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# Chondroitin Sulfate Proteoglycans Down-regulate Spine Formation in Cortical Neurons by Targeting Tropomyosin-related Kinase B (TrkB) Protein

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**Background:** CSPGs are major players that inhibit experience-dependent plasticity.
**Results:** We show that CSPGs dephosphorylate TrkB. Whereas BDNF promoted dendritic spine formation in cortical neurons, CSPGs abolished the effects.
**Conclusion:** The inhibitory activity of CSPGs operates through the targeting of neurotrophins at the receptor level.
**Significance:** Our findings unveil unexpected cross-talk between BDNF and CSPG signals for the plastic changes of the synapses.

**Chondroitin sulfate proteoglycans (CSPGs) are components of the extracellular matrix that inhibit axonal sprouting and experience-dependent plasticity. Although protein-tyrosine phosphatase $\sigma$ (PTP$\sigma$) has been proven to be a receptor for CSPGs, its downstream signaling has remained a mystery. Here, we show that CSPGs target and dephosphorylate tropomyosin-related kinase B, the receptor of brain-derived neurotrophic factor (BDNF), via PTP$\sigma$ in embryonic cortical neurons *in vitro*. Whereas BDNF promoted dendritic spine formation in embryonic cortical neurons, CSPGs abolished the effects of BDNF and eliminated existing dendritic spines when BDNF was present. The latter effect was dependent on the p75 receptor, presumably because BDNF binding to the p75 receptor elicits elimination of dendritic spines. These results suggest that the inhibitory activity of CSPGs on dendritic spine formation operates through the targeting of neurotrophins at the receptor level.**

The extracellular matrix of the adult central nervous system (CNS) has a unique composition rich in hyaluronic acid and chondroitin sulfate proteoglycans (CSPGs)[2] rather than collagens, laminin-1, and fibronectin (1). CSPGs are inhibitory molecules that are reinduced after injury and inhibit regrowth of neuronal axons. Digestion of CSPGs by chondroitinase ABC treatment enhances functional recovery after spinal cord injury in rats (2). Furthermore, CSPGs in the extracellular matrix are involved in the control of neuronal plasticity (3). CSPGs are required for closure of the critical period in the developing visual cortex. Indeed, the increase of CSPGs in the CNS coincides with the end of the critical period, and chondroitinase ABC treatment increases spine dynamics after the end of the critical period (4–6). Despite a strong understanding of the roles of CSPGs, the molecular mechanisms underlying growth inhibition and limitation of neuronal plasticity by CSPGs remain poorly understood.

In contrast to CSPGs, brain-derived neurotrophic factor (BDNF) positively regulates neuronal plasticity via its receptor, tropomyosin-related kinase B (TrkB). In the visual cortex, BDNF influences ocular dominance column formation and plasticity before the critical period (7, 8). BDNF also increases apical dendritic spine density and synapse number in hippocampal CA1 pyramidal neurons (9). Finally, in cerebellar cultures, BDNF increases the spine density of Purkinje cells without affecting dendritic complexity (10).

Protein-tyrosine phosphatase $\sigma$ (PTP$\sigma$) has been shown to be the receptor of CSPGs (11). The observation that PTP$\sigma$ dephosphorylates TrkB (12) hinted at a connection between CSPGs and TrkB. The opposite effects of CSPGs and BDNF on experience-dependent plasticity of neurons further support this notion. In this study, we show that CSPGs inhibit phosphorylation of TrkB via PTP$\sigma$ activation. The opposing effect of CSPGs against BDNF was observed in dendritic spine formation of cortical neurons *in vitro*. We also identified involvement of p75 receptor in this signal transduction pathway.

## EXPERIMENTAL PROCEDURES

*Antibodies and Reagents*—The following antibodies were used: anti-$\alpha$-tubulin clone B-7 (Santa Cruz Biotechnology), anti neurotrophin receptor p75 (anti-p75NTR; Millipore), anti-phosphotyrosine clone 4G10 (Millipore), anti-PTP$\sigma$ clone 17G7.2 (MEDIMABS), anti-Shc (Millipore), biotinylated anti-TrkB (R&D), and anti-phospho-TrkB (Tyr$^{706/707}$; Cell Signaling Technology). The following reagents were used: recombinant human BDNF (PeproTech) and CSPG mixture (Millipore).

*Plasmid Constructs*—For generation of murine PTP$\sigma$ (Accession number BC052462) shRNA constructs, the following oligonucleotides were used: 5′-GATCTCCGCATCATGGGTAGTG-

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[2] The abbreviations used are: CSPG, chondroitin sulfate proteoglycan; BDNF, brain-derived neurotrophic factor; DIV, days *in vitro*; E18, embryonic day 18; p75NTR, neurotrophin receptor p75; PTP$\sigma$, protein-tyrosine phosphatase $\sigma$; TrkB, tropomyosin-related kinase B.

ATTATTCAAGAGATAATCACTACCCATGATGCTTTTT-A-3′ and 5′-AGCTTAAAAAGCATCATGGGTAGTGATTAT-CTCTTGAATAATCACTACCCATGATGCGGA-3′ (shRNA #1: 2978–2996 nucleotide position of murine PTP$\sigma$); 5′-GATCT-CCGAAATCACGGATCAAATGTTTCAAGAGAACATTTG-ATCCGTGATTTCTTTTTA-3′ and 5′-AGCTTAAAAAGAA-ATCACGGATCAAATGTTCTCTTGAAACATTTGATCCG-TGATTTCGGA-3′ (shRNA #2: 3144–3162 nucleotide position of murine PTP$\sigma$). Sense and antisense oligonucleotides were annealed and cloned into BglII and HindIII sites of the pSUPER gfp/neo vector (oligoengine). For generation of murine PTP$\sigma$ constructs, the full-length murine Ptprs and GFP expression cassette in the pSUPER gfp/neo vector was cloned into cDNA3.1 (+) vectors (Invitrogen). The catalytically inactive mutant PTP$\sigma$-D/A was generated by site-directed mutagenesis of $Asp^{1110}$ into Ala.

*Neuronal Culture*—Cortical neurons obtained from mouse pups on embryonic day (E) 18 were dissociated by trypsinization (treatment with 0.25% trypsin in PBS for 15 min at 37 °C) followed by resuspension in DMEM/F12 (Invitrogen) containing 10% FBS and trituration. Dissociated neurons were plated on poly-L-lysine-coated dishes. The culture medium was replaced with serum-free DMEM/F12 supplemented with B27 (Invitrogen) 2 h after plating and maintained at 37 °C in 5% $CO_2$.

*Nucleofection*—Freshly isolated mouse cortical neurons were suspended in 100 $\mu$l of transfection solution (Amaxa Biosystems) containing 2.5 $\mu$g of the various plasmid DNA. The cell suspension was nucleofected using program O-005 (Amaxa Biosystems) according to the manufacturer's protocol. After the addition of 500 $\mu$l of DMEM/F12 supplemented with 10% FBS, neurons were plated on poly-L-lysine-coated dishes. The culture medium was replaced with serum-free DMEM/F12 supplemented with B27 2 h after plating.

*Lipofection*—Lipofection was performed using Lipofectamine 2000 (Invitrogen) as described previously (13) with minor modifications. Mouse cortical neurons grown on poly-L-lysine-coated 14-mm diameter glass coverslips (Deckgläser) in 24-well plates (Greiner bio-one) were transfected with 2 $\mu$g of plasmid DNA and 8 $\mu$l of Lipofectamine 2000 reagent per well. The culture medium was replaced with conditional medium 4 h after transfection.

*Immunoprecipitation*—For immunoprecipitation with TrkB, mouse cortical neurons were lysed in 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1.0 mM EGTA, 10% glycerol, and 1% Triton X-100 supplemented with protease inhibitor mixture tablets (Roche). The lysates were incubated on a rocking platform at 4 °C for 20 min and clarified by centrifugation at 20,000 × *g* for 10 min. The supernatants collected were precleared for 1 h by incubating with 20 $\mu$l of streptavidin-agarose resins (Thermo). After a brief centrifugation to remove the precleared beads, cell lysates were incubated for 3 h at 4 °C with 1 $\mu$g of biotinylated anti-TrkB antibody. The immunocomplexes were collected for 1 h at 4 °C by using the streptavidin-agarose resins. The immunoprecipitation experiment with anti-PTP$\sigma$ antibody did not work in our hands. For immunoprecipitation with phophotyrosine, cells were lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, supplemented with protease inhibitor mixture, 10 mM sodium fluoride, and 1 mM orthovanadate. The lysates were incubated on a rocking platform at 4 °C for 20 min and clarified by centrifugation at 20,000 × *g* for 10 min. The supernatants collected were incubated for 3 h at 4 °C with 2 $\mu$g of anti-phosphotyrosine antibody. The immunocomplexes were collected for 1 h at 4 °C by using the protein A-Sepharose beads (GE Healthcare). The beads were washed four times with the lysis buffer, and bound proteins were solubilized with 2× sample buffer and subjected to SDS-PAGE followed by immunoblotting.

*Western Blot Analysis*—The protein samples were boiled in sample buffer for 5 min, run on SDS-PAGE, and transferred to PVDF membranes. The membranes were blocked for 1 h at room temperature with 5% skim milk and incubated for 1 h at room temperature with the primary antibody. HRP-conjugated secondary antibodies and ECL plus reagents or ECL advance (GE Healthcare) were used for detection. Membrane was exposed to an image system (LAS-3000; Fujifilm) according to the manufacturer's specifications. The intensity (area × density) of the individual bands on Western blots was quantitated by ImageJ software 1.42q (National Institutes of Health, Bethesda, MD). The background was subtracted from the calculated area, and the results were calculated as ratio changes compared with the corresponding control bands.

*Immunofluorescence*—For immunostaining against PTP$\sigma$, cultured neurons were fixed with 4% paraformaldehyde and 4% sucrose at room temperature for 10 min. After 1 h of blocking in 2% BSA and 0.2% Tween 20 in PBS, cells were incubated with anti-PTP$\sigma$ antibody (1:100 dilution) overnight at 4 °C. Cells were washed in PBS and visualized using Alexa Fluor 568-coupled secondary antibody (Invitrogen).

*Spine Density Analysis*—Cortical neurons cultured on coverslips were transfected with pSUPER gfp/neo or pSUPER gfp/neo-PTP$\sigma$ by lipofection at 7 days *in vitro* (DIV) and fixed at 15 DIV with 4% paraformaldehyde in 0.1% phosphate buffer, pH 7.4, for 1 h at room temperature. After washing three times with PBS, coverslips were mounted on glass slides (Matsunami glass) using Fluorescent Mounting Medium (Dako). Images of neurons expressing GFP were acquired using a microscope (BX51; Olympus) equipped with a camera (DP71; Olympus) that used a controller software (version 3.1.1.267; DP; Olympus). The UPlanSApo 40×/0.90 objectives (Olympus) were used. The proximal dendrites were selected for analysis of the number of dendritic spines. Ten GFP-expressing neurons were randomly selected for each experimental group, and two proximal dendrites per each neuron were analyzed. Spine density was calculated by dividing the number of spines by the length of dendrites.

*Statistical Analyses*—Statistical comparisons between two independent groups of samples were performed using Student's *t* test. For comparisons among three or more groups of samples, one-way ANOVA was used. When post hoc tests were required, Dunnett's test was used to compare the means of each experimental group with those of the control group or Turkey-Kramer's test to compare all possible pairs of means. In all figures, means ± S.E. are indicated. The level of significance is indicated by *asterisks*: *, $p < 0.05$; **, $p < 0.01$. The number of independent examinations conducted is indicated in the figure legends.

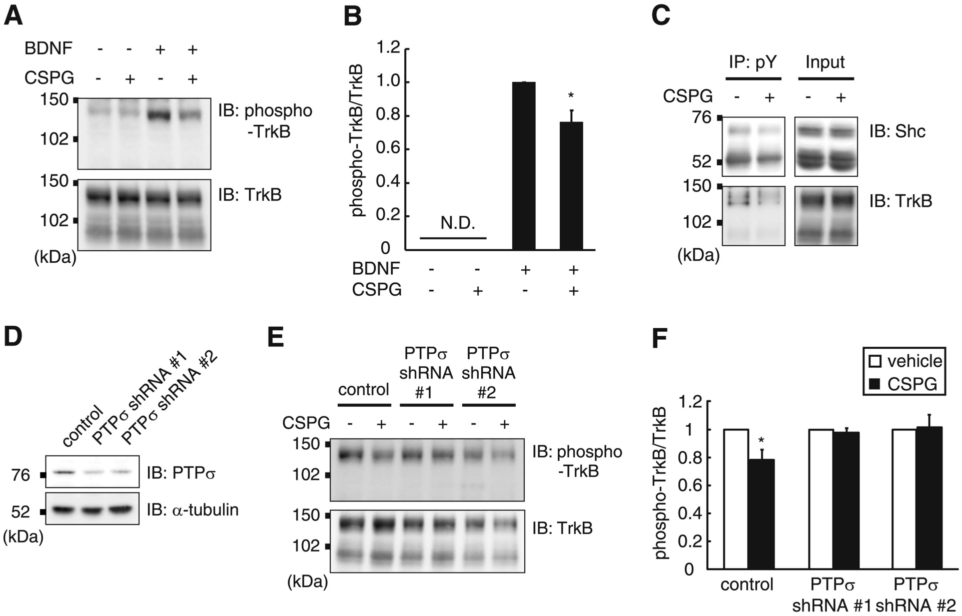


FIGURE 1. **CSPGs dephosphorylate TrkB by a PTPσ-dependent mechanism.** *A*, Western blots showing the levels of phosphorylated TrkB. Mouse cortical neurons at 10–14 DIV were preincubated with vehicle or 2 μg/ml CSPG for 15 min, and each of these treatments was continued for an additional 30 min in the presence of vehicle or 20 ng/ml BDNF. Cell lysates were subjected to immunoblotting (*IB*) with the indicated antibodies. *B*, quantification of the data in *A* from four independent experiments. Significant difference between BDNF (+)/CSPG (−) and BDNF (+)/CSPG (+) are indicated by * ($p < 0.05$, two-tailed Student's *t* test). *N.D.*, not determined. *C*, Western blots showing the levels of phosphorylated Shc. Mouse cortical neurons at 14 DIV were preincubated with vehicle or 2 μg/ml CSPG for 15 min. Then, the cells were treated for 15 min with vehicle or 20 ng/ml BDNF in the preincubated medium. Cell lysates were immunoprecipitated (*IP*) with anti-phosphotyrosine (*pY*) antibody and immunoblotted with the indicated antibodies. *D*, Western blots for the detection of PTPσ. Expression of endogenous PTPσ was decreased in PTPσ shRNA-expressing neurons. E18 mouse cortical neurons were nucleofected with pSUPER or pSUPER-PTPσ and lysed at 10 DIV. Lysates were immunoblotted with antibodies against PTPσ and α-tubulin. *E*, Western blots showing the levels of phosphorylated TrkB and total TrkB. Cortical neurons were nucleofected with pSUPER, pSUPER-PTPσ #1 or #2 and maintained for 10 days. Neurons were preincubated with vehicle or 2 μg/ml CSPG for 15 min; each of these treatments was continued for an additional 30 min in the presence of 20 ng/ml BDNF. Cell lysates were subjected to immunoblotting with antibodies against phospho-TrkB and total TrkB. *F*, quantification of the data in *E* from four independent experiments. Significant difference between control and CSPG (+) is indicated by * ($p < 0.05$, two-tailed Student's *t* test). *Error bars*, S.D.

## RESULTS

*CSPG Stimulation Induces Dephosphorylation of TrkB by PTPσ in Cortical Neurons*—We first examined whether CSPGs affected the phosphorylation status of TrkB. E18 mouse cortical neurons were cultured with or without CSPGs in the presence of vehicle or BDNF. Although BDNF enhanced tyrosine phosphorylation of TrkB, stimulation by CSPGs attenuated it (Fig. 1, *A* and *B*). We also checked whether CSPGs affected phosphorylation status of Shc, which is the downstream signaling effector of TrkB. Pretreatment with CSPGs attenuated BDNF-induced phophorylation of Shc as well as TrkB (Fig. 1*C*). These results demonstrate that CSPGs inhibit the TrkB activity in the cortical neurons. We then assessed whether PTPσ, the CSPG receptor (11), was involved in this effect. We used shRNA-expressing constructs, pSUPER-PTPσ #1 and #2, to knock down the expression of endogenous PTPσ in cultured cortical neurons. Both the 19-nucleotide sequences corresponding to nucleotides 2978–2996 (shRNA #1) and 3144–3162 (shRNA #2) of the murine PTPσ decreased the expression of endogenous PTPσ in the cortical neurons (Fig. 1*D*), indicating that we successfully achieved shRNA-mediated knockdown of PTPσ. Knockdown of PTPσ did not modulate the phosphorylation level of TrkB in neurons stimulated with BDNF (one-way ANOVA, $p = 0.076$, $n = 5$; data not shown), whereas it resulted in disappearance of down-regulation of phosphorylated TrkB induced by CSPGs (Fig. 1, *E* and *F*).

*Constitutive Interaction of PTPσ with TrkB*—The above results suggest that PTPσ contributes to tyrosine dephosphorylation of TrkB. Thus, we next examined whether PTPσ interacted with TrkB in the cortical neurons. Supporting this hypothesis, it was previously reported that only trace amounts of PTPσ was co-immunoprecipitated with TrkB in HEK 293T cells overexpressing PTPσ and TrkB (12); however, whether endogenous proteins interacted in a similar fashion is unknown. Dissociated mouse cortical neurons were treated with or without CSPGs and/or BDNF, and the cell extracts were immunoprecipitated with an anti-TrkB antibody. We found that PTPσ co-immunoprecipitated with TrkB, indicating that PTPσ interacts with TrkB in the cortical neurons (Fig. 2*A*). The amount of PTPσ co-immunoprecipitated with TrkB significantly increased after the application of BDNF. Stimulation of BDNF-treated cortical neurons with CSPGs also significantly increased the amount of PTPσ co-immunoprecipitated with TrkB compared with control neurons not treated with BDNF, whereas CSPGs did not induce a further increase in the binding of these molecules in BDNF-treated neurons (Fig. 2*B*). These results suggest that the interaction of PTPσ with TrkB is enhanced by phosphorylation of TrkB. Because CSPG stimulation tended to decrease the binding of PTPσ with TrkB in the BDNF-treated neurons, phosphorylation levels of TrkB may be a key determinant of the binding of TrkB with PTPσ.

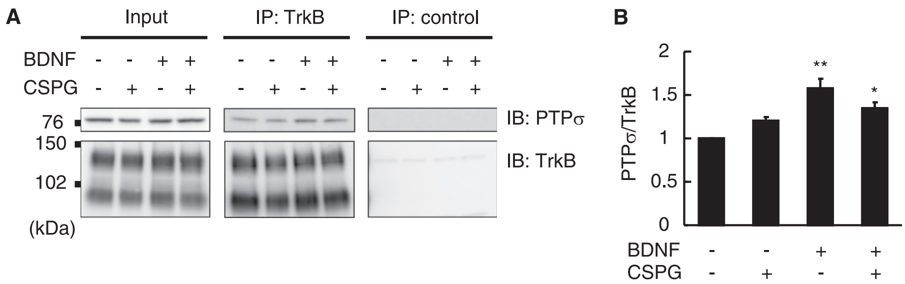


FIGURE 2. **PTP$\sigma$ interacts with TrkB.** *A*, co-immunoprecipitation of PTP$\sigma$ with TrkB in lysates of mouse cortical neurons. Cortical neurons at 10–14 DIV were preincubated with vehicle or 2 $\mu$g/ml CSPGs for 15 min; each of these treatments was continued for an additional 30 min in the presence of vehicle or 20 ng/ml BDNF. Cell lysates were immunoprecipitated (*IP*) with anti-TrkB antibody and immunoblotted (*IB*) with antibodies against PTP$\sigma$ and total TrkB. Control experiments were carried out in parallel by excluding the antibody for immunoprecipitation. *B*, amount of co-immunoprecipitated PTP$\sigma$ with TrkB quantified by measuring the intensity of individual bands of the data in *A* from four independent experiments. Statistically significant differences between control and each experimental group are indicated by * ($p < 0.05$), ** ($p < 0.01$; one-way ANOVA followed by Dunnett's post hoc test).

*CSPG Stimulation Suppresses Spine Formation in Cortical Neurons*—The above observations suggest that CSPGs have a negative effect on BDNF-TrkB signaling. Because BDNF increases the density of dendritic spines in cultured neurons (10), we tested whether CSPGs negatively regulate dendritic spine formation. Cortical neurons were cultured for 14 DIV and treated with CSPGs in the presence or absence of BDNF for 24 h. Dendritic spine density was then measured. Although neurons treated with BDNF had a higher density of spines than did control neurons, stimulation with CSPGs attenuated the effect of BDNF (Fig. 3, *A* and *B*). These results demonstrate that CSPGs counteracted the effect of BDNF on the spine formation in the cortical neurons. Interestingly, CSPG treatment of BDNF-stimulated neurons resulted in decreased spine density compared with the basal level of spine density in neurons without BDNF stimulation, whereas CSPG treatment of BDNF-untreated neurons did not alter spine density (Fig. 3, *A* and *B*). CSPGs may have a role in eliminating the spines in the BDNF-treated cortical neurons, but not in the BDNF-untreated neurons.

We next examined whether these effects of CSPGs on dendritic spine density were mediated by PTP$\sigma$. Neurons were transfected with pSUPER gfp/neo-PTP$\sigma$ at 7 DIV, treated with or without CSPGs and BDNF for 24 h at 14 DIV, and the dendritic spine density was measured. Immunostaining against PTP$\sigma$ demonstrated that the PTP$\sigma$ immunoreactivities in GFP-positive neurons were lower in pSUPER gfp/neo-PTP$\sigma$-transfected neurons than in pSUPER gfp/neo neurons or neighboring GFP-negative neurons (Fig. 3*C*). Thus, we successfully achieved shRNA-mediated knockdown of PTP$\sigma$. In the neurons where PTP$\sigma$ was knocked down, we observed that BDNF increased spine density even after CSPGs stimulation (Fig. 3*D*), as observed in the absence of CSPG stimulation. We also investigated the effects of overexpression of wild-type or catalytically inactive PTP$\sigma$ on dendritic spine formation. The spine density of neurons transfected with wild-type PTP$\sigma$ did not significantly differ from that of control neurons, suggesting that PTP$\sigma$ was expressed at an adequate level in the control neurons. Neurons transfected with catalytically inactive PTP$\sigma$ had higher density of dendritic spine than the control neurons after stimulation with CSPGs and BDNF (Fig. 3*E*). This result is consistent with the observation using PTP$\sigma$ knocked down neurons. It has been shown that catalytically inactive PTP$\sigma$ acts as a dominant negative mutant (14). Therefore, PTP$\sigma$ is necessary for the effect of CSPGs on BDNF-treated cortical neurons.

*Involvement of p75NTR in Effects of CSPGs on Spine Formation*—Whereas TrkB promotes dendritic spine formation (10), p75NTR, another BDNF receptor, mediates spine elimination (15, 16). Thus, we investigated whether p75NTR is involved in decreased spine formation elicited by CSPGs in BDNF-treated neurons. We employed anti-p75NTR antibody, which is known to neutralize the function of p75NTR (16–18). Cortical neurons (14 DIV) were treated with CSPGs and BDNF in the presence or absence of anti-p75NTR antibody for 24 h, and dendritic spine density was measured. Anti-p75NTR antibody treatment completely attenuated the effect of CSPGs on BDNF-treated neurons (Fig. 4, *A* and *B*). These results indicate that spine elimination induced by CSPGs in BDNF-treated neurons is dependent on p75NTR. It was possible that CSPGs activated p75NTR directly. To assess this issue, we investigated the effects of CSPGs on dendritic spine density in the presence or absence of anti-p75NTR antibody. CSPGs by themselves did not have significant effects on spine density either in the presence or absence of anti-p75NTR antibody (Fig. 4*C*). This result indicates that CSPGs does not stimulate p75NTR directly.

## DISCUSSION

Here, we showed that CSPGs dephosphorylate TrkB via PTP$\sigma$ and suppress spine formation induced by BDNF in cortical neurons. Furthermore, we found that BDNF causes spine elimination through p75NTR when TrkB is inactivated by CSPGs. We also found that phosphorylation of TrkB induced by BDNF is inhibited by CSPGs and is fully rescued by knockdown of PTP$\sigma$ (Fig. 1). In addition, TrkB associates with PTP$\sigma$, and this association is regulated by CSPGs and BDNF (Fig. 2). These results suggest that PTP$\sigma$ recognizes phosphorylated TrkB as a substrate and dephosphorylates it in a ligand-dependent fashion. In this study, we examined the phosphorylation status of TrkB at the $Tyr^{706/707}$ sites. We also checked the phosphorylation status of the $Tyr^{515}$ site and found that it was also dephosphorylated by CSPGs (data not shown). Thus, it is possible that PTP$\sigma$ recognizes more than one site. Precise molecular determinant of the interaction will be the subject of future studies. Inconsistent with our findings, it was previously reported that PTP$\sigma$ binds TrkA and TrkC, but rarely binds TrkB in HEK293T cells overexpressing PTP$\sigma$ with TrkA, TrkB,

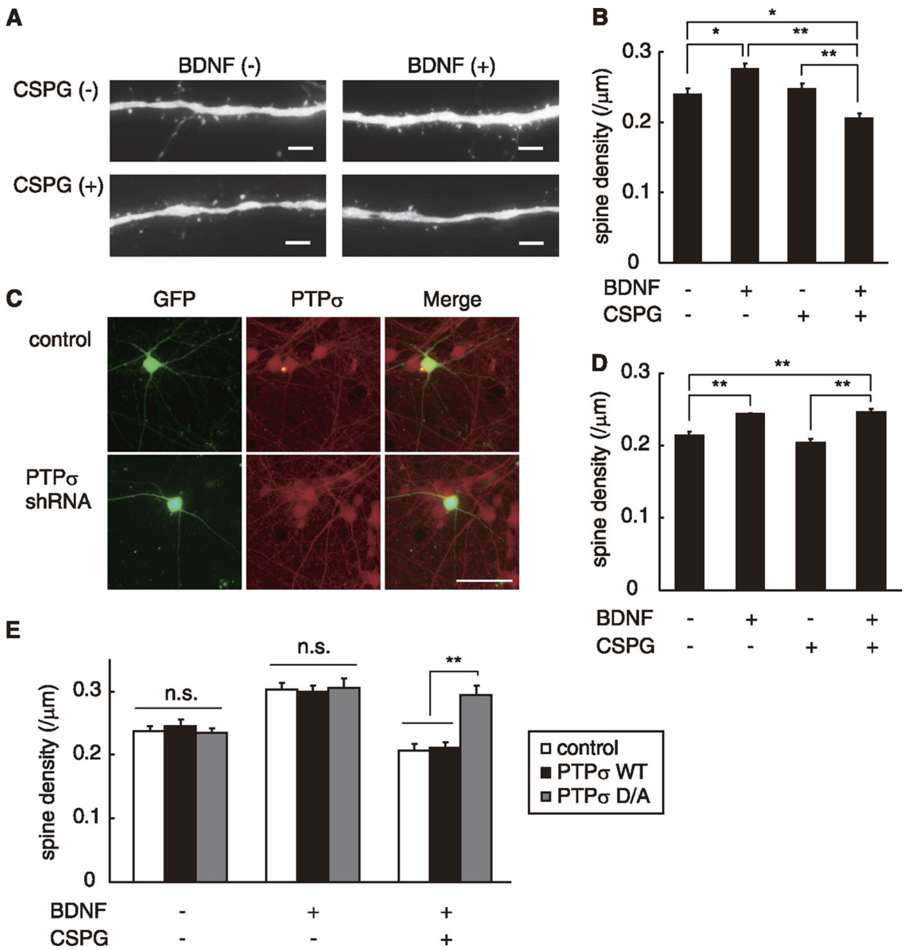


FIGURE 3. **CSPGs stimulation suppresses spine formation in cortical neurons.** *A*, representative micrographs of cortical neuron dendrite segments. The neurons at 14 DIV, transfected with pSUPER gfp/neo at 7 DIV, were treated with vehicle or 2 μg/ml CSPG for 15 min, and cultured for an additional 24 h in the presence of vehicle or 100 ng/ml BDNF. *Scale bars*, 5 μm. *B*, *D*, and *E*, graphs showing dendritic spine density in the cortical neurons. Statistically significant differences between all possible pairs of means are indicated by * ($p < 0.05$), ** ($p < 0.01$; one-way ANOVA followed by Turkey-Kramer's post hoc test). *B*, application of BDNF increasing dendritic spine density and CSPG stimulation decreasing spine density in BDNF-treated neurons below the control level. The numbers of independent experiments were five. *C*, representative micrographs of cortical neuron transfected with pSUPER gfp/neo or pSUPER gfp/neo-PTPσ #1. The cells were immunostained for PTPσ. Expression level of endogenous PTPσ was decreased in pSUPER gfp/neo-PTPσ-transfected neurons. *Scale bar*, 50 μm. *D*, involvement of PTPσ in the effects of BDNF and CSPGs on dendritic spine density. The neurons at 14 DIV, transfected with pSUPER gfp/neo-PTPσ #1 at 7 DIV, were treated with vehicle or 2 μg/ml CSPG for 15 min and cultured for an additional 24 h in the presence of vehicle or 100 ng/ml BDNF. The number of independent experiments performed was three. *E*, effects of overexpression of wild-type PTPσ or catalytically inactive PTPσ-D/A on spine density. The neurons at 14 DIV, transfected with pcDNA-GFP, pcDNA-PTPσ WT-GFP, or pcDNA-PTPσ D/A-GFP at 10 DIV, were treated with vehicle or 2 μg/ml CSPG for 15 min and cultured for an additional 24 h in the presence of vehicle or 100 ng/ml BDNF. The number of independent experiments performed was five; *n.s.*, not significant.

or TrkC (12). This discrepancy might be due to molecules that facilitate binding of PTPσ to TrkB being present in cortical neurons but not in HEK293T cells. However, it is important to note that our data clearly demonstrate the interaction of these endogenous proteins in neurons.

In the present study, we focused on dendritic spine density as a representation of neuronal plasticity. Changes in spine density, morphology, and motility have been shown to occur with programs that induce synaptic plasticity (19). For example, BDNF treatment elicits a rapid potentiation of excitatory synapse transmission (20, 21) and increases dendritic spine density (9, 10). Consistent with these observations, we observed an increase in dendritic spine density after application of BDNF in cortical neurons *in vitro*. Stimulation with CSPGs inhibited the increase in spine density induced by BDNF through PTPσ (Fig. 3). This effect may be mediated by suppression of BDNF-induced TrkB phosphorylation by CSPGs. We showed that phosphorylation level of Shc decreased by stimulation with CSPGs (Fig. 1). Shc regulates ERK and PI3K-Akt signaling pathway. ERK activation is necessary for increase in the spine density induced by BDNF in hippocampal CA1 pyramidal neurons (22), and PI3K-Akt is also involved in spine formation (23). Thus, CSPGs may regulate spine formation by suppressing these pathways. Furthermore, our observation that application of CSPGs concomitantly with BDNF resulted in decreased spine density below control levels was of particular interest (Fig. 3). This effect is attributable to p75NTR. It has been shown that p75NTR is involved in the long lasting decremental form of synaptic plasticity. For example, p75NTR-deficient mice have a higher spine density than wild-type mice, and conversely, p75NTR overexpression in wild-type neurons decreases spine density (15). A high affinity ligand for p75NTR, pro-BDNF, eliminates dendritic spines via p75NTR (16). In this study, we showed that BDNF decreased dendritic spine density when CSPGs were added and that this effect was attenuated by neutralization by anti-p75NTR antibody (Fig. 4). Because CSPGs

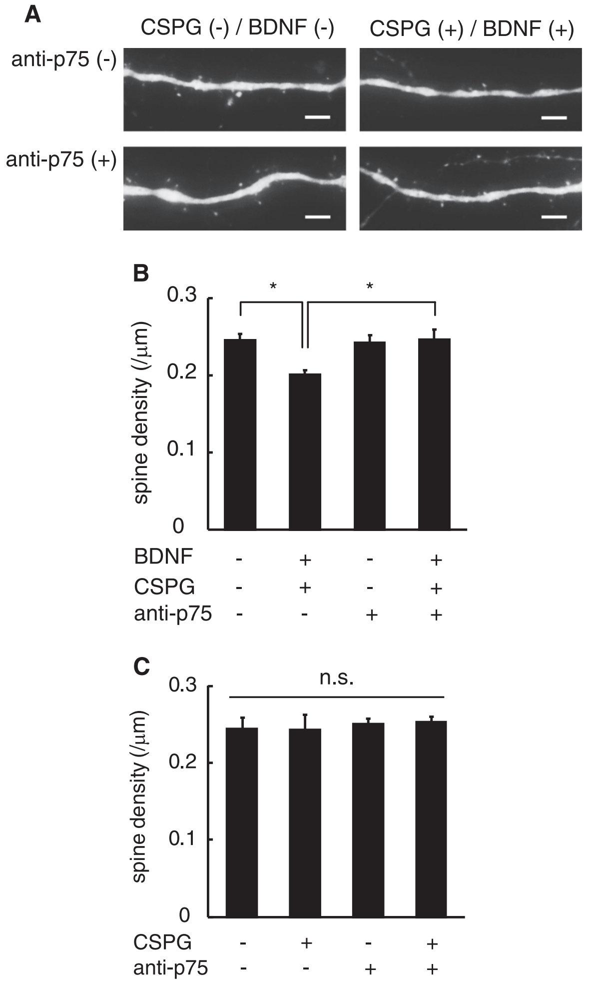


FIGURE 4. **Involvement of p75NTR in reduction of dendritic spine density induced by CSPGs in BDNF-treated cortical neurons.** *A*, representative micrographs of cortical neuron dendrite segments. The neurons at 14 DIV transfected with pSUPER gfp/neo at 7 DIV were treated with vehicle, 2 μg/ml CSPG, and/or 0.2% anti-p75NTR antibody for 15 min, and cultured for an additional 24 h in the presence of vehicle or 100 ng/ml BDNF. *Scale bars*, 5 μm. *B*, graphs showing dendritic spine density in the cortical neurons. Anti-p75NTR antibody treatment abolished the spine elimination induced by CSPGs in BDNF-treated neurons. The number of independent experiments performed was five. *C*, CSPGs alone did not affect dendritic spine density. The neurons at 14 DIV, transfected with pSUPER gfp/neo at 7 DIV, were treated with vehicle, 2 μg/ml CSPG, and/or 0.2% anti-p75NTR antibody for 24 h. The number of independent experiments performed was three. Statistically significant differences between all possible pairs of means are indicated by * ($p < 0.05$; one-way ANOVA followed by Turkey-Kramer's post hoc test); *n.s.*, not significant.

block activation of TrkB, BDNF might predominantly activate p75NTR signaling, thus leading to elimination of dendritic spines in cortical neurons. The effect of CSPGs was not dependent on p75NTR. Specifically, primary neurons derived from p75NTR-deficient mice are still sensitive to CSPGs (24). Our data do not contradict this. In the presence of anti-p75NTR antibody, the spine density in neurons stimulated by CSPGs and BDNF was not different from that in control neurons (Fig. 4), which was lower than that in neurons stimulated by BDNF alone (Fig. 3). Therefore, CSPGs still have negative effects on BDNF-induced spine formation in the presence of anti-p75NTR antibody. Concerning the possibility that CSPGs activate p75NTR directly, we showed that CSPGs themselves did not have significant effects on spine density either in the presence or absence of anti-p75NTR antibody (Fig. 4). In addition, it is reported that CSPGs do not interact with the p75NTR-NgR receptor complex (24). These data suggest that CSPGs do not stimulate p75NTR directly. CSPGs may modulate the binding equilibrium of BDNF to TrkB and p75NTR. However, it is very difficult to measure binding affinity of BDNF to TrkB and p75 separately because TrkB and p75NTR interact with each other to form a high affinity site for BDNF (25).

Cortical spine density drastically increases with age during the postnatal period and then gradually declines to reach the mature value (26, 27). The expression of BDNF is low during the embryonic stage and dramatically increases after birth (28–30). The expression of CSPGs increases from postnatal day 22 and reaches adult levels at day 70 (4). These expression analyses correlated with our observations and provide an interesting hypothesis for synapse formation during development; namely, that the increased level of BDNF induces the drastic spine formation, and appearance of CSPGs leads to closure of the critical period by blocking TrkB-mediated signaling and gradual elimination of spines by stimulatory p75NTR-mediated signaling.

In conclusion, our findings elucidate the cross-talk between CSPGs and BDNF at the receptor level in neurons. The relevance of these findings *in vivo* should be assessed in the future.

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