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Themed Section: Recent Advances in Targeting Ion Channels to Treat Chronic Pain

RESEARCH PAPER

Gabapentin prevents synaptogenesis between sensory and spinal cord neurons induced by thrombospondin-4 acting on pre-synaptic $Ca_v\alpha_2\delta_1$ subunits and involving T-type Ca^{2+} channels

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BACKGROUND AND PURPOSE

Nerve injury induces concurrent up-regulation of the voltage-gated calcium channel subunit $Ca_v\alpha_2\delta_1$ and the extracellular matrix protein thrombospondin-4 (TSP4) in dorsal root ganglia and dorsal spinal cord, leading to the development of a neuropathic pain state. Interactions of these proteins promote aberrant excitatory synaptogenesis that contributes to neuropathic pain state development through unknown mechanisms. We investigated the contributions of $Ca_v\alpha_2\delta_1$ subunits and TSP4 to synaptogenesis, and the pathways involved *in vitro*, and whether treatment with gabapentin could block this process and pain development *in vivo*.

EXPERIMENTAL APPROACH

A co-culture system of sensory and spinal cord neurons was used to study the contribution from each protein to synaptogenesis and the pathway(s) involved. Anti-synaptogenic actions of gabapentin were studied in TSP4-injected mice.

KEY RESULTS

Only presynaptic, but not postsynaptic, $Ca_v\alpha_2\delta_1$ subunits interacted with TSP4 to initiate excitatory synaptogenesis through a pathway modulated by T-type calcium channels. $Ca_v\alpha_2\delta_1$/TSP4 interactions were not required for maintenance of already formed synapses. *In vivo*, early, but not delayed, treatment with low-dose gabapentin blocked this pathway and the development of the pain state.

CONCLUSIONS AND IMPLICATIONS

$Ca_v\alpha_2\delta_1$/TSP4 interactions were critical for the initiation, but not for the maintenance, of abnormal synapse formation between sensory and spinal cord neurons. This process was blocked by early, but was not reversed by delayed, treatment with gabapentin. Early intervention with gabapentin may prevent the development of injury-induced chronic pain, resulting from $Ca_v\alpha_2\delta_1$/TSP4-initiated abnormal synapse formation.

LINKED ARTICLES

This article is part of a themed section on Recent Advances in Targeting Ion Channels to Treat Chronic Pain. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v175.12/issuetoc

Abbreviations

AAV, adeno-associated virus vector; DRG, dorsal root ganglia; FrdU, 5-fluorodeoxyuridine; i.t., intrathecal; MAP2, microtubule-associated protein 2; PSD95, postsynaptic density 95; VGCC, voltage-gated calcium channel; VGlut2, vesicular glutamate transporter 2; shRNA, small hairpin RNA; TSP, thrombospondin; U, uridine

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Introduction

The gabapentinoids (**gabapentin** and **pregabalin**) are anti-hyperalgesic drugs with relatively mild side effects but only effective in approximately 30% of patients with neuropathic pain, or painful sensation derived from injuries to the central or peripheral nervous systems (Gordh *et al.*, 2008). Understanding the mechanisms of gabapentinoids' anti-neuropathic pain actions would allow the expansion of their clinical implications and further development of better neuropathic pain medications. Gabapentinoids bind to the α-2-δ-1 and α-2-δ-2 accessory subunits ($Ca_v\alpha_2\delta$) of voltage-gated calcium channels (**VGCCs**) (Gee *et al.*, 1996; Marais *et al.*, 2001). There are four members in the $Ca_v\alpha_2\delta$ family: $Ca_v\alpha_2\delta_1$, $Ca_v\alpha_2\delta_2$, $Ca_v\alpha_2\delta_3$ and $Ca_v\alpha_2\delta_4$. Both the $Ca_v\alpha_2$ (~150 kDa) and $Ca_v\delta$ (~25 kDa) proteins are encoded by the same gene, post-translationally cleaved and subsequently linked *via* disulfide bonds. The $Ca_v\alpha_2$ subunit is entirely extracellular, while the $Ca_v\delta$ subunit is membrane bound *via* a glycosylphosphatidylinositol anchor (Davies *et al.*, 2010). Peripheral nerve injury only induces up-regulation of $Ca_v\alpha_2\delta_1$, but not $Ca_v\alpha_2\delta_2$, in dorsal root ganglia (DRG) and dorsal spinal cord that correlates with neuropathic pain development (Luo *et al.*, 2001; Newton *et al.*, 2001; Li *et al.*, 2004; Bauer *et al.*, 2009) and the anti-neuropathic pain efficacy of gabapentin (Luo *et al.*, 2002) in animal models. These findings support that injury-induced $Ca_v\alpha_2\delta_1$ dysregulation in the sensory pathway contributes critically to the development of neuropathic pain.

The $Ca_v\alpha_2\delta_1$ subunit is normally associated with high-voltage-activated L-type, N-type, P/Q-type and R-type VGCCs and expressed in many tissues including skeletal, cardiac and smooth muscles as well as central and peripheral nervous systems (Dolphin, 2012; Dolphin, 2013). Even though $Ca_v\alpha_2\delta_1$ dysregulation has been shown to play a causal role in neuropathic pain processing in animal models (Luo *et al.*, 2001; Li *et al.*, 2004; Li *et al.*, 2006; Boroujerdi *et al.*, 2008; Bauer *et al.*, 2009; Nguyen *et al.*, 2009; Zhou and Luo, 2013; Zhou and Luo, 2015), the detailed mechanisms of $Ca_v\alpha_2\delta_1$-mediated central sensitization remain elusive. The $Ca_v\alpha_2\delta_1$ subunit is the binding site for the thrombospondin (TSP) family of extracellular matrix proteins secreted by astrocytes, and TSP binding to $Ca_v\alpha_2\delta_1$ promotes excitatory synaptogenesis in the CNS (Eroglu *et al.*, 2009).

The TSP family can be divided into two groups based on structure homologies. Group A includes trimeric TSP1 and TSP2, and group B includes pentameric TSP3, TSP4 and TSP5 (Adams, 2001). TSPs are widely distributed in different tissues suggesting diversified functions that include wound healing, angiogenesis, connective tissue organization and synaptogenesis (Adams and Lawler, 2011). Interestingly, one member of the TSP family, TSP4, is also up-regulated in DRG and dorsal spinal cord after peripheral nervous system and CNS injuries that correlate with increased presynaptic excitatory input into dorsal spinal cord and pain state development (Kim *et al.*, 2012; Zeng *et al.*, 2013; Li *et al.*, 2014a; Pan *et al.*, 2015). Apparently, injury-induced TSP4 and $Ca_v\alpha_2\delta_1$ subunits interact to promote aberrant excitatory synaptogenesis in dorsal spinal cord that contributes to neuron sensitization and the development of pain states (Li *et al.*, 2014b; Park *et al.*, 2016). However, the mechanism(s) underlying synaptogenesis mediated by $Ca_v\alpha_2\delta_1$ and TSP4 remains unknown. In this study, we explored how $Ca_v\alpha_2\delta_1$ and TSP4 modulate excitatory synaptogenesis between sensory and spinal cord neurons and examined the potential pathway(s) involved as well as its sensitivity to blockade by gabapentin in relation to the development of pain states.

Methods

Animals

All animal care and experimental procedures complied with protocols approved by the Institutional Animal Care Committee of the University of California, Irvine. The assessments of hyperalgesia also followed the Guidelines of the IASP. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). To check sex-dependent sensitivity to TSP4-induced behavioural hypersensitivity and to confirm our *in vitro* findings, we used 129sv adult male and female mice (20-30 g; Charles River Laboratories, Wilmington, MA, USA). On arrival, the mice were housed 4 to a cage (Zyfone^{TM} , Lab Products Inc., Seaford, DE, USA) in a temperature (22 ± 1.5^{o}C) controlled environment with a light/dark cycle of 12/12h, and free access to food (standard mouse diet Envigo 2020x) and tap water, for at least 2 days before they were used for the experiments. This mouse model system of nociception has been in use for studying the nociceptive effects of TSP4 for some years (Kim *et al.*, 2012; Park *et al.*, 2016) and is not replaceable with *in vitro* culture systems.

TSP4/gabapentin injection and behaviour test

Bolus recombinant TSP4 (5 μg per mouse, Kim *et al.*, 2012) was injected intrathecally (i.t.) into naïve adult 129sv male and female mice as described previously (Park *et al.*, 2016) at day 0. Some animals were treated daily with saline or gabapentin (25 μg per mouse, i.t.) for up to 4 days after the bolus TSP4 injection, either starting at day 0 (day 0–3 treatment) or delayed for 2 days (day 2–3 treatment).

Behavioural testing was performed, blindly, before the TSP4 injection and before each daily gabapentin or saline injection, for 4 days, which correlated with peak behavioural hypersensitivity induced by i.t. TSP4 injections (Kim *et al.*, 2012). Briefly, mice were acclimatized for at least 30 min in a wire-mesh floor in Plexiglas chambers. Plantar surfaces of both hindpaws were stimulated by applying a series of von Frey filaments (Stoelting, Wood Dale, IL, USA), starting with a buckling weight of 0.41 g, perpendicularly to the hindpaw with enough force to bend the filament slightly. A rapid withdrawal and/or licking of the paw were considered a positive response that prompted the use of the next weaker filament. A negative response resulting from an absence of paw withdrawal after 5 s prompted the use of the next heavier filament. This paradigm continued for four more measurements after the initial change of the behavioural response, or until four consecutive positive (assigned a score of 0.01 g) or five consecutive negative (assigned a score of 2 g) responses had occurred. The 50% paw withdrawal thresholds to von Frey filament stimulation were calculated using the up–down method of Dixon (1980).

As gabapentin has a short lasting anti-hyperalgesic effect (Boroujerdi *et al.*, 2011; Li *et al.*, 2014b) due to its rapid clearance *via* renal secretion (Radulovic *et al.*, 1995), testing the gabapentin effects on TSP4-induced hypersensitivity approximately 24 h after each injection allowed us to assess its long-term effect distinguished from its short-term effect. Spinal cord samples were collected after behavioural testing at day 4 post-TSP4 injection for immunohistochemical studies.

Spinal cord immunohistochemistry

Mice were decapitated under deep isoflurane analgesia, and samples of L4/L5 lumbar spinal cord were collected at the designated times *via* hydraulic extrusion, fixed in 4% paraformaldehyde overnight and cryoprotected in 30% sucrose. Samples were then mounted in Tissue-Tek optimum cutting temperature compound (Sakura Finetek Inc., Torrance, CA, USA) and sectioned into 10 μm slices by a cryostat (Leica Microsystems, Wetzlar, Germany). Samples were pretreated with heat-based antigen retrieval as described previously (Park *et al.*, 2016) and then incubated with a combination of primary antibodies against the presynaptic marker – **vesicular glutamate transporter 2 (VGlut2)** (guinea pig; Synaptic Systems, Goettingen, Germany) and the postsynaptic marker – postsynaptic density 95 protein (PSD95) (rabbit; Invitrogen, Carlsbad, CA, USA) overnight at 4°C, followed by incubation with species-specific secondary antibodies conjugated to unique fluorophores for 2 h at room temperature. Images were taken using a Zeiss LSM700 confocal microscope (University of California Irvine Optical Biology Core) in 0.3-μm-thick Z stacks and cropped down to the seven consecutive Z stacks with the best signal, merged and used for analysis using Volocity 6.0 (PerkinElmer, Waltham, MA, USA).

DRG and spinal cord neuron culture

The DRG/spinal cord co-culture protocol was adapted mainly from a series of published protocols (Burkey *et al.*, 2004; Seybold and Abrahams, 2004; Albuquerque *et al.*, 2009b; Albuquerque *et al.*, 2009a) along with some modifications from published methods (Varon and Raiborn, 1971; Delree *et al.*, 1989; Ohshiro *et al.*, 2007; Bauer *et al.*, 2009; Joseph *et al.*, 2010). Briefly, at least six embryos (for spinal cord neurons) and three adult mice (for DRG neurons) were used for each multiwell culture plate. Spinal cords were collected from mouse embryos (embryonic days 14–19) after making a longitudinal incision along the ventral side of the spinal vertebrae. Neurons were dissociated with 0.25% trypsin, plated onto glass coverslips coated with 0.1 $mg{\cdot}mL^{-1}$ poly-D-lysine (Thermo Fisher, Scientific Inc., Huntington Beach, CA, USA) and 0.04 $mg{\cdot}mL^{-1}$ laminin (Sigma-Aldrich, St Louis, MO, USA) and cultured in DMEM (Thermo Fisher) supplemented with 10% horse serum (Thermo Fisher) and 10% FBS (Thermo Fisher). After 24 h, the media were replaced with Neurobasal media (Thermo Fisher) supplemented with B27 supplement (NB/B27) (Thermo Fisher). Three days later, 100 nM uridine (U; Sigma) and 20 nM 5-fluorodeoxyuridine (FrdU; Sigma) were added to inhibit proliferation of non-neuronal cells. Using immunostaining of astrocyte-specific antibody against glial fibrillary acidic proteins, we confirmed that astrocytes were greatly depleted in culture after U/FrdU treatment (data not shown).

For collecting mouse adult DRG neurons, mice were decapitated under deep isoflurane analgesia, and the spinal cord was removed first *via* hydraulic extrusion, and the vertebral column segments approximately from T7-L5 were dissected out. After laminectomy, DRGs were collected and dissociated in 1.25 $mg{\cdot}mL^{-1}$ collagenase in F12 media. Cells were plated onto glass coverslips coated with poly-D-lysine and laminin and cultured in NB/B27 media with U/FrdU.

For spinal cord/DRG co-cultures, spinal cord neurons were first allowed to grow for 3 days for maturation and neurite sprouting. DRG neurons were then added to the spinal cord neuron cultures at the time of medium change at day 4, and cultures were maintained in NB/B27 media with U/FrdU.

Campenot chamber cultures

Campenot chambers (Tyler Research Co., Edmonton, AB, Canada) were set up as described by Pazyra-Murphy and Segal (2008). Briefly, 35 mm cell culture dishes were coated with 1 $mg{\cdot}mL^{-1}$ collagen (Sigma). Grooves were scored on the dish surface using insect pins at approximately 200 μm intervals and wetted with a drop of NB/B27 with 0.6 $mg{\cdot}mL^{-1}$ methylcellulose (Sigma). Campenot chamber bottoms were then coated with autoclaved silicone grease and mounted onto a culture dish, which was stored overnight in a cell culture incubator with media in the middle chamber to ensure a leak-proof seal created with silicone grease. DRG neurons were then cultured in the middle chamber for 7 days to allow axon sprouting into the outer chambers. Spinal cord neurons were then plated into the two outer chambers and incubated for additional 6 days. Chambers were ready to use when sufficient number of DRG axons fully cross the divider between chambers to reach the outer chambers.

Selective knockdown of $Ca_v\alpha_2\delta_1$ subunits

To knock down the $Ca_v\alpha_2\delta_1$ subunit in spinal cord neurons, adeno-associated virus (AAV) vectors encoding small hairpin RNA (shRNA) against $Ca_v\alpha_2\delta_1$ mRNA (3 μL, 2×10^{12} $virus{\cdot}mL^{-1}$, gift from Dr Perez-Reyes, University of Virginia School of Medicine) were used to treat the spinal cord neurons for 10 days. To knock down $Ca_v\alpha_2\delta_1$ from DRG neurons, $Ca_v\alpha_2\delta_1^{flox/flox}$ conditional knockout mice (Park *et al.*, 2016) were crossed with Advillin–Cre mice (Zurborg *et al.*, 2011) so that Cre-inducible $Ca_v\alpha_2\delta_1$ ablation occurred in over 90% of DRG neurons (Park *et al.*, 2016). These mice were further crossed with a Rosa-tdTomato Cre-reporter mouse line (Jackson Labs, Bar Harbor, ME, USA) to generate $Adv^{+/Cre}/Ca_v\alpha_2\delta_1^{flox/flox}/tdTomato^{+/-}$ mice.

Cell culture immunohistochemistry

Cultured neurons were fixed in methanol at −20°C for 15 min and then incubated for 24 h at 4°C with the following primary antibodies diluted in Dako antibody diluent (Dako, North America, Inc., Carpinteria, CA, USA): microtubule-associated protein 2 (MAP2) (chicken, Abcam, Cambridge, MA, USA), PSD95 (mouse, Thermo Fisher) and VGlut2 (guinea pig, Synaptic Systems). After incubation overnight with respective Alexafluor 488, 594 or 647 secondary antibodies, cells on coverslips were mounted onto glass slides using Vectashield DAPI hardmount media (Vector Labs, Burlingame, CA, USA). Images from co-cultures were taken using a 63× objective on the LSM700 confocal microscope (University of California Irvine Optical Biology Core) and analysed using Volocity 6.0. Image from Campenot chambers

were acquired with a 40× objective on the LSM700 confocal microscope.

Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Images captured from L4/L5 spinal cord sections (n = 36 over three animals per treatment type, 100 μm apart) that were co-stained with VGlut2/PSD95 antibodies were analysed using Volocity to determine the number of total $VGlut2^+$ puncta, $VGlut2^+$/$PSD95^+$ and $PSD95^+$ puncta. As functional synapses should contain presynaptic and postsynaptic components, a ratio of $VGlut2^+/PSD95^+$ to $VGlut2^+/PSD95^-$ was used to account for the number of functional synapses and minimize the animal and sampling differences between groups.

Synapses in neuron co-cultures were identified by counting the number of co-localized immunoreactivity to PSD95 and VGlut2 marker antibodies on individual dendrites (stained with MAP2) of each spinal cord neuron. To correct for variations in the numbers and size of dendrites among individual neurons that could markedly affect the synapse numbers per neuron, the synapse count from a given spinal cord neuron was calculated as per μm^2 area of dendrites (shown as MAP2 immunoreactivity) of that neuron before data comparison analysis.

Collected data were analysed for statistical significance using Student's *t*-test for pairwise comparisons, ANOVA analysis with *post hoc* tests for multiple comparisons and non-parametric Kruskal–Wallis test with multiple comparisons for fold over control values, as specified using statistical software in Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA).

Materials

The thrombospondin was expressed and purified as described by Kim *et al.*, (2012). Other compounds were supplied as follows: gabapentin by MEDISCA, Inc. (Plattsburgh, NY, USA); nifedipine and ω-conotoxin GVIA by Sigma-Aldrich; ω-conotoxin MVIIC and TTA-P2 by Alomone Labs (Jerusalem, Israel).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b).

Results

TSP4 acts on $Ca_v\alpha_2\delta_1$ subunits to initiate synaptogenesis between DRG and spinal cord neurons

We investigated if $Ca_v\alpha_2\delta_1$ channel subunits played a role in TSP4-induced excitatory synapse formation between mouse DRG sensory neurons and spinal cord neurons using a co-culture system. DRG neurons and axons were visualized by Cre-induced expression of tdTomato red fluorescent proteins (RFPs) (Figure 1A, left), and spinal cord neurons and dendrites were visualized by MAP2 immunoreactivity (Figure 1A, right). Excitatory synapse formation between sensory neuron axons and spinal cord neuron dendrites was monitored by counting puncta of co-localized immunoreactivities of VGlut2 and PSD95 within dendritic spine-like structure on top of MAP2 immunoreactivity along with or surrounded by red DRG neuron axons (Figure 1A, right, and B). As VGlut2 immunoreactivity is mainly at the terminals, but not axons, and tdTomato fluorescence is mainly expressed along the axons, but not in VGlut2 positive presynaptic vesicles, it was highly likely that the quantified VGlut2 signal within the dendritic spine-like structure along MAP2 immunoreactivity derived from VGlut2 immunoreactivity but not from tdTomato fluorescence. Upon maturation, different concentrations of recombinant TSP4 proteins in a range reported previously (Eroglu *et al.*, 2009) were added, and synapse formation analysis carried out 4 days later, which correlated with the peak pro-nociceptive and synaptogenic effects of TSP4 *in vivo* (Kim *et al.*, 2012; Park *et al.*, 2016). The same volume of PBS was used for control treatments.

Our data indicated that TSP4 could dose dependently increase the number of $VGlut2^+/PSD95^+$ synapses associated with spinal cord neurons (Figure 1B, C). Treatments with 8 and 20 nM, but not 2 nM, TSP4 for 4 days increased the number of $VGlut2^+/PSD95^+$ synapses by roughly 75%, which was less than the twofold to threefold increases in synapse formation induced by similar TSP4 concentrations previously reported in retinal ganglion cell cultures (Christopherson *et al.*, 2005; Eroglu *et al.*, 2009). This discrepancy may be attributed to the difference between these culture systems as retinal ganglion neurons are functionally distinctive from DRG and spinal cord neurons. Nevertheless, these data indicate that the spinal cord and DRG neuron co-culture system, which mimics sensory neuron synapse formation in dorsal spinal cord, is suitable for our studies.

To determine if the $Ca_v\alpha_2\delta_1$ subunits contributed to the TSP4-induced excitatory synapse formation, 100 nM gabapentin was added into the co-culture system at the same time as the TSP4 treatment. This bolus gabapentin treatment for 4 days did not affect the baseline synapse formation but totally blocked the TSP4-induced excitatory synaptogenesis (Figure 1D, E). This suggests that $Ca_v\alpha_2\delta_1$ played a role in TSP4-induced sensory synapse formation, similar to that reported for TSP-induced synaptogenesis in other neuronal culture systems (Eroglu *et al.*, 2009; Xu *et al.*, 2009). To determine if both $Ca_v\alpha_2\delta_1$ subunits and TSP4 were required for initiation and/or maintenance of excitatory synapse formation in this co-culture system, TSP4 was removed after 2 days in culture, and synapse analysis was performed at day 4 after the initial bolus TSP4 treatment. Our data indicated that TSP4 removal at day 3 did not significantly decrease synapse formation compared with 4 day TSP4 treatment (Figure 1D, E), supporting that TSP4 acted on $Ca_v\alpha_2\delta_1$ subunits to initiate, but not maintain, sensory synaptogenesis *in vitro*. This is supported by findings that delaying gabapentin treatment for 2 days, when TSP4-induced synapses have been formed, did not block TSP4-induced synaptogenesis (Figure 1D, E). This finding is consistent with data from a different *in vitro* system (Eroglu *et al.*, 2009). Taken together, these data support that the presence of both $Ca_v\alpha_2\delta_1$

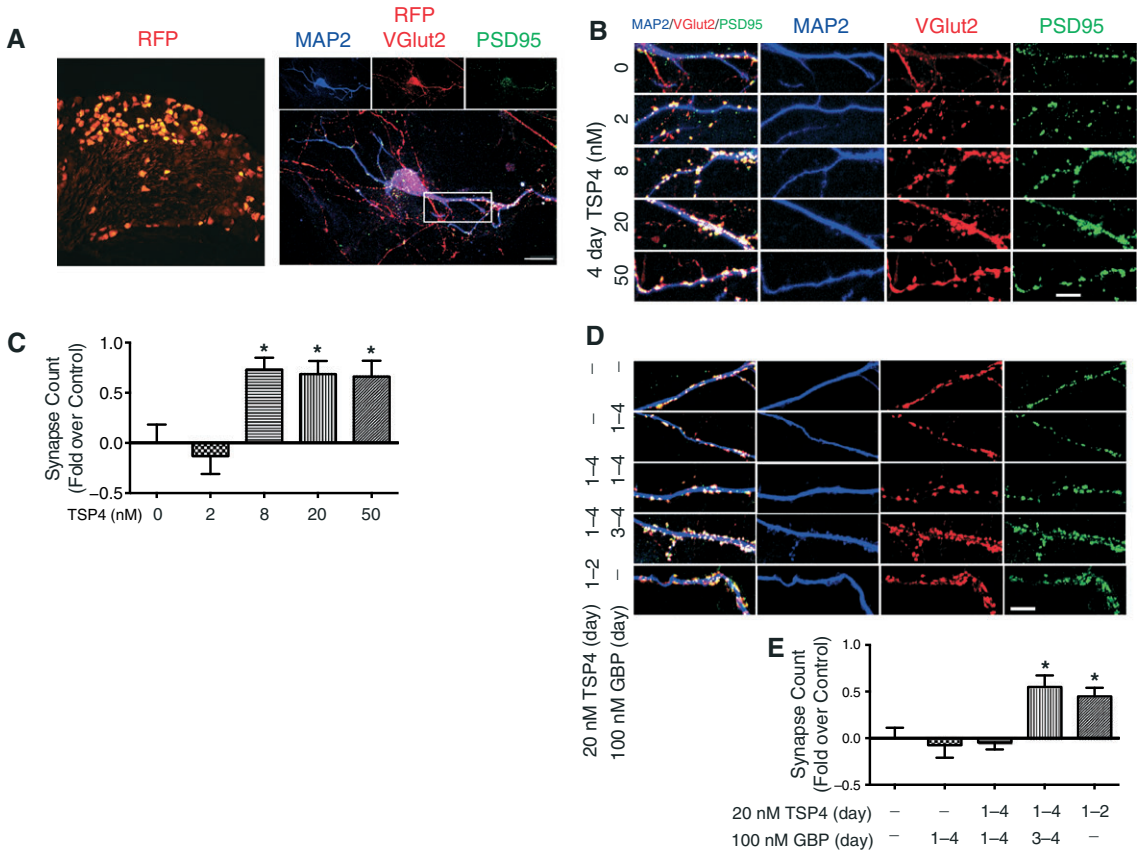

Figure 1

TSP4 induces excitatory synaptogenesis between sensory and spinal cord neurons that can be blocked by early, but not delayed, gabapentin treatment. (A) Representative images of a mouse DRG (10×) showing RFP-labelled neurons (left) and a stained spinal cord neuron in co-culture (right). The box denotes a sampling area taken for close-up analysis of co-localized dendritic (MAP2, blue) and synaptic (VGlut2, red; PSD95, green) marker immunoreactivities shown in the top panels, which are also shown in the top row panels in (B). Scale bar = 20 μm. (B) Close up images of spinal cord neuron dendrite segments and associated synapses after treatment with different concentrations of TSP4 for 4 days. Scale bar = 10 μm. (C) Quantification of synapse counts on individual spinal cord neurons showing that 8–50 nM TSP4 strongly induce synaptogenesis. Means ± SEM from 20 neurons per group, five neurons per well randomly selected from multiple culture plates of independent experiments. *$P < 0.05$; significantly different from control (no TSP4 treatment); non-parametric Kruskal–Wallis test with multiple comparisons. (D) Close-up images of spinal cord neuron dendrite segments and associated excitatory synapses after early or delayed gabapentin (GBP) treatment. Scale bar = 10 μm. (E) Quantification of synapse counts on individual spinal cord neurons showing that TSP4-induced excitatory synaptogenesis can be blocked by early, but not delayed, gabapentin treatment. Means ± SEM from 20 neurons per group, five neurons per well randomly selected from multiple culture plates of independent experiments. *$P < 0.05$, significantly different from control (PBS) treatment; non-parametric Kruskal–Wallis test with multiple comparisons.

and TSP4 is critical in the initiation, but not maintenance, of excitatory synapse formation *in vitro*.

DRG, but not spinal cord, neuronal $Ca_v\alpha_2\delta_1$ subunits are critical in promoting synaptogenesis

Under normal physiological conditions, $Ca_v\alpha_2\delta_1$ subunits are widely expressed in different locations and cell types throughout the body, including somata and axons of DRG neurons and somata, and axons and dendrites of spinal cord neurons. Following peripheral nerve injury, the $Ca_v\alpha_2\delta_1$ subunits can be up-regulated in DRG sensory neurons and then undergo axonal transport to their central axon terminals in dorsal spinal cord (Li *et al.*, 2004; Bauer *et al.*, 2009). However, it is not known if $Ca_v\alpha_2\delta_1$ promotes synaptogenesis in spinal cord at a postsynaptic level (spinal cord neurons and their axons/dendrites) or at a presynaptic level (DRG neurons and their axons) or both. We addressed this question by selectively knocking out the $Ca_v\alpha_2\delta_1$ subunits from spinal cord neurons or DRG neurons in the co-culture system and investigating its influence on TSP4-induced synaptogenesis. $Ca_v\alpha_2\delta_1$ expression in spinal cord neurons was knocked down [SC(KO)] by treating cultured neurons with shRNA against $Ca_v\alpha_2\delta_1$ mRNA in AAV vectors (University of North Carolina Vector Core; see Methods), which is known to knock down $Ca_v\alpha_2\delta_1$ expression efficiently *in vivo* and *in vitro,* as reported in our previous studies (Park *et al.*, 2016). $Ca_v\alpha_2\delta_1$ ablation from over 90% DRG neurons (Zurborg *et al.*, 2011) [DRG(KO)] was achieved with Cre recombinase-induced $Ca_v\alpha_2\delta_1$ knockout in a triple transgenic ($Adv^{+/Cre}/Ca_v\alpha_2\delta_1^{flox/flox}/tdTomato^{+/-}$) mouse line as reported in our previous studies

(Park *et al.*, 2016). As a result, DRG neurons with $Ca_v\alpha_2\delta_1$ subunit ablation were marked with RFPs encoded by a Cre-specific tdTomato reporter gene.

We found that TSP4 (20 nM) treatment increased synaptogenesis significantly (above 50% over control PBS treatment) with normal presynaptic and postsynaptic $Ca_v\alpha_2\delta_1$ subunit expression in the SC(WT)/DRG(WT) group. A similar TSP4 treatment in the SC(KO)/DRG(WT) group where $Ca_v\alpha_2\delta_1$ was knocked down in spinal cord neurons also caused a significant increase of excitatory synaptogenesis (around 35% over control PBS treatment). In contrast, a similar TSP4 treatment in the SC(WT)/DRG(KO) or SC(KO)/DRG(KO) groups where $Ca_v\alpha_2\delta_1$ ablation occurred in DRG neurons resulted in a complete blockade of TSP4-induced excitatory synaptogenesis, such that synapse count after TSP4 treatment was similar to that of PBS control treatment (Figure 2). One-way ANOVA analysis comparing TSP4 treated groups indicated that the synaptogenic effects of TSP4 were significantly diminished in both groups with $Ca_v\alpha_2\delta_1$ ablation in DRG neurons [SC(WT)/DRG(KO) or SC(KO)/

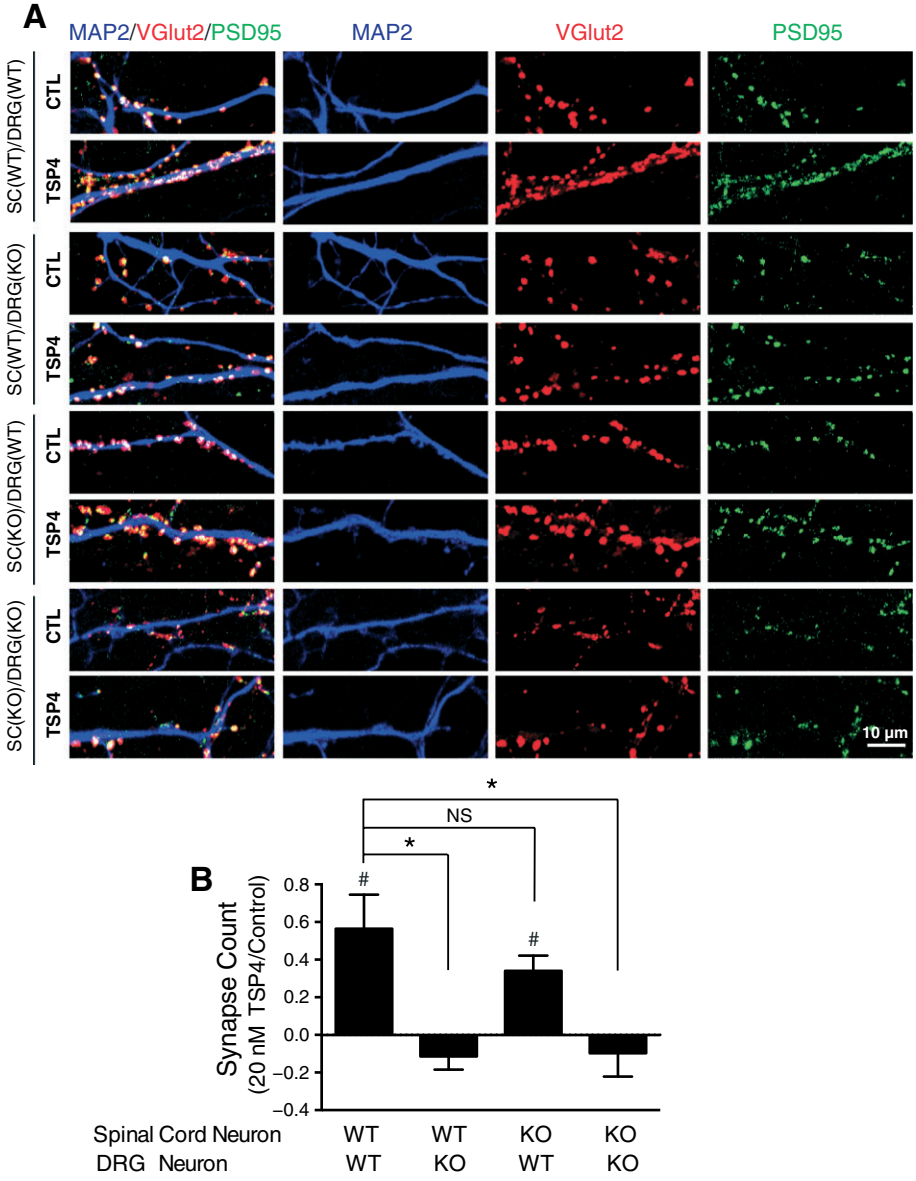

Figure 2

Presynaptic, but not postsynaptic, $Ca_v\alpha_2\delta_1$ subunits are required for TSP4-induced excitatory synaptogenesis. (A) Representative images showing immunoreactivities to dendritic and synaptic markers in DRG/spinal cord neuron co-cultures in the presence of TSP4 (20 nM) for 4 days with or without $Ca_v\alpha_2\delta_1$ ablation from DRG (presynaptic) or spinal cord (postsynaptic) neurons. CTL, control; scale bar = 10 μm. (B) Quantification of synapse counts showing that only presynaptic (DRG) $Ca_v\alpha_2\delta_1$ subunits are required for TSP4-induced excitatory synaptogenesis. Means ± SEM from 15 neurons each group that were randomly selected from five wells of multiple culture plates of independent experiments. $^{\#}P < 0.05$, significantly different from respective PBS control (no TSP4) treatment; Student's *t*-test. $^{*}P < 0.05$, significantly different from the TSP4-treated control group; one-way ANOVA with Dunnett's multiple comparisons test. NS, not significant.

DRG(KO)] compared with the control group SC(WT)/DRG(WT). However, the synaptogenic effects of TSP4 were not significantly affected in the group with $Ca_v\alpha_2\delta_1$ knockdown in spinal cord neurons [SC(KO)/DRG(WT)] compared with the control group (Figure 2B). A limitation in the latter comparison is that variations in $Ca_v\alpha_2\delta_1$ ablation/knockdown efficiency between AAV-induced knockdown *in vitro* and Cre-induced ablation *in vivo* could affect the interpretation of the analysis. However, this limitation is not present in the former analysis in which the only variable is the TSP4 treatment. Thus, combining these analyses allowed us to conclude that the presynaptic $Ca_v\alpha_2\delta_1$ subunits expressed in DRG, but not the postsynaptic $Ca_v\alpha_2\delta_1$ subunits expressed in spinal cord, were required for TSP4-induced synaptogenesis.

Presynaptic $Ca_v\alpha_2\delta_1$ subunits from DRG are responsible for TSP4-induced synaptogenesis in spinal cord

As nerve injury induces TSP4 up-regulation in both DRG (Pan *et al.*, 2015) and dorsal spinal cord (Kim *et al.*, 2012) that correlates with abnormal synapse formation and neuropathic pain development (Park *et al.*, 2016), it is possible that $Ca_v\alpha_2\delta_1$ proteins in DRG neuron surface and/or presynaptic terminals in dorsal spinal cord interact with TSP4 to promote excitatory synaptogenesis. To address this issue, we used a Campenot chamber set-up where DRG neurons were cultured in the middle chamber but their axons could grow through a fluid-sealed methylcellulose barrier to reach spinal cord neurons in the outer chambers (Figure 3A). This *in vitro* environment closely mimics the *in vivo* environment in which peripheral DRG neurons are anatomically separated from central spinal cord neurons but send long axons into the dorsal spinal cord to form the first synapse connection with centrally located spinal cord neurons. The action site of $Ca_v\alpha_2\delta_1$/TSP4-induced synaptogenesis thus could 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.

In these experiments, a significant increase of excitatory synapses was only observed when TSP4 (20 nM) was added into the outer chambers but not to the middle chamber

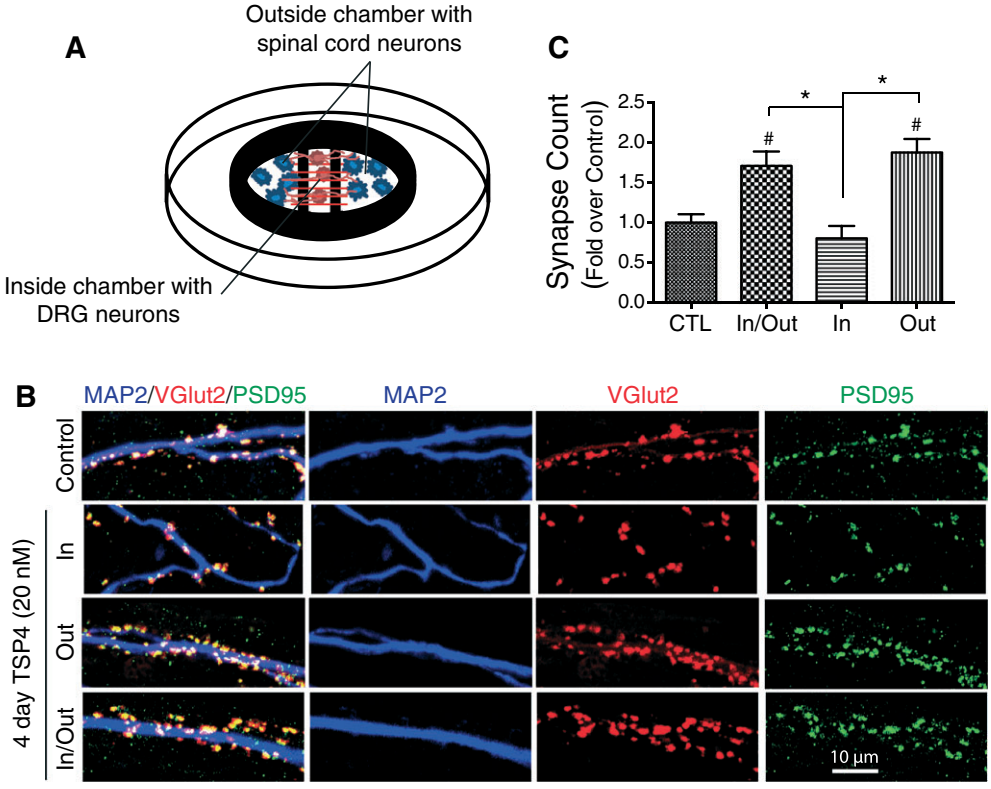

Figure 3

TSP4 induces synaptogenesis by interacting with $Ca_v\alpha_2\delta_1$ subunits at the presynaptic terminals but not DRG neuron somata. (A) Diagram of Campenot chamber set-up showing spinal cord neurons were cultured in the outer chambers and DRG neurons were in the inner chamber, where their axons could grow through the inner chamber wall to reach the outer chambers. (B) Representative images showing immunoreactivities to dendritic and synaptic markers in DRG/spinal cord neuron co-cultures in the presence or absence of TSP4 (20 nM) for 4 days in different chambers. Scale bar = 10 µm. (C) Summarized data showing that only TSP4 application to the outer chamber could induce excitatory synaptogenesis while TSP4 application to the inner chamber alone had no effect compared with control (no TSP4 treatment). Means ± SEM from 15 neurons per group that were randomly selected from five culture dishes of independent experiments. $^{\#}P < 0.05$, significantly different from control (PBS) treatment; $^{*}P < 0.05$, significantly different as indicated, among TSP4-treated groups; non-parametric Kruskal–Wallis test with multiple comparisons. CTL, control.

(Figure 3B, C). This suggested the $Ca_v\alpha_2\delta_1$ subunits expressed in central presynaptic terminals, but not that on soma surface of DRG neurons, was critical for TSP4-induced excitatory synaptogenesis. Data from this experiment alone would not allow us to exclude the contribution from spinal cord neuron $Ca_v\alpha_2\delta_1$ in the outside chamber to synaptogenesis. However, our findings (Figure 2) from the SC(KO)/DRG(WT) group, provided additional evidence that the $Ca_v\alpha_2\delta_1$ subunits in spinal cord neurons did not play a critical role in TSP4-induced synaptogenesis.

$Ca_v\alpha_2\delta_1$/TSP4-mediated synaptogenesis is dependent on T-type VGCC function

We next examined the potential mechanisms underlying the role of the $Ca_v\alpha_2\delta_1$/TSP4-mediated pathway in synaptogenesis. As the $Ca_v\alpha_2\delta_1$ protein is an accessory subunit of VGCCs with important roles in mediating channel assembly and stabilization, it is possible that $Ca_v\alpha_2\delta_1$ mediates synaptogenesis through a VGCC-dependent pathway. Although blocking some high-voltage-activated calcium channels fails to prevent the synaptogenic action of TSP in cultured retinal ganglion cells (Eroglu *et al.*, 2009), these cells differ from DRG and spinal cord neurons. In addition, the effects of low-voltage-activated T-type calcium channel blockers in $Ca_v\alpha_2\delta_1$/TSP4-mediated synaptogenesis have not yet been tested. T-type VGCCs are of particular interest because they are not only highly expressed in primary afferents (Todorovic *et al.*, 2001; Jacus *et al.*, 2012) but also important in injury-induced hypersensitivity (Dogrul *et al.*, 2003; Bourinet *et al.*, 2005; Choi *et al.*, 2007; Todorovic and Jevtovic-Todorovic, 2013). Accordingly, the effects of the following VGCC subtype-specific blockers in blocking TSP4-induced synaptogenesis were tested in the co-culture system: 0.5 μM **nifedipine** (L-type), 0.2 μM **ω-conotoxin GVIA** (N-type), 0.2 μM **ω-conotoxin MVIIC** (P/Q-type) and 0.5 μM **TTA-P2** (T-type). Excitatory synapse formation was analysed after treating the co-cultures with 20 nM TSP4 and each of these VGCC blockers for 4 days. The concentration of each drug used was determined to be sufficient in blocking corresponding channel-type conductance in isolated DRG neurons (Pan *et al.*, 2016) (personal communication with Dr Quinn Hogan, Medical College of Wisconsin).

Our data indicated that only the T-type VGCC blocker TTA-P2, but not other types of VGCC blockers tested, blocked TSP4-induced synaptogenesis to a level similar to that of control (PBS) treatment (Figure 4A, B). This suggests that the $Ca_v\alpha_2\delta_1$/TSP4 pathway promoted excitatory synaptogenesis through a T-type VGCC-dependent mechanism. However, it is not clear if T-type VGCCs act at a site in close proximity to $Ca_v\alpha_2\delta_1$/TSP4, or on a different location along the $Ca_v\alpha_2\delta_1$/TSP4-mediated pathway, or on an independent pathway.

$Ca_v\alpha_2\delta_1$ subunits mediate initiation of synaptogenesis in vivo that can be prevented by early treatment with low-dose gabapentin

Our *in vitro* data showed that activation of the $Ca_v\alpha_2\delta_1$/TSP4 pathway mediates the initiation, but not maintenance, of excitatory synaptogenesis that can be blocked by early but not delayed gabapentin treatments (Figure 1). If confirmed *in vivo*, the translational impact of these findings may include that early application of gabapentin could block abnormal synaptogenesis, such as that derived from peripheral nerve injuries and lead to prevention of chronic pain development. To validate this, we examined if early gabapentin treatment could prevent TSP4-induced excitatory synaptogenesis and the development of pain states *in vivo*. While we could use a neuropathic pain model of peripheral nerve injury in which injury induction of TSP4 has been known to cause abnormal synaptogenesis and neuropathic pain states (Kim *et al.*, 2012; Park *et al.*, 2016), it is also inevitable that nerve injuries cause increased expression of many different proteins (Wang *et al.*, 2002; Valder *et al.*, 2003; Kim *et al.*, 2009), many of which, including brain-derived neurotropic factor (Obata and Noguchi, 2006), TNF-α, IL-6, IL-1β (Inoue, 2006; Schafers and Sorkin, 2008; Ren and Torres, 2009) and fractalkine (Zhuang *et al.*, 2007), are found to be critical in mediating neuropathic hypersensitivities. In order to study the role of $Ca_v\alpha_2\delta_1$/TSP4 in mediating synaptogenesis and behavioural hypersensitivity without the influence from other injury factors, we injected TSP4 i.t. into naïve mice (5 μg per mouse) and monitored daily the development of behavioural hypersensitivity and analysed excitatory synaptogenesis 4 days after the bolus TSP4 injection, an optimal time point for TSP4-induced behavioural hypersensitivity and excitatory synaptogenesis *in vivo* (Kim *et al.*, 2012; Park *et al.*, 2016). To determine if the $Ca_v\alpha_2\delta_1$/TSP4 pathway is critical in the initiation and/or maintenance of synaptogenesis, we injected gabapentin daily (25 μg per mouse, i.t.) after each daily behavioural testing, started either immediately after bolus TSP4 injection (from day 0–3) or with a 2 day delay (from day 2–3) (Figure 5A). The gabapentin dose used (approximately 1 $mg{\cdot}kg^{-1}$) was a subclinical dose that was about 50-fold below the recommended maximum daily dosage for neuropathic pain management (Dworkin *et al.*, 2007) and the effective dose of pain state reversal in neuropathic pain models (Luo *et al.*, 2002). In addition, the effects of gabapentin on TSP4-induced pain states were examined approximately 24 h after each injection, which should only assess the chronic, but not acute, effects of a low-dose gabapentin treatment as the effective duration of gabapentin for pain relief is about 6 h (Gordh *et al.*, 2008). Because the nociceptive effects of TSP4 was not sex-related (Figure 5B), only male mice were used for gabapentin treatment and synaptogenesis studies.

Our data indicated that i.t. TSP4 injection resulted in a time-dependent development of behavioural hypersensitivity (Figure 5A), which peaked at day 4 after TSP4 injection, as we have shown previously (Kim *et al.*, 2012; Park *et al.*, 2016). This peak behavioural hypersensitivity correlated with around a 37% increase in excitatory synapses in dorsal spinal cord compared with saline-injected controls (Figure 5C, D). Importantly, both TSP4-induced synaptogenesis and behavioural hypersensitivity could be blocked by daily low-dose gabapentin treatments for the same duration (Figure 5A, C, D). These data support that i.t. TSP4 interacts with $Ca_v\alpha_2\delta_1$ subunits to induce excitatory synaptogenesis and behavioural hypersensitivity that can be blocked by low-dose gabapentin treatment. However, delaying gabapentin

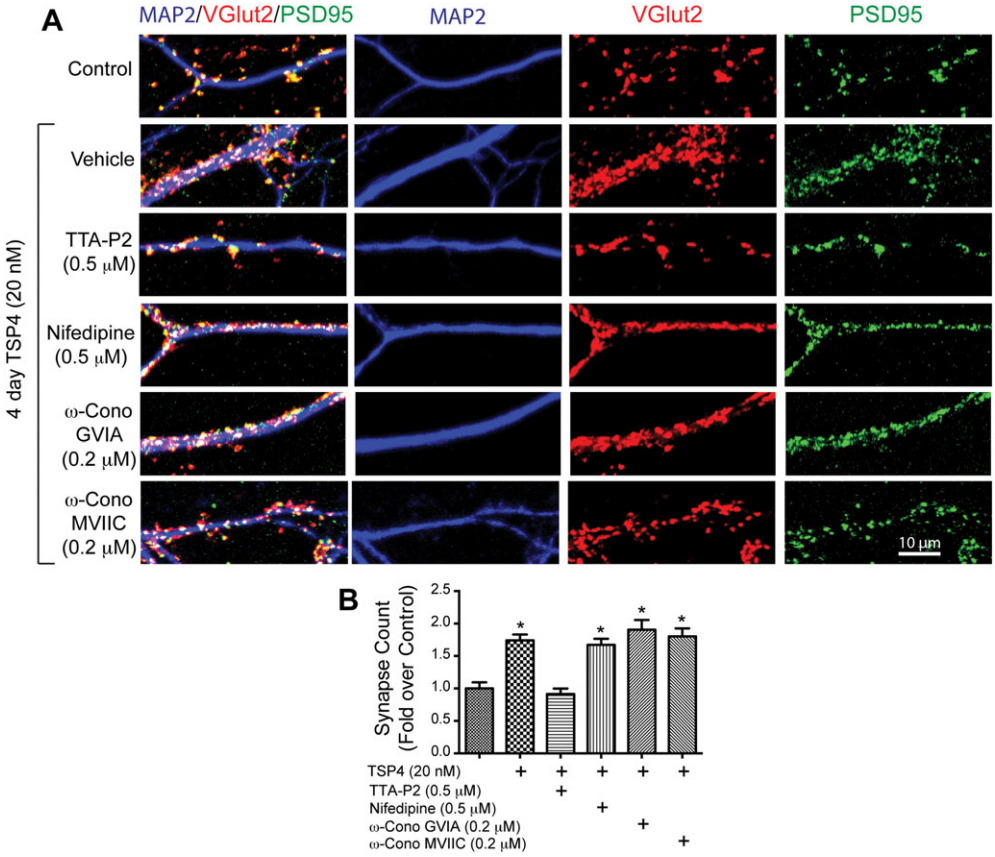

Figure 4

$Ca_v\alpha_2\delta_1$/TSP4-mediated excitatory synaptogenesis through a T-type VGCC sensitive pathway. (A) Representative images showing immunoreactivities to dendritic and synaptic markers in DRG/spinal cord neuron co-cultures in the presence or absence of TSP4 (20 nM) and with or without indicated VGCC blockers for 4 days. Scale bar = 10 μm. (B) Summarized data showing that only T-type VGCC blocker TTA-P2, but not any other VGCC blockers tested, could block $Ca_v\alpha_2\delta_1$/TSP4-mediated excitatory synaptogenesis. Means ± SEM from 20 neurons per group that were randomly selected from four wells of multiple culture plates of independent experiments. *$P < 0.05$ significantly different from control (no TSP4 treatment); non-parametric Kruskal–Wallis test with multiple comparisons.

treatment for 2 days after the TSP4 injection failed to block TSP4-induced synaptogenesis and behavioural hypersensitivity (Figure 5A, C, D). This suggests that activation of the $Ca_v\alpha_2\delta_1$/TSP4 pathway plays a critical role in the initiation, but not maintenance, of excitatory synapses in vivo, and blocking TSP4-induced synaptogenesis by early low-dose gabapentin treatments can prevent the development of behavioural hypersensitivity. Blockade of injury-induced synaptogenesis and behavioural hypersensitivity by early treatment with low-dose gabapentin has also been reported in an injury-induced joint pain model (Crosby *et al.*, 2015).

Discussion

Using a co-culture system of sensory and spinal cord neurons that models the *in vivo* environment for dorsal spinal cord synapse formation between sensory neurons and spinal cord neurons, we have shown here that presynaptic, but not postsynaptic, $Ca_v\alpha_2\delta_1$ subunits play a critical role in promoting excitatory synaptogenesis between sensory neurons and spinal cord neurons by interacting with synaptogenic TSP4. This process was dependent on T-type VGCC. Early, but not delayed, low-dose gabapentin treatment blocked $Ca_v\alpha_2\delta_1$/TSP4-mediated excitatory synaptogenesis *in vitro* and *in vivo*, and pain state development *in vivo*, supporting a preventive action of gabapentin in blocking synaptogenesis and development of chronic pain states.

Role of $Ca_v\alpha_2\delta_1$ subunits in excitatory synaptogenesis between sensory and spinal cord neurons

It is not clear if normal expression of $Ca_v\alpha_2\delta_1$ and TSP4 under physiological conditions contributes to normal synapse formation and behavioural sensitivity. Our findings are more applicable to pathological conditions in which up-regulation of $Ca_v\alpha_2\delta_1$ and/or TSP4 can promote abnormal excitatory synaptogenesis and synaptic neurotransmission. Noticeable pathological conditions leading to up-regulation of $Ca_v\alpha_2\delta_1$ and/or TSP4 include peripheral nerve injuries (Newton *et al.*, 2001; Luo *et al.*, 2002; Li *et al.*, 2004), trigeminal nerve injuries (Li *et al.*, 2014a; Li *et al.*, 2014b), diabetic neuropathy

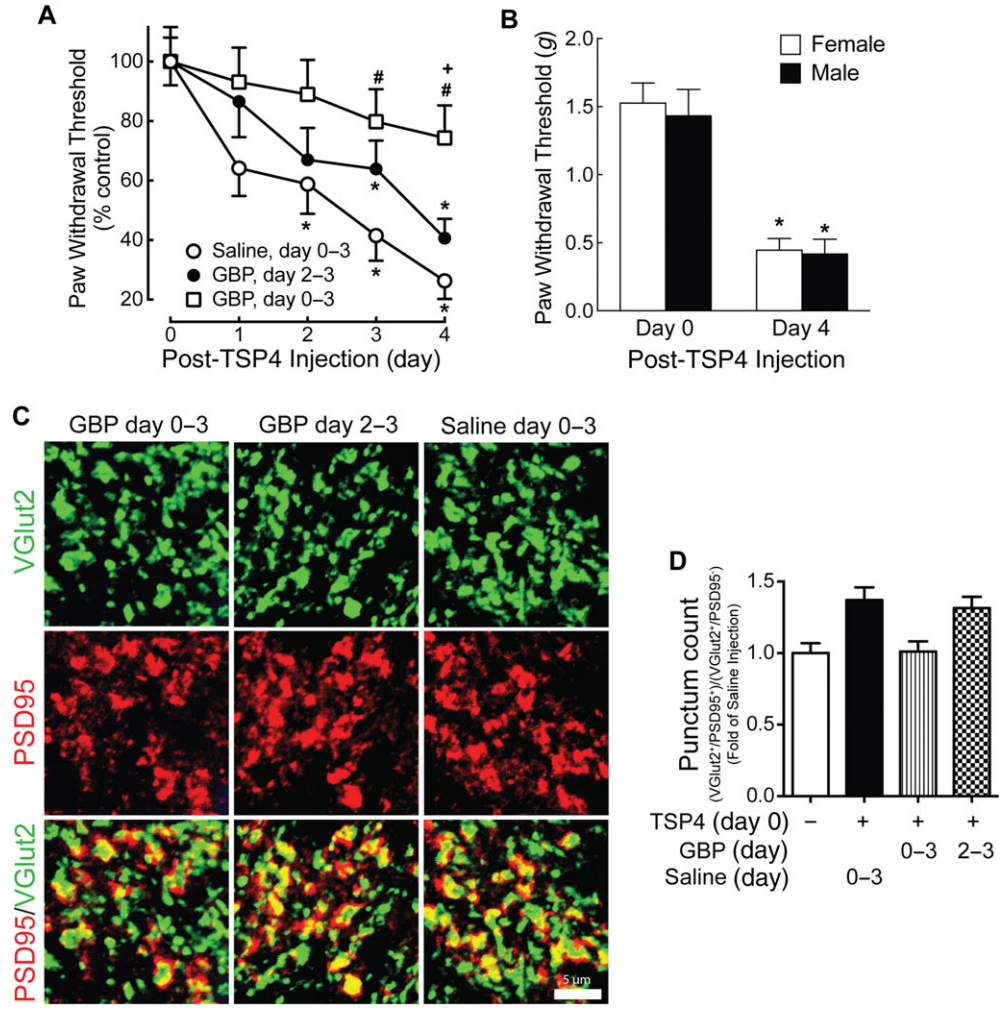

Figure 5

Early, but not delayed, i.t. gabapentin treatment can block TSP4-induced excitatory synaptogenesis in dorsal spinal cord and behavioural hypersensitivity development. (A) Summarized data of hindpaw mechanical sensitivity to daily von Frey filament stimulation after bolus TSP4 injection (day 0, 5 μg per mouse, i.t.) and early (day 0–3) or delayed (day 2–3) daily gabapentin (GBP; 25 μg per mouse, i.t.) treatments. Means ± SEM from eight mice for each treatment group. $^{\#}P < 0.05$, significantly different from the saline treatment group on the same day; $^{+}P < 0.05$, significantly different from the delayed gabapentin treatment group in the same day; $^{*}P < 0.05$, significantly different from pre-TSP4 injection baseline value of each group; two-way ANOVA with Tukey's multiple comparisons test. (B) Hindpaw mechanical sensitivity to von Frey filament stimulation before (day 0) and 4 days after bolus TSP4 injection (5 μg per mouse, i.t.) in adult male or female mice. Means ± SEM from five mice each group. $^{*}P < 0.05$ significantly different from pre-TSP4 injection baseline values at day 0; two-way ANOVA with Sidak's multiple comparisons test. (C) Representative images from dorsal spinal cord samples showing immunoreactivities to synaptic markers 4 days after bolus TSP4 injection with or without early (day 0–3) or delayed (day 2–3) daily gabapentin (25 μg per mouse, i.t.) treatments. Scale bar = 5 μm. (D) Summarized synaptic counts quantified as ratio of VGlut2^{+}/PSD95^{+} over VGlut2^{+}/PSD95^{-} punctum counts. Means ± SEM from 36 images over three animals per treatment group.

(Luo *et al.*, 2002), chemotherapy-induced neuropathy (Luo *et al.*, 2002; Xiao *et al.*, 2007), spinal cord contusion injury (Boroujerdi *et al.*, 2011; Zeng *et al.*, 2013) and mechanical joint injury (Crosby *et al.*, 2015). All these conditions can lead to chronic pain state development. Our previous findings support that abnormal synaptogenesis resulting from elevated $Ca_v\alpha_2\delta_1$ and/or TSP4 expression in the sensory pathway underlies the development of chronic pain states (Park *et al.*, 2016). Based on data presented here from our *in vitro* model, presynaptic $Ca_v\alpha_2\delta_1$ interacts with spinal cord TSP4 to promote excitatory synaptogenesis through a T-type VGCC-dependent mechanism, which in turn may cause dorsal horn neuron sensitization and ultimately underlie the development of behavioural hypersensitivity. As gabapentin was only effective in blocking synaptogenesis when it was administered at the same time as TSP4, but not started 2 days later, it is likely that direct or indirect interactions between TSP4 and $Ca_v\alpha_2\delta_1$ that could be affected by gabapentin binding to $Ca_v\alpha_2\delta_1$ subunits play a critical role in the initial formation, but not maintenance, of excitatory synapses. This is supported by the recent findings that mutations in the TSP4-binding domain of $Ca_v\alpha_2\delta_1$ protein block the effects of TSP4 on gabapentin binding to $Ca_v\alpha_2\delta_1$ subunits (Lana *et al.*, 2016). Interactions of TSP4/$Ca_v\alpha_2\delta_1$ at the presynaptic

level, however, may require tissue-type-specific or cell-type-specific co-factors, as *in vitro* co-transfection experiments could not detect extracellular TSP4/$Ca_v\alpha_2\delta_1$ interactions (Lana *et al.*, 2016).

Potential role of T-type VGCC on $Ca_v\alpha_2\delta_1$/TSP4-mediated synaptogenesis

Because application of TSP4 to isolated DRG neurons enhances T-type VGCC currents but reduces HVA calcium channel currents that require the presence of $Ca_v\alpha_2\delta_1$ subunits (Pan *et al.*, 2016), it is likely that TSP4 is a modulator for VGCC functions in DRG neurons through acting on $Ca_v\alpha_2\delta_1$ subunits (Pan *et al.*, 2016). As it is generally accepted that ancillary subunits, including $Ca_v\alpha_2\delta_1$, only associate with and modulate HVA calcium channels, but it is not so clearly defined for LVA calcium channels (Dolphin *et al.*, 2013), it is possible that activation of the $Ca_v\alpha_2\delta_1$/TSP4 pathway modulates HVA and LVA calcium channel functions through distinct mechanisms. Findings from the current study further demonstrate that TSP4-induced T-type calcium channel activity may contribute to abnormal synapse formation because only T-type but not L-type, N-type and P/Q-type calcium channel blockers can prevent TSP4-induced excitatory synaptogenesis. Further detailed investigations are required to determine the contribution of other calcium channels, such as the R type, and subtypes of T-type calcium channels on TSP4-induced synaptogenesis.

How increased T-type VGCC conductance contributes to increased excitatory synaptogenesis remains to be established. It has been reported that TSP4 modulates T-type calcium channel currents through the $Ca_v\alpha_2\delta_1$ subunits as TSP4-induced T-type calcium channel currents and intracellular calcium can be blocked by the $Ca_v\alpha_2\delta_1$ ligand gabapentin (Pan *et al.*, 2016). In addition, TSP4 modulates intracellular calcium signalling through $Ca_v\alpha_2\delta_1$ subunits as that can be blocked by genetic ablation of $Ca_v\alpha_2\delta_1$ (Guo *et al.*, 2017). These data support that TSP4 modulates T-type calcium channel activities through $Ca_v\alpha_2\delta_1$, which may directly or indirectly interact with T-type calcium channels. Further detailed investigations are needed to distinguish between these possibilities. Nevertheless, activation of presynaptic T-type VGCCs and the associated increase of intracellular calcium can mediate spontaneous synaptic release of glutamate (Jacus *et al.*, 2012). These changes may increase postsynaptic modulation and prime the postsynaptic density for maturation or remodelling. Alternatively, increased calcium influx through T-type VGCCs at the presynaptic terminals could activate transcriptional pathways, leading to changes in expression of factors required for synapse maturation. These hypotheses are supported by recent findings that presynaptic $Ca_v\alpha_2\delta$ is required for trans-synaptic homeostatic modulation, which relies on its interactions with other proteins (Wang *et al.*, 2016). If T-type VGCCs are involved in the synaptogenic activity of TSP4, then the TSP4-induced increase of T-type calcium channel currents at presynaptic terminals, as that reported in DRG neurons (Pan *et al.*, 2016), could facilitate the formation, increase the excitability of the presynaptic terminals or promote stabilization and maturation of functional synapses. The finding that absence of TSPs leads to increased mismatch of presynaptic and postsynaptic components (Christopherson *et al.*, 2005) supports a role of TSP4 in stabilization and maturation, but not formation, of synapses. However, we cannot exclude the possibility that TSP4 mediates presynaptic and postsynaptic stabilization and maturation *via* a mechanism independent of T-type VGCC.

The T-type VGCC blocker TTA-P2 has been reported to have short-term analgesic properties in formalin-induced and diabetic neuropathy-induced pain models (Choe *et al.*, 2011). Furthermore, T-type VGCCs have been implicated in synaptogenesis (Ikeda *et al.*, 2003; Jacus *et al.*, 2012; Todorovic and Jevtovic-Todorovic, 2013), which could be modulated by a $Ca_v\alpha_2\delta_1$/TSP4-related pathway as shown in this study, at least in neuropathic pain models with increased $Ca_v\alpha_2\delta_1$/TSP4 expression in the sensory pathway (Li *et al.*, 2014b; Park *et al.*, 2016). These findings provide mechanistic insights into the proposed mechanism of $Ca_v\alpha_2\delta_1$/TSP4-induced formation of abnormal synapses following peripheral nerve injuries (Gong *et al.*, 2017). It is possible that TTA-P2 may have both an acute analgesic effect, which is mediated by a mechanism independent of synapse formation, and a chronic anti-synaptogenic effect, which involves blocking TSP4-induced changes in T-type VGCCs. This is consistent with findings from a recent study that TSP4 only modulates VGCC currents in sensory neurons after chronic, but not acute, exposure (Pan *et al.*, 2016), which supports a chronic mechanism of action of TSP4 in modulating VGCC functions. Further studies with TTA-P2 treatments in animal models would be needed to test whether the observed TTA-P2 effects in blocking $Ca_v\alpha_2\delta_1$/TSP4-induced synaptogenesis *in vitro* translate into similar effects *in vivo*, which could provide valuable information towards new therapeutic options for pain management.

Preventing abnormal synaptogenesis by GBP

The translational values of our data include the finding that early, low-dose gabapentin treatment can prevent abnormal excitatory synaptogenesis and pain state development. After further preclinical and clinical validations, this could be an attractive approach for preventing development of chronic pain due to injury-induced up-regulation of $Ca_v\alpha_2\delta_1$ and/or TSP4 in the sensory pathway and abnormal synapse formation. There is already some evidence that this approach is effective in clinical practice. For instance, gabapentin was effective in blocking post-operative pain when administered before surgery (Field *et al.*, 1997; Clivatti *et al.*, 2009; Mardani-Kivi *et al.*, 2013; Ravindran, 2014; Hwang *et al.*, 2015). In some cases, pain relief can last for months (Brogly *et al.*, 2008; Khurana *et al.*, 2014), much longer than the half-life of gabapentin or its acute anti-hyperalgesic effects (Radulovic *et al.*, 1995; Gee *et al.*, 1996; Gordh *et al.*, 2008). It remains to be shown that blocking synaptogenesis contributes, at least partly, to the long-term mechanism of action of gabapentin. Even though gabapentin is a relatively safe drug, it does exhibit some unwanted side effects, including cognitive or gait impairment (Dworkin *et al.*, 2007). Treatment with low-dose gabapentin could further minimize its side effects. Based on our findings, there seems to be a critical time window to achieve gabapentin efficacy in blocking synaptogenesis, which may lead to prevention of chronic pain development after injury. Alternatively, revealing the mechanism

underlying the maintenance of synapses could lead to the development of novel drugs that could disrupt already formed aberrant synapses thus providing a wider therapeutic window for the management of chronic pain.

In conclusion, our findings indicate that interactions of presynaptic, but not postsynaptic, $Ca_v\alpha_2\delta_1$ subunits with TSP4 are required for initiation, but not maintenance, of excitatory synaptogenesis between sensory and spinal cord neurons. T-type calcium channels modulate this synaptogenic pathway. Early, but not delayed, low-dose treatment with gabapentin blocks this pathway and pain state development, which can be an alternative approach for preventing injury-induced synaptogenesis and neuropathic pain development after further validation.

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Author contributions

Y.P.Y. designed and performed experiments of co-cultures, synaptogenesis and confocal imaging, analysed and interpreted data, wrote the first draft and participated in editing of the manuscript. N.G., T.D.K. and B.V. performed *in vivo* behavioural pharmacology experiments and data analysis. Z.D.L. conceived, designed and supervised the study and contributed to data analysis, interpretation, drafting and editing of the paper.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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