of pain. On the other hand, dysregulation of α2δ-1 has been shown to play a critical role in neuropathic pain processing [14]. The α2δ-1 subunit is normally associated with high-voltage L-, N- and P/Q/R-type VGCC complexes [46]. Increases of both α2δ-1 mRNA and proteins were found to correlate with the time course of onset and maintenance of hypersensitive states in the SNL injury model [47]. In contrast, α2δ-2 and α2δ-3 are down-regulated after SNL [48, 49] while the α1 and β subunits remain unchanged [14]. The importance of spinal α2δ-1 in SNL-induced behavioral hypersensitivity was further demonstrated by blocking α2δ-1 protein synthesis via intrathecal α2δ-1 antisense oligonucleotide treatment or by preventing α2δ-1 trafficking from dorsal root ganglia to dorsal spinal cord via dorsal rhizotomy. Both treatments led to a reversal of behavioral hypersensitivity in nerve injured animals [8]. Compared to wild-type (WT) mice, transgenic mice over-expressing α2δ-1 in neuronal cells exhibited increased duration and amplitude of dorsal spinal cord neuron firing in response to peripheral thermal or mechanical stimuli, as well as behavioral hypersensitivity sensitive to gabapentin blockade [14].

Although the main function of VGCC is to allow calcium influx after membrane depolarization, calcium current independent functions are also reported, many of which are mediated by the β and α2δ subunits [39]. One such function involves the α2 subunit's Von Willebrand Factor A (VWF-A) domain that is hypothesized to be the site for protein-protein interactions responsible for VGCC independent excitatory synaptogenesis [50]. Mechanistically, α2δ-1 mediates synaptogenesis even when HVA VGCC function is blocked with type-specific toxins [50]. These synapses may contribute to increased miniature Excitatory Post-Synaptic Current (mEPSC) frequency that can be blocked by antagonists of AMPA and N-methyl-D-aspartate (NMDA), but not metabotropic glutamate, receptors [51]. This suggests that α2δ-1 may contribute to neuropathic pain states by increasing glutamate transmission through aberrant excitatory synapses that can lead to a hypersensitized spinal circuitry. Recently, it has been shown that the Epidermal Growth Factor like (EGF-like) domain of thrombospondins (TSPs) interacts with the VWF-A region of α2δ-1

in regulating excitatory synaptogenesis in the CNS [50]. The potential importance of this α2δ-1/TSP interaction in pain processing is reinforced by the finding that gabapentin treatment can block synapse formation *in vivo* and *in vitro* but has no effect on already-formed CNS synapses [50].

TSPs belong to a family of extracellular matrix proteins secreted in various cell types including cancer cells, platelet cells and astrocytes. The TSP family can be divided into two groups based on structure homology, which share about 60% homology: group A includes trimeric TSP1, 2 and group B includes pentameric TSP3, 4, 5 [52] (Figure 1.3). TSPs are calcium-binding glycoproteins that play a role in wound healing, angiogenesis, connective tissue organization and synaptogenesis through interaction with other extracellular matrix proteins, growth factors, proteases and membrane bound receptors [53]. TSP has been implicated to play a role in vascular diseases, malaria and cancer [53, 54].

TSPs are widely distributed. TSP1 protein has been found in skeletal muscle, spinal cord, cornea, platelet cells and ovary. TSP2 protein is predominantly seen in the adrenal cortex and bone marrow stromal cells. TSP3 is expressed in the intestine. TSP4 protein is found in skeletal muscle, tendon, neuromuscular junction, blood vessels, retina and cerebellum. TSP5 protein is found predominantly in tendon, cartilage, skeletal muscle, arteries, eye and heart [53]. The synaptogenic property of TSP was first identified through findings that TSP treated neurons had increased pre-synaptic activity mediated by NMDA but not AMPA receptors [55]. Furthermore, it was observed that retinal ganglion cell cultures treated with TSP had higher incidence of aligned pre- and post-synaptic components compared to untreated controls [55]. Based on this observation, it was proposed that TSP might have a structural rather than a transcriptional effect. However, it was subsequently found that antibodies against α2δ-1's VWF-A domain mimicked the synaptogenic effect of TSP treatment [50]. The exact mechanism of TSP interaction with α2δ-1 in promoting synaptogenesis has not yet been reported.

Previous work in the Luo Lab and collaborators' labs indicated that peripheral and central nerve injuries also induced upregulation of TSP4, but not other TSPs, in sensory neurons and

dorsal spinal cord through unknown mechanisms [56-59]. Findings by Eroglu et al. [50] provide an intriguing possibility that α2δ-1 may play a critical role in behavioral hypersensitivity by increasing excitability in the dorsal spinal cord through interactions with TSP4 that promote excitatory synaptogenesis. I proposed to further elucidate this pathway by studying whether and how α2δ-1/TSP4 interaction signaling played a role in synaptogenesis in pain models

Chapter 1: Chronic Constriction Injury of the Infraorbital Nerve induces α2δ-1-associated aberrant excitatory synaptogenesis that leads to increased spinal circuit excitability and behavioral hypersensitivity

Introduction

The neuropathic pain model of Chronic Constriction Injury of the Infraorbital Nerve (CCI-IoN) is a trigeminal nerve injury model of orofacial neuropathic pain. It involves making two loose ligations on the infraorbital nerve, which innervates the vibrissal pad, the upper teeth and the dorsal part of the oral cavity, to cause nerve injury and induce orofacial hypersensitivity [60]. CCI-IoN has been used as a model for trigeminal neuralgia and temporomandibular disorders [61]. CCI-IoN is different from other nerve injury models because it involves injury to entirely sensory nerve fibers instead of to a mixture of sensory and motor fibers such as in SNL. Furthermore, CCI-IoN rats exhibit both induced mechanical hypersensitivity as well as spontaneous "pain" associated with increased face grooming [60].

Previous data from the Luo lab have shown that after CCI-IoN, α2δ-1 protein level is increased starting from 1 week post injury in the trigeminal ganglion (TG), before the onset time of orofacial hypersensitivity. The increase is only seen starting at 3 weeks post injury in the associated spinal cord subnucleus caudalis and C1/C2 segments (Vc/C2). However, α2δ-1 induction in Vc/C2 correlates with the appearance of peak orofacial hypersensitivity. This delayed α2δ-1 induction in the Vc/C2 may be due to the time required for α2δ-1 trafficking from TG to Vc/C2 post injury. CCI-IoN induced increases of α2δ-1 in both TG and Vc/C2 return to the pre-injury level at 10 weeks post injury when the injured rats recover from orofacial pain states even though CCI-IoN ligations are still present. This evidence suggests that the change in Vc/C2 α2δ-1 levels is important to the development of behavioral hypersensitivity. α2δ-1 increases in Vc/C2 of CCI-IoN rats lead

to increased frequency but not amplitude of mESPC. This increase is α2δ-1 dependent as it can be ablated by gabapentin treatment [62]. *In vivo*, blocking α2δ-1 with gabapentin or treatment with α2δ-1 antisense can reverse CCI-IoN induced orofacial hypersensitivity [62].

These results suggest that increased α2δ-1 at the spinal cord level leads to enhanced pre-synaptic excitatory input. It is not clear however whether injury-induced α2δ-1 increases mEPSC frequency through aberrant synaptogenesis [50], which may form the basis for orofacial hypersensitivity.

Methods

CCI-IoN

CCI-IoN surgeries were done by Dr. Li as described and also detailed in the following publication by the Luo lab [57, 62]. Briefly, an incision was made parallel to the frontal bone above the left eye. The frontal bone is then exposed and the orbital content retracted to expose the infraorbital nerve. A modified syringe needle is then used to put two loose ligations 3-4mm apart using 5-0 silk sutures. The incision is then closed with silk sutures and the rat allowed to recover on a warm heating pad. [60]

Immunohistochemistry

The immunohistochemistry (IHC) experiments were modified from already published methods detailing procedures on the analysis of synapses via co-staining and detection with confocal microscopy [50, 55, 63, 64]. Vc/C2 spinal cord and TG samples were collected from three animals after 1-week, 3-week CCI-IoN or 3-week sham ligation. Samples were fixed in 4% paraformaldehyde solution (PFA) overnight and cryoprotected in 30% sucrose in water. Samples are then mounted in Optimum Cutting Temperature medium (OCT) and sectioned in 10μm slices by cryostat. Samples on glass slides were then pretreated with heat-based antigen retrieval before

staining with combinations of rabbit α2δ-1 antibody (ABR) or mouse α2δ-1 antibody (Sigma), Isolectin B4 (Invitrogen), goat VGlut2 (Synaptic Systems), rabbit PSD95 (Invitrogen) and goat Synaptophysin (Santa Cruz). Images were taken using a Zeiss LSM710 (Air 10X lens) or Zeiss LSM780 (Air 20X, Oil 63X, Oil 100X lens) (UC Irvine Optical Biology Core) in 0.3µm thick Z stacks. Images were then cropped down to the 3 consecutive Z stacks with the best signal, merged and used for analysis using Volocity 6.0 (PerkinElmer, Waltham).

TG sensory neurons were identified based on their morphology and nucleus size from captured images (2µm sections, n = 30 over 3 animals, >6000 cells analyzed, 100µm apart), and quantified for size and average intensity of immunoreactivity using the Volocity program. Signal intensity was normalized by subtracting the average intensity of each neuron with the lowest intensity in each image.

α2δ-1 immunoreactivity was selected from 1-week, 3-week CCI-IoN and 3-week sham rat Vc/C2 spinal cord sections (2µm sections, n= 9 over 3 animals, 100µm apart) using Volocity thresholding function and analyzed for fluorescent signal. Captured images from VGlut2/α2δ-1 antibody co-stained samples (n = 54 over 3 animals per surgery group, 100µm apart) and VGlut2/PSD95 antibody co-stained samples (n=36 over 3 animals, 100µm apart) were analyzed using Volocity to determine the number of total $VGlut2^+$, $VGlut2^+/\alpha2\delta\text{-}1^+$, $VGlut2^+/PSD95^+$ and $PSD95^+$ puncta. Due to the rat Vc/C2 spinal segment being too large to capture at high resolution in one frame, shots of three different regions of the superficial spinal dorsal horn were taken: the inner region near the dorsal column, the outer region near the lateral column and a region in between the two other regions. Images of the three areas were pooled together to calculate the synapse numbers in the superficial dorsal horn (SDH) because there was no indication that α2δ-1 expression in the SDH changed across from the dorsal to the lateral column.

The Luo lab has previously reported that the contralateral side of CCI-IoN rats does not exhibit any difference in behavior sensitivity or α2δ-1 expression compared to sham. Therefore, using the contralateral side as the control to the nerve injured ipsilateral side allowed me to reduce

variability arising from differences in individual rats and also from Vc/C2 sample collection. Statistical significance was calculated using 2-tailed paired t-test.

Results

α2δ-1 is increased in TG sensory neurons after CCI-IoN

It has been reported that α2δ-1 is increased across all sensory neuron subtypes after SNL [65], however no such study has been done in the CCI-IoN model. Although both SNL and CCI-IoN involve ligation induced injury to the primary afferent, CCI-IoN differs from SNL in that it involves two loose ligations compared to one tight ligation, and the ligated infraorbital nerve is only a part of the maxillary nerve, one of the three major nerves originating from the TG. In addition, the TG-Vc/C2 structure is different from the DRG-spinal cord structure and ligation injury induced orofacial hypersensitivity occurs much slower in the CCI-IoN model (3 weeks [57]) than that in the SNL model (within 1-week [8, 47]). To check whether CCI-IoN also induced increase in α2δ-1 across all TG sensory neuron subtype, I performed IHC staining on TG sections from 3 week CCI-IoN rat using anti-α2δ-1 antibody to examine α2δ-1 expression levels in individual TG neurons. By comparing the number of α2δ-1 positive neurons on the injured (ipsilateral) side compared to that in the uninjured (contralateral) side across different neuronal size categories, I could determine whether any particular subpopulation of TG sensory neurons had increased α2δ-1 expression in response to injury [65-67].

I found that CCI-IoN injury induced increased α2δ-1 protein production in TG neurons. Although I observed a more pronounced increase in α2δ-1 immunoreactivity in larger diameter neurons, the increase of α2δ-1 immunoreactivity intensity (greater than one fold over baseline) still spanned through all sizes of sensory neurons (Figure 1.1 A, B). This suggests that injury induced α2δ-1 production is not restricted to any subset of TG sensory neurons.

In comparison to the change reported in dorsal root ganglia (DRG) neurons after SNL,

which shows a nearly three-fold increase in α2δ-1 across all neuron types [65], injury induced α2δ-1 expression change in the TG is less drastic. This may be due in part to some differences between DRG neurons and TG neurons. In my experiments, the TG shows a higher basal level α2δ-1 expression compared to that in DRG. Another possibility is that CCI-IoN only injures the infraorbital nerve while leaving other trigeminal nerves such as Mandibular and Ophthalmic nerves intact. This creates a TG that contains a mixture of healthy neurons associated with the uninjured nerves and injured neurons associated with the injured infraorbital nerve. In my opinion, the change in α2δ-1 expression would be more pronounced if other trigeminal nerves were also injured.

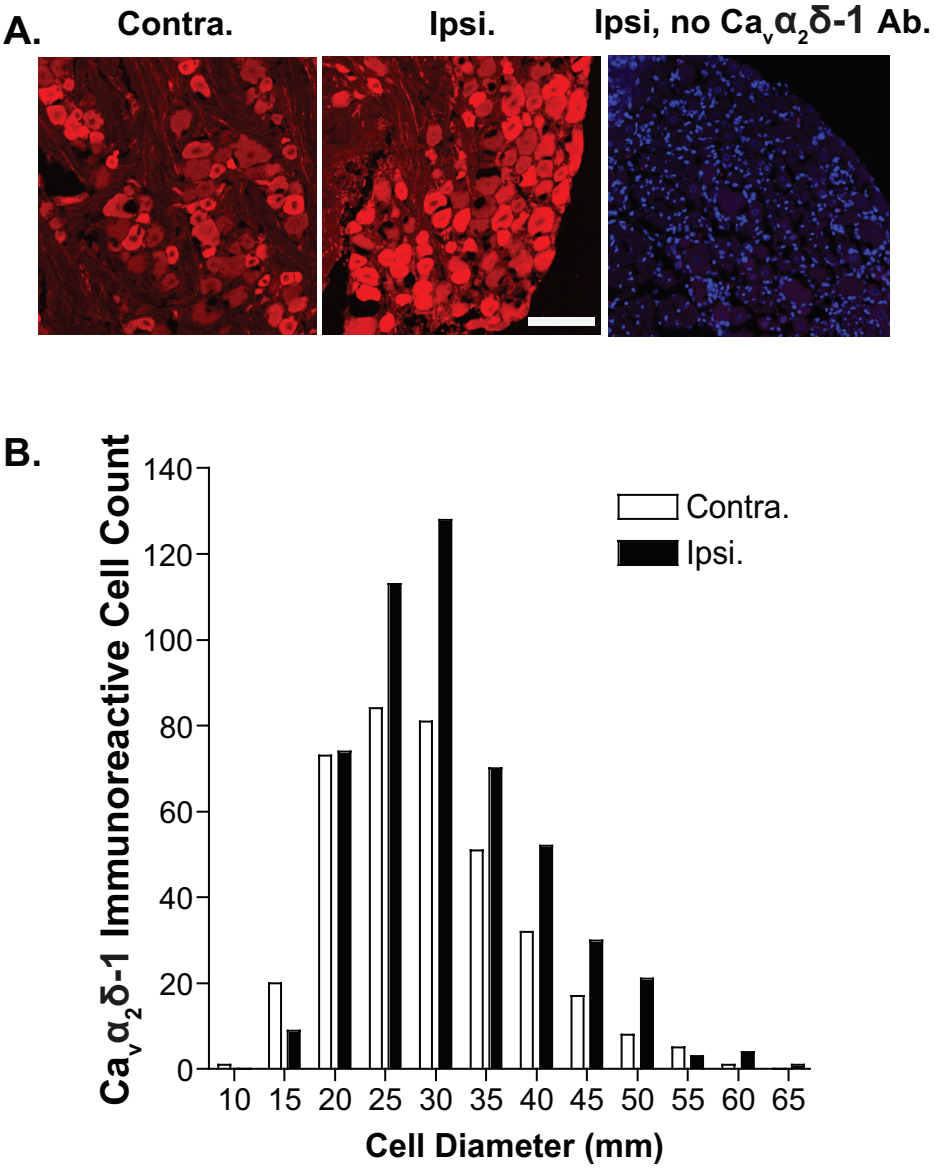

Figure 1.1: A. Representative α2δ-1 staining showing trigeminal ganglion neurons produced increased α2δ-1 after 3 week CCI-IoN compared to the contralateral side. Scale = 100μm. **B.**Quantification of α2δ-1 signal in individual neurons showed that α2δ-1 is increased after nerve injury in all but the smallest diameter neuron population. (n = 30 sections over 3 animals, > 6000 cells analyzed)

Increase in TG α2δ-1 leads to an increase of α2δ-1 in the superficial dorsal horn of Vc/C2

It has been reported in the lumbar spinal nerve injury models that α2δ-1 is produced in the DRG and then trafficked to the superficial dorsal horn of the spinal cord [8, 65]. Due to the location of the TG under the brain, a dorsal rhizotomy to conclusively confirm α2δ-1 trafficking between the TG and Vc/C2 could not be performed. However, our published Western blot (WB) data show that the α2δ-1 protein increase in TG one week after CCI-IoN is followed by an α2δ-1 increase in the spinal cord three weeks post CCI-IoN [62]. This presents circumstantial evidence suggesting that the α2δ-1 increase observed in the dorsal horn may originate from the TG. However, WB analysis of the Vc/C2 SDH only shows the increased level but gives no indication to the localization of the increase, which could provide valuable insight into the source and location of increased α2δ-1 in Vc/C2. For example, punctum immunoreactivity would suggest possibly synaptic distribution of α2δ-1 while α2δ-1 immunoreactivity concentrated in a particular type of cell bodies would suggest the origin of α2δ-1 induction in Vc/C2. If the increased punctum immunoreactivity is mainly localized in laminae I-II, that would suggest that increased α2δ-1 mainly comes from TG as central terminals of Aδ and C-nociceptors form synapses with postsynaptic dorsal horn neurons in the superficial dorsal horn [68]. Changes in α2δ-1 punctum immunoreactivity in laminae III-V could mean that CCI-IoN induces α2δ-1 synthesis in large Aβ or some Aδ sensory neurons with sensory fibers projecting into deep dorsal horn [68].

To verify this, I performed IHC staining of the dorsal horn from 3 week CCI-IoN rats, when orofacial hypersensitivity has been established, to clarify the exact location of dorsal horn α2δ-1 increase. I used *Isolectin* B4 (IB4) that stain for non-peptidergic sensory neurons as a landmark to delineate superficial dorsal horn (Lamina I-II) from deep dorsal horn (Lamina III-V) because it has been shown that IB4 fibers are projecting primarily onto the inner edge of lamina II [69].

My data support that CCI-IoN injury-induced increase of α2δ-1, similar to that reported in SNL [65], is translocated from the TG to the Vc/C2 superficial dorsal horn. There was a roughly 50% increase in α2δ-1 intensity in the ipsilateral superficial dorsal horn from 3 week CCI-IoN rats

compared to that in the contralateral side (P = .006). This increase was localized in the SDH as the increased α2δ-1 signal overlapped with that of IB4. In comparison, there was no significant change in α2δ-1 signal in the deep dorsal horn (P = .852) (Figure 1.2 A, B). The increase in α2δ-1 intensity was mainly due to an increase in α2δ-1 immunoreactivity area in the SDH (P = .01), although a slightly but statistically significant increase in mean intensity was also observed (P = .015) (Figure 1.2 C, D). Since a punctate rather than neuronal or axonal staining pattern was observed, it is highly likely that α2δ-1 is present at sensory fiber terminals. Furthermore, injury induced α2δ-1 increase was observed predominantly in increased surface area in the SDH rather than increased intensity, suggesting that α2δ-1 is likely trafficked to new locations rather than to locations already containing α2δ-1.

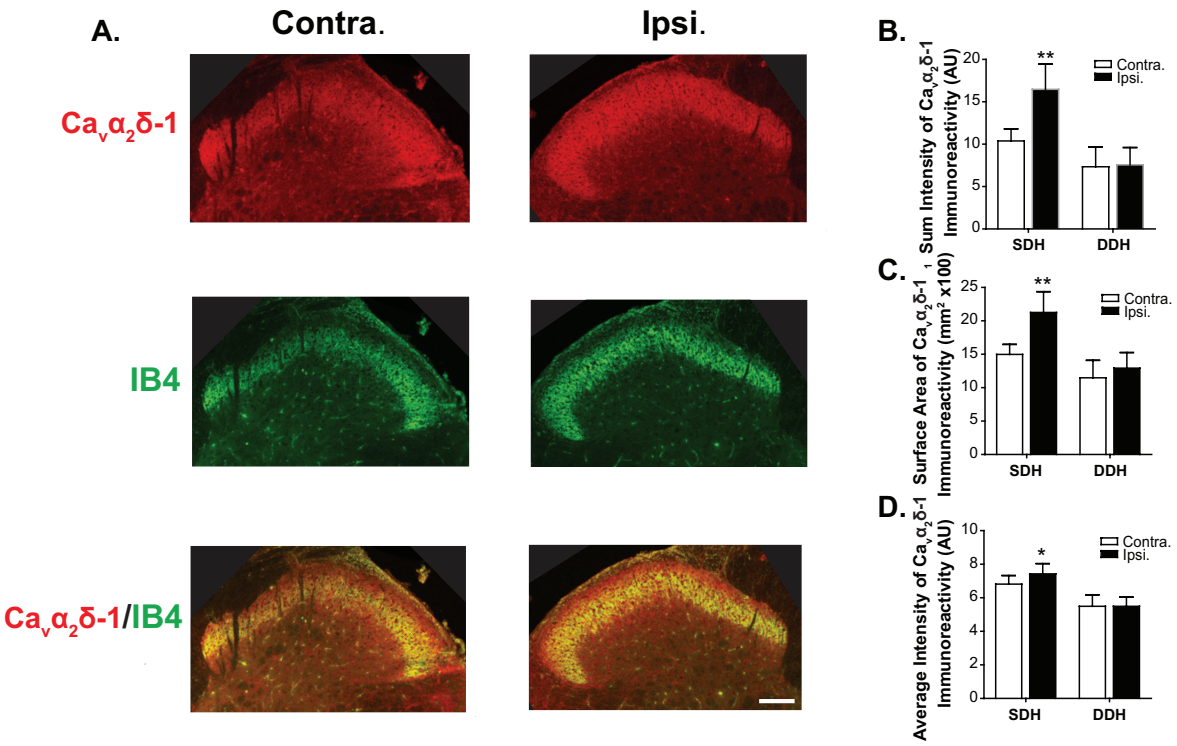

Figure 1.2: A. Representative image of α2δ-1 (Red) and IB4 (Green) staining in 3 week CCI-IoN rat spinal cord. Most of the α2δ-1 increase due to nerve injury occured in the superficial dorsal horn (Lamina I-II). Scale = 140μm **B.** Quantification of α2δ-1 signal showed that most of the 50% increase ($P < .006$) is due to a 30% increase in surface area of expression ($P < .01$) **(C)** with a small but significant increase in intensity ($P < .015$) **(D)**. (n = 9 sections over 3 animals)

Increase of α2δ-1 in the superficial dorsal horn associates with new synapses rather than existing α2δ-1 negative terminals

If α2δ-1 is expressed at the synaptic level, the increase in the surface area of α2δ-1 fluorescence can be due to two reasons: α2δ-1 is either increased in newly formed synapses or trafficked into previously α2δ-1 negative synapses. To address whether α2δ-1 plays a role in synaptogenesis after CCI-IoN injury or if the increased α2δ-1 simply comes from trafficking of α2δ-1 to previously α2δ-1 negative terminals, I performed IHC co-staining of α2δ-1 with pre-synaptic marker synaptophysin (SYN). Synaptophysin is a protein present on synaptic vesicles and in most neurons regardless of neurotransmitter type [70]. Increased SYN puncta count on the nerve injury side would suggest novel synapse formation. Co-localization of α2δ-1 with SYN in dorsal spinal cord would suggest that α2δ-1 is synaptic. In addition, by comparing changes in the number of co-localized SYN/α2δ-1 puncta between ipsilateral and contralateral sides in the SDH, I could determine whether α2δ-1 is positively correlated with the appearance of new synapses. Increased co-localization of SYN/α2δ-1 with no change to total SYN would suggest that α2δ-1 is likely being trafficked to previously non-α2δ-1 containing synapses. Conversely, an increase in SYN associated with increased co-localization with α2δ-1 would suggest that α2δ-1 is likely trafficked to newly formed synapses.

My data indicated a 50% increase in total SYN count ($P = .01$) in the injury side of Vc/C2 3-week post CCI-IoN injury. Furthermore, when I separated out α2δ-1 negative synaptophysin puncta ($SYN^+/\alpha2\delta\text{-}1^-$) from α2δ-1 positive synaptophysin puncta ($SYN^+/\alpha2\delta\text{-}1^+$), I found that the change mainly occurred in the α2δ-1-containing synapse population since there was a 1.5 fold increase in $SYN^+/\alpha2\delta\text{-}1^+$ immunoreactivity ($P = .003$), but no statistically significant change in the $SYN^+/\alpha2\delta\text{-}1^-$ immunoreactive population ($P = .830$) (Figure 1.3 A, B). These results suggest that IoN injury leads to new synapse formation and that these newly formed synapses associate with α2δ-1.

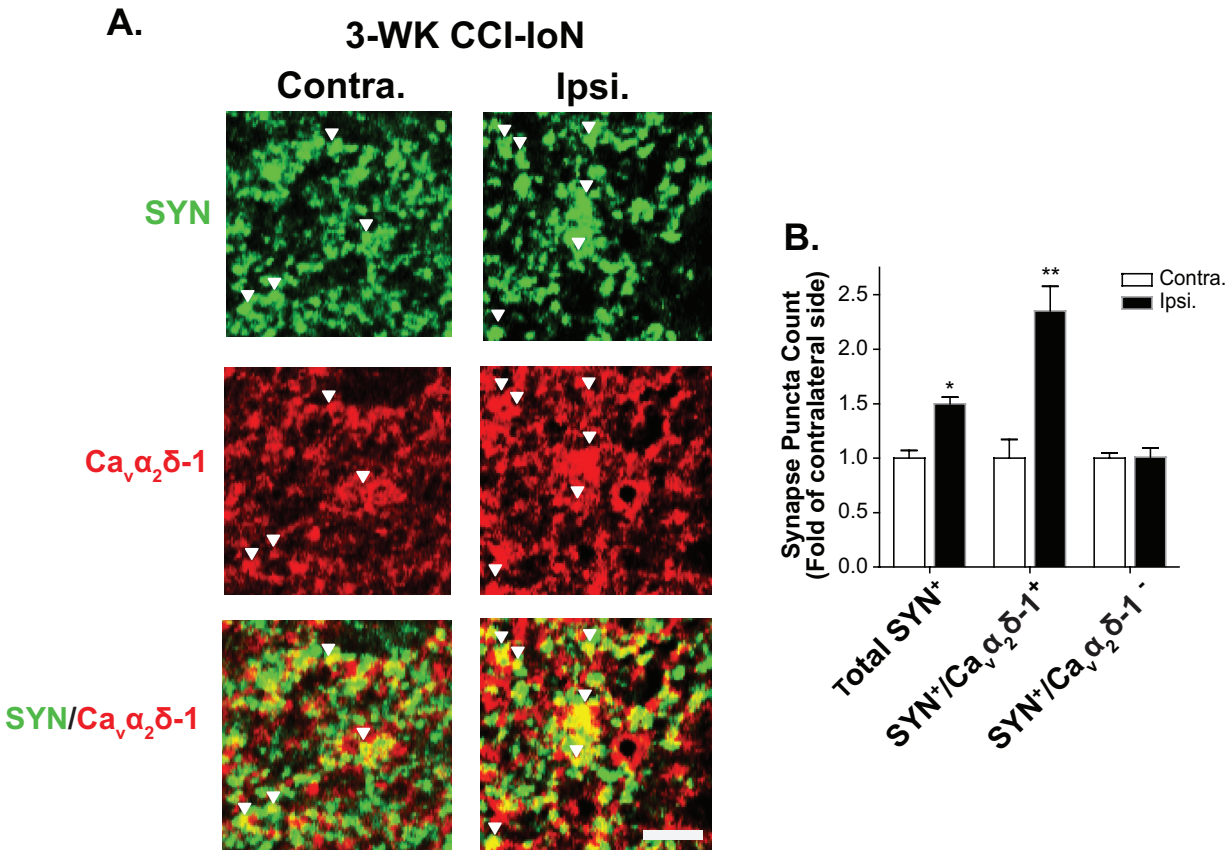

Figure 1.3: A. Representative image of synaptophysin (Green) and α2δ-1 (Red) on the ipsilateral IoN compared to the contralateral IoN. Scale = 5μm **B.** Quantification of total synaptophysin count showed a 50% increase ($P < .01$) compared to the contralateral side. This increase was due to a 150% increase ($P < .003$) in the synaptophysin positive, α2δ-1-positive synapse population (SYN+/α2δ-1+) while the α2δ-1-negative synapse population count remained unchanged. (n = 18 sections over 3 animals)

Increased α2δ-1+ excitatory synapses correlate with behavioral hypersensitivity

Having shown that nerve injury induced α2δ-1 expression that associated with increased synapse formation, I next looked at whether this increase in SDH synapses was correlated with behavior hypersensitivity. The time course of behavioral hypersensitivity development after CCI-IoN involves a two-week hyposensitivity period before the onset of maximal orofacial hypersensitivity around three weeks post-injury [62]. This delay in hypersensitivity onset allows me to draw a temporal correlation between the appearance of α2δ-1 positive synapses and the onset of orofacial hypersensitivity. At 1 week post CCI-IoN, α2δ-1 levels are unchanged in the Vc/C2 and no orofacial hypersensitivity is observed. In contrast, α2δ-1 levels in Vc/C2 are increased and behavioral hypersensitivity is observed at 3 weeks post CCI-IoN [62].

To determine if increased α2δ-1 was associated with excitatory synapses, I performed IHC staining on Vc/C2 spinal cord sections using antibodies against α2δ-1 and pre-synaptic excitatory marker Vesicular Glutamate Transporter 2 (VGlut2). I decided to only focus on excitatory synapses as sensory neurons are all excitatory. There are three members in the VGlut family: VGlut1 [71], VGlut2 [72] and VGlut3 [73], all are highly homologous. In spinal cord dorsal horn, VGlut1 is expressed predominantly from the inner edge of the substantia gelatinosa (lamina II) to the magnocellular layer (lamina III) while VGlut2 is expressed predominantly in the marginal layer and the substantia gelatinosa (laminae I and II) [74]. About 80% of TG neurons express either VGlut1 or VGlut2 [74]. Although there is no clear cut distribution of VGlut1 and VGlut2 across sensory fiber subtypes, it has been reported that VGlut2 is the predominant transporter for unmyelinated primary afferent fibers terminating in lamina II [74]. I chose to focus on VGlut2 because VGlut1 knockout did not significantly alter nerve injury-induced behavioral hypersensitivity [75], while hemizygous knockout of VGlut2 [75] as well as selective knockout of VGlut2 in DRG neurons [76, 77] lead to attenuation of neuropathic pain hypersensitivity. In addition, VGlut2 expression pattern in the spinal cord shows predominantly in laminae I and II [74], which overlaps with the expression pattern of α2δ-1. Moreover, over-expressing α2δ-1 in neurons of a transgenic mouse

line results in VGlut2 expression in these neurons that would not normally express VGlut2 in WT mice [50, 63]. VGlut3 was found to localize in lamina I and the inner edge of lamina II and are expressed by low-threshold mechanoreceptors in the DRG [78]. Expression of VGlut3 in TG has not been studied to date, but I was not able to look at VGlut3 due to the lack of good commercial antibodies.

My data indicated that at 3 week post CCI-IoN, total VGlut2 positive ($VGlut2^+$) synapses were increased around 40% on the injury side ($P < .001$). This change was not seen in either the 1 week CCI-IoN ($P = .127$) or the 3 week sham ($P = .416$) groups (Figure 1.4 A, B). This suggests that increased $VGlut2^+$ synapses correlate temporally with the increase of α2δ-1 and the development of orofacial hypersensitivity. Further analysis by separating total $VGlut2^+$ puncta into $\alpha2\delta\text{-}1^+$ and $\alpha2\delta\text{-}1^-$ groups showed that the $VGlut2^+/\alpha2\delta\text{-}1^+$ puncta population increased by over 1-fold ($P < .001$) while $VGlut2^+/\alpha2\delta\text{-}1^-$ synapses showed a 15% decrease ($P = .01$). The 1-week CCI-IoN surgery group showed a more pronounced 35% decrease ($P = .002$) in $VGlut2^+/\alpha2\delta\text{-}1^-$ synapses (Figure 1.4 C,D). Taken together, my data shows that nerve injury leads to an increase in $VGlut2^+/\alpha2\delta\text{-}1^+$ synapses but a loss in $VGlut2^+/\alpha2\delta\text{-}1^-$ synapses. The loss in synapses may be due to loss of innervations resulting from dying back of axons post nerve injury [6]. However, I cannot rule out the possibility that α2δ-1 is translocated to synapses that previously did not contain α2δ-1.

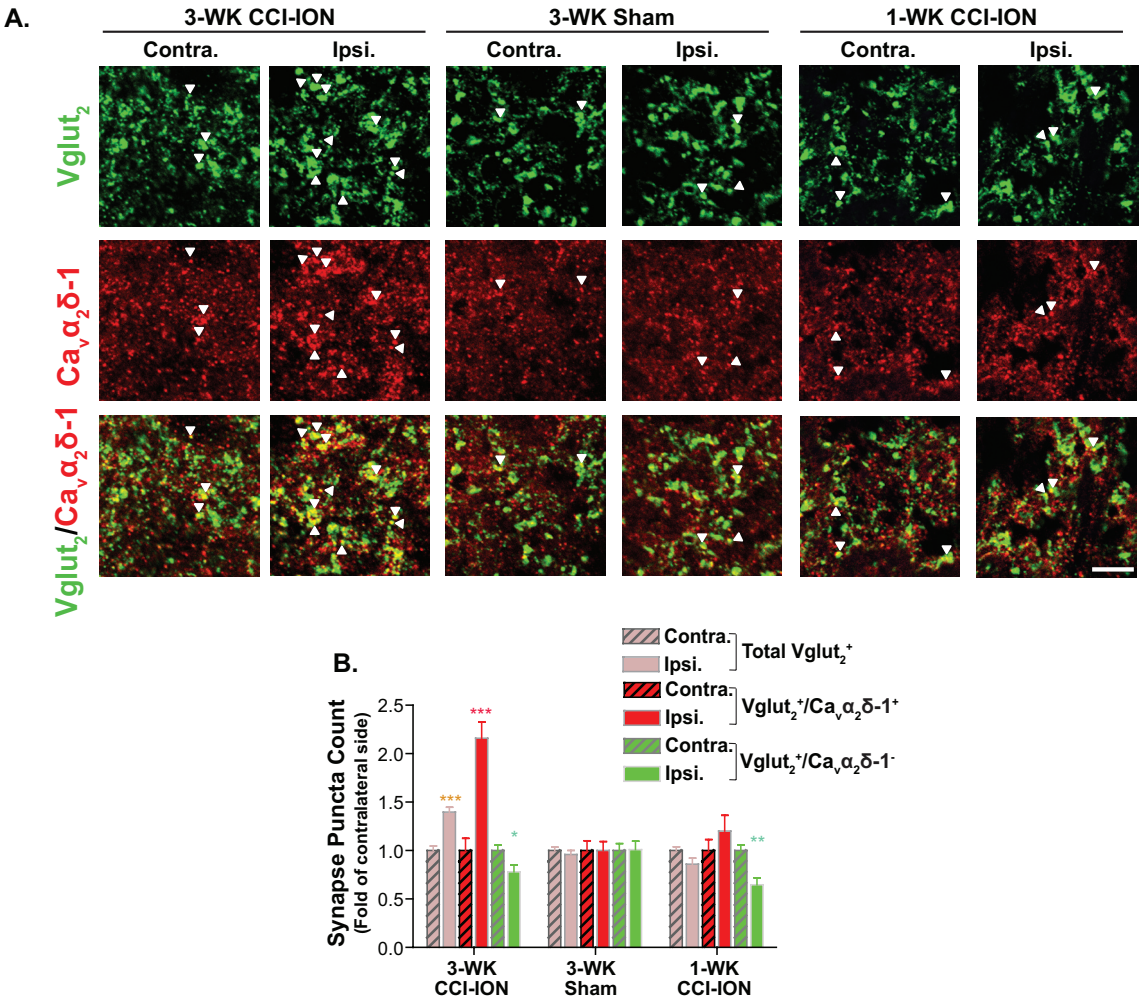

Figure 1.4: A. Representative image of VGlut2 (Green) and α2δ-1 (Red) co-localization showing increased α2δ-1-containing VGlut2 synapses 3 weeks after CCI-IoN, when mechanical hypersensitivity has developed. Scale = 5μm **B.** Quantification of VGlut2/ α2δ-1 co-staining showed the 40% increase in total VGlut2 synapses ($P < .001$) on the ipsilateral side is due to a 100% ($P < .0001$) increase in α2δ-1 containing VGlut2 synapses at 3 week CCI-IoN. There was also a 35% ($P = .002$) and 15% ($P < .01$) decrease in VGlut2 synapses not containing α2δ-1 at 1 and 3 week CCI-IoN respectively. No change was observed in 3 week sham CCI-IoN rats. (n = 54 sections over 3 animals per surgery group)

CCI-IoN also leads to increased post-synaptic protein PSD95

Previous data from the Luo laboratory has indicated that miniature Excitatory Post-Synaptic Current (mEPSC) frequency but not amplitude is increased in the Vc/C2 neurons in 3 week but not in 1 week CCI-IoN rats. This CCI-IoN induced change temporally correlates with orofacial hypersensitivity and with increased VGlut2^{+}/α2δ-1^{+} synapses. This temporal correlation along with the dose-dependent normalization of mEPSC frequency by gabapentin [79] suggests that increased VGlut2^{+}/α2δ-1^{+} synapses may contribute to the increased mEPSC frequency. If so, we should observe increased post-synaptic proteins that are required for functional synapses. Accordingly, I examined co-localization of VGlut2 with post-synaptic marker Post-Synaptic Density protein 95 (PSD95) by IHC staining on Vc/C2 from 3 week CCI-IoN rats.

My data indicated that CCI-IoN ligation increased PSD95 immunofluorescence surface area by more than 1-fold (P = .004) (Figure 1.5 A, B). While VGlut2 staining yielded very clear single puncta, the staining for PSD95 was less defined and clusters of PSD95 puncta in close proximity would be counted as a single puncta. This would underestimate total PSD95 count. Given this problem, although PSD95 count was also significantly increased by 50% (P = .011), I chose to instead report PSD95 as total surface area denoting the relative abundance of the protein (Fig. 1.5 C). In conjunction with the increase in PSD95, the co-localization of VGlut2^{+}/PSD95^{+} puncta was also increased by 60% (P = .013). This suggests that at least a part of the newly formed VGlut2^{+} synapses is functional as they contain both pre- and post-synaptic elements (Figure 1.5 D). Although the importance of PSD95 in CCI-IoN model has not been reported, PSD95 as a post-synaptic scaffolding protein plays a critical role in development of behavioral hypersensitivity in the SNL model of nerve injury [80-82], likely by stabilizing NMDA receptors at the post-synapse terminals [83, 84]. Based on these findings, I believe that CCI-IoN injury-induced PSD95 expression along with increased pre-synaptic VGlut2 and α2δ-1 contributes to increased excitatory synapses that underlie the increased mEPSC frequency, which in turn contributes to dorsal horn neuron hyperexcitability and orofacial hypersensitivity. What remains unclear is whether α2δ-1 in

newly formed VGlut2^{+} synapses plays a role in the development of these synapses.

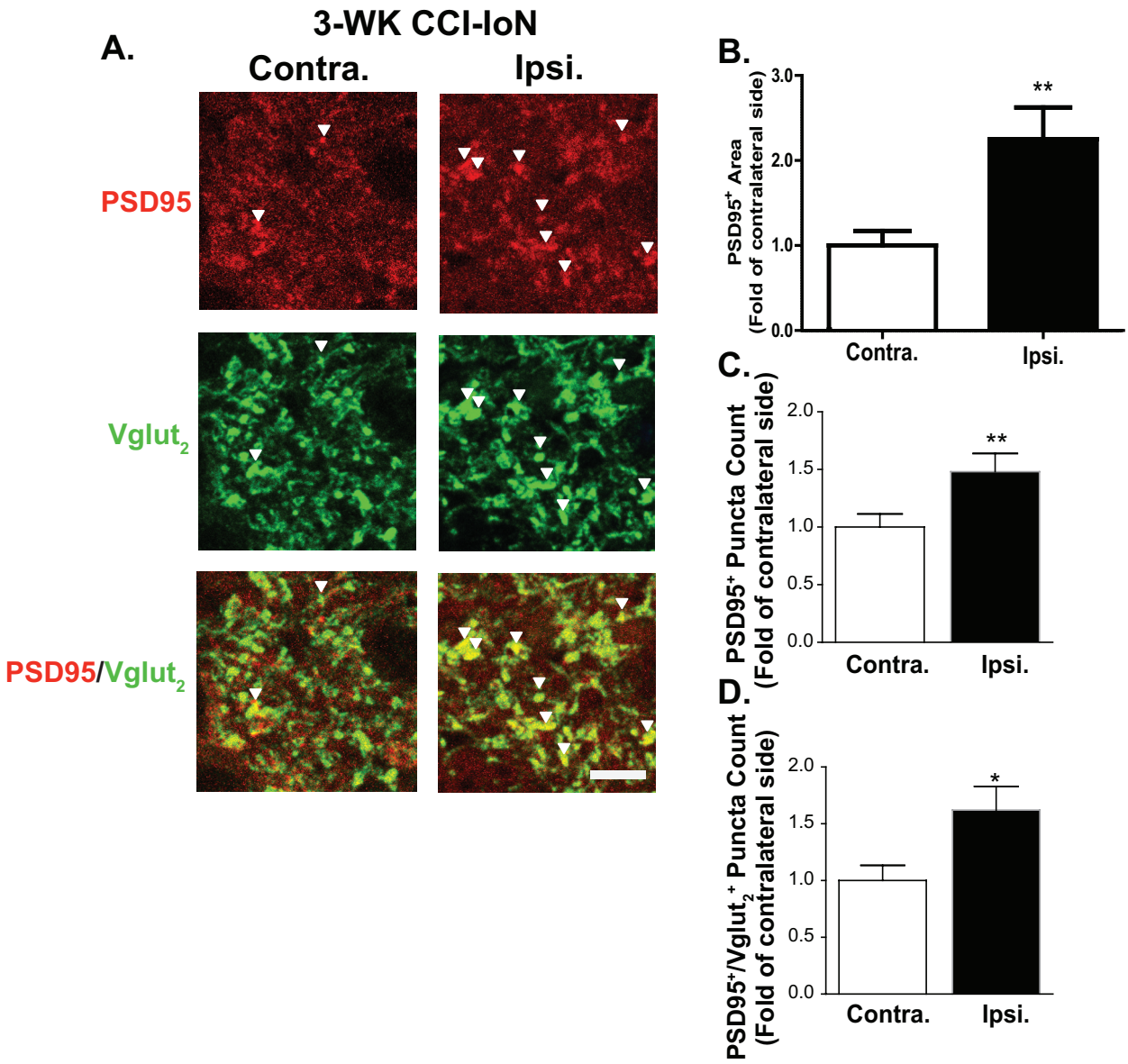

Figure 1.5: A. Representative image of VGlut2 (Green) and PSD95 (Red) showing that 3 week after CCI-IoN, occurances of both pre- and post-synaptic components were increased. Scale = 5μm **B.** Total area of PSD95 immunofluorescence was increased by 125% (P = .004). **C.** Similarly, PSD95 count was increased by 50% (P = .011). **D.** In conjunction with PSD95 increase, co-localized VGlut2+/PSD95+ puncta also increased by 60% (P = .013) suggesting that newly formed synapses have both the pre- and post-synaptic proteins necessary to become functional. (n = 36 sections over 3 animals)

Discussion

Mechanism of α2δ-1 contribution to behavior hypersensitivity

My findings suggest the following mechanism underlying the contribution of α2δ-1 to behavior hypersensitivity. CCI-IoN injury leads to increased expression of α2δ-1 proteins in TG neurons, which undergo axonal transport to the DH of the Vc/C2. Increased SDH α2δ-1 leads to increased functional excitatory synapses containing pre-synaptic VGlut2 and post-synaptic PSD95 proteins. These increased excitatory synapses may contribute to Vc/C2 neuron sensitization and behavioral hypersensitivity. Since gabapentin can block α2δ-1-induced increase in mEPSC frequency and behavior hypersensitivity within hours after administration [62], it is likely that elevated α2δ-1 in the Vc/C2 is actively modulating synaptic activity through a yet identified mechanism. Blocking this α2δ-1-mediated process by gabapentin may underlie a short-term effect of gabapentin in pain relief. On the other hand, my data support that elevated α2δ-1 in Vc/C2 may also play a role in promoting abnormal excitatory synaptogenesis, which can contribute to spinal circuit hyperexcitability, leading to chronic pain states. Understanding the mechanism of α2δ-1-mediated abnormal synaptogenesis as presented in later chapters of my thesis research would provide helpful information in designing novel interventions for achieving target specific pain relief.

Limitations in the *in vivo* model

While I have presented circumstantial evidences suggesting that injury-induced α2δ-1 may lead to synaptogenesis and ultimately behavioral hypersensitivity, detailed information of this process is still unknown. Sensory neurons are heterogeneous and different subtypes of sensory neurons mediate different behavior modalities [85-88]. However, it would be difficult to address sensory neuron subtype specificity with the CCI-IoN model because CCI-IoN creates a mixed population of injured and uninjured neurons. In addition, the TG contains neurons that form three major branches of trigeminal nerves: Mandibular, Maxillary and Ophthalmic nerves, and the

injured infraorbital nerve is only a portion of the Maxillary nerve. Thus, unless some form of tracing is used to identify which neurons are part of the infraorbital nerve, it is hard to draw any conclusions regarding the role of α2δ-1 in mediating territorial orofacial neuropathic pain states. A model such as SNL where all primary afferents from a DRG are injured would be a better model to study α2δ-1's role in mediating different sensory modalities. This can be achieved by knocking out α2δ-1 in specific subpopulations of DRG neurons through recombination induced by Cre-recombinase expressed under the control of a sensory neuron subtype specific promoter, followed by modality specific testing and biochemical studies.

From a mechanistic point of view, although my data support that CCI-IoN induced α2δ-1 is located at the synapse level, the current experimental setup cannot determine whether α2δ-1 plays a causal role in synaptogenesis nor can it rule out the possibility that α2δ-1 is only trafficked in after the synapses are already formed. Furthermore, the present finding is insufficient to determine if elevated α2δ-1 alone is sufficient to cause the synapse increase. Findings from previously reported studies suggest that α2δ-1 requires TSP4 to mediate synaptogenesis [50, 55]. One way to address these mechanistic questions would be to isolate α2δ-1 and TSP4 expression after nerve injury while leaving all other pathways intact. If the interaction between upregulated α2δ-1 and TSP4 post nerve injury is critical for synaptogenesis, knocking out either α2δ-1 or TSP4 in mice should block injury-induced synaptogenesis. Knocking out α2δ-1 would also answer whether α2δ-1 induces synaptogenesis or if it is just translocated to synapses after they are formed. If synapses are still increased in α2δ-1 knockout mice post injury, then α2δ-1 may not play a permissive role in synaptogenesis and likely be trafficked to newly formed synapses. These questions were addressed in details in next chapters using genetically modified mice and co-culture systems.

Chapter 2: Elevated TSP4 induces synaptogenesis and behavioral hypersensitivity that requires its interaction with α2δ-1

Introduction

Following the landmark paper by Eroglu et al. showing that the TSP family of protein binds to α2δ-1 to promote synaptogenesis [50], I wanted to look at whether synaptogenesis also plays a role in the development of behavioral hypersensitivity after nerve injury. The Luo lab had already investigated the role of TSPs in the SNL model of pain and published a gene chip paper showing that TSP4 mRNA was increased in the DRG after SNL [89]. Subsequently, the lab reinforced the importance of TSP4 in SNL-induced hypersensitivity when we reported that TSP4 KO mice developed diminished thermal and mechanical hypersensitivity after SNL [56], and blocking TSP4 using antibody or antisense treatment could reverse established behavioral hypersensitivity [56].

Although I have established the correlation between the CCI-IoN induced increase of α2δ-1 and VGlut2^{+} synapses, the data was insufficient to conclusively determine whether α2δ-1 played a role in inducing excitatory, VGlut2 positive synapse formation. Furthermore, given the importance of TSP4 in SNL-induced hypersensitivity and its reported interaction with α2δ-1 in promoting synaptogenesis, I wanted to look at whether α2δ-1 interaction with TSP4 was critical to α2δ-1-mediated formation of VGlut2 positive synapses at the spinal cord level after nerve injury by looking at whether selective ablation of α2δ-1 or TSP4 expression could block SNL-induced synaptogenesis and behavioral hypersensitivity. My data support that both α2δ-1 and TSP4 are critical for injury-induced excitatory synaptogenesis and that α2δ-1/TSP4 signaling induced synaptogenesis contributes to injury-induced behavioral hypersensitivity.

Methods

Advillin-Cre/α2δ-1 CKO mice generation

Since α2δ-1 can be found in skeletal muscles, smooth muscles, heart, neurons and Purkinje cells [32-35], an animal with α2δ-1 globally knocked out may exhibit phenotypic deficiencies that may change or mask any nerve injury-induced effects. To confirm the role of α2δ-1 in synaptogenesis, we have generated a mouse line with DRG sensory neuron specific knockout of α2δ-1 (in collaboration with Dr. Feng at MIT). This was achieved by breeding mice that have exon 6 of the α2δ-1 gene flanked by LoxP sites (α2δ-1 CKO) with animals that produce Cre recombinase under a DRG neuron specific promoter Advillin (Adv-Cre) to produce Adv-Cre $^{Cre/WT}$/ α2δ-1 CKO$^{Flox/Flox}$ (Adv/α2δ-1 CKO) mice.

Spinal Nerve Ligation

I performed spinal nerve ligation (SNL) according to the procedure described by Chung et al. [48]. Dr. Li, from the Luo lab performed the surgeries for the TSP4-eGFP data. Due to anatomical differences between the species [90], a slight modification of the rat protocol was made so that the L4-L5, instead of L5-L6, spinal nerves, were ligated. Only male mice were used for SNL for behavioral and biochemical experiments as gonadal hormones such as estrogen play a role in pain modulation [91].

Briefly, mice were anesthetized and kept in deep anesthesia using mixed air and isofluorane and the hair near their lower back and hip was clipped. The skin was then sterilized using iodine applied with a cotton swab. Under a dissection microscope, a 1cm incision was made on the left side near the midline beginning from the iliac crest to the sacrum. After blunt end dissection, muscle layers were retracted with surgical retractors until the L5 spinal transverse process was revealed. The left L5 transverse process was removed with forceps, and then L4/L5 nerves were individually isolated and tightly ligated using 6-0 silk thread. Muscle layers and skin were sutured

back up respectively. The animals were then administered with 1 mL of warm saline intraperitonally and put on pre-warmed padding under a heat lamp until they wake up. The animals were then monitored for health for 1 week after surgery. In general, the animals were healthy and did not exhibit any outward signs of pain or distress such as vocalization, weight loss or lack of grooming.

Success of the surgery was determined by mechanical allodynia testing via von Frey filaments where the side ipsilateral to the surgery was hypersensitive compared to that from the contralateral side, which should be unchanged from the pre-surgery level. The sham control for SNL involved performing the above steps except that the nerve was not ligated. Our lab has shown extensively that the contralateral side sensitivity remains unchanged after SNL surgery so an internal control using the contralateral side is often used for biochemical studies.

TSP4/gabapentin Injection and Behavior Test

To see whether interaction of TSP4 with α2δ-1 led to synaptogenesis, and if so, whether the TSP4/α2δ-1 signaling is critical in the initiation and/or maintenance of synaptogenesis, bolus 5μg TSP4 was injected intrathecally into naïve mice in conjunction with daily saline or gabapentin (25μg/mouse, i.t.) treatment (by Ben Vo in the Luo Lab) for designated durations, either started at the beginning (day 0-3) or delayed for two days (day 2-3). Daily behavioral test was performed before daily injection, if any, for 4 days, which correlated with peak behavioral hypersensitivity after intrathecal TSP4 injection [56]. Spinal cord samples were collected at day 4 for immunochemical studies. Gabapentin has a short analgesic effect [14] due to its rapid clearance via renal secretion [92]. By testing the gabapentin effects 24 hours after injection, one could assess the long-term effect of gabapentin treatment independent of its short-term analgesic effect.

Immunohistochemistry

L4/L5 spinal cord at the lumbar enlargement and associated DRG samples were collected from three animals after 2-week SNL, or at day 4 post intrathecal TSP4 injections. Samples were

fixed in 4% PFA overnight and cryoprotected in 30% sucrose. Samples were then mounted in OCT and sectioned in 10μm slices by a cryostat. Samples were pretreated with heat-based antigen retrieval before being stained with combinations of rabbit α2δ-1 antibody (ABR) or mouse α2δ-1 antibody (Sigma), guinea pig VGlut2 (Synaptic Systems) and rabbit PSD95 (Invitrogen). For eGFP data, 10μm sections were cut and stained with rabbit GFAP (BD Bioscience), Iba-1 (Dako), MBP (Abcam) or NF70 (Millipore). Images were taken using a Zeiss LSM780 confocal microscope (Air 20X, Oil 63X, Oil 100X lens) (UC Irvine Optical Biology Core) in 0.3 μm thick Z stacks. Images were then cropped down to the 3 consecutive Z stacks with the best signal, merged and used for analysis using Volocity 6.0 (PerkinElmer).

Data Analysis

For eGFP signal quantification, mirroring regions of interest spanning from the midline to both sides of the dorsal spinal cord were selected. eGFP fluorescence above the background level was selected for analysis using Volocity's thresholding function. Surface area and intensity data (n = 24 sections over 4 mice, 50μm interval minimum) were then pooled and analyzed. For quantifying the number of eGFP-expressing cells, cells with nuclei with eGFP signal above and below mean cell intensity were counted in both the dorsal column and the dorsal horn from the injured and non-injured sides. Statistical significance was calculated using paired 2-tailed T-test. For co-localization data, thresholded Pearson's correlation coefficient values were obtained from sections stained with cell marker (n = 9 over 3 animals per marker) using the Volocity colocalization function [93].

L4/5 DRG neurons were selected based on morphology from captured images. Increase in α2δ-1 immunoreactive signals were quantified and compared between the ipsilateral side and contralateral side after SNL (2μm sections, n = 9 over 3 animals, 100μm apart).

α2δ-1 fluorescent signal was analyzed from L4/L5 mouse spinal cord sections (2μm sections, n = 9 over 3 animals, 100μm apart) using Volocity thresholding function. Captured

images from Vglut2/α2δ-1 antibody co-stained samples (n = 36 over 3 animals per genotype, 100μm apart) or Vglut2/PSD95 antibody co-stained samples (n=36 over 3 animals, 100μm apart) were analyzed using Volocity to determine number of total $Vglut2^+$ puncta, $Vglut2^+/\alpha2\delta\text{-}1^+$ puncta, $Vglut2^+/PSD95^+$ and $PSD95^+$ puncta in the ipsilateral side versus the contralateral side control. Statistical significance between injury and non-injury sides was calculated using 2-tailed paired t-test.

A slight modification was made in data presentation for the TSP4 injection experiment. Since intrathecal injection produces a bilateral effect, a ratio of $PSD95^+/VGlut2^+$ to $PSD95^-/VGlut2^+$ (PV/V) was used to account for changes in the number of functional synapses and minimize the animal and sampling differences between groups. Total PSD95 area and VGlut2 count were also looked at separately in case both PSD95 and VGlut2 are increased at a similar rate as that could increase synapse count without changing the ratio.

Results

TSP4 is produced and secreted by astrocytes located in the white matter of the dorsal horn and in the DRG

Although TSP4 has been shown to play an important role in neuropathic pain, little is known about the location and cell types where TSP4 is being produced after SNL injury. Does SNL increase TSP4 production in existing cells or does it induce TSP4 production in new cell types? In the nervous system, thrombospondins are secreted by astrocytes [50, 55, 94, 95], coincidentally, astrocytes are activated after nerve injury and undergo hypertrophy after SNL [96, 97]. To address this question, I used a transgenic mouse line with enhanced Green Fluorescent Protein (eGFP) expression driven by a TSP4 promoter (GENSAT) to look at the location and cell-type specific expression of TSP4 in spinal cord after 2-week SNL. A bacterial artificial chromosome containing the TSP4 promoter region followed by 8 copies of eGFP transcript is inserted into these mice.

Activation of TSP4 transcription will also activate eGFP transcription leading to eGFP labeling of TSP4 producing cells.

My data indicated that nerve injury increased TSP4 production in the spinal cord more than 3-fold ($P < .0001$) (Figure 2.1 A, B, C), most of this increase came from an increase in eGFP surface area as these cells show hypertrophy ($P < .0001$) while there was also a smaller but statistically significant increase in average intensity ($P < .0001$) (Figure 2.1 D). In addition, the total number of eGFP positive cells did not change on the injured side compared to the uninjured side after nerve injury, supporting that no new TSP4 expressing cells are induced by nerve injury. Thus, SNL induced eGFP expression is likely from existing TSP4-expressing cells undergoing hypertrophy and increased TSP4 transcription. This is supported by the finding that the number of cells with weak eGFP intensity (less than mean cell intensity) was reduced while those with strong eGFP intensity (above mean cell intensity) were increased ($P < .0001$) (Figure 2.1 E). These TSP4 transcribing cells originate in the white matter of the dorsal horn and column rather than in the grey matter of the superficial dorsal horn. Further analysis through colocalization of the eGFP signal with immunoreactivity from neuronal marker Neurofilament 70 (NF70), microglial marker Ionized calcium-Binding Adapter molecule-1 (Iba-1), astrocytic marker Glial Fibrillary Acidic Protein (GFAP) and oligodendrocyte marker Myelin Basic Protein (MBP) showed that eGFP co-localized exclusively with GFAP as shown by the Pearson's linear correlation (Figure 2.2 A-E). This data showed that white matter astrocytes are the main source of TSP4 in the spinal cord, which is upregulated after nerve injury. In collaboration with the lab of Dr. Quinn Hogan, we have shown that Schwann cells in the DRG are also a significant source of TSP4 [59].

A limitation of this set of experiments is that the eGFP signal is amplified 8 times over each transcribed TSP4 mRNA molecule, but TSP4 proteins are not labeled by eGFP and can be secreted. eGFP fluorescence can be used to detect where TSP4 is transcribed, but it does not allow to determine the location and concentration of synthesized TSP4 proteins. The latter information can be obtained once a good TSP4 antibody for IHC becomes available.

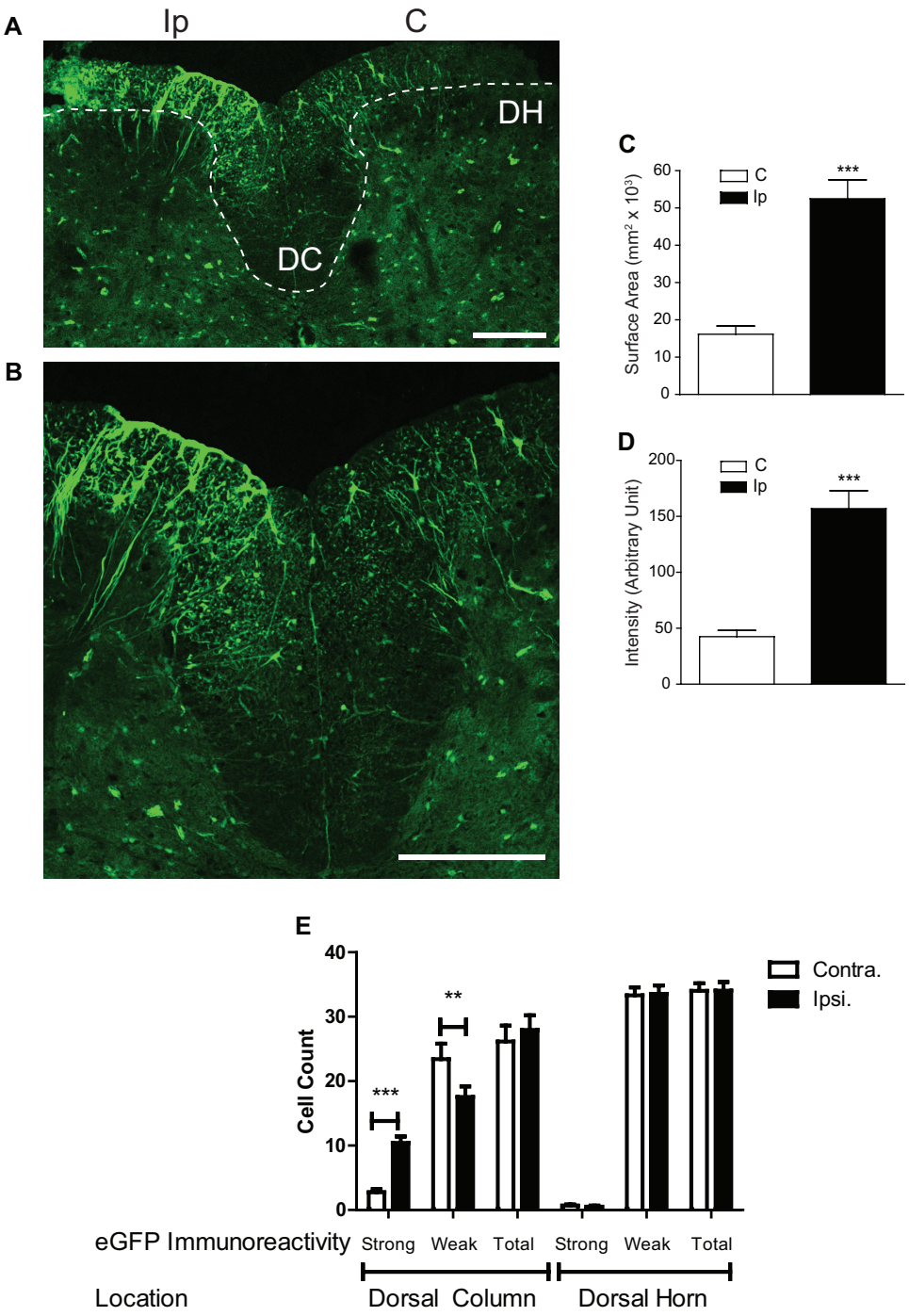

Figure 2.1: A. Representative image of 2wk SNL TSP4-eGFP mice L5 dorsal horn with enlarged view of the dorsal spinal cord **(B)**. C - Contralateral, Ip - Ipsilateral, DC - Dorsal Column, DH - Dorsal Horn. Scale = 200µm. SNL induced a 3-fold increase in eGFP surface area ($P < .0001$) **(C)** and 3-fold increase in intensity ($P < .0001$) **(D)**. **E.** SNL did not alter the total number of eGFP expressing cells but shifted eGFP expression in the dorsal column so that more cells had an intensity above the mean cell intensity ($P < .0001$). (n = 24 sections over 4 animals)

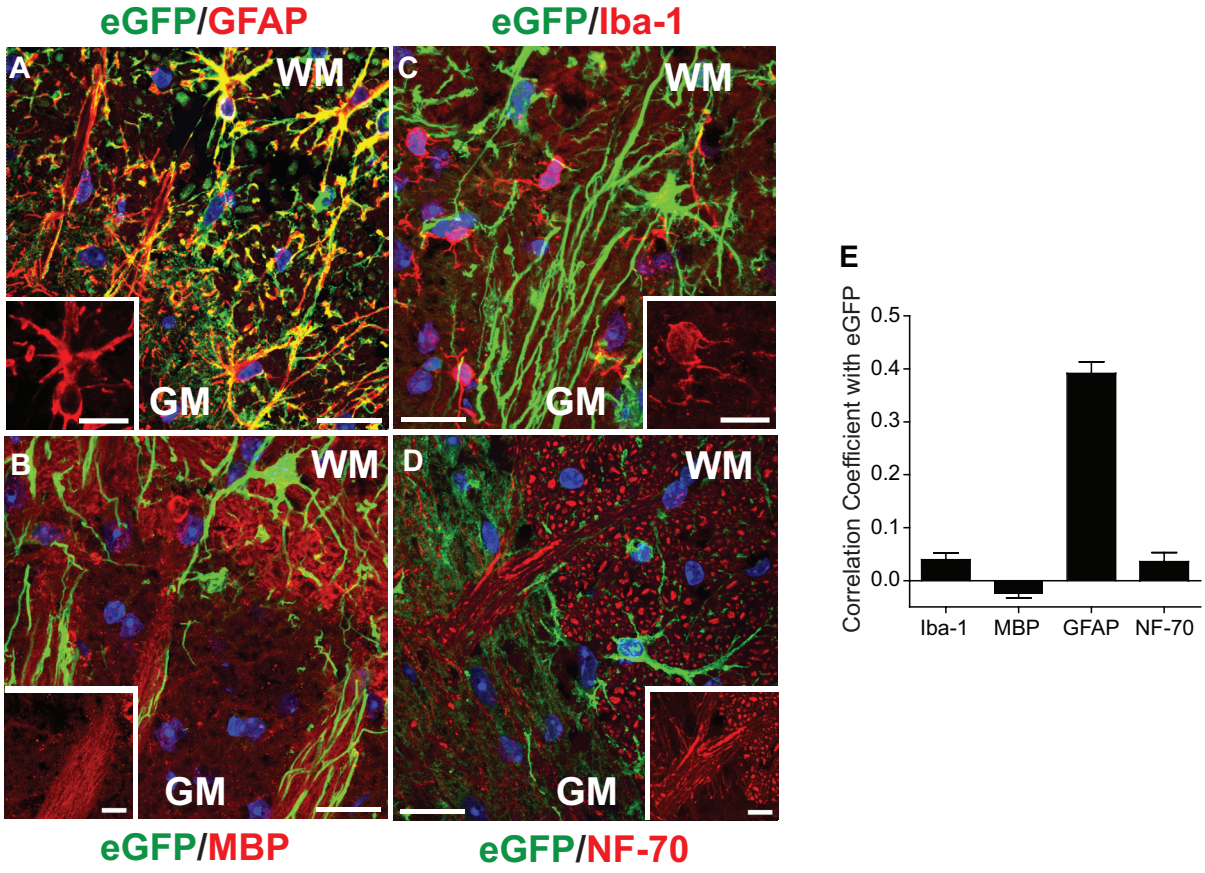

Figure 2.2: eGFP signal (Green) in 2wk SNL TSP4-eGFP mice co-localized with stained cell markers for astrocytes (GFAP, Red) **(A)**, oligodendrocytes (MBP, Red) **(B)**, microglia (Iba-1, Red) **(C)** and neurons (NF70, Red) **(D)** show eGFP expressing cells only co-localizes with astrocytic marker GFAP **(E)**. Scale = 20μm. **Insert:** Enlarged image showing cell mophology. Scale = 10μm. GM - Grey Matter, WM - White Matter (n = 9 over 3 animals per marker)

Absence of TSP4 blocked nerve injury-induced excitatory synaptogenesis in dorsal spinal cord

Having established that TSP4 synthesis is increased in the dorsal horn after SNL, I next examined the importance of TSP4/α2δ-1 interaction signaling in excitatory synaptogenesis in dorsal spinal cord. Specifically, I looked at if nerve injury induced an increase in VGlut2 positive synapse count and if TSP4 ablation could block these changes. Co-localization of immunoreactivities to VGlut2 and α2δ-1 antibodies in the superficial dorsal horn of 2-week SNL TSP4 KO and WT were examined.

Similar to my findings in the CCI-IoN model, nerve injury induced a 15% increase of $VGlut2^+$ puncta in the injury side superficial dorsal horn of the L4/L5 spinal cord from WT mice compared to that in the uninjured side ($P = .004$). α2δ-1 containing synapses accounted entirely for this increase since there was a 30% increase in $\alpha2\delta\text{-}1^+/VGlut2^+$ puncta ($P < .001$), but no significant change in $\alpha2\delta\text{-}1^-/VGlut2^+$ puncta ($P = .296$) (Figure 2.3 A, B). The relative increase of synapses on the injury side is less than that observed in the CCI-IoN model. This may be due to the SNL model's tight ligation yielding damage to all L4/5 primary afferents compared to the CCI-IoN model's loose ligation yielding a mixed population of healthy and damaged primary afferents. A tight ligation would cause more damage leading to increased loss of synapses due to more severe dying back of sensory fibers [5, 6]. However, absence of TSP4 in TSP4 KO mice blocked injury-induced increases of total $VGlut2^+$ puncta counts ($P = .140$) or $\alpha2\delta\text{-}1^+/VGlut2^+$ puncta counts ($P = .952$). The lack of injury-induced increase of $VGlut2^+$ puncta/synapses in the 2-week SNL TSP4 KO mice was not due to influence of absence of TSP4 on α2δ-1 expression since α2δ-1 expression in the injury side of SDH after nerve injury was similar to that in the contralateral side ($P = .011$) (Figure 2.3 C). The lack of injury-induced synaptogenesis in the TSP4 KO mice correlates with the reported deficits in injury induced mechanical allodynia and thermal hyperalgesia in the TSP4 KO mice [56].

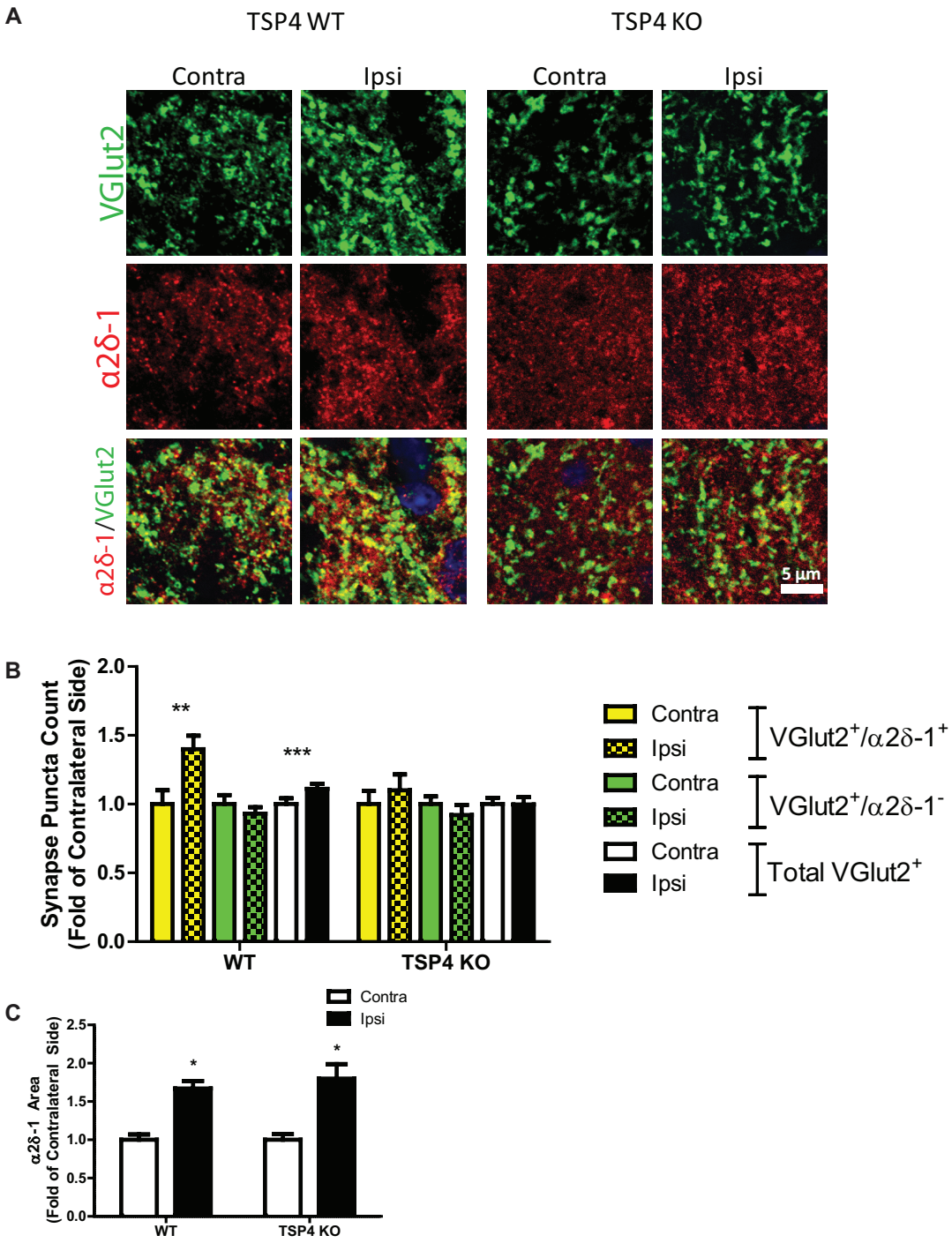

Figure 2.3: A. Representative image of VGlut2 (Green) co-stained with α2δ-1 (Red) in 2wk SNL TSP4 WT or TSP4 KO mice. **B.** SNL caused a 15% increase in total VGlut2 synapses (P = .004) due to a 30% increase in VGlut2+/α2δ-1+ synapse count (P < .001) in WT mice while TSP4 KO mice showed no change. (n = 18 sections per side (Contra/Ipsi) over 3 animals per genotype) **C.** Loss of TSP4 did not affect SNL-induced α2δ-1 increase (P = .011) (n = 9 sections over 3 animals per genotype)

Since functional synapses need to have the necessary post-synaptic machinery to become functional, I examined whether α2δ-1/TSP4 signaling induced synapses are functional by looking at the post-synaptic marker PSD95 in the number of synapses two weeks after SNL in WT and TSP4 KO mice. My data indicated that SNL induced significant (35%) increase of total VGlut2^{+} puncta ($P < .0001$) in dorsal spinal cord of the injury side that translated to about a 40% increase in VGlut2^{+}/PSD95^{-} puncta ($P < .001$) and about 30% increase of VGlut2^{+}/PSD95^{+} functional synapses after SNL in WT ($P = .007$), but not in SNL TSP4 KO mice ($P = .148$) (Figure 2.4 A, B). In comparison, CCI-IoN increased functional synapses by 60% in dorsal Vc/C2. This discrepancy may be due to differences between these models as discussed previously.

Taken together, these data support that VGlut2^{+}/α2δ-1^{+} signaling-induced synaptogenesis post SNL injury is dependent upon the injury induction of TSP4 and α2δ-1. This is supported by the fact that α2δ-1 increase alone in the SDH of TSP4 KO mice is not sufficient to induce excitatory synaptogenesis. Since only VGlut2^{+} synapses were examined, we could not exclude the possibility that TSP4 might mediate injury-induced behavioral hypersensitivity through other roles independent from VGlut2^{+} synapses. Given the importance of VGlut2 in mediating hypersensitive states [75-77], these findings support that TSP4/α2δ-1 signaling induced excitatory synaptogenesis is likely to contribute, at least partially, to SNL injury-induced behavioral hypersensitivity.

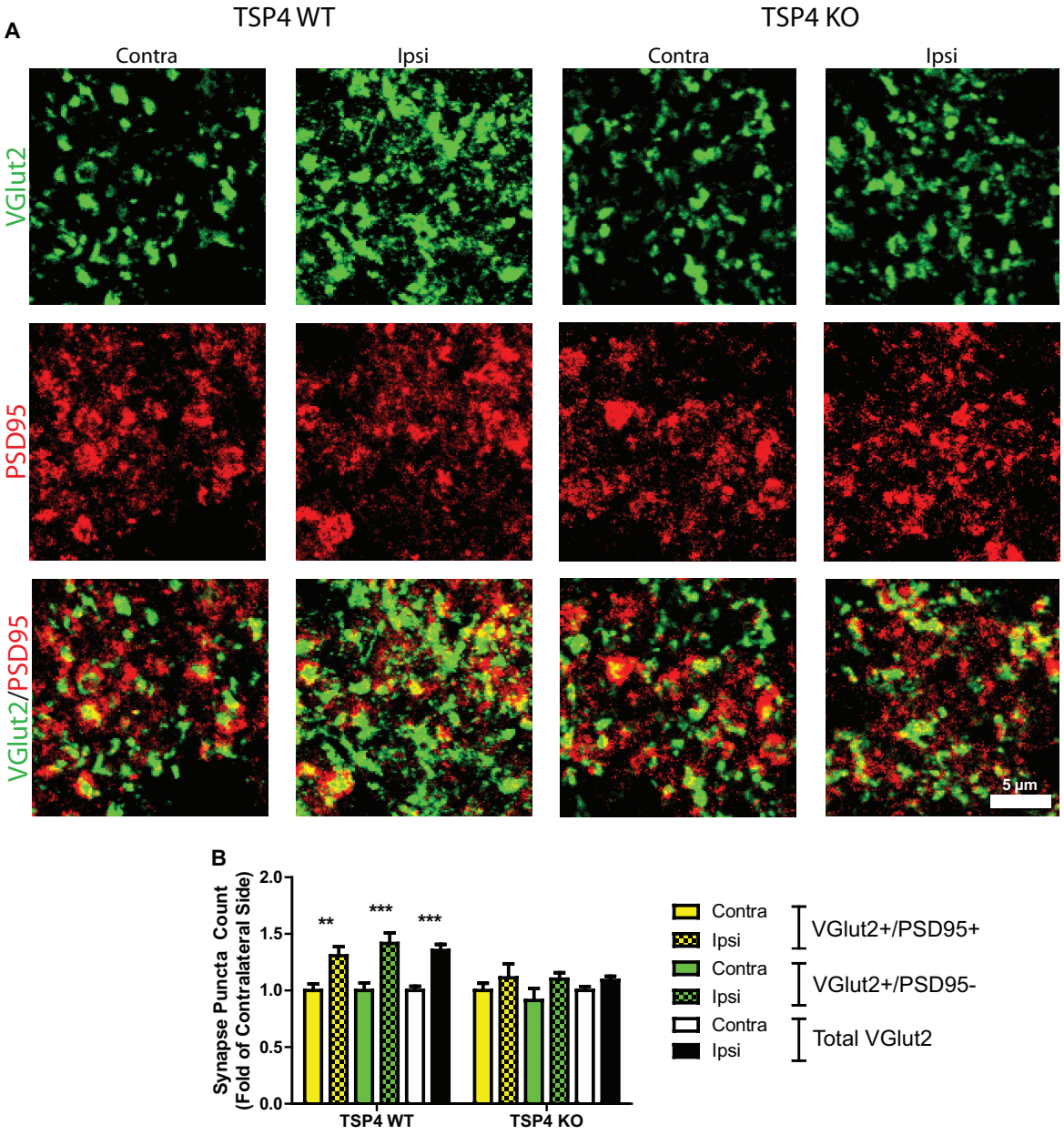

Figure 2.4: A. Representative image of VGlut2 (Green) co-stained with PSD95 (Red) in 2wk SNL TSP4 WT or TSP4 KO mice. **B.** SNL caused a 35% increase in total VGlut2 synapses ($P < .0001$) due to a 30% increase in VGlut2+/PSD95+ synapse count ($P = .007$) and also a 40% increase in VGlut2+/PSD95- synapse count ($P < .001$) in WT mice while TSP4 KO mice showed no change. (n = 18 sections per side over 3 animals per genotype)

DRG neuron specific knock out of α2δ-1 can block TSP4-induced excitatory synaptogenesis

Conversely, I examined if knocking out α2δ-1 could block TSP4-induced excitatory synaptogenesis. α2δ-1 knock out in 80-90% of DRG neurons was achieved by crossing the α2δ-1 conditional knockout (α2δ-1 CKO) with the Advillin-Cre line (Adv-Cre, gift from Dr. F. Wang at Duke University) that has been characterized in several papers using a red fluorescent protein (RFP) as well as a LacZ reporter line to show that Adv is expressed in 80-90% of DRG neurons but absent in some small diameter neurons [98, 99]. This crossing generated a α2δ-1 conditional knock out line in which α2δ-1 expression is ablated in most DRG neurons (Adv/α2δ-1 CKO). I performed IHC staining to check α2δ-1 expression in L4/5 DRG neurons from SNL Adv/α2δ-1 CKO mice to confirm that α2δ-1 was properly knocked out. α2δ-1 expression should be increased after SNL [9, 14] but should be diminished or absent in the SNL Adv/α2δ1 CKO mice if the α2δ-1 knockout was successful. α2δ-1 CKO mice without Cre expression were used as a control. My data indicated that there was a one-fold increase in α2δ-1 expression in injured DRG after 2-week SNL injury compared to that in the contralateral (unligated control) side ($P = .024$) (Figure 2.5 A, B1-2). This injury-induced increase was not seen in Adv/α2δ-1 CKO mice ($P = .829$), supporting that α2δ-1 was properly knocked out. The one-fold increase after nerve injury is less than the roughly 10 to 20-fold increase we have previously reported in Western blots. However, the IHC data is focused only on α2δ-1 expression in DRG neuron cell bodies so the one-fold increase does not reflect total α2δ-1 in the DRG.

The Adv/α2δ-1 CKO mice were used to study the influence of α2δ-1 KO in TSP4-induced excitatory synaptogenesis. My data indicated that intrathecal TSP4 injection (5μg/mouse) was able to increase synaptogenesis in L4 dorsal spinal cord from non-Cre expressing control α2δ-1 CKO mice by almost 40% ($F(2, 107) = 12.05$, $P < .0001$) (Figure 2.5 C, D). However, similar TSP4 injection into the Adv/α2δ-1 CKO mice did not significantly increase synapse count suggesting that α2δ-1 expression in the DRG is required for TSP4-induced synaptogenesis in the

SDH. This change correlated with deficiency in TSP4 induced mechanical allodynia and thermal hyperalgesia in the Adv/α2δ-1 CKO mice, confirming that TSP4/α2δ-1 signaling induced excitatory synaptogenesis plays a role in the development of behavioral hypersensitivity after peripheral nerve injury [Submitted, in revision].

We have tested the Adv/α2δ-1 CKO mice for behavioral changes after SNL. However our findings were inconclusive with unknown reasons (still under active investigation) as one group tested was partially resistant to injury-induced hypersensitivity while another group from a later generation was found to develop hypersensitivity normally. This is surprising as the Luo lab has previously found that α2δ-1 antisense treatment could reverse SNL induced mechanical allodynia [8]. One possible reason for the conflicting data in behavioral sensitivity to SNL in the Adv/α2δ-1 CKO mice may be due to residual activity in the 10-15% of neurons that do not express Adv and therefore do not lose α2δ-1 expression. These neurons may play a critical role in pain signal transduction post SNL. Moreover, Adv/α2δ-1 CKO mice may have yet identified compensatory pathways due to the loss of α2δ-1, which can affect the pain phenotype in the SNL model. However, these limitations of *in vivo* experiments would have less influence on isolated DRG neuron culture systems. Therefore, the Adv/α2δ-1 CKO line was used as a tool for isolating α2δ-1 CKO DRG neurons by crossing them with a tdTomato RFP Cre reporter line (Adv/α2δ-1 CKO/RFP) to study the interaction between TSP4 and α2δ-1 in synaptogenesis *in vitro*.

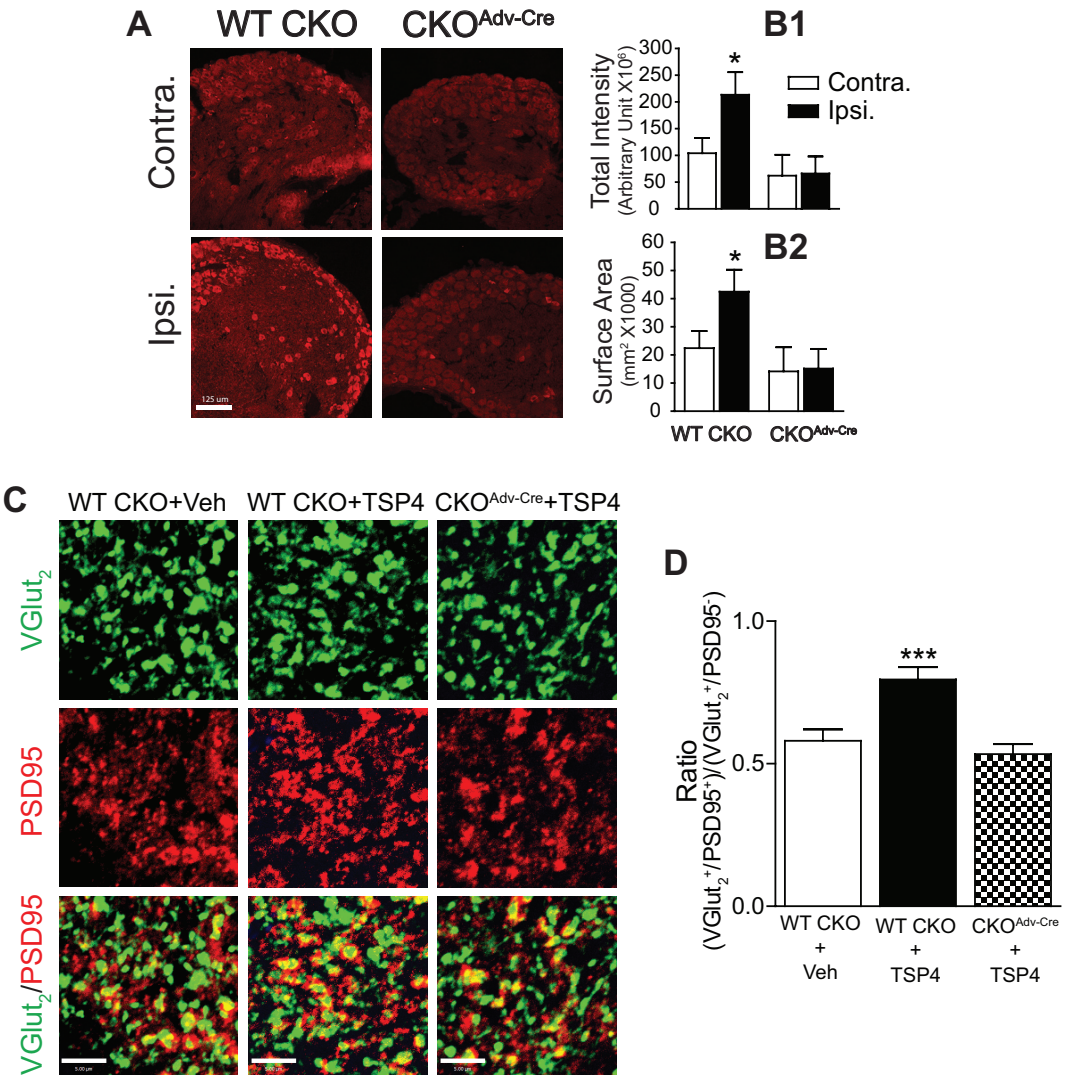

Figure 2.5: A. Representative image of α2δ-1 staining (Red) in DRG specific α2δ-1 knockout mice (Adv/α2δ-1 CKO) and WT mouse L4/5 DRG after 2 week SNL. While SNL could induce α2δ-1 expression in WT mouse DRG neurons, no increase was observed in Adv/α2δ-1 CKO mice. Scale = 125μm. **B1-2.** Quantification of α2δ-1 immunofluoresence showed a 1-fold increase in WT mouse DRG ($P < .024$) while immunofluorescence remained unchanged in Adv/α2 δ-1 CKO mice. (n = 9 over 3 animals per genotype) **C.** Representative image of VGlut2 (Green) and PSD95 (Red) co-localization showing that knocking out α2δ-1 in DRG neurons could block TSP4-injection induced synaptogenesis ($F(2, 107) = 12.05$, $P < .0001$) **(D)**. Scale = 5μm. (n = 36 sections over 3 animals per treatment group)

TSP4/α2δ-1 signaling mediates increased formation but not maintenance of synapses

Nerve injury causes increased production of many different proteins including extracellular matrix proteins, inflammatory factors and other synaptogenic factors [89]. Many of these proteins such as Brain Derived Neurotropic Factor [21], Tumor Necrosis Factor α, Interleukin 6, Interleukin 1β [15-17], Fractalkine [100] are all found to be critical in nerve injury induced behavioral hypersensitivity. In order to study the role of TSP4 in nerve injury induced synaptogenesis and behavioral hypersensitivity without the influence from other injury factors, we injected TSP4 intrathecally into naïve mice (5μg/mouse) followed by daily test for behavioral hypersensitivity and analysis of excitatory synaptogenesis at day 4 post TSP4 injection. To address the question of whether TSP4/α2δ-1 signaling is critical in the initiation and/or maintenance of synaptogenesis, gabapentin was injected daily (25 μg/mouse, i.t.) after behavioral testing, started either right after bolus TSP4 injection (from day 0-3) or with a 2-day delay (from day 2-3), and TSP4-induced synaptogenesis was analyzed at day 4 post TSP4 injection that correlated with TSP4-induced peak behavioral hypersensitivity [56].

My data indicated that TSP4 injection increased excitatory synapses around 35% compared with saline injected control ($F(4, 179) = 7.528$, $P < .0001$) (Figure 2.6 A, B). This change correlated with behavioral hypersensitivity in the TSP4 injected mice ($F(1,6) = 33.92$, $P < .0011$) (Figure 2.6 C). Furthermore, TSP4 induced synaptogenesis and behavioral hypersensitivity were blocked by daily treatment with gabapentin for the same duration (Figure 3.5 A, B). These data support that injected TSP4 interacts with α2δ-1 to induce excitatory synaptogenesis and behavioral hypersensitivity. Interestingly, delayed gabapentin treatment for the first 2 days after TSP4 injection failed to block TSP4-induced synaptogenesis and behavioral hypersensitivity (Figure 2.6 A-C). This suggests that the signaling pathway activated by TSP4/α2δ-1 interaction plays a critical role in the formation, but not maintenance, of excitatory synapses. This time-dependent blockade of TSP4-induced synaptogenesis and behavioral hypersensitivity with gabapentin has also been reported by our collaborators in a whiplash model of pain [101].

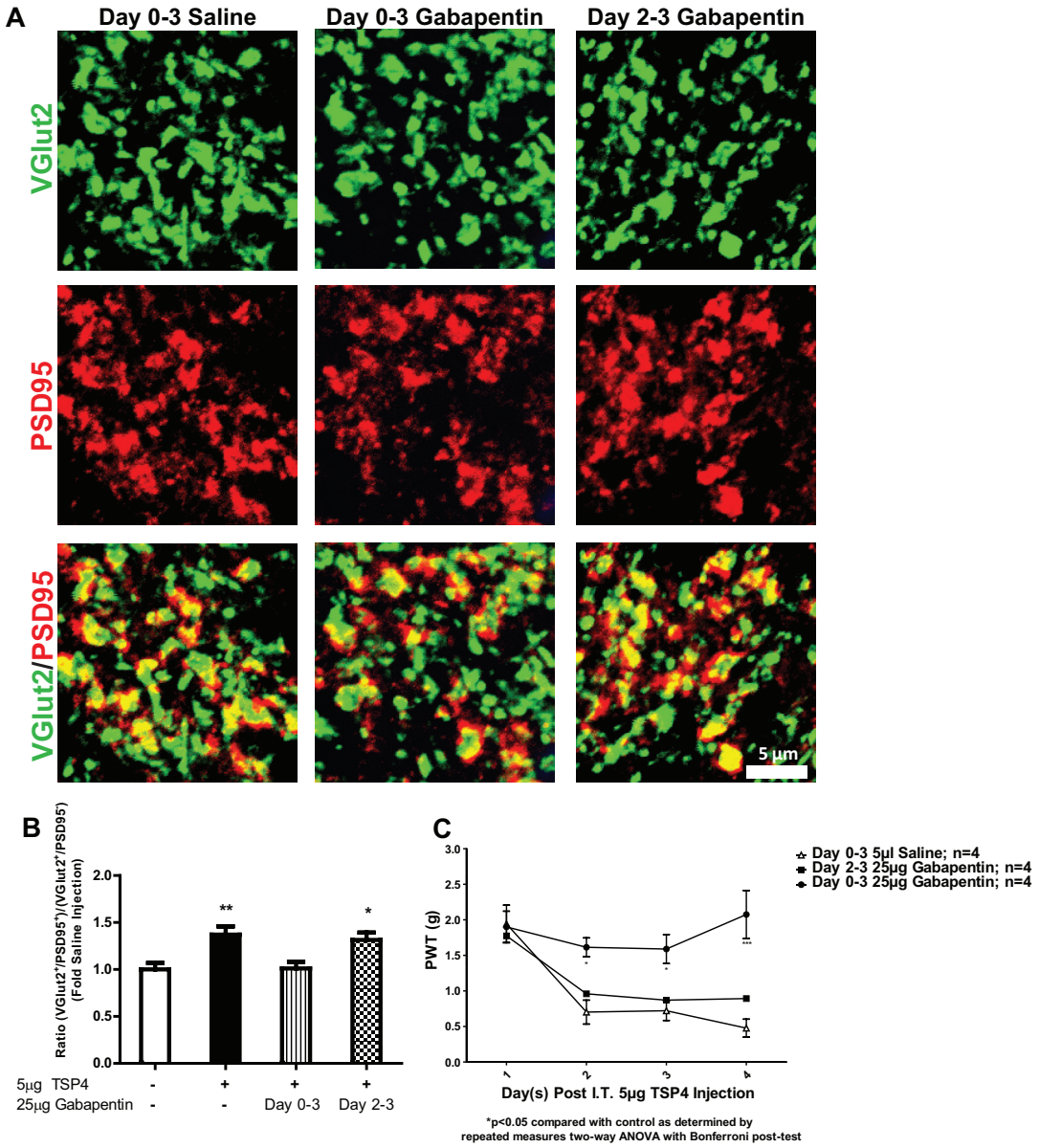

Figure 2.6: A. Representative image of VGlut2 (Green) and PSD95 (Red) staining after one time 5µg TSP4 i.t. injection and daily saline or 25µg gabapentin injection for 4 days. **B.** Quantification of synapse ratio based on VGlut2+/PSD95+ to VGlut2+/PSD95- co-localization showed increased synapse ratio after TSP4 injection which can be blocked by daily (Day 0-3), but not by delayed (Day 2-3), gabapentin injection ($F(4, 179) = 7.528$, $P < .0001$) (n = 36 images over 3 animals per treatment group). **C.** Von Frey test for mechanical allodynia showed that daily, but not delayed, gabapentin injection could reverse TSP4 induced mechanical allodynia ($F(1,6) = 33.92$, $P < .0011$). (n = 4 per treatment group)

Discussion

White matter astrocytes are the source of TSP4 in the spinal dorsal horn

Surprisingly, although astrocytes seems to be the only source of TSP4 in the spinal dorsal horn, my data support that white matter "fibrillar" astrocytes but not the more commonly studied gray matter "protoplasmic" astrocytes are the source of elevated TSP4. These astrocytes seem to travel with the nerve and extend long processes through the dorsal horn entry zone and into the dorsal horn. In contrast to gray matter astrocytes, whose role in pain has been extensively studied, very little research has been done on white matter astrocytes. Given the importance of TSP4 in nerve injury induced pain, perhaps more attention should be given to white matter astrocytes in order to understand their role in neuropathic pain development.

Thrombospondin-4 interaction with α2δ-1 leads to synaptogenesis

Using genetically modified mice, findings from my studies confirmed that a signaling pathway activated by TSP4/α2δ-1 interaction is required for injury-induced excitatory synaptogenesis. I have defined "functional" synapses by the co-localization of the pre-synaptic VGlut2 marker with the post-synaptic PSD95 marker without examining functional properties of synapses. Electrophysiological recording studies about the effects of TSP4 application in DRG and SC neuron co-cultures, with or without α2δ-1 ablation, on synaptic activities, such as miniature Excitatory Post-Synaptic Current (mEPSC) and evoked EPSC, could help to make more conclusive determination.

However, my findings are consistent with other findings suggesting that these synapses are electrophysiologically functional. The Luo lab has previously reported that increased α2δ-1 expression in SNL mice or transgenic mice over-expressing α2δ-1 causes increased mEPSC frequency compared to control and that these changes in mESPC can be blocked with gabapentin treatment [8, 14]. TSP4 ablation from the α2δ-1 over-expressing mice could normalize mESPC

frequency back to the basal level observed in WT mice without affecting basal level of synapse expression [Submitted, in review]. Furthermore, intrathecal injection of TSP4 leads to an increase in mEPSC frequency with no change to amplitude while acute application of TSP4 to spinal cord slices has no effect on mEPSC frequency or amplitude [56], suggesting a chronic effect of TSP4 in synaptic modulation. Gabapentin is able to block the TSP4-mediated increase in mEPSC frequency [Submitted]. All together, these findings support that TSP4/α2δ-1 interactions activate a pathway that is critical in promoting excitatory synaptogenesis, which in turn can change synaptic excitability leading to central sensitization, and ultimately resulting in behavioral hypersensitivity.

My data provide important findings that activation of the TSP4/α2δ-1 signaling pathway is critical for the initiation, but less so for the maintenance of excitatory synaptogenesis. While pre-emptive gabapentin treatment can effectively block TSP4-induced excitatory synaptogenesis, delayed gabapentin treatment is without similar effect. This finding is similar to that reported for synaptogenesis in retinal ganglion cells *in vitro* and in lesioned mouse barrel cortex *in vivo* [50]. For pain research, this finding supports a new therapeutic action of gabapentin in which early application of the drug can prevent aberrant synaptogenesis that could contribute to chronic pain development. Thus, pre-emptive or early application of gabapentin to surgical or injury patients could prevent future chronic pain development [102, 103]. This could be different from the acute effects of gabapentin in pain relief, which occur within one hour in neuropathic pain models [14, 104, 105], most likely through blocking pre-synaptic neurotransmitter release [106, 107].

Mechanism of TSP4/α2δ-1 mediated synaptogenesis

The exact mechanism underlying TSP4/α2δ-1 signaling in synaptogenesis is not known. α2δ-1 is expressed both pre- and post-synaptically and can associate with all members of the VGCC family [43]. It is unknown whether pre-, post- or pre- and post-synaptic α2δ-1 is required for synaptogenesis. Similarly, TSP4 expressing cells are present in both the DRG and SC. The following questions are important to address the mechanisms of TSP4/α2δ-1 signaling in synaptogenesis: Does TSP4 produced in the peripheral nervous system act on DRG neuron soma

or axons to promote synaptogenesis? Does TSP4 produced in the central nervous system acts at the synaptic terminals in spinal cord? What downstream pathways are activated by the TSP4/α2δ-1 signaling pathway? I will address some of these questions in Chapter 3.

Chapter 3: Mechanisms of TSP4/α2δ-1 signaling mediated excitatory synaptogenesis

Introduction

As discussed previously, there are some limitations in the *in vivo* models for studying detail mechanisms of TSP4/α2δ-1 signaling mediated excitatory synaptogenesis. For example, it is not clear what is the synaptogenic domain of TSP4 in mediating synaptogenesis at the spinal cord level; whether injury-induced TSP4 acts on peripheral (DRG) or central (spinal cord) sites to promote synaptogenesis; whether pre-synaptic and/or post-synaptic α2δ-1 molecules are involved in TSP4-induced synaptogenesis; and what is the down-stream mediators involved in TSP4/α2δ-1 signaling mediated excitatory synaptogenesis. With studies presented in this chapter, I explored these cellular mechanisms related to TSP4/α2δ-1 signaling mediated excitatory synaptogenesis in co-culture systems of isolated DRG spinal cord neurons.

As for the down-stream mediator for this process, I hypothesized that α2δ-1's role in synaptogenesis could involve modulation of calcium currents. Although it has been previously shown that α2δ-1 mediates its synaptogenic effect independent of N-, L- and P/Q-type VGCC function [50], data from our collaboration with the lab of Dr. Quinn Hogan indicated that TSP4 treatment in isolated dorsal root ganglion (DRG) sensory neurons caused increased T-type VGCC currents and decreased high-voltage activated currents such as N-, L-, and P/Q-type currents [submitted]. This suggests that synaptogenesis resulting from activation of the TSP4/α2δ-1 signaling pathway is mediated by modulation of the T-type VGCC function.

T-type VGCCs are low-voltage activated, fast inactivating channels present in sensory neurons [108, 109]. It is currently unclear what is the role of T-type VGCCs in neuropathic pain as literature has evidences both for and against the involvement of T-Type VGCC. T-type VGCCs

Cav3.2 knockout mice show deficiencies in pain response and exhibit reduced hypersensitive behavior in inflammation and tissue injury models of pain but developed normal behavioral hypersensitivity after SNL [110]. Somewhat paradoxically, others have reported that blocking T-type channel function in nerve injury models can alleviate hypersensitivity [111, 112]. Systemic but not intrathecal injection of selective T-type VGCC antagonist Mibefradil can block SNL-induced mechanical and thermal hypersensitivity [111]. Similarly, another selective T-type blocker ethosuximide has been shown to alleviate both mechanical allodynia and thermal hyperalgesia in vincristine-induced and Chronic Constriction Injury of the sciatic nerve (CCI) models of neuropathic pain [112, 113]. However, while both ethosuximide and Mibefradil have high affinity to T-type VGCC, they also have affinity for L-type VGCC [114, 115]. More recently, T-type VGCCs have been shown to play a role in modulating superficial dorsal horn synapse plasticity through an unknown mechanism in the diabetic model of neuropathic pain [116-119]. In this chapter, I examined a possible action for T-type VGCC in synaptogenesis. Using an *in vitro* model of nerve injury induced synaptogenesis, I found that the interaction of TSP4 and α2δ-1 promotes synaptogenesis via a T-type VGCC current dependent mechanism.

Methods

Cell Culture

In order to study the TSP4/α2δ-1 interaction in more details and in a controlled environment, I designed a DRG/spinal cord primary neuron co-culture system. This co-culture system would allow me to study the interaction of TSP4 and α2δ-1 in a controlled environment by studying changes in synapse formation after controlled exogenous TSP4 treatments. Although a cell culture system does not wholly replicate the *in vivo* milieu, I believe that this system could mimic approximately SC/DRG neuron interactions and synapse formation in the SDH.

Protocol was derived from primarily a series of published protocols [120-123] along with

some method sections from several papers [65, 86, 124-126]. Spinal cord neurons were harvested from E14-E19 mice embryo by making a lengthwise incision along the ventral side of the spinal vertebrae and collecting the spinal cord. Following dissociation using 0.25% trypsin, the cells were plated onto 0.1 mg/mL poly-D-lysine and 0.04 mg/mL laminin coated glass coverslips. Cells were then grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% horse serum and 10% fetal bovine serum. After 24 hours, the media was replaced with Neurobasal media supplemented with B27 supplement (NB/B27). After 3 days, 100nM uridine and 20nM 5-fluorodeoxyuridine are added to inhibit non-neuronal cell proliferation.

One potential caveat of using laminin in culture is that laminin also contains an EGF-like domain. However DRG culture in the absence of laminin results in neurons with only a few short neurites. In preliminary studies, DRG and SC neurons cultured in laminin-coated wells did not produce significant number of synapses. In contrast, treatment with TSP4 led to a marked increase in synapse count. Therefore the EGF-like domain of laminin is either structurally different enough from TSP4 or laminin is not present in sufficiently high concentrations (coating concentration of 48nM) to have a significant effect. Furthermore, I performed all my experiments in laminin-coated wells, which should minimize any changes due to contributions from laminin in TSP4 treatment data.

Adult DRG neurons were harvested by first removing the spinal cord via hydraulic extrusion. The vertebral column was then dissected out from the T7-8 vertebrae segment to around the L4/L5 segment. A laminectomy was performed to remove the dorsal half of the vertebrae exposing the spinal cavity. The T9 to the L5 DRG were then collected and dissociated in 1.25 mg/mL collagenase. Cells were plated onto poly-D-lysine and laminin coated glass coverslips and grown in NB/B27 mediaand 100nM uridine/20nM 5-fluorodeoxyuridine (U/FrdU).

In SC/DRG co-cultures, SC neurons were first allowed to grow for 3 days to mature and sprout neurites. In my experience, embryonic SC neurons take a few days after plating to sprout neurites while adult DRG neurons would be able to sprout neurites overnight. DRG neurons were

added to the SC neuron cultures at the time of SC culture media change at Day 3. From Day 3 onwards, neurons were grown in NB/B27 media and U/FrdU. U/FrdU was required to control the proliferation of non-neuronal cells in both DRG and SC cultures. Uncontrolled proliferation of non-neuronal cells could overwhelm the culture and reduce neuronal survival rates. In addition, astrocytes have been found to secrete other synaptogenic proteins including Thrombospondin-1 [50, 55, 94], so controlling the proliferation of astrocytes is important in isolating the function of TSP4. However, U/FrdU also affects the proliferation and survival of embryonic SC neurons. By day 3 in culture, embryotic SC neurons are mature enough that many survive after U/FrdU anti-mitotic treatment. Using a GFAP staining, I was able to confirm that astrocytes are greatly depleted in culture after U/FrdU treatment.

Campenot Chamber Cultures

Campenot chambers (Tyler Research, Edmonton) were set up as described by Pazyra-Murphy & Segal [127]. Briefly, 35mm cell culture dish were coated with 1 mg/mL collagen. Grooves were scored on the dish surface using insect pins at approximately 200µm intervals. Grooves were then wetted with a drop of NB/B27 + 0.6 mg/mL methylcellulose solution. Campenot chamber bottoms were then coated with autoclaved silicone grease and mounted onto the culture dish. Media was added into the middle chamber and the chamber was stored overnight in the incubator to make sure the silicone grease created an adequate seal and that there were no leaks. In practice, the methylcellulose/silicone layer created a seal that allows axons to grow through the medium while minimizing media diffusion from the middle chamber to outer chambers. The grooves provided a physical barrier preventing axons from looping and growing back towards the center chamber. DRG neurons were then plated into the middle chamber and allowed to grow for 7 days so that their axons could cross to the outer chambers. After around 7 days, when DRG axons were almost completely crossing under the divider from the middle chamber and to the outer chambers. SC neurons were then plated into the two outer chambers. Chambers are ready to use when sufficient number of DRG axons fully cross the divider between chambers to the SC neuron

(outer) side.

Adeno-associated Virus Treatment

To chemically knock down α2δ-1 from SC neurons, I used Adeno-Associated Virus (AAV) vectors encoding small hairpin RNA (shRNA) against the α2δ-1 mRNA (Gift from Dr. Perez-Reyes) to induce α2δ-1 knocking down in cultures. To determine the optimal dose for *in vitro* studies, I did a dose-dependent AAV treatment in SC neuron cultures with 1.5μL, 3μL and 6μL AAV per well. This dose-range was chosen based on Dr. Perez-Reyes' suggestion that 3μL of the AAV stock (rAAV8/SYNI-CSRHAD1 (AAV-α2δ-1), 2x10e12 viral particles/mL) per well could infect 100% of hippocampal neurons in culture. As a control, I used an AAV-CTL vector with the shRNA sequence but no promoter (rAAV5/CSRHFS (AAV-CTL), 6x10e12 viral particles/mL). I omitted DRG neurons from preliminary AAV experiments because the AAV-shRNA was only going to be used to knock down α2δ-1 in SC neurons. To compensate for the lack of DRG axons, the SC neurons were cultured at a higher density of 500k cells per well so that SC-SC neuron connections could be made.

For differential SC/DRG α2δ-1 knockout experiments, I knocked out α2δ-1 from SC neurons by treating the SC neuron culture with 3μL AAV-α2δ-1 for 10 days, and from DRG neurons by isolating DRG neurons from the Adv/α2δ-1 CKO/RFP mice. SC neurons were allowed to mature for 4 days before they were treated with AAV-α2δ-1 for 10 days, followed by adding DRG neurons from Adv/α2δ-1 CKO/RFP or WT mice for 2 more days. TSP4 was then added to cultures for a final concentration of 20nM per well for 4 days, equivalent volume of PBS was used for control treatment. The cells were harvested and stained for VGlut2/PSD95/MAP2 immunoreactivities to identify functional synapses.

Cell Culture Immunohistochemistry

Cultured neurons were fixed in -20^{o}C methanol for 15 minutes. They were then incubated for

24 hours with primary antibodies diluted in Dako antibody diluent (Dako): chicken Microtubule-Associated Protein 2 (MAP2) (Abcam), mouse PSD95 (Pierce), guinea pig VGlut2 (Synaptic Systems). The fixed cells were then incubated for overnight in corresponding Alexafluor 488, 594 and 647 secondary antibodies. Cells were mounted onto glass slides using Vectashield DAPI hardmount media (Vector Labs). Images were taken from the appropriate areas using a 63X objective on the LSM700 confocal microscope (UCI Optical Biology Core) and analyzed using Volocity 6.0. For Campenot chamber image acquisition, images were taken from the appropriate areas using a 40X objective on the LSM700 confocal microscope.

Data Analysis

In order to identify synapses from interactions between DRG and spinal cord neurons, a three color immunocytochemistry (ICC) staining was performed to identify the synapse type of interest and distinguish DRG to SC synapses from SC to SC synapses. Since both DRG and SC neurons have axons, I distinguished DRG neuron axons from SC neuron axons by using red fluorescent protein-labeled (RFP) DRG neurons from transgenic Advillin-Cre/Rosa-tdTomato mice (Adv/RFP), a mouse line expressing RFP driven by Cre recombinase expression under the control of a promoter from the Advillin gene, which is present in about 90% of DRG neurons [98, 99]. By looking at RFP expression, I could trace DRG axons. I then used the dendritic marker MAP2 as a marker for spinal cord neurons because DRG neurons are pseudo-unipolar and do not have dendrites. Functional synapses are identified by co-localizing PSD95 and VGlut2 immunoreactivity. Since tdTomato RFP are not expressed in VGlut2 positive pre-synaptic vesicles, I could visualize VGlut2 expression using the same spectral channel as that for tdtomato proteins. I also plated SC neurons at a low density (250k cells/mL) and DRG neurons at a high density (10k cells/mL) so that for a given SC neuron, most of their inputs are from DRG axons. This method of analysis allowed me to isolate and mainly count excitatory (VGlut2 positive) synapses formed between DRG axons and SC dendrites as only these synapses would have overlapping PSD95, VGlut2, and MAP2 immunoreactivity along RFP-labeled DRG axons. To account for the heterogeneity of SC

neuron morphology, I normalized the synapse count from a given SC neuron with the total MAP2 surface area of that neuron. This would help account for any neuron size-differences in synapse counts since larger neurons or neurons with more dendrites have more surface area available for synapse formation.

Collected data were analyzed for statistical significance using one-way ANOVA with a Dunnett's post-hoc test in Prism 5.0.

Results

TSP4/α2δ-1 interaction increases synapse formation in DRG/Spinal Cord neuron co-cultures

I first tried to replicate my *in vivo* findings using the *in vitro* culture systems. I applied different concentrations of recombinant TSP4 proteins, as reported previously *in vivo* [50], to the culture system and assessed synapse formation 4 days after treatment, which correlated with the peak pro-nociceptive and synaptogenic effects of TSP4 in vivo.

I found that TSP4 treatment in our culture system could dose dependently increase the number of $VGlut2^{+}/PSD95^{+}$ synapses associated with spinal cord neurons (Figure 3.1 A, B, C). Treatments of 8nM and 20nM, but not 2nM, TSP4 were able to increase the number of $VGlut2^{+}$ synapses by roughly one-fold ($F(5, 119) = 7.38$, $P = 0.0003$). These concentrations were similar to that reported to promote robust synaptogenesis *in vitro* [50, 55]. In those papers, TSP4 treatment in retinal ganglion cell cultures was reported to increase synapse formation by two to three-fold. This difference may be attributed to the difference in culture systems as retinal ganglion neurons are functionally different from SC or DRG neurons. Another explanation could be a difference in cell density. A retinal ganglion neuron culture contains a relatively homogenous population of neurons so the neuron densities are higher than that of heterogeneous neuron populations. Higher

cell density would increase axon and dendrite densities and synapses formed.

TSP4 treatment-induced synapse increase could be blocked by a single treatment of 100nM gabapentin (Figure 3.1 C), which should be stable in aqueous solution since the main method of gabapentin clearance in the body is through renal secretion [26, 92]. Thus, the concentration of gabapentin over the 4 day treatment duration should remain constant. The sensitivity to gabapentin blockade in TSP4 treated cultures suggests that TSP4 induced synaptogenesis is likely mediated through α2δ-1. TSP4 treatment also increased PSD95 expression by around 70% in spinal cord neuron dendrites ($F(5, 119) = 4.52$, $P = .0009$), which could be blocked by gabapentin (Figure 3.1 D). VGlut2 quantification was not performed because RFP proteins shared the same spectral channel as the VGlut2 immunoreactivity. The reason for the observed 70% increase in PSD95 remains unclear and it can result from clustering of post-synapse densities in response to pre-synaptic input, activity dependent translocation of PSD95 to the post-synapse densities, or increased *de novo* synthesis of PSD95 [128-130], which can be determined by examining PSD95 mRNA and protein levels after TSP4 treatment. Nevertheless, these data support that the co-culture system is suitable to model the TSP4 effects *in vivo* because TSP4 can induce synaptogenic effects *in vitro* similar to that observed *in vivo*. Since gabapentin can block TSP4's synaptogenic effects both *in vivo* and *in vitro,* it suggests that both model systems share the same mechanism of TSP4-induced synaptogenesis.

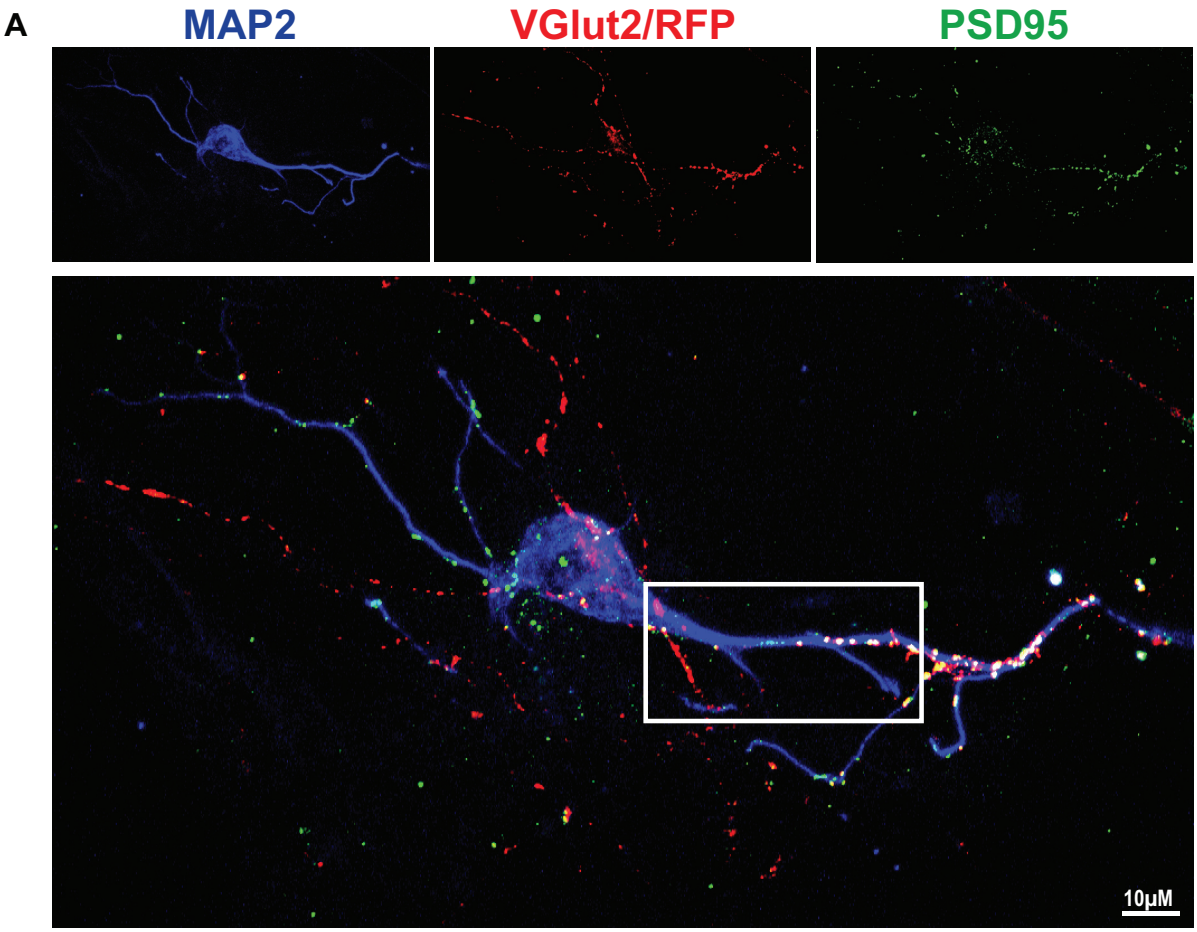

Figure 3.1: A. Representative image of an analyzed spinal cord neuron in co-culture stained for VGlut2 (Red), PSD95 (Green) and Map2 (Blue). White box denote area taken for close up image to better show synapse change.

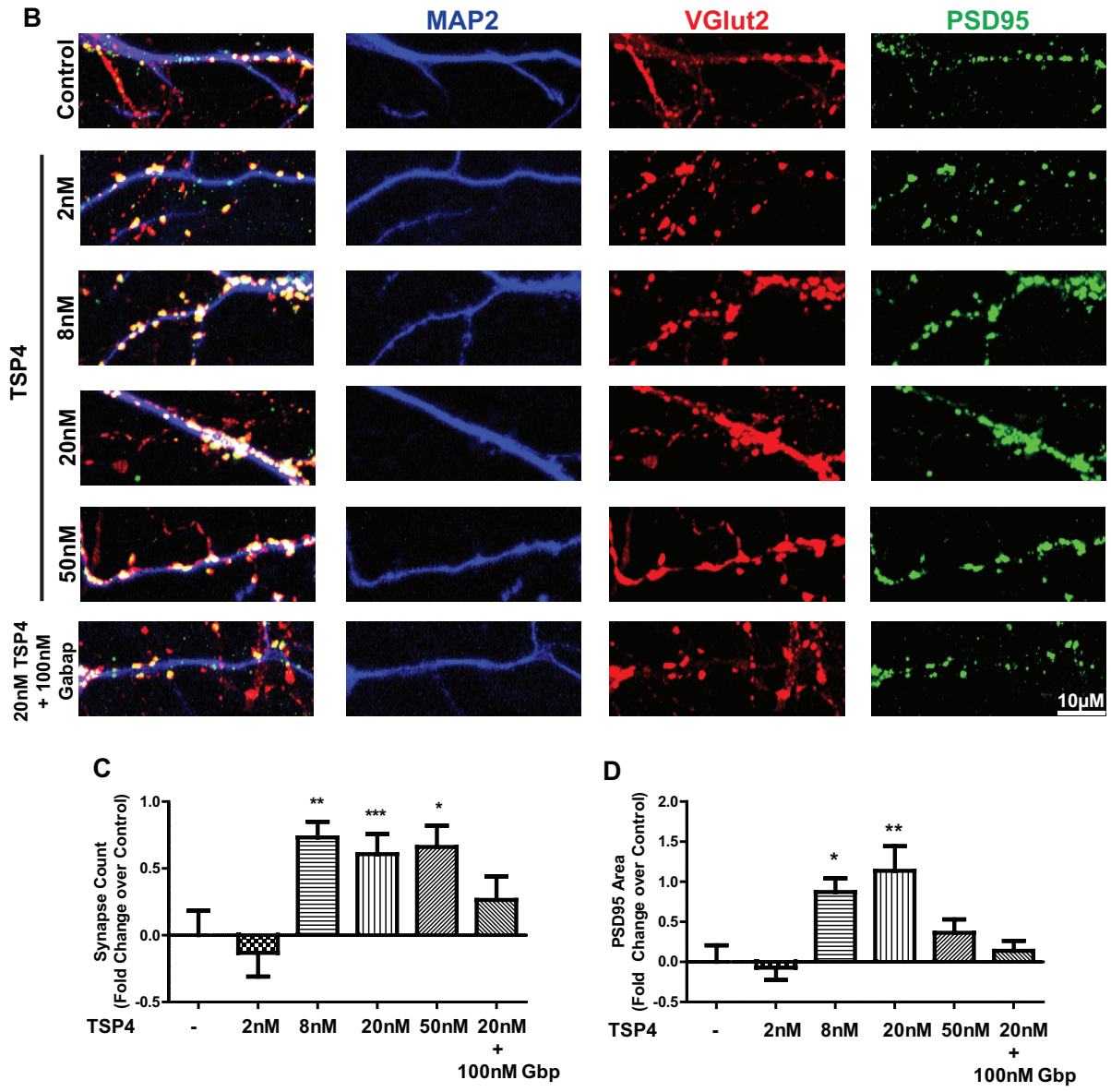

Figure 3.1 Continued: B. Close up image of segment of spinal cord neuron dendrite and associated synapses at 0, 2, 8, 20, 50nM TSP4 treatment for 4 days. **C.** Quantification of synaptic connections made on individual spinal cord neurons showed that the 8nM, 20nM, 50nM concentrations of TSP4 can strongly induce synaptogenesis ($F(5,119) = 7.378$, $P < .001$). TSP4 induced synaptogenesis could be blocked by 5-fold concentration treatment of gabapentin (GBP). **D.** Similarly, 8 and 20nM concentrations of TSP4 could also significantly increase PSD95 ($F(5,119) = 6.254$, $P < .0001$). (n = 20 cells analyzed per treatment group)

EGF-Like Region of TSP4 is responsible for TSP4/α2δ-1 interaction mediated synaptogenesis

I next examined what was the synaptogenic domain of TSP4 in DRG/spinal cord co-cultures. It has been previously reported in retinal ganglion cell cultures that the EGF-like domain of TSP1 and 2 is responsible for synaptogenesis [50]. Specifically, it has been shown that the EGF-like region interacts with the von Willebrand Factor A domain of α2δ-1. Pertaining to pain models, our lab has shown that injection of only the EGF-like domain, without any other domains, of TSP4 is sufficient to cause mechanical allodynia and thermal hyperalgesia at a level similar to that seen post SNL or full-length TSP4 injection [manuscript in preparation]. All these data provided the rationale supporting my study.

To determine the TSP4 domain important for mediating spinal synaptogenesis, I treated DRG/SC neuron co-cultures with 20nM each of different TSP4 mutant proteins, encoded by the constructs described below, for 4 days before fixing and staining the slides. The constructs were made by Dr. John Park, expressed in a mammalian cell line and purified using a poly-Histidine tag using a Sephadex nickel column. The TSP4 constructs included the following. The "NT+CC" construct contains the N-terminal region and the coiled-coil domain of TSP4. These domains have been found to play an important role in the assembly of TSP4 into its pentameric form [53]. The "EGF" construct contains the EGF-like repeat domain of TSP4. The "Type3" construct contains the calcium binding Type3 and L-type lectin-like domains of TSP4. These domains have been shown to play an important role in the proper folding of the protein [53]. The "NT(-)" construct contains all but the N-terminal and coiled-coiled domains (Figure 3.2 B). The 20nM concentration was used since full-length TSP4 was able to produce a significant increase in synapses at this concentration in my dose-dependent experiments.

Similar to what was reported for TSP1 and 2 [50], the EGF-like domain of TSP4 also mediates synaptogenesis. Both the EGF and the NT(-) constructs (with EGF-like domain) were able to increase synapse count significantly by one-fold over control (Dunnett's post hoc test (F(5,

119) = 15.73, P < .0001 compared to the untreated control group; or Bonferroni's post hoc test (F(5, 119) = 15.73, P = .0278 comparing all groups to each other) (Figure 3.2 A, C). This increase is similar to that seen from treatment with equal molar concentration of TSP4. In contrast, the NT+CC and the Type3 constructs (lacking the EGF-like domain) did not significantly alter synapse count. Taken together, these results support that the EGF-like domain is critical for TSP4's synaptogenic effects since constructs lacking the EGF-like domains failed to significantly increase synapse count. These data support that the EGF-like domain of TSP4 is critical in promoting excitatory synaptogenesis in DRG/SC neuron co-cultures, most likely through the proposed interaction with the Von Willebrand Factor A domain of α2δ-1 [50].

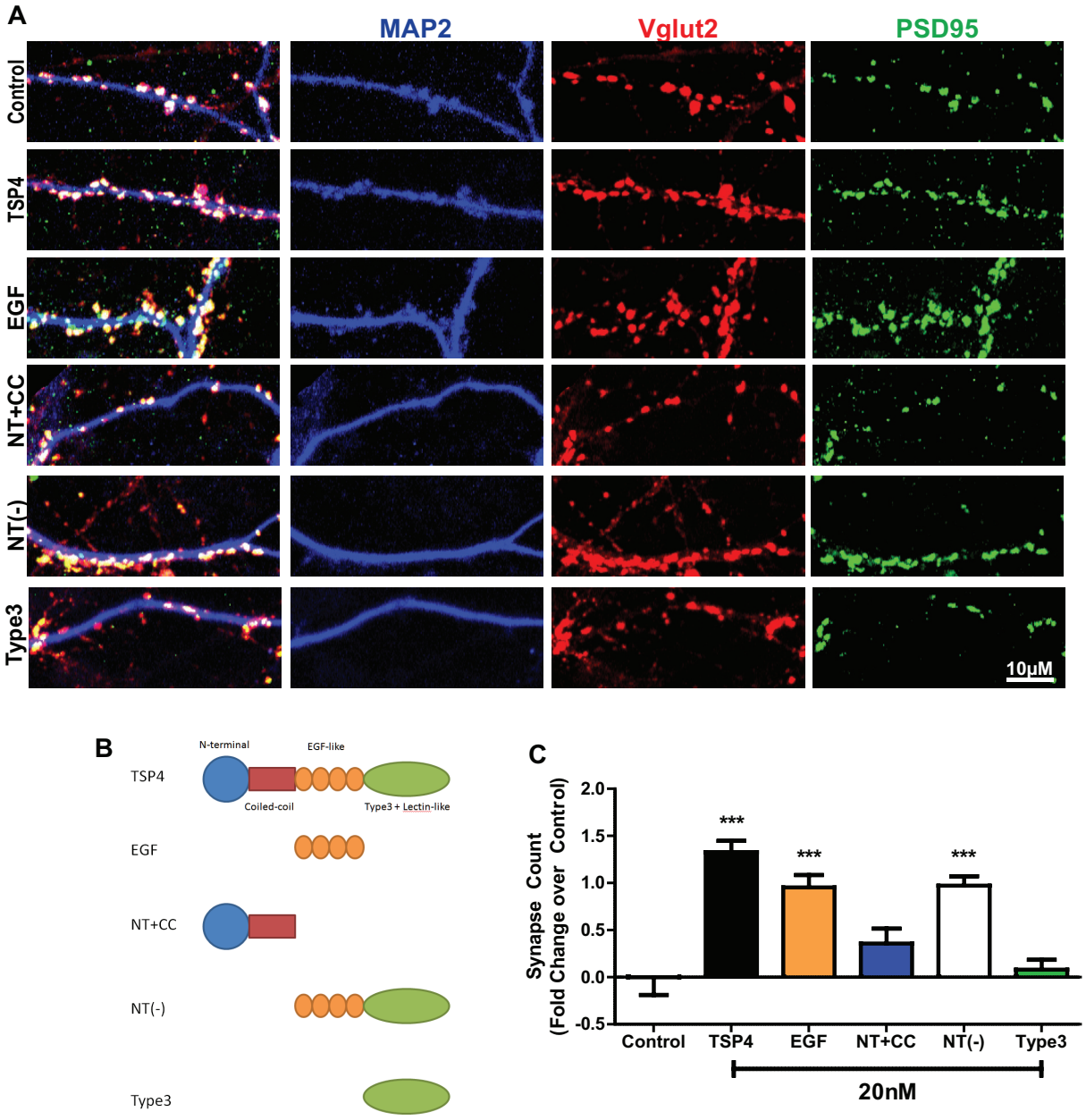

Figure 3.2: A. Close up image of spinal cord neuron dendrite and associated synapses after TSP4 construct treatment for 4 days. **B.** Diagram showing the TSP4 domains contained within each construct. **C.** Quantification of synaptic connections made on individual spinal cord neurons showed that only constructs containing the EGF-like domain (EGF, NT(-)) could significantly increase synapse count ($F(5,119) = 15.73$, $P < .0001$). (n = 20 cells analyzed per treatment group)

Synaptogenic action of TSP4 is localized in spinal cord but not DRG

The exact site of TSP4's synaptogenic action is unknown. Intrathecal TSP4 injection can affect both the spinal cord and the DRG [131]. TSP4 is mainly upregulated in the DRG [59] and in the white matter of the dorsal spinal cord after nerve injury [56]. To determine if TSP4 exerts its synaptogenic activity in SC or DRG, I use a Campenot chamber setup where DRG neurons are cultured in the middle chamber but their axons can grow through a methycellulose barrier to reach the outer chambers containing SC neurons. The methylcellulose barrier creates a fluidic seal that minimizes any diffusion of media (Figure 3.3 A, B). This roughly mimics the *in vivo* environment where DRG neurons are anatomically separate from SC neurons but send long axons into the spinal cord to form synapses there. The location of TSP4's action can be determined by selectively applying TSP4 either to the middle chamber containing DRG neuron soma and axons or to the outer chambers containing spinal cord neurons and DRG neuron axon terminals. If TSP4 applied to the middle chamber increases synaptogenesis in the outer chambers, the mode of action of TSP4 would be on the DRG soma or along the primary afferents. This would suggest that TSP4 may affect axonal transport, the conductive properties of the axons, or protein transcription at the cell soma, which may indirectly affect synapse formation in the outer chambers. On the other hand, if applying TSP4 to the outer chambers increases synaptogenesis, it would suggest that TSP4 exerts its synaptogenic action either on spinal cord neurons or at synaptic terminals. These results would translate into whether TSP4 works at the DRG or primary afferent level or at the spinal dorsal horn level to induce synaptogenesis *in vivo*.

Using this setup, I found that adding 20nM TSP4 to the outer chambers, but not to the middle chamber, could significantly increase synapse formation ($F(3, 59) = 15.34$, $P < .0001$) (Figure 3.3 C, D). This suggests that TSP4 in the spinal dorsal horn, but not in DRG, is required for synaptogenesis.

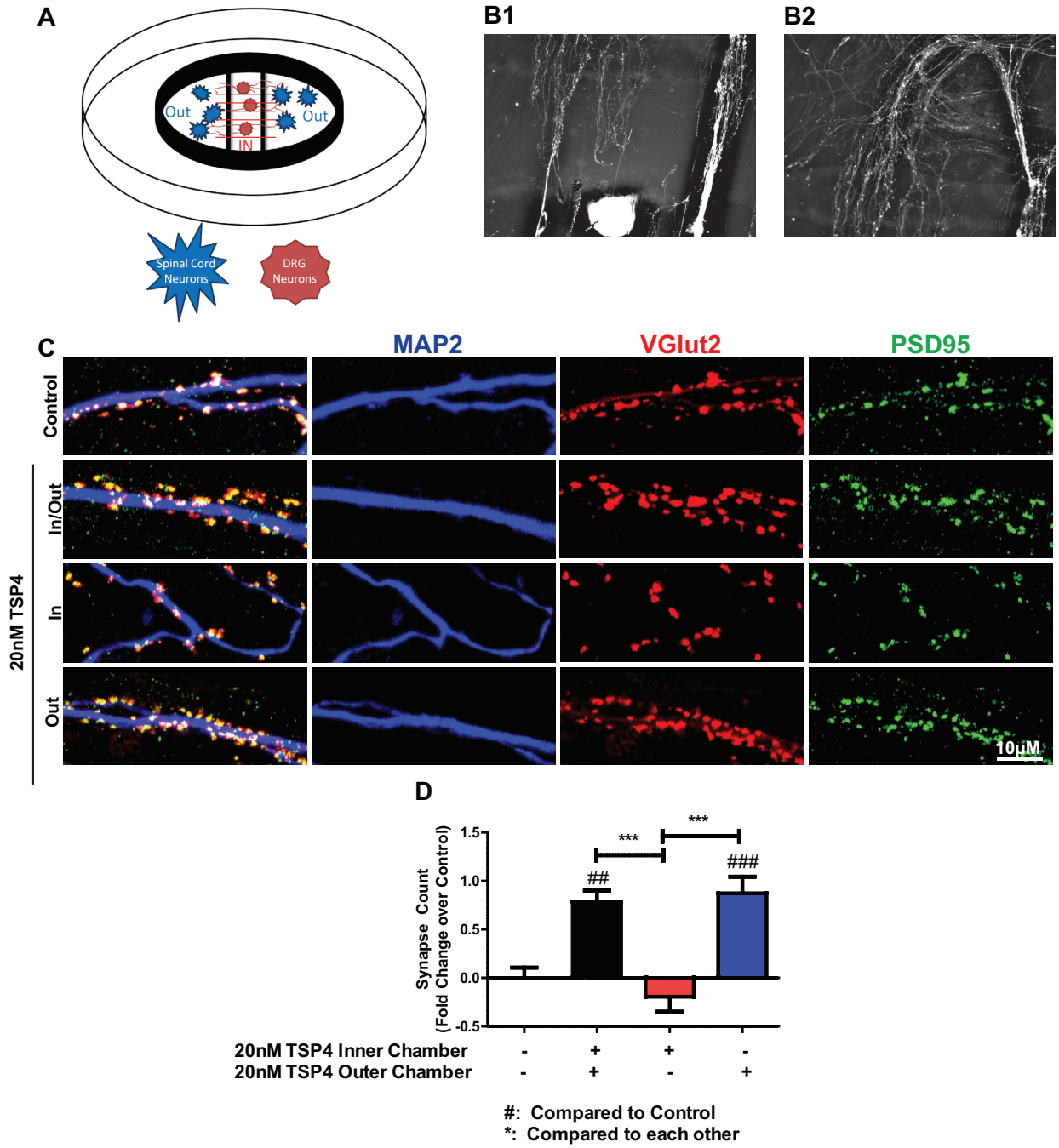

Figure 3.3: A. Diagram of Campenot chamber setup where spinal cord neurons were cultured in the inner chamber and DRG neurons in the other chambers. DRG neuron axons but not soma would cross the inner chamber wall **(B1)** into the outer chambers **(B2)**. **C.** Close up image of spinal cord neuron dendrite and associated synapses after TSP4 treatment for 4 days. **D.** Application of TSP4 into the outer chamber could induce synaptogenesis while application into the inner chamber had no effect ($F(5,59) = 15.34$, $P < .0001$). (n = 15 cells analyzed per group)

TSP4 interacts with pre-synaptic but not post-synaptic α2δ-1 to promote synaptogenesis

While literature suggests that TSP4 interacts with α2δ-1 to induce synaptogenesis in the brain [50], it is not clear if that is true in TSP4-induced synaptogenesis in spinal cord, and if so, whether TSP4/a2d1 interactions occur at the post-synaptic or pre-synaptic level. I wanted to verify this in my culture system by asking the following question: if TSP4 can dose-dependently increase synapse count, would knocking out its putative receptor α2δ-1 post-synaptically or pre-synaptically block its synaptogenic properties? To address this question, I used an AAV vector to deliver a short hairpin RNA (shRNA) against α2δ-1 mRNA (UNC Gene Therapy Center, gift from Dr. E. Perez-Reyes, University of Virginia School of Medicine) to knock down α2δ-1 expression from spinal cord neurons (post-synaptic knockdown). The AAV vector has high infection rate to all the neurons. Once in the cell, the shRNA is transcribed and silences α2δ-1 expression by binding to α2δ-1 mRNA, recruiting the RNA-induced silencing complex and ultimately leading to α2δ-1 mRNA degradation by the endoribonuclease Dicer [132]. This method has been successfully employed to knockout α2δ-1 both in vivo [Submitted, in revision] and in vitro (Communications with collaborator).

My data indicated that 20nM TSP4 was able to increase synapse count by 70% over control as measured by co-localization of VGlut2/PSD95/MAP2 immunoreactivity denoting functional synapses ($F(6, 69) = 4.483$, $P = .0008$). This increase could be ablated by treatment with all doses of AAV-α2δ-1 tested (Figure 3.4 A, B). The lack of dose-dependent curve of AAV-α2δ-1 treatment is likely due to the fact that shRNA production is continuous so it reaches the maximal effectiveness at the end of the 10 day treatment period. The effects of AAV-α2δ-1 were α2δ-1 specific since AAV-CTL vector treatment (3μL/well) could not reverse the synaptogenic effect of TSP4. At the highest dose (6μL/well), cytotoxicity was observed at the end of 10-day treatment period reflected as non-specific loss of synapses in both AAV-α2δ-1 and AAV-CTL groups. Thus, for future experiments, I chose the 3μL/well dose (6.6x10e11 viral particles/mL) as the 1.5μL/well dose did not reach a full infection rate while the 6μL/well dose was cytotoxic.

Having established a working AAV-α2δ-1 dose, I next determined the time course of treatment. Specifically, I needed to know when α2δ-1 silencing occurs after bolus AAV treatment and how long the effective duration lasts. This information is also critical in determining when AAV-α2δ-1 must be removed from SC neuron cultures before adding DRG neurons. Accordingly, I treated SC neuron cultures with 3μL/well AAV-α2δ-1 and replacing the media with fresh media after 6 or 10 days, waited for two days and then treated the cultures at day 12 after the bolus AAV-α2δ-1 treatment with 20nM TSP4 for 4 days (Figure 3.4 C). This would allow me to determine whether AAV-α2δ-1 could achieve α2δ-1 knock down after 6 days of treatment and maintain the effective knockdown for 10 days, so that I could have a 6-day window for plating DRG neurons, which requires 2-days for maturation, and start the treatment with TSP4 for 4 days before analysis of synaptogenesis.

My data indicated that both the 6 day and the 10 day AAV treatments resulted in similar blockade of synaptogenesis after 20nM TSP4 treatment compared with control ($F(5, 119) = 5.034$, $P = .0003$) (Figure 3.4 D, E). This supports that AAV-α2δ-1 silencing begins as soon as 6 days after treatment initiation and lasts for at least 10 days. In addition, no toxic effect for AAV at this dose was observed for up to 16 days in culture. These findings suggest that the AAV-shRNA is an excellent tool to study the role of α2δ-1 *in vitro*.

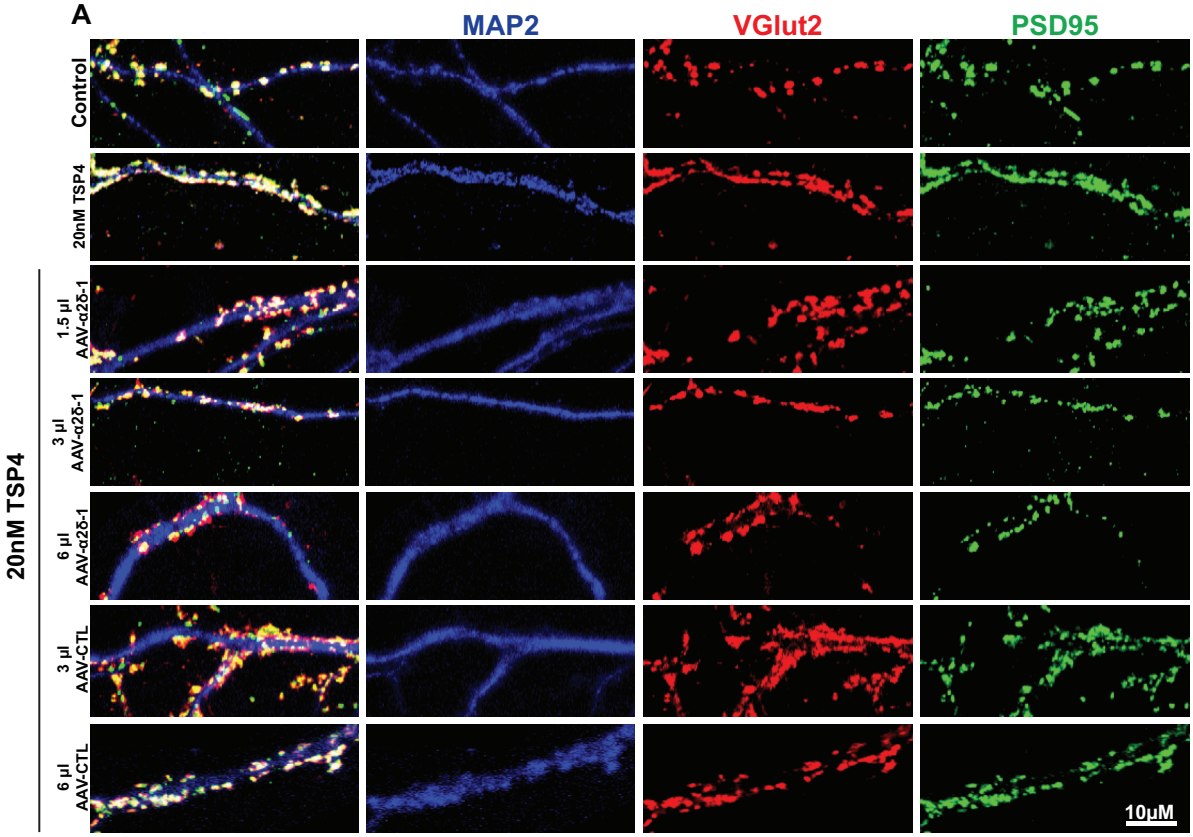

Figure 3.4: A. Close up image of spinal cord neuron dendrite and associated synapses after 10 day AAV pre-treatment followed by 4 day TSP4 treatment. **B.** Quantification of synapse count showed that all doses of AAV-α2δ-1 were able to reverse TSP4 induced synaptogenesis. However at 6µL AAV, cytotoxicity was observed in culture as synapse count was decreased even in the AAV-CTL treated group. ($F(6,69) = 4.483$, $P = .008$). ($n = 10$ cells analyzed per group)

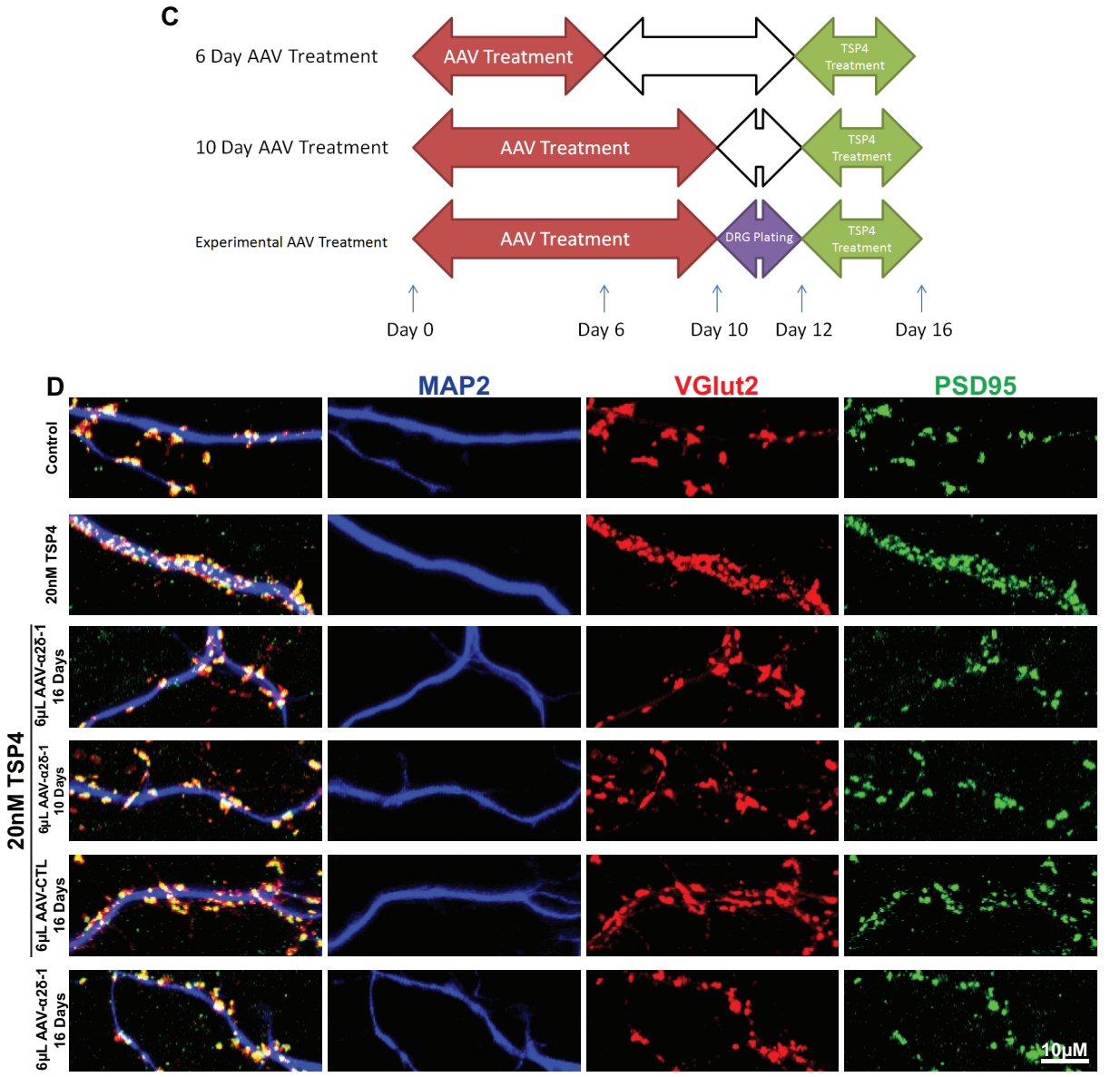

Figure 3.4 Continued: C. Diagram showing time course of AAV treatment. **D.** Close up image of spinal cord neuron dendrite and associated synapses after time-dependent AAV-α2δ-1 treatment. **E.** Quantification of synapse count showed that AAV-α2δ-1 treatment for 6 days could block TSP4 induced synaptogenesis for at least 10 days. ($F(5,119) = 5.034$, $P = .0003$). (n = 20 cells analyzed per group)

My data indicated that both the 6 day and the 10 day AAV treatments resulted in similar blockade of synaptogenesis after 20nM TSP4 treatment compared with control ($F(5, 119) = 5.034$, $P = .0003$) (Figure 3.4 D, E). This supports that AAV-α2δ-1 silencing begins as soon as 6 days after treatment initiation and lasts for at least 10 days. In addition, no toxic effect for AAV at this dose was observed for up to 16 days in culture. These findings suggest that the AAV-shRNA is an excellent tool to study the role of α2δ-1 *in vitro*.

Using this system, I addressed the question if TSP4 interacts with α2δ-1 in SC neurons or in pre-synaptic terminals to promote synaptogenesis by differentially knocked out α2δ-1 from SC or DRG neurons before treating them with TSP4 for analyses of excitatory synaptogenesis. My data indicated that only pre-synaptic α2δ-1 was required for TSP4 induced synaptogenesis. Synapse count increased significantly when α2δ-1 expression was intact in the DRG: around 50% over control after TSP4 treatment ($P = .011$) in the SC (WT)/DRG (WT) group and around 35% over control ($P = .05$) in the SC (KO)/DRG (WT) group. In contrast, knocking out α2δ-1 expression in DRG neurons led to no increase in synapse count after TSP4 treatment (Figure 3.5 A, B, C).

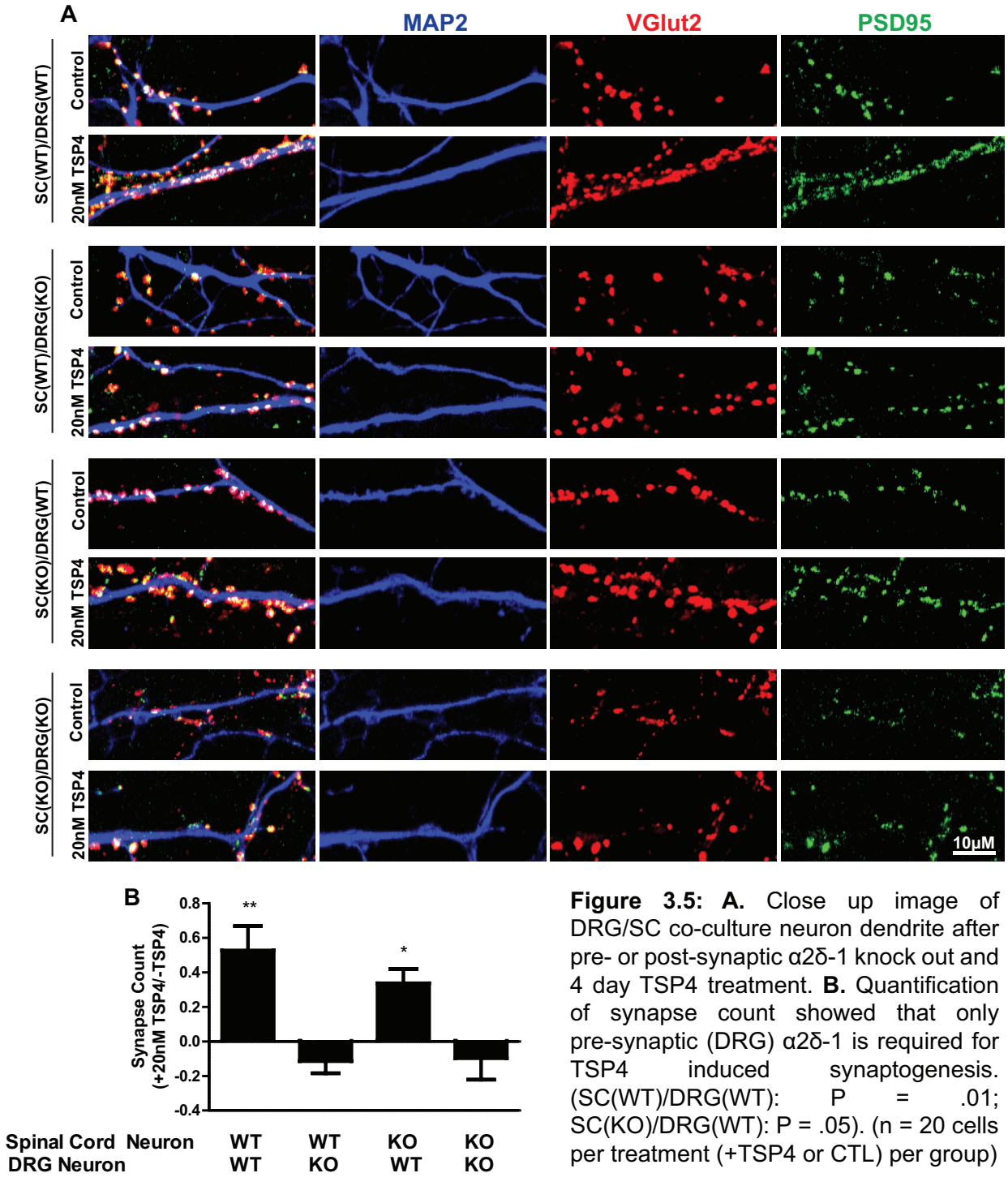

Figure 3.5: A. Close up image of DRG/SC co-culture neuron dendrite after pre- or post-synaptic α2δ-1 knock out and 4 day TSP4 treatment. **B.** Quantification of synapse count showed that only pre-synaptic (DRG) α2δ-1 is required for TSP4 induced synaptogenesis. (SC(WT)/DRG(WT): P = .01; SC(KO)/DRG(WT): P = .05). (n = 20 cells per treatment (+TSP4 or CTL) per group)

TSP4/α2δ-1 interaction mediated synapse formation is T-type VGCC dependent

I next looked at potential mechanisms underlying the role of TSP4/α2δ-1 signaling in synaptogenesis. Since α2δ-1 is an accessory subunit of VGCCs [43], I checked to see whether blocking VGCC ion channel function using VGCC subtype specific blockers could block TSP4/α2δ-1 induced synaptogenesis. Although effects of VGCC channel blockers in TSP4's synaptogenic action has already been reported in retinal ganglion cells [50], it has not been tested in synapse formation between DRG and spinal cord neurons. In addition, the effects of T-Type VGCC blockers have not yet been tested. T-Type VGCC is of interest because it is not only highly expressed in primary afferents [133, 134] but also important in injury induced hypersensitivity [110, 111, 116, 135]. I assessed for synapse formation after treating the co-cultures with 20nM TSP4 and each of the following VGCC blockers: 0.5μM Nifedipine (L-type), 0.2μM ω-conotoxin GVIA (N-type), 0.2μM ω-conotoxin MVIIC (P/Q-type) to block HVA VGCC, and 0.5μM TTA-P2 (T-type) VGCC to block LVA VGCC. These concentrations were found to be sufficient in blocking channel conductance form each channel type in isolated DRG neurons by our collaborator, Dr. Quinn Hogan's lab. All four blockers were stable in aqueous solution so the drugs were left in culture for 4 days.

My data indicated that only TTA-P2 could significantly reduce synapse formation to the control level while other blockers failed to block TSP4 induced synapse formation ($F(5,119) = 15.97$, $P < .0001$) (Figure 3.6 A, B). This suggests that TSP4/α2δ-1 signaling promotes synapse formation through a T-type VGCC dependent mechanism. This further supports the possibility that α2δ-1, TSP4 and T-Type VGCC may interact to play a role in synaptogenesis as blocking T-Type VGCC yields similar result as blocking α2δ-1 in TSP4/α2δ-1 induced synapse formation. However, we cannot exclude the possibility that T-Type VGCC act on a different site along the TSP4/α2δ-1 signaling pathway, or on an independent pathway.

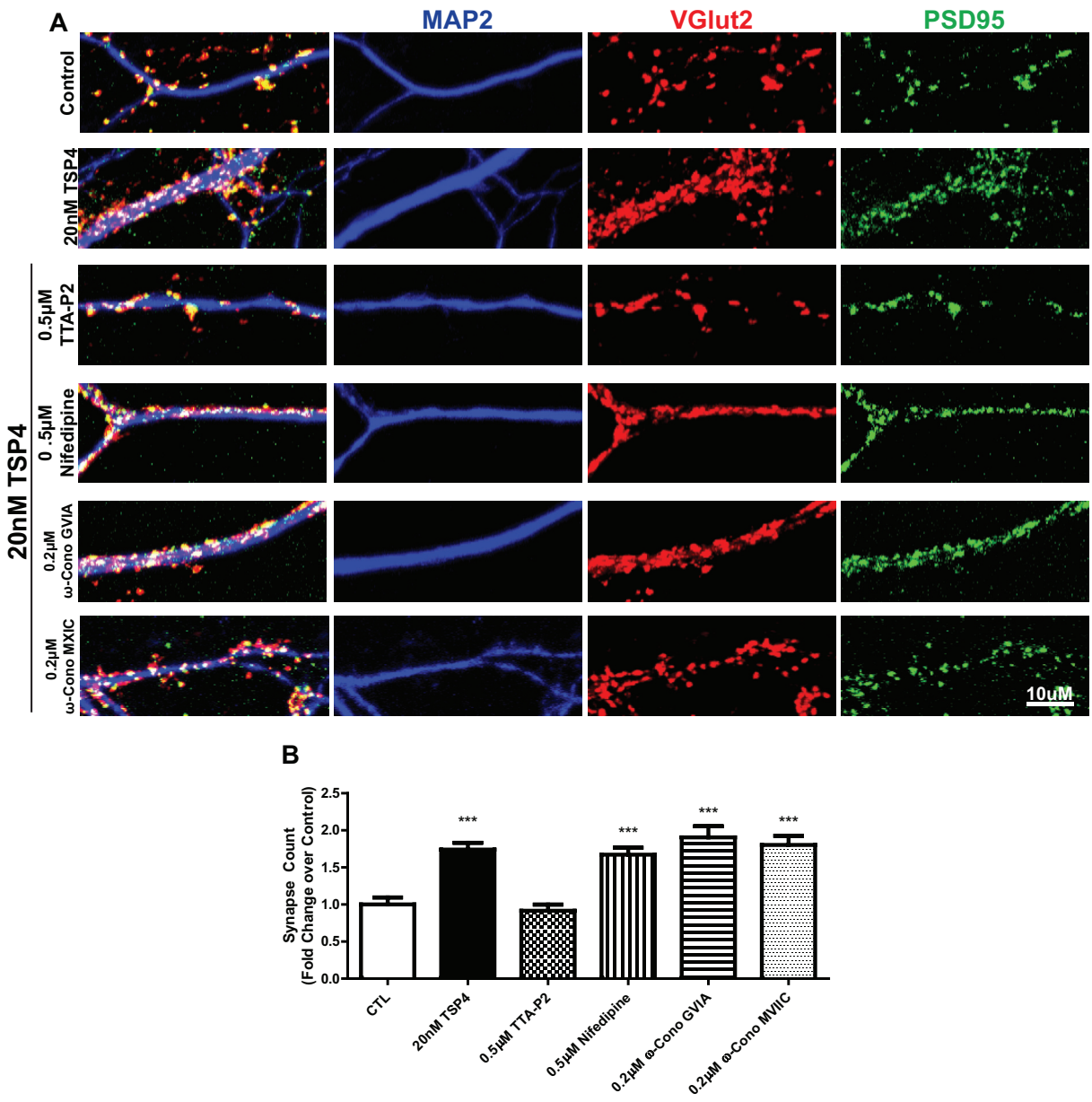

Figure 3.6: A. Close up image of spinal cord neuron dendrite and associated synapses after co-treatment of TSP4 and VGCC blocker for 4 days. **B.** T-type blocker TTA-P2 but not any other VGCC blockers was able to block TSP4 induced synaptogenesis. ($F(5,119) = 15.97$, $P < .0001$). (n = 20 cells analyzed per treatment group)

Discussion

My data support that nerve injury-induced increases of TSP4 in the dorsal horn [56] and α2δ-1 in sensory neuron pre-synaptic terminals [8, 14] interact to promote excitatory synapse formation through a T-type VGCC dependent mechanism, which may cause dorsal horn neuron hyper-excitability and ultimately underlie the behavioral hypersensitivity. In addition, the action site for the TSP4 synaptogenic effects is likely at the spinal cord level where increased TSP4 is likely to interact with increased α2δ-1 at the pre-synaptic terminals of sensory fibers, a favorable environment generated in dorsal spinal cord post peripheral nerve injury. Since gabapentin is only effective in blocking synaptogenesis when it was administered at the same time as TSP4, but not 2-days later, it is likely that TSP4/α2δ-1 signaling plays a critical role in the formation but not in the maintenance of synapses.

Significance of EGF-like domain

TSP4 can be cleaved by extracellular proteases such as A Disintegrin And Metalloprotease with Thrombospondin Motifs (ADAMTS) and thrombin. While TSP4 is a pentamer consisting of 100 kDA subunits, the EGF-like region is only around 23 kDA. The reduced size may facilitate diffusion from the dorsal horn white matter into the dorsal horn gray matter as well as facilitate diffusion into the synaptic cleft. I have shown here that the EGF-like domain alone can promote synaptogenesis to a level similar to that promoted by full length TSP4. This supports the possibility that a TSP4 fragment containing the EGF-like region, rather than full length TSP4, interacts with α2δ-1 to promote synaptogenesis. Interestingly, both ADAMTS and thrombin have been implicated in neuropathic pain although literature is limited on the subject, and none have been published in the SNL model. Thrombin is increased after nerve injury and has been proposed to play a role in modulation of inflammatory cytokines such as Il-1β and TNFα [136, 137]. However, it is possible that thrombin may also play a role in cleaving TSP4, contributing to neuropathic pain development. Similarly, the ADAMTS family of proteins has also been found to play a role

in pain state development in models of disc degeneration through a hypothetical action involving modifications to the extracellular matrix [138, 139]. While it is not known if cleaved TSP4 interacts with α2δ-1 *in vivo*, injecting a stable form of TSP4 or preventing TSP4 degradation followed by assessing for synapse formation and behavior hypersensitivity in vivo could provide some insight to address this question.

Role of α2δ-1 on T-type VGCC modulation

Since α2δ-1 is an accessory subunit of VGCC that lacks the necessary transmembrane pore-forming domain, any change to current property must come from modulating the pore-forming α1 subunit. However, whether α2δ-1 associates with and modulates T-type VGCC function is still a matter of debate. It has been reported that T-type VGCC expressed without accessory subunits has current properties similar to physiological T-type VGCC. In addition, α2δ-1 co-expressed with T-type VGCC α1 subunit does not affect the gating properties of the α1 subunit [140]. On the other hand, it has also been reported that over-expression of α2δ-1 with T-type VGCC α1 subunit can increase the channel currents 2-fold [141]. This seems to suggest that α2δ-1 does not have a high affinity to T-type VGCC α1 subunit compared to members of the HVA VGCC α1 subunit and may only bind to T-type VGCC at high concentrations. This shift in concentration is observed in the SNL model where 15-fold α2δ-1 protein increase in the DRG and 1-fold increase in the dorsal spinal cord [9] provide sufficiently high concentrations of α2δ-1 for interaction with T-type VGCC α1. Since we found that application of TSP4 to isolated DRG neurons enhances T-type VGCC currents while reducing HVA VGCC currents [Submitted], it is likely that TSP4 is the trigger for α2δ-1-mediated T-type current changes. The von Willebrand Factor-A domain on α2δ-1 that TSP4 binds to is known to act as a protein conformational switch [142]. The T-type VGCC current changes could then be due to conformational changes in α2δ-1 after TSP4 binding that either directly affect current properties or cause a conformational change in the α1 subunit that modifies current properties. One possible scenario could be that TSP4 binds directly to a α2δ-1/T-type VGCC α1 protein complex, causing a conformational change and leading to increased conductance. Another

possibility could be that TSP4 first needs to bind to α2δ-1 to induce a conformational change in α2δ-1. This in turn then either opens up a binding site or increases α2δ-1's affinity to T-type VGCC α1 subunit, leading to increased conductance. Since α2δ-1 can already associate with the α1 subunit and increase T-type current in the absence of TSP4 [141], the former possibility may be more likely. This could be confirmed by immunoprecipitating the T-type α1 subunits in the presence or absence of TSP4 and probing for α2δ-1. If TSP4 affects the binding of these two T-type VGCC subunits, I would expect more α2δ-1 in the immuno-complexes from the TSP4 treated group.

Potential role of T-type VGCC on TSP4/α2δ-1 signaling and synaptogenesis

A subject of further inquiry is how increased T-type VGCC conductance leads to increased synapse formation. Even though TSP4 interacts with pre-synaptic α2δ-1, their interactions also promote an increase in post-synaptic densities that are required to form functional synapses. It has been shown that pre-synaptic T-type VGCC mediates spontaneous synaptic release of glutamate [133]. Perhaps increased glutamate release mediated by T-type currents increases post-synaptic modulation and primes the post-synaptic density for maturation or remodeling. Another possibility could be that, given calcium's role as a secondary messenger, the increased calcium influx could activate transcriptional pathways required for synapse maturation.

If TSP4 mediates its synaptogenic activity through VGCC, the T-type currents could either facilitate the formation or increase the excitability of the pre-synaptic terminals in addition to promoting stabilization and maturation of functional synapses. The finding that absence of TSPs leads to increased mismatched pre- and post-synaptic components [55] support a role of TSP4 in stabilization and maturation, but not of formation, of synapses. However, we cannot exclude the possibility that TSP4 mediates pre- and post-synaptic stabilization and maturation via a yet undiscovered mechanism independent of T-type VGCC.

It is interesting to note that TTA-P2 blocker has been reported to have short-term analgesic

properties in formalin- and diabetic neuropathy-induced pain models [143]. Furthermore, T-type VGCC has been implicated in synaptogenesis [116, 117, 133]. It is possible that similar to gabapentin, TTA-P2 also has both a short-term analgesic effect and a long-term anti-synaptogenic effect that may translate into long-term effect in blocking behavioral hypersensitivity. Further studies with TTA-P2 treatments in animal models would be needed to test whether the observed TTA-P2 blockage of TSP4-induced synaptogenesis *in vitro* translates to similar effects *in vivo*, which could provide valuable information towards new therapeutic solutions to pain management.

The association of the α2δ-1 subunit with T-Type VGCC is still a matter of debate with conflicting evidences from cell expression systems [140, 141]. However, even if T-Type VGCC does not associate with α2δ-1 in the native state, it should not preclude the possibility that T-Type VGCC are associated with α2δ-1 in a pathological condition. It is supported by findings that T-type VGCC expression is increased after SNL injury that contributes to neuropathic pain development [144].

Significance of synaptogenesis in pain therapy:

More work is needed to translate these results into clinical applications. Some evidences suggest that the α2δ-1/TSP4 signaling pathway may be conserved in humans. Gabapentin has shown efficacy, when administered before surgeries to block post-operative pain [102, 103, 145-147], with some pain relief lasting on the order of months [27, 148], much longer than the half-life of gabapentin or the acute effect of gabapentin [4, 26, 92]. It could be possible that gabapentin's long-term mechanism of action is through blocking synaptogenesis. Based on my study, there seems to be a critical time to achieve gabapentin efficacy in blocking synaptogenesis. My data support pre-emptive gabapentin treatment to prevent injury-induced abnormal excitatory synaptogenesis and pain state development. However, pain etiology varies dramatically so finding the critical time for gabapentin administration may be difficult. An alternative approach would be to study the mechanism underlying the maintenance of TSP4-mediated synaptogenesis. Disrupting already

formed aberrant synapses could be an alternative way for achieving chronic pain relief – one that may have a larger therapeutic window for administration.

Alternatively, the critical role of T-type VGCC in synaptogenesis would suggest that T-type VGCC would be an interesting target for pain therapy. So far, T-type VGCC blockers such as ethosuximide, Mibefradil and TTA-P2 have shown promise in reversing hypersensitivity in nerve injury, diabetes and vincristine-induced models of neuropathic pain [111, 113, 114, 143, 149]. The idea of using T-type VGCC blockers for pain therapy is currently under active evaluation in clinical trials. For example, Ethosuximide is currently in phase 2 clinical trials. Another T-type blocker ABT-639 was found to be effective in several animal models [150] but failed to show any efficacy in clinical trials for treating diabetic neuropathy [151, 152]. Hopefully, T-type VGCC blockers could be further developed and validated as alternative analgesics for pain management in the near future.

Limitations

While the cultures contain a heterogeneous population of SC and DRG neurons, I did not attempt to differentiate them due to technical limitations of using a fourth fluorophore to identify cell type specific markers. Conventional fluorescent microscope detectors can handle three distinct spectral channels well, but adding a fourth channel creates significant overlap and degrades signal quality. Furthermore, it is currently unclear that subtypes of DRG and SC neurons are involved in TSP4/α2δ-1 interactions. At this point, attempting to identify any cell type specificity in TSP4/α2δ-1 interaction *in vitro* would provide very little conclusive findings as neurons in cell culture are dissociated and make random connections, thus fail to model connections found in the SDH. Therefore, neuron subtype specificity should be addressed after understanding the overall interactions between TSP4 and α2δ-1. This can be done by using different lines of transgenic mice with DRG neuron subtype specific Cre-expression in combination with conditional knockout of genes of interest, such as α2δ-1, in a neuron type specific manner to generate *in vivo* and *in vitro*

models, followed by similar *in vivo* and *in vitro* investigations. These experiments are ongoing in the Luo laboratory.

While I have shown that TSP4/α2δ-1 signaling leads to abnormal excitatory synaptogenesis through a T-type VGCC function dependent mechanism, continued research is required to fully understand the significance of the TSP4/α2δ-1 signaling pathway in neuropathic pain processing. Following are some potential experiments to further studies.

- Searching for the downstream target of TSP4/α2δ-1 induced synaptogenesis could answer how increased excitatory synapses leading to spinal circuitry hyperexcitability and behavioral hypersensitivity.
- Understanding if TSP4/α2δ-1 signaling mediated synaptogenesis is injury model specific could help to understand its mechanism and its relevance in different pain pathologies.
- Understanding the transcriptional controls and trigger molecules governing TSP4 and α2δ-1 expression could offer new targets for intervention and provide new therapeutic avenues to pain management.

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