



The EGF-LIKE domain of thrombospondin-4 is a key determinant in the development of pain states due to increased excitatory synaptogenesis

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Up-regulation of thrombospondin-4 (TSP4) or voltage-gated calcium channel subunit $\alpha_2\delta_1$ ($\text{Ca}_v\alpha_2\delta_1$) proteins in the spinal cord contributes to neuropathic pain development through an unidentified mechanism. We have previously shown that TSP4 interacts with $\text{Ca}_v\alpha_2\delta_1$ to promote excitatory synaptogenesis and the development of chronic pain states. However, the TSP4 determinants responsible for these changes are not known. Here, we tested the hypothesis that the $\text{Ca}_v\alpha_2\delta_1$ -binding domains of TSP4 are synaptogenic and pronociceptive. We mapped the major $\text{Ca}_v\alpha_2\delta_1$ -binding domains of TSP4 within the coiled-coil and epidermal growth factor (EGF)-like domains *in vitro*. Intrathecal injection of TSP4 fragment proteins containing the EGF-like domain (EGF-LIKE) into naïve rodents was sufficient for inducing behavioral hypersensitivity similar to that produced by an equal molar dose of full-length TSP4. Gabapentin, a drug that binds to $\text{Ca}_v\alpha_2\delta_1$, blocked EGF-LIKE-induced behavioral hypersensitivity in a dose-dependent manner, supporting the notion that EGF-LIKE interacts with $\text{Ca}_v\alpha_2\delta_1$ and thereby mediates behavioral hypersensitivity. This notion was further supported by our findings that a peptide within EGF-LIKE (EGFD355–369) could block TSP4- or $\text{Ca}_v\alpha_2\delta_1$ -induced behavioral hypersensitivity after intrathecal injections. Furthermore, only TSP4 proteins that contained EGF-LIKE could promote excitatory synaptogenesis between sensory and spinal cord neurons, which could be blocked by peptide EGFD355–369. Together, these findings indicate that EGF-LIKE is the molecular determinant that mediates aberrant excitatory synaptogenesis and chronic pain development. Blocking interactions between EGF-LIKE and $\text{Ca}_v\alpha_2\delta_1$ could be an alternative approach in designing target-specific pain medications.

Neuropathic pain, or pain states derived from injuries to the peripheral or central nervous systems, includes spontaneous

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pain and evoked pain such as tactile allodynia (exaggerated response to otherwise innocuous tactile stimuli) and hyperalgesia (exaggerated pain sensations to mildly noxious stimuli) (1, 2). Most current pain medications are not efficacious against neuropathic pain and are often associated with intolerable side effects. Identifying new targets and pathways involved in neuropathic pain processing is an unmet medical need that can provide mechanistic insights leading to development of target-specific pain medication. In the search for new targets/pathways critical in pain state development, our group and others have reported that peripheral nerve injury induces a parallel up-regulation of the astrocyte-secreted extracellular matrix protein thrombospondin-4 (TSP4)³ and voltage-gated calcium channel $\alpha_2\delta_1$ subunit ($\text{Ca}_v\alpha_2\delta_1$) in the dorsal root ganglia (DRG) and dorsal spinal cord that correlates with the development of behavioral hypersensitivities (3–9).

TSPs are large oligomeric, multidomain, extracellular matrix proteins that mediate cell/cell and cell/matrix interactions through binding to other extracellular matrix proteins, membrane proteins, and cytokines (10, 11). The TSP family consists of two subfamilies, subgroup A (TSP1 and -2) and subgroup B (TSP3–5), which are distinguished by their oligomerization states and domain structures. TSP4 has been shown to play a role in the development of neuropathic pain states. Intrathecal injection of TSP4 recombinant proteins into naïve rodent animals causes exaggerated presynaptic excitatory input into dorsal spinal cord and behavioral hypersensitivity (9, 12). TSP4 blockade by intrathecal treatments with antisense oligodeoxynucleotides, antibodies, or genetic ablation of the TSP4 gene reverses or prevents behavioral hypersensitivity induced by injuries to the peripheral and central nervous systems or intrathecal injection of TSP4 recombinant proteins (9, 13, 14).

Interestingly, TSP4-induced spinal cord neuron sensitization and behavioral hypersensitivity can be blocked by gabapentin, a gabapentinoid drug that binds to $\text{Ca}_v\alpha_2\delta_1$ (15, 16), suggesting that TSP4 induces spinal cord sensitization

³ The abbreviations used are: TSP4, thrombospondin-4; EGF, epidermal growth factor; Ca_v , voltage-gated calcium channel; EGF-LIKE, EGF-like domain; DRG, dorsal root ganglia; TG, transgenic; HEK, human embryonic kidney; i.t., intrathecal; VWF-A, von Willebrand factor A; NMDA, *N*-methyl-D-aspartate; DMEM, Dulbecco's modified Eagle's medium; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; HRP, horseradish peroxidase; PWT, paw withdrawal threshold; VGlut2, vesicular glutamate transporter 2; PSD95, postsynaptic density protein 95; MAP2, microtubule-associated protein 2; ANOVA, analysis of variance.

Synaptogenic and pronociceptive domains of TSP4

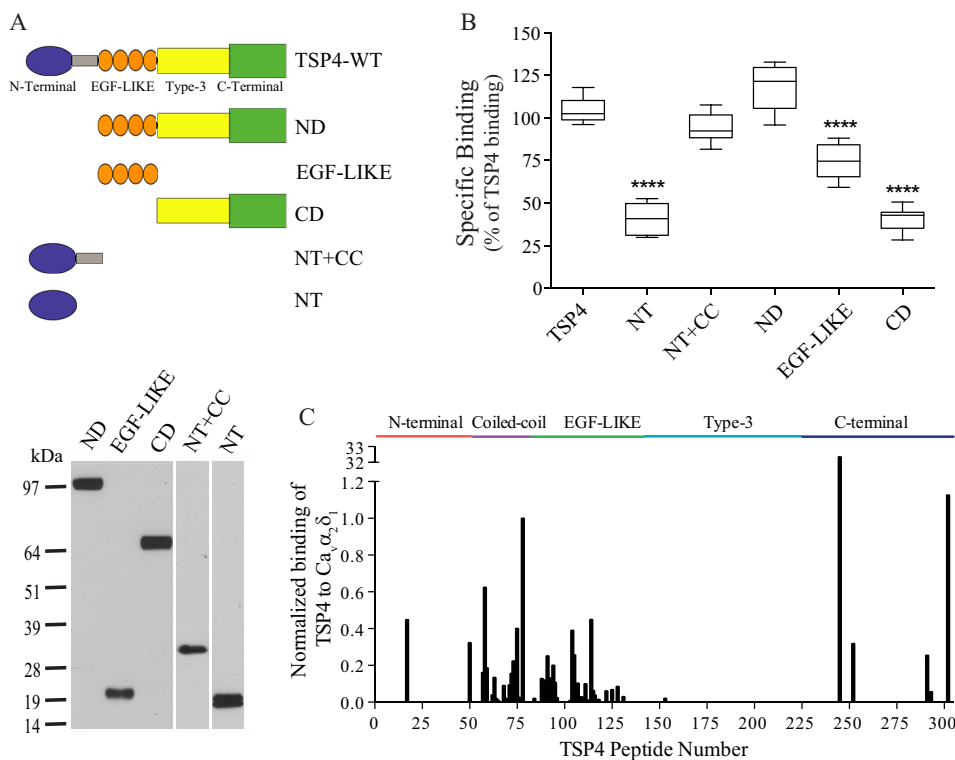


Figure 1. Identification of $\text{Ca}_v\alpha_2\delta_1$ -binding domains of TSP4. *A*, top, domain structure of full-length TSP4 and recombinant TSP4 truncation proteins. Shown are the N-terminal domain (blue), coiled-coil oligomerization domain (gray), four type II EGF-like domain repeats (orange), seven type-3 calcium-binding repeats (yellow), and the C-terminal domain (green). Bottom, representative Western blots under reducing conditions showing each purified recombinant TSP4 truncated protein detected by anti-His antibodies. The approximate molecular mass (kDa) is shown on the left. *B*, binding of FLAG- $\text{Ca}_v\alpha_2\delta_1$ to immobilized TSP4 or truncated TSP4 proteins at equal molar concentrations to TSP4 (80 $\mu\text{g}/\text{ml}$) in an ELISA-based ligand binding assay. Data presented in the box-and-whisker plot are from six to nine independent determinations; ****, $p < 0.0001$ compared with full-length TSP4 by one-way ANOVA with Bonferroni's multiple comparisons test. *C*, summary of normalized binding of $\text{Ca}_v\alpha_2\delta_1$ lysates to 15-mer peptides (overlapping by 12 amino acids) encompassing the entire TSP4 amino acid sequence. Summarized data are normalized to peptide 78.

and behavioral hypersensitivity through its interactions with $\text{Ca}_v\alpha_2\delta_1$ (12). Multiple lines of evidence also support this notion. Transgenic (TG) mice overexpressing $\text{Ca}_v\alpha_2\delta_1$ in neuronal tissues exhibit dorsal horn neuron hyperexcitability (17–19) and behavioral hypersensitivity (20) without nerve injury that can be blocked by TSP4 antibodies or viral or genetic ablation of TSP4 (12). In addition, genetic ablation of $\text{Ca}_v\alpha_2\delta_1$ or gabapentin treatment can block TSP4-induced spinal neuron sensitization and behavioral hypersensitivity (12, 21). Furthermore, TSP4 proteins interact with $\text{Ca}_v\alpha_2\delta_1$ to promote excitatory synaptogenesis in dorsal spinal cord that can be blocked by gabapentin or genetic ablation of $\text{Ca}_v\alpha_2\delta_1$ (12, 21). All these findings support that activation of a TSP4/ $\text{Ca}_v\alpha_2\delta_1$ pathway is critical in promoting central sensitization and pain states, at least partially through aberrant excitatory synaptogenesis (12, 21). Thus, blocking the activated TSP4/ $\text{Ca}_v\alpha_2\delta_1$ pathway could be an alternative approach in developing specific interventions for pain relief. However, both TSP4 and $\text{Ca}_v\alpha_2\delta_1$ are large proteins, so it is difficult to design small molecules or other drugs to block their interactions for pain relief without knowing the $\text{Ca}_v\alpha_2\delta_1$ -interacting and nociceptive domains of TSP4. Accordingly, we tested the hypothesis in this study that the $\text{Ca}_v\alpha_2\delta_1$ -interacting domains of TSP4 play a critical role in promoting behavioral hypersensitivity, at least partially through enhancing spinal cord excitatory synaptogenesis.

Results

Mapping $\text{Ca}_v\alpha_2\delta_1$ -binding motifs of TSP4

We have shown previously that injury-induced spinal TSP4 contributes to the development of neuropathic pain states (9) through a direct interaction with $\text{Ca}_v\alpha_2\delta_1$ proteins (12). To identify the TSP4-binding domain to $\text{Ca}_v\alpha_2\delta_1$, we created five recombinant truncation constructs of TSP4: the N-terminal domain deletion construct (ND) encoding the EGF-LIKE domains, type-3 calcium-binding domain, and C-terminal domain; the EGF-like domain construct (EGF-LIKE) encoding EGF-LIKE; the C-terminal domain construct (CD) encoding the type-3 calcium-binding domain and C-terminal domain; the N-terminal domain alone construct (NT); and the N-terminal domain plus the coiled-coil domain construct (NT+CC) (Fig. 1A, top). TSP4 mutant proteins from these constructs were expressed in the human embryonic kidney (HEK) cell line 293-EBNA and purified through His-tag columns (Fig. 1A, bottom). Binding of the recombinant TSP4 proteins to recombinant $\text{Ca}_v\alpha_2\delta_1$ proteins was tested in an ELISA-based ligand binding assay. Our data indicated that domain proteins containing EGF-LIKE (ND and EGF-LIKE) and coiled-coil domain of TSP4 bound to $\text{Ca}_v\alpha_2\delta_1$ with a similar or over 70% capacity as the full-length TSP4, whereas binding of domain proteins containing the N-terminal domain, type-3 calcium-binding domain, and C-terminal domain to $\text{Ca}_v\alpha_2\delta_1$ was about 35% of

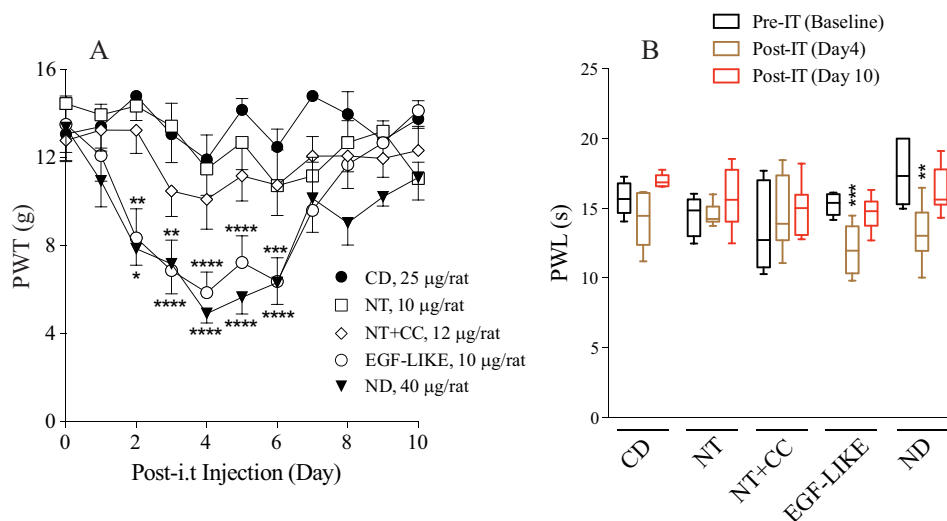


Figure 2. Induction of tactile allodynia and thermal hyperalgesia in naive rats after bolus intrathecal injection of TSP4 truncation proteins containing the EGF-LIKE domains. PWTs to mechanical stimulation (A) were tested daily, and paw withdrawal latencies (PWL) to thermal stimulation (B) were tested at days 4 and 10 after bolus i.t. injection of TSP4 truncated proteins at day 0 following baseline tests. A, data presented are the means from at least five rats in each group with error bars representing S.E. B, data presented in the box-and-whisker plot are from at least five rats in each group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ compared with pretreatment level in each group by two-way ANOVA with Tukey's multiple comparisons test in A and one-way ANOVA with Sidak's multiple comparisons test in B.

that observed for full-length TSP4 (Fig. 1B). Thus, the coiled-coil and EGF-LIKE domains of TSP4 likely contained the major $\text{Ca}_v\alpha_2\delta_1$ -binding sites.

We further examined the $\text{Ca}_v\alpha_2\delta_1$ -binding motifs of TSP4 with SPOT peptide array analysis by measuring binding of recombinant $\text{Ca}_v\alpha_2\delta_1$ purified from transfected HEK 293 cells to immobilized overlapping 15-mer peptides that covered the entire length of TSP4. Similar to data from protein binding assays, TSP4 linear peptides with high affinity to $\text{Ca}_v\alpha_2\delta_1$ were clustered in the coiled-coil and EGF-LIKE domains but not in the type-3 calcium-binding domain (Fig. 1C). A few peptides from the N-terminal and C-terminal domains also bound to $\text{Ca}_v\alpha_2\delta_1$, which might contribute to the low binding affinity to $\text{Ca}_v\alpha_2\delta_1$ observed from the NT and CD truncated proteins (Fig. 1, B and C).

EGF-LIKE domain of TSP4 is pronociceptive

Previous findings have shown that increased spinal TSP4 protein alone is sufficient to induce tactile allodynia and thermal hyperalgesia (9) through interactions with $\text{Ca}_v\alpha_2\delta_1$ (12). To identify the pronociceptive domain of TSP4, we injected the recombinant truncated TSP4 proteins into L5/6 spinal region of naive adult rats and tested their hind paw sensitivity to mechanical and thermal stimuli. Intrathecal (i.t.) injection of EGF-LIKE-containing proteins (EGF-like and ND), at an equal molar dose to the pronociceptive dose of full-length TSP4 (45 µg/rat) (9), induced a reduction in paw withdrawal thresholds to von Frey filaments (tactile allodynia) (Fig. 2A) and paw withdrawal latency to radiant heat (thermal hyperalgesia) (Fig. 2B). The pronociceptive effects of EGF-LIKE-containing proteins started within 2 days and lasted for about 1 week post-i.t. injection, similar to that induced by full-length TSP4 proteins (9). In contrast, neither lowering the dose of EGF-LIKE proteins to a molar dose (5 µg/rat) equivalent to the sub-pronociceptive dose of full-length TSP4 (20 µg/rat) (Fig. 3A) (9) nor injecting

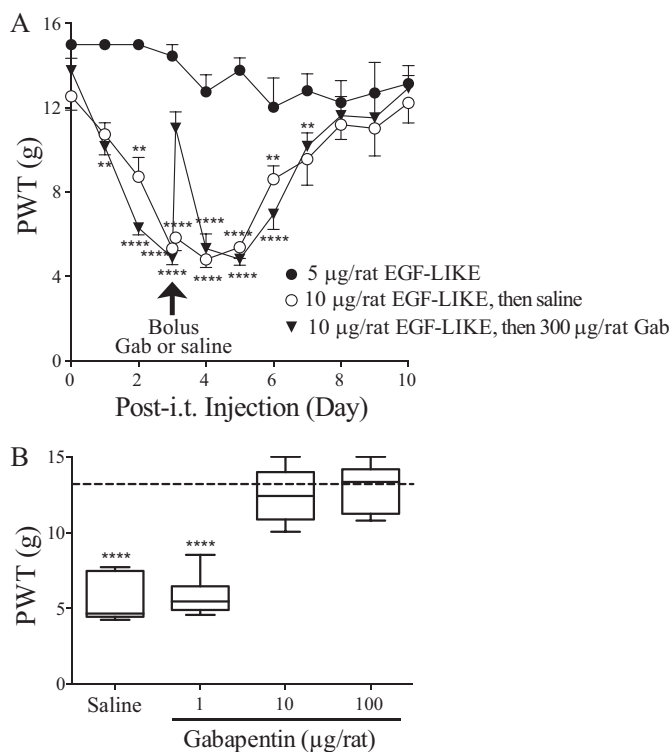


Figure 3. Gabapentin could block tactile allodynia induced by intrathecal injection of EGF-LIKE domain proteins of TSP4 into naive rats. A, daily PWTs to von Frey filament stimulation were tested blindly after bolus i.t. injection of EGF-LIKE proteins into naive rats at time 0. Bolus intrathecal gabapentin (Gab) (300 µg/rat) or saline was administered at day 3 postinjection of EGF-LIKE (indicated by the arrow) followed by behavioral tests after 1 h and then daily. Data presented are the means from six rats in each group with error bars representing S.E. **, $p < 0.01$; ****, $p < 0.0001$ compared with pretreatment level by repeated measures one-way ANOVA with Bonferroni's multiple comparisons test. B, dose-dependent effects of gabapentin 1 h after intrathecal injections in reversal of EGF-LIKE-induced tactile allodynia described in A. The dotted line represents the baseline level of PWT (before i.t. injections of EGF-LIKE). Data presented in the box-and-whisker plot are from five to seven rats in each group. ****, $p < 0.0001$ compared with pretreatment level by one-way ANOVA with Bonferroni's multiple comparisons test.

Synaptogenic and pronociceptive domains of TSP4

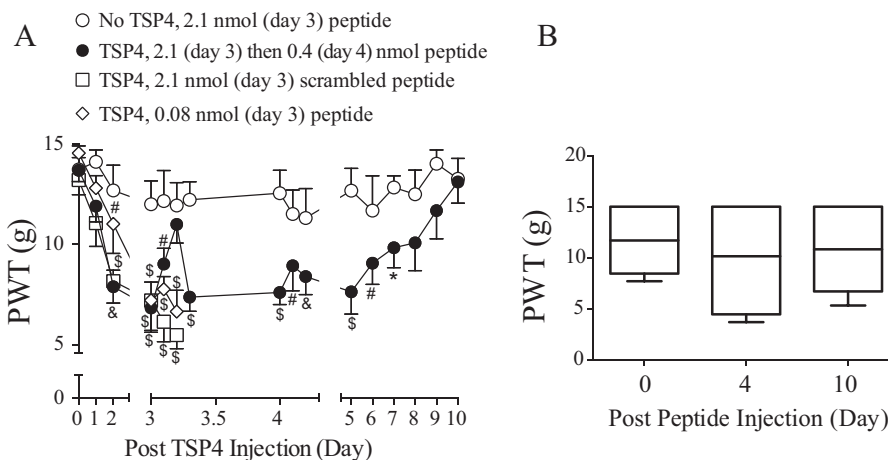


Figure 4. TSP4-induced tactile allodynia could be attenuated by intrathecal injection of peptide EGFD355–369 from the EGF-LIKE domain of TSP4. *A*, TSP4 proteins (45 μ g/rat; \sim 1.6 nM considering averaged volume of rat cerebrospinal fluid of 290 μ l (56)) or sterile saline were injected into the L5/6 spinal region of naïve rats at day 0 after baseline testing followed by daily behavioral tests using von Frey filaments. As shown previously (9), tactile allodynia peaked between 3 and 4 days post-TSP4 injection. Bolus peptide EGFD355–369 or its scrambled control peptide was administered at day 3 or 4 post-TSP4 injection as indicated followed by behavioral tests every 2 h for up to 6 h after peptide treatment. The peptide doses used were at 5 \times molar excess (2.1 nmol/rat), molar equivalent (0.4 nmol/rat), or 1/5 molar dose (0.08 nmol/rat) of the pronociceptive dose of TSP4 (45 μ g/rat). Data presented are the means from six to seven rats in each group with error bars representing S.E. *, $p < 0.05$; #, $p < 0.01$; &, $p < 0.001$; \$, $p < 0.0001$ compared with pretreatment level in each group by repeated measures two-way ANOVA with Dunnett's multiple comparisons test. *B*, PWTs to von Frey filament stimuli were tested blindly before, at day 4, and at day 10 after bolus i.t. injection of peptide EGFD355–369 (2.1 nmol/rat) into naïve rats. Data presented in the box-and-whisker plot are from seven rats in each group. 15 g was the maximum value assigned to a rat as described under "Experimental procedures."

recombinant truncated proteins without EGF-LIKE (CD, NT, and NT+CC) at a molar dose equivalent to the pronociceptive dose of full-length TSP4 (45 μ g/rat) (Fig. 2) (9) cause behavioral hypersensitivity. Similar to its anti-nociceptive effects post-TSP4 injection (12), $\text{Ca}_v\alpha_2\delta_1$ ligand gabapentin could dose-dependently reverse tactile allodynia induced by EGF-LIKE within an hour post-i.t. injection (Fig. 3, *A* and *B*). Together, these findings support that EGF-LIKE is the functional determinant of TSP4's pronociceptive effects, and its interaction with the $\text{Ca}_v\alpha_2\delta_1$ proteins is likely required for TSP4's pronociceptive effects.

Effects of intrathecal injection of peptide EGFD355–369 on behavioral hypersensitivities induced by elevated TSP4 and/or $\text{Ca}_v\alpha_2\delta_1$

Because EGF-LIKE is the pronociceptive domain, we tested whether a peptide within the EGF-LIKE domain of TSP4 could interfere with behavioral hypersensitivity induced by increased TSP4 and/or $\text{Ca}_v\alpha_2\delta_1$ *in vivo*. Based on the SPOT peptide array analysis (Fig. 1C), we identified peptide EGFD355–369 within EGF-LIKE as highly conserved in rodents and having high binding affinity to $\text{Ca}_v\alpha_2\delta_1$. This peptide was synthesized and injected i.t. into the L5/6 spinal region of TSP4-injected rats when the animals had severe allodynia (day 3 or 4 after bolus TSP4 injection). A blind behavioral sensitivity test to von Frey filament stimulation was performed daily after the TSP4 injection before and every 2 h for up to 6 h after each peptide injection. Our data indicated that peptide EGFD355–369, but not its scrambled peptide control, blocked TSP4-induced tactile allodynia dose-dependently. This effect lasted for >6 h (Fig. 4A). Bolus injection of peptide EGFD355–369 alone into naïve rats at the highest dose tested (2.1 nmol/rat) did not cause significant changes in baseline behavioral sensitivity acutely (Fig. 4A) or chronically (Fig. 4B). These data support that peptide EGFD355–369 can block TSP4-induced tactile allodynia.

To confirm that the anti-nociceptive effects of peptide EGFD355–369 were mediated by interfering with the activation of a pathway due to TSP4/ $\text{Ca}_v\alpha_2\delta_1$ interactions, we examined its effect on mechanical allodynia in a $\text{Ca}_v\alpha_2\delta_1$ TG mouse line with increased neuronal $\text{Ca}_v\alpha_2\delta_1$ overexpression (20). This model has been shown to enhance excitatory synaptic transmission in dorsal spinal cord that contributes to behavioral hypersensitivity (12, 17–20). Our data indicated that intrathecal injection of the effective dose (0.047 nmol/mouse) of peptide EGFD355–369, but not its scrambled peptide, could reverse allodynia in the $\text{Ca}_v\alpha_2\delta_1$ TG mice with an onset time of 2 h and a duration <6 h (Fig. 5). These data indicate that peptide EGFD355–369 can attenuate tactile allodynia induced by $\text{Ca}_v\alpha_2\delta_1$, most likely through its interactions with TSP4 (12).

EGF-LIKE domain of TSP4 is synaptogenic

To determine whether EGF-LIKE induces excitatory synaptogenesis that may underlie its nociceptive effects, we examined the synaptogenic effects of TSP4 domain proteins derived from the constructs shown in Fig. 1 in cocultures of DRG and spinal cord neurons. Treatments of cocultures with an equal molar concentration of recombinant proteins containing EGF-LIKE, such as that encoded by full-length TSP4, ND, and EGF-LIKE domain constructs, resulted in significant increases of excitatory synapse numbers compared with control (PBS treatment). There was no significant difference in synaptic numbers among treatments with these EGF-LIKE-containing proteins (Fig. 6). In contrast, treatments with proteins lacking EGF-LIKE, such as those encoded by the CD and NT+CC constructs, did not induce significant excitatory synapse formation compared with control (Fig. 6). These data indicate that EGF-LIKE is synaptogenic and promotes excitatory synapse formation between sensory neurons and spinal cord neurons.

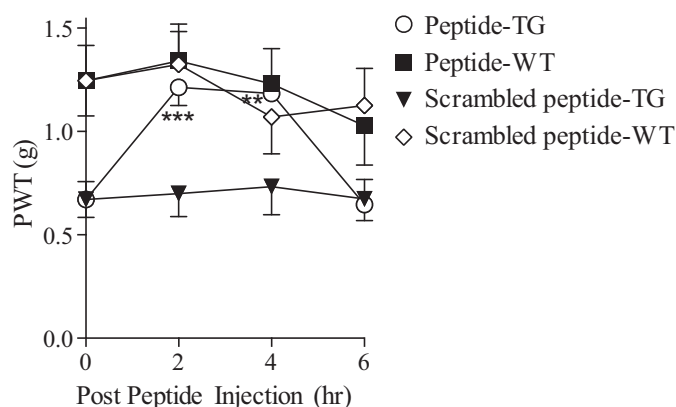


Figure 5. $\text{Ca}_v\alpha_2\delta_1$ -induced tactile allodynia could be attenuated by intrathecal injection of peptide EGFD355–369. A bolus i.t. injection of peptide EGFD355–369 or its scrambled control peptide (0.047 nmol/mouse) at an equal molar dose to the pronociceptive dose of TSP4 (5 μg /mouse) was administered to $\text{Ca}_v\alpha_2\delta_1$ -overexpressing TG mice and their age- and sex-matched WT littermates at time 0 followed by a blind behavioral test for PWTs to von Frey filament stimuli at 2, 4, and 6 h after bolus peptide treatments. Data presented are the means from 10 mice in each group with error bars representing S.E. **, $p < 0.01$; ***, $p < 0.001$ compared with pretreatment level by repeated measures one-way ANOVA with Bonferroni's multiple comparisons test.

EGFD355–369 can block synaptogenesis induced by EGF-LIKE domain of TSP4

To determine whether peptide EGFD355–369 could block excitatory synaptogenesis induced by EGF-LIKE, we examined the effects of peptide EGFD355–369 on excitatory synaptogenesis induced by EGF-LIKE in the cocultures. Similar to that shown in Fig. 6, treatments with EGF-LIKE resulted in a significant increase of excitatory synapses compared with the control. Concurrent treatments with peptide EGFD355–369, but not the scrambled control peptide, blocked the increase of synapse formation induced by EGF-LIKE (Fig. 7). These data support that peptide EGFD355–369 can block the synaptogenic effects of EGF-LIKE.

Discussion

Peripheral nerve injury induces concurrent up-regulation of TSP4 and $\text{Ca}_v\alpha_2\delta_1$ in dorsal spinal cord (5, 9), and TSP4/ $\text{Ca}_v\alpha_2\delta_1$ interaction promotes excitatory synaptogenesis and chronic pain states (12). To gain molecular insight into these processes, here we have identified the $\text{Ca}_v\alpha_2\delta_1$ -binding motifs of TSP4 and that EGF-LIKE is prosynaptogenic and nociceptive. Administration of gabapentin blocks behavioral hypersensitivity induced by EGF-LIKE. Administration of the EGFD355–369 peptide from within the EGF-LIKE motif blocks excitatory synaptogenesis induced by EGF-LIKE as well as tactile allodynia induced by elevated TSP4 or $\text{Ca}_v\alpha_2\delta_1$. Together, our findings support that EGF-LIKE is a functional determinant in promoting aberrant excitatory synaptogenesis and sensory hypersensitivity resulting from activation of a TSP4/ $\text{Ca}_v\alpha_2\delta_1$ pathway.

Multidomain interactions between TSP4 and $\text{Ca}_v\alpha_2\delta_1$

We used SPOT peptide synthesis method and an ELISA-based binding assay to map the linear and discontinuous binding sites of TSP4 to $\text{Ca}_v\alpha_2\delta_1$. Our data indicate that most TSP4

linear peptides with relatively high binding affinity to $\text{Ca}_v\alpha_2\delta_1$ are located within the coiled-coil and EGF-LIKE domains, but not the type-3 calcium-binding domain, of TSP4. However, a few discontinuous peptides from the TSP4 N-terminal and C-terminal domains also bind to $\text{Ca}_v\alpha_2\delta_1$ with high affinity and may be responsible for the low-affinity binding of TSP4 truncated N- or C-terminal domain proteins to $\text{Ca}_v\alpha_2\delta_1$. Thus, the binding interaction between TSP4 and $\text{Ca}_v\alpha_2\delta_1$ is likely mediated through multidomain interactions, which may lead to subsequent conformational changes of the binding proteins. Recent data from a cotransfection study suggest that TSP4/ $\text{Ca}_v\alpha_2\delta_1$ interactions occur intracellularly (22). Therefore, the exact location of TSP4/ $\text{Ca}_v\alpha_2\delta_1$ interactions *in vivo* remains to be established.

Specific interactions between the TSP4 EGF-LIKE domain and $\text{Ca}_v\alpha_2\delta_1$ are critical in TSP4-induced behavioral hypersensitivity

Functionally, we show that only truncated TSP4 proteins containing EGF-LIKE promote behavioral hypersensitivities like full-length TSP4 proteins. Intriguingly, those containing the N-terminal, coiled-coil, calcium-binding, and C-terminal domains fail to elicit behavioral hypersensitivity even though some of these proteins, such as that encoded by the NT+CC construct, have similar $\text{Ca}_v\alpha_2\delta_1$ binding affinity as full-length TSP4. Thus, multidomain-binding interactions between TSP4 and $\text{Ca}_v\alpha_2\delta_1$ may facilitate specific interactions between EGF-LIKE and $\text{Ca}_v\alpha_2\delta_1$, which are critical in mediating behavioral hypersensitivity. It is reported that EGF-LIKE interacts with von Willebrand factor A (VWF-A) domain of $\text{Ca}_v\alpha_2\delta_1$ in promoting central nervous system excitatory synaptogenesis (23); a similar mechanism in spinal cord can lead to behavioral hypersensitivity. Because gabapentin binds to a site upstream of the VWF-A domain of $\text{Ca}_v\alpha_2\delta_1$ (24), it is less likely that the inhibitory effects of gabapentin on EGF-LIKE-induced behavioral hypersensitivity are mediated through direct blockage of TSP4 binding to $\text{Ca}_v\alpha_2\delta_1$. Instead, binding of small molecules such as gabapentin to $\text{Ca}_v\alpha_2\delta_1$ may cause conformational changes of the protein that may interfere with TSP4/ $\text{Ca}_v\alpha_2\delta_1$ interactions. This is supported by recent findings that TSP4 binding to $\text{Ca}_v\alpha_2\delta_1$ can reduce gabapentin binding to $\text{Ca}_v\alpha_2\delta_1$, but this effect is diminished with mutations in the VWF-A domain of $\text{Ca}_v\alpha_2\delta_1$ (22).

We hypothesized that a peptide from the EGF-LIKE sequence might interfere with EGF-LIKE/ $\text{Ca}_v\alpha_2\delta_1$ interactions, thereby blocking TSP4/ $\text{Ca}_v\alpha_2\delta_1$ pathway activation and attenuating behavioral hypersensitivity. To test this, we designed peptide EGFD355–369 and tested its effects in blocking behavioral hypersensitivity induced by elevated TSP4 and/or $\text{Ca}_v\alpha_2\delta_1$. Intrathecal injection of this peptide can block tactile allodynia induced by intrathecal TSP4 injection in rats and $\text{Ca}_v\alpha_2\delta_1$ overexpression in transgenic mice without affecting baseline behavioral sensitivity in naive animals. Therefore, peptide EGFD355–369 can block behavioral hypersensitivity induced by elevated TSP4 or $\text{Ca}_v\alpha_2\delta_1$ alone. Its anti-nociceptive effects are specific because neither a high-affinity binding peptide from the coiled-coil domain (data not shown) nor the scrambled control peptide has any functional effect in blocking

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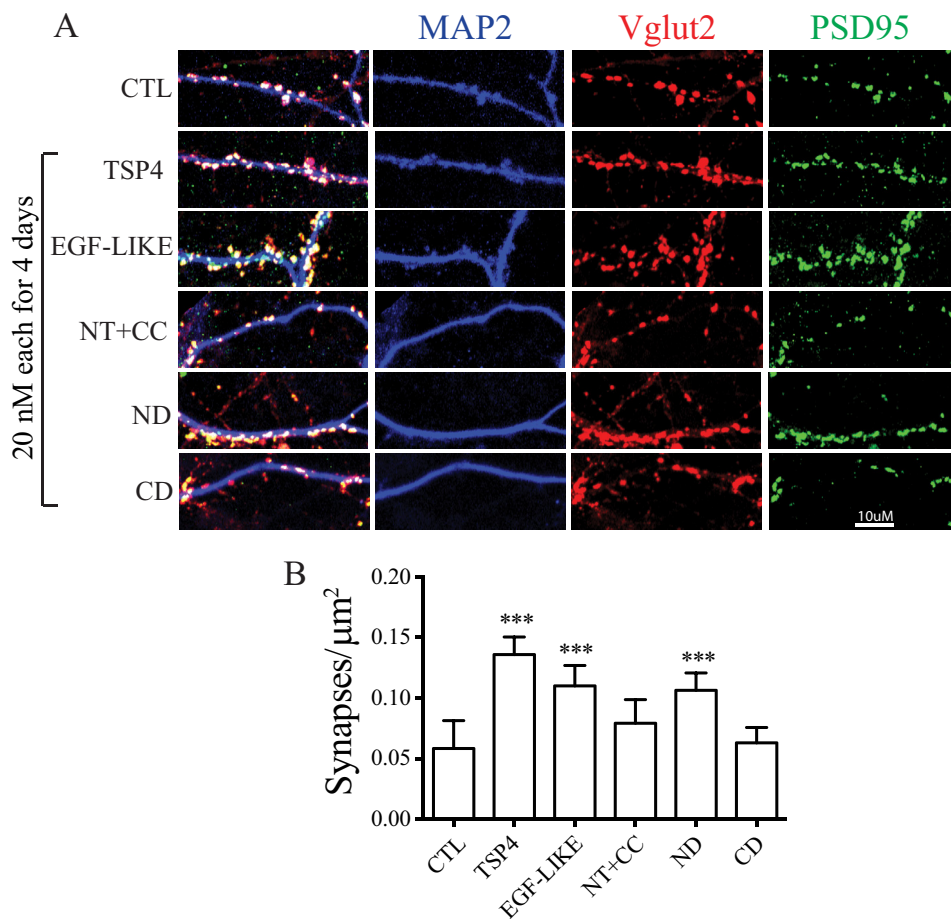


Figure 6. TSP4 EGF-LIKE domain proteins promote synapse formation between sensory neurons and spinal cord neurons. Synaptogenesis analysis was performed in cocultures of DRG and spinal cord neurons after treatment for 4 days with PBS (CTL) or an equal molar concentration of either full-length or different domain proteins of TSP4 in multiple-well plates of independent experiments. *A*, representative confocal images showing immunoreactivities to antibodies of synaptic markers (Vglut2, red; PSD95, green) along spinal neuron dendrites (MAP2, blue). Puncta of colocalized synaptic marker immunoreactivity were quantified as synapse numbers. *B*, summarized synapse numbers in cocultures after treatment with TSP4 or its domain proteins. Data presented are the means from 20 neurons per group of three independent experiments with error bars representing 95% confidence interval. ***, $p < 0.001$ compared with control by repeated measures one-way ANOVA with a Dunnett's post hoc test.

nociception. Because intrathecal injection of EGF-LIKE-containing proteins (TSP4, ND, and EGF-LIKE) in naïve animals is pronociceptive but that of EGF-LIKE linear peptides alone is not, our data suggest that the nociceptive effect of TSP4 may require multiple binding sites and an optimal protein interface upon its interactions with $\text{Ca}_v\alpha_2\delta_1$.

Potential mechanisms underlying TSP4/ $\text{Ca}_v\alpha_2\delta_1$ -induced pain transduction and drug actions

Bolus injection of peptide EGFD355–369 blocks $\text{Ca}_v\alpha_2\delta_1$ -induced tactile allodynia, confirming the requirement of a basal level of TSP4 for the pronociceptive effects of elevated $\text{Ca}_v\alpha_2\delta_1$ (12). Enhanced excitatory transmission and behavioral hypersensitivity induced by $\text{Ca}_v\alpha_2\delta_1$ overexpression in neuronal cells can be attenuated by gabapentin (20) through inhibiting $\text{Ca}_v\alpha_2\delta_1$ and voltage-gated calcium channel trafficking to presynaptic axon terminals of nerve-injured DRG neurons in the dorsal horn (25, 26). Future studies to explore whether peptide EGFD355–369 could modulate excitatory neurotransmission and reduce $\text{Ca}_v\alpha_2\delta_1$ trafficking to the presynaptic sites in dorsal spinal cord would shed some light on the mechanistic contri-

bution of TSP4 to behavioral hypersensitivity associated with $\text{Ca}_v\alpha_2\delta_1$.

Conversely, the ability of gabapentin to inhibit TSP4- (12) or EGF-LIKE-induced central sensitization and behavioral hypersensitivity highlights a critical role of the TSP4/ $\text{Ca}_v\alpha_2\delta_1$ pathway in mediating chronic pain states. Because gabapentin can block TSP-induced excitatory synapse formation (21, 23), dorsal horn neuron sensitization, and behavioral hypersensitivities (12), it is likely that activation of the TSP4/ $\text{Ca}_v\alpha_2\delta_1$ pathway by EGF-LIKE contributes to aberrant excitatory synaptogenesis, central sensitization, and pain states. This is supported by our recent findings that trigeminal nerve injury causes increased expression of $\text{Ca}_v\alpha_2\delta_1$ and TSP4 in dorsal spinal cord that correlates with aberrant excitatory synaptogenesis and enhances presynaptic excitatory input and neuropathic pain states (14, 27). Our findings reveal that only TSP4 proteins containing EGF-LIKE are synaptogenic and pronociceptive, but NT+CC domain proteins lacking EGF-LIKE are not even though they have similar $\text{Ca}_v\alpha_2\delta_1$ binding affinity as full-length TSP4. Together, these data support that specific interactions between EGF-LIKE and $\text{Ca}_v\alpha_2\delta_1$, but not multidomain-binding

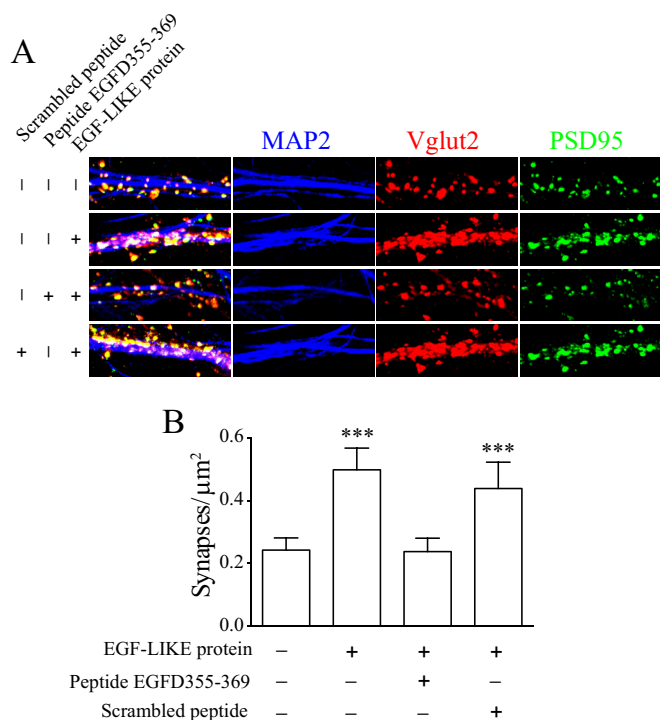


Figure 7. Synaptogenic effects of the TSP4 EGF-LIKE domain protein were blocked by peptide EGFD355–369. Synaptogenesis analysis was performed in cocultures of DRG and spinal cord neurons after treatment for 4 days with PBS (CTL) or 20 nM EGF-LIKE proteins in the absence or presence of an equal molar concentration of scrambled control peptide or peptide EGFD355–369 in multiple-well plates of independent experiments. *A*, representative confocal images showing immunoreactivities to antibodies of synaptic markers (Vglut2, red; PSD95, green) along spinal neuron dendrites (MAP2, blue). Puncta of colocalized synaptic marker immunoreactivity were quantified as synapse numbers. *B*, summarized synapse numbers in cocultures after each treatment. Data presented are the means from 13–17 neurons per group in each of three independent experiments with error bars representing 95% confidence interval. ***, $p < 0.001$ compared with control by repeated measures one-way ANOVA with a Dunnett's post hoc test.

interactions between TSP4 and $\text{Ca}_v\alpha_2\delta_1$, are critical in mediating synaptogenesis and behavioral hypersensitivity.

Our findings reveal that gabapentin and peptide EGFD355–369 can block excitatory synaptogenesis and behavioral hypersensitivity induced by EGF-LIKE without affecting basal level values, supporting that aberrant synapse formation induced by interactions of EGF-LIKE with $\text{Ca}_v\alpha_2\delta_1$ contributes to behavioral hypersensitivity. Interestingly, both gabapentin and peptide EGFD355–369 have a rapid onset time (within 2 h) and short duration (<4 h) in blocking pain states. This time frame coincides with that of new excitatory synapse formation *in vitro* and *in vivo*. For example, new excitatory synapses can be assembled *in vitro* within 1–2 h of initial axodendritic contact (28, 29), which correlates with the time for new synaptic spine formation after high frequency stimulation (30–32). *In vivo* data indicate that activity-dependent synaptic stabilization and elimination are dynamic processes that can occur as rapidly as <30 min to 2 h poststimulation (33, 34). These rapidly assembled excitatory synapses are initiated by presynaptic remodeling through recruitment and stabilization of presynaptic packets containing calcium channel subunits (28). Thus, it is possible that binding of TSP4, or EGF-LIKE, proteins to $\text{Ca}_v\alpha_2\delta_1$ promotes the assembly/recruitment of presynaptic protein precursors

and/or stabilization of presynaptic remodeling after nerve injury that allows a rapid initiation of new excitatory synapse formation. Supportive findings include that injury-induced $\text{Ca}_v\alpha_2\delta_1$ proteins are translocated from injured DRG neurons to their presynaptic terminals in dorsal spinal cord (5, 26) and play a role in axonal trafficking and presynaptic assembly (26, 35). This in turn can lead to increased presynaptic excitatory input into dorsal horn neurons (18, 19, 35) that can accelerate stimulation-dependent aberrant synapse formation (30–32). Gabapentin or peptide EGFD355–369 can bind to $\text{Ca}_v\alpha_2\delta_1$, likely induce conformational changes of target proteins, and prevent rapid aberrant synaptogenesis initiated by TSP4/ $\text{Ca}_v\alpha_2\delta_1$ interactions, leading to pain state reversal. This is supported by our recent findings that only early gabapentin treatment can prevent TSP4-induced excitatory synaptogenesis and behavioral hypersensitivity (21).

The transient effects of gabapentin and EGFD355–369 in reversing pain state may be due to the short half-life of these agents *in vivo* because gabapentin has a mean elimination half-life about 2 h in rats (36), and the half-life of peptides in rats can range from minutes to 2 h (37, 38). Modifications to improve the stability and pharmacokinetics of these agents may provide better therapeutic values. However, we cannot rule out the possibility that the effects of gabapentin and the peptide are independent from its binding to $\text{Ca}_v\alpha_2\delta_1$ –TSP4 complexes. Detailed studies using mutational analyses across $\text{Ca}_v\alpha_2\delta_1$ and/or EGF-LIKE should provide further mechanistic insights. A recent study reports that gabapentin's anti-nociceptive effects could be mediated by its selective inhibition of $\text{Ca}_v\alpha_2\delta_1$ -bound NMDA receptors on the cell membrane surface (39). Because TSP4 and $\text{Ca}_v\alpha_2\delta_1$ also form complexes in spinal cord (12), it will be interesting to investigate whether TSP4 plays a contributory role to events associated with $\text{Ca}_v\alpha_2\delta_1$ –NMDA receptor complex formation.

Summary and conclusion

In summary, our findings demonstrate that EGF-LIKE is the molecular determinant in mediating excitatory synaptogenesis and behavioral hypersensitivity and requires its interaction with $\text{Ca}_v\alpha_2\delta_1$. Blocking EGF-LIKE/ $\text{Ca}_v\alpha_2\delta_1$ interaction can be an alternative approach in designing target-specific medications for neuropathic pain management.

Experimental procedures

Construction of recombinant TSP4 truncated cDNA

The recombinant truncated TSP4 constructs were prepared by the PCR-driven overlap extension technique (40) using full-length rat TSP4 cDNA (GenBankTM accession number X89963) in a pCEP-Pu vector as template (a gift from Dr. Frank Zaucke, University of Cologne). Segments of the TSP4 cDNA were amplified using two flanking master primers and internal primers that introduce the mutation for truncation and create an overlapping nucleotide sequence. The construct with the N-terminal domain of TSP4 deletion was created with the internal primers CACGCGCTAGTCTCTCTGTTCCAG (forward), CTGGAACAGAGAGACTAGCGCGTG (reverse) and master primers CGCAGAACTGGTAGGTATGG (forward) and CTTATCATGTCTGGATCCGGC (reverse). The con-

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struct containing only the EGF-like domains of TSP4 (EGF-LIKE) was obtained with internal primers CGCGCTAGTCCCAACACGTC (forward) and GCTTCTTGGTTTATCCACAGACATAG (reverse) and CTATGTCTGTGGATAAACCAAGAAGC (forward) and GACGTGTTGGGACTAGCGCG (reverse) and master primers CGGGACTTTCCTACTTG-GCAG (forward) and CCCGACACCCGCCAACACC (reverse). The construct containing the type-3 calcium-binding domain and C-terminal domain of TSP4 was created with the internal primers CACGCGCTAGTCAAGGATGTGGAC (forward) and GTCCACATCCTTGGACTAGCGCGTG (reverse) and master primers CGCAGAACTGGTAGGTATGG (forward) and CTTATCATGTCTGGATCCGGC (reverse). The N-terminal domain-only construct was created with the internal primers GTGGTGAGGGGCTAAACCAAGAAG (forward) and CTTCTTGGTTTAGCCCCTCACCAC (reverse) and master primers CTAGAAGCTGGGTACCTTAAGGC (forward) and CAAGCTGTGACCGTCTCCG (reverse). The construct containing the N-terminal domain and coiled-coil domain of TSP4 was obtained with the internal primers CTCCTCCAGCATAAACCAAGAAGC (forward) and GCTTCTTGGTTTATGCTGGAGGAG (reverse). The same master primers for the N-terminal domain-only construct were used. The constructs were inserted back into the pCEP-Pu vector containing the BM40 signal peptide (41, 42) and N-terminal His₆ tag using restriction sites KpnI and BamHI. The constructs were confirmed by sequencing (Eton Biosciences, San Diego, CA).

Expression and purification of recombinant TSP4 proteins

The recombinant rat TSP4 cDNA (GenBank accession number X89963) with a His₆ tag at the N-terminal was transfected into human embryonic kidney cell line 293-EBNA (Invitrogen) using the calcium phosphate transfection method. The transfected cells were selected with 0.5 μ g/ml puromycin and grown to confluence. Secretion of the full-length TSP4-His and truncated TSP4-His proteins into Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Mediatech, Manassas, VA) was confirmed by Western blotting using anti-penta-His monoclonal antibodies (Qiagen, Valencia, CA). The recombinant TSP4-His proteins were purified using a nickel-nitrilotriacetic acid column based on the manufacturer's instructions (Invitrogen), concentrated with an Amicon Ultra-4 centrifugal filter unit based on the cutoff molecular weight (Millipore, Billerica, MA), verified using Western blot analysis, aliquoted, and stored at -80°C until use.

Western blot analysis

Protein samples were denatured in the presence of DTT, heated to 70°C for 10 min, and then loaded onto a 4–12% NuPAGE Bis-Tris gel (Invitrogen) for gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride membrane and blocked with 5% skim milk powder in PBS-T (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 0.1% Tween 20, pH 7.4) at room temperature. The immunoblot was probed with anti-penta-His monoclonal antibodies (Qiagen; 1:1000) overnight at 4°C and then incubated with secondary anti-mouse IgG antibody conjugated with horseradish peroxi-

dase (HRP) (Cell Signaling Technology; 1:2000) for 1 h at room temperature followed by enhanced chemiluminescence detection of the protein–antibody complexes in an Eastman Kodak Co. image station (2000MM).

Purification of FLAG-Ca_v $\alpha_2\delta_1$

FLAG-Ca_v $\alpha_2\delta_1$ cDNA was transiently transfected into HEK 293 cells using Lipofectamine 2000 (Invitrogen). Transfected cells were washed twice with PBS and then extracted in protein extraction buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, pH 7.4) 2–3 days after the transfection. The cell lysate was incubated on ice for 15 min followed by centrifugation at $13,000 \times g$ for 20 min at 4°C . The supernatant was incubated with anti-FLAG M2 agarose affinity resin (Sigma-Aldrich) on a rotating mixer for 2 h at 4°C and then washed three times with protein extraction buffer. FLAG-Ca_v $\alpha_2\delta_1$ was eluted in elution buffer (0.1 M glycine, pH 3.5) and stored at -20°C for future use.

ELISA-based binding assay

The reagents for solid-phase binding were from Invitrogen (catalog number CNB0011). Recombinant TSP4 proteins were immobilized onto a 96-well polystyrene plate (Thermo Fisher Scientific) overnight at 4°C in coating buffer A (10 mM phosphate buffer, 0.1% azide, pH 7.4). All further incubations were carried out at room temperature for 1 h, and proteins or antibodies were diluted in assay buffer containing bovine serum albumin (BSA). After blocking, the plates were incubated with affinity-purified FLAG-Ca_v $\alpha_2\delta_1$ for 1 h, washed, and then incubated with mouse monoclonal anti-FLAG antibodies (1:1000; Sigma-Aldrich) followed by HRP-conjugated secondary antibodies. The bound FLAG-Ca_v $\alpha_2\delta_1$ complexes were detected by measuring a color reaction (yellow product) at 450 nm after adding tetramethylbenzidine for 15 min followed by adding sulfuric acid to stop the reaction.

SPOT peptide array and far-Western blotting

Peptide arrays containing overlapping peptides (15-mers; overlapping by 12 amino acids) covering the entire length of rat TSP4 protein were synthesized and immobilized onto a cellulose membrane (Sigma-Aldrich custom SPOT service). The peptide array was rinsed with methanol for 5 min, washed with TBS-T (50 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, pH 8.0), and blocked with 5% dry milk in TBS-T for 1 h at room temperature. Plasma membranes from tsA-201 cells stably expressing Ca_v2.2e ($\Delta 24a,31a$), Ca_v β_3 , and Ca_v $\alpha_2\delta_1$ (43) (a gift from Dr. Diane Lipscombe at Brown University) were collected in Tris buffer (5 mM Tris/HCl, 5 mM EDTA, pH 7.4, containing PMSF, leupeptin, and pepstatin A), incubated on ice for 15 min, sonicated, and then centrifuged at $1000 \times g$ for 10 min. The supernatant was collected and centrifuged at $50,000 \times g$ for 30 min at 4°C . The resulting pellet was resuspended in Tris buffer (0.1 μ g/ml) and incubated with the peptide array in 5% dry milk for 1 h at room temperature. Binding of Ca_v $\alpha_2\delta_1$ to the peptide array was detected by incubating the peptide array for 2 h at room temperature with primary antibodies against Ca_v $\alpha_2\delta_1$ (1:1000; mouse; Sigma-Aldrich) and then HRP-conjugated secondary antibodies against mouse IgG

for 1 h at room temperature (1:2000; Cell Signaling Technology) followed by enhanced chemiluminescence detection.

Peptides

The following peptides were synthesized and verified by MS and HPLC analysis by Genscript (Piscataway, NJ): EGF-LIKE domain peptide of TSP4 (PGVRCNTLAPGFRCD) (EGFD355–369; GenBank accession number CAA62002) and its scrambled control peptide (GLAFVNCPRRDTGCP). The lyophilized peptides were dissolved in sterile water before use.

Animals

Male adult Harlan Sprague-Dawley rats (<150 g) were from Harlan Sprague-Dawley Industries (Indianapolis, IN). Adult male and female mice (20–30 g) with 129sv background were from Charles River Laboratories (Wilmington, MA). They were bred internally to obtain pregnant females for spinal cord cell collection for cell cultures. The $Ca_v\alpha_2\delta_1$ -overexpressing transgenic mice were generated and characterized as described in our previous publication (20). Briefly, a neuronal specific thy-1 promoter (44) was used to drive overexpression of the mouse brain $Ca_v\alpha_2\delta_1$ cDNA (GenBank accession number U73484) in the transgenic mice. These genetically modified mice were fertile and showed normal growth, grooming, social interactions, and feeding. They were backcrossed to the 129sv background for over 10 generations before use. Mouse genotyping was performed by TransnetYX, Inc. (Cordova, TN). All animals were housed in separate cages and exposed to a 12-h light/dark cycle with food and water *ad libitum*. All animal care and experiments were performed according to protocols approved by the Institutional Animal Care Committees of the University of California, Irvine.

Tactile allodynia

The animals were placed in Plexiglass chambers on a wire mesh-bottomed cage for acclimatization (at least 60 min). von Frey filaments (Stoelting, Wood Dale, IL) were used to determine the 50% paw withdrawal threshold (PWT) using the up-down method of Dixon (45). A series of von Frey filaments were applied to both plantar surfaces of the hind paws, starting with a buckling weight of 2.0 or 0.41 g for a rat or mouse, respectively, with sufficient force to cause the filament to buckle. A positive response was defined as a rapid withdrawal and/or licking of the paw upon application of the stimulus, which prompted the use of the next weaker filament. Absence of a paw withdrawal response after 5 s prompted the use of the next filament of increasing weight. This paradigm was continued until four more measurements have been made after the initial change of the behavioral response or until five consecutive negative (assigned a score of 15 g for rats or 2 g for mice) or four consecutive positive (assigned a score of 0.25 g for rats or 0.01 g for mice) responses had occurred. The resulting scores of six (starting from the one before the first change in response) were used to calculate the 50% response threshold as described previously (3).

Thermal hyperalgesia (Hargreaves method)

The animals were placed in Plexiglass chambers on a glass panel that was maintained at 30 °C for acclimatization (at least

60 min). A heat stimulus projecting through a small aperture below the glass panel from a high intensity light bulb was applied to both plantar surfaces of the hind paws. When the animals moved their paw away from the thermal stimulus, the light beam was shut off automatically. The length of time between the start of the light beam and the hind paw withdrawal was defined as the paw withdrawal latency. A cutoff time of 20 s was used to avoid tissue damage to the hind paw.

Cell culture

A DRG/spinal cord primary neuron coculture system derived from published protocols (46–49) with minor modifications (26, 50–53) was used for *in vitro* synaptogenesis analysis. Briefly, at least six E14–E19 mouse embryos (for spinal cord neurons) and three adult mice (for DRG neurons) were used for each multiwell culture plate in independent experiments. Spinal cord neurons were dissociated with 0.25% trypsin in DMEM, plated onto 0.1 mg/ml poly-D-lysine- and 0.04 mg/ml laminin-coated glass coverslips, and grown in DMEM supplemented with 10% horse serum and 10% fetal bovine serum. After 24 h, the media were replaced with neurobasal medium supplemented with B27 supplement (NB/B27) to allow spinal cord neuron maturation and neurite sprouting for 3 days. Adult DRG (T9–L5) were harvested after hydraulic extrusion of spinal cord and laminectomy from Advillin-Cre/Rosa-tdTomato mice, which expressed red fluorescent proteins driven by the Advillin promoter in about 90% of DRG neurons (54, 55). Neurons were dissociated in DMEM containing 1.25 mg/ml collagenase (Sigma) and added to the spinal cord neuron cultures at the time of culture media change at day 4. The cocultures were maintained in NB/B27 supplement with 100 nM uridine and 20 nM 5-fluorodeoxyuridine to control nonneuronal cell proliferation. The culture cell treatments started about 2–3 days after DRG cell addition.

Immunohistochemistry

Excitatory synapse numbers in cultured cells were analyzed as described previously (12, 21, 51). Because a single investigator performed all the immunohistochemical experiments, data analyses were not double blinded. Briefly, after heat-based antigen retrieval as described previously, cell samples were stained with primary antibodies against vesicular glutamate transporter 2 (VGlut2; guinea pig; Synaptic Systems), postsynaptic density protein 95 (PSD95; rabbit; Thermo Fisher Scientific), and microtubule-associated protein 2 (MAP2) (chicken; Abcam) at 4 °C overnight. Our laboratory and others have done validation of these antibodies as reported previously (12). After washing, samples were incubated with species-specific secondary antibodies conjugated to unique fluorophores for 2 h at room temperature and washed again. Fluorescence images were acquired with a confocal microscope (Zeiss LSM700, University of California Irvine Optical Biology Core) in 0.3- μ m-thick Z stacks. DRG axons could be traced by tdTomato red fluorescence. Because VGlut2 immunoreactivity is mainly in presynaptic vesicles, but not axons, and tdTomato fluorescence is mainly expressed along DRG neuron axons, but not in presynaptic vesicles, it was highly likely that VGlut2 immunoreactivity detected within the dendritic spinelike structure along

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MAP2 immunoreactivity was from VGlut2 immunostaining and not from tdTomato fluorescence (21). This allowed measurement of excitatory synapses between sensory axons and individual dendrites of each spinal cord neuron with overlapping VGlut2, PSD95, and MAP2 immunoreactivity along with or surrounded by red DRG axons. Corrections for variations in dendrite size and numbers among individual spinal cord neurons were done by calculating synapse count/ μm^2 area of MAP2-immunoreactive dendrites of that neuron. Data analyses were performed on merged seven consecutive Z stacks with the best signal using Volocity 6.0 (PerkinElmer Life Sciences).

Author contributions—J. F. P. data curation; J. F. P., Y. P. Y., and N. G. formal analysis; J. F. P., Y. P. Y., N. G., and V. N. T. investigation; J. F. P. writing-original draft; Z. D. L. conceptualization; Z. D. L. supervision; Z. D. L. funding acquisition; Z. D. L. validation; Z. D. L. writing-review and editing.

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