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

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Chondroitin sulfate proteoglycans (CSPGs) are components of the extracellular matrix that inhibit axonal sprouting and experience-dependent plasticity. Although protein-tyrosine phosphatase ζ (PTPζ) 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 (TrkB), the receptor of brain-derived neurotrophic factor (BDNF), via PTPζ in embryonic cortical neurons in vitro. Whereas BDNF promoted dendritic spine formation in embryonic cortical neurons, CSPGs abolished the effects. 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) rather than collagen, 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 ζ (PTPζ) has been shown to be the receptor of CSPGs (11). The observation that PTPζ 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 PTPζ 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-α-tubulin clone B-7 (Santa Cruz Biotechnology), anti-neurotrophin receptor p75 (anti-p75NTR; Millipore), antiphosphotyrosine clone 4G10 (Millipore), anti-PTPζ clone 17G7.2 (MEDIMABS), anti-Shc (Millipore), biotinylated anti-TrkB (R&D), and anti-phospho-TrkB (Tyr706/707; Cell Signaling Technology). The following reagents were used: recombinant human BDNF (PeproTech) and CSPG mixture (Millipore).

**Plasmid Constructs**—For generation of murine PTPζ (Accession number BC052462) shRNA constructs, the following oligonucleotides were used: 5’-GATCTCCGACATCATGGGTAGTG-3’.
ATTATCAAGAGATAATCAGCTCCGATGCTTTTTTA-3′ and 5′-AGCTTAAGACATCTGGTATGTATTTCCTCGAAATATCATC-3′ (shRNA #1: 2978–2996 nucleotide position of murine PTPσ; 5′-GATCTCCCGAAATCACGGATCAAATGTTTCAAGAGAACATTTGATCCCTGATTTCTTTTTA-3′ and 5′-AGCTTAAGACATCTGGTATGTATTTCCTCGAAATATCATC-3′ (shRNA #2: 3144–3162 nucleotide position of murine PTPσ). Sense and antisense oligonucleotides were annealed and cloned into BglII and HindIII sites of the pSUPER gfp/neo vector (Invitrogen). For generation of murine PTPσ constructs, the full-length murine PTPσ and GFP expression cassette in the pSUPER gfp/neo vector was cloned into cDNA3.1 (Invitrogen) 2 h after plating and maintained at 37 °C in 5% CO2, 8% FBS, 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% CO2.

Neuroelectected mouse cortical neurons were suspended in 100 μl of transfection solution (Amaza Biosystems) containing 2.5 μg of the various plasmid DNA. The cell suspension was neuroelectected using program O-005 (Amaza Biosystems) according to the manufacturer’s protocol. After the addition of 500 μl 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 (Invitrogen) 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 μg of plasmid DNA and 8 μl 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 MgCl2, 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 incubated for 3 h at 4 °C with 2 μg 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 PTPσ, 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-PTPσ 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-PTPσ 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 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.
CSPGs Down-regulate Spine Formation by Targeting TrkB

RESULTS

CSPG Stimulation Induces Dephosphorylation of TrkB by PTPs 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 phosphorylation of Shc as well as TrkB (Fig. 1C). These results demonstrate that CSPGs inhibit the TrkB activity in the cortical neurons. We then assessed whether PTPs, the CSPG receptor (11), was involved in this effect. We used shRNA-expressing constructs, pSUPER-PTP#1 and #2, to knock down the expression of endogenous PTPs in cultured cortical neurons. Both the 19-nucleotide sequences corresponding to nucleotides 2978–2996 (shRNA #1) and 3144–3162 (shRNA #2) of the murine PTPs decreased the expression of endogenous PTPs in the cortical neurons (Fig. 1D), indicating that we successfully achieved shRNA-mediated knockdown of PTPs. Knockdown of PTPs 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 PTPs with TrkB—The above results suggest that PTPs contributes to tyrosine dephosphorylation of TrkB. Thus, we next examined whether PTPs interacted with TrkB in the cortical neurons. Supporting this hypothesis, it was previously reported that only trace amounts of PTPs was co-immunoprecipitated with TrkB in HEK 293T cells overexpressing PTPs 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 antibodies against phosphotyrosine and \( \alpha \)-tubulin. E, Western blots showing the levels of phosphorylated TrkB and total TrkB. Cortical neurons were nucleofected with pSUPER, pSUPER-PTP#1 or #2 and maintained for 10 days. Neurons were preincubated with vehicle or 2 \( \mu \)g/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 immunoprecipitation with an anti-TrkB antibody. Western blots for the detection of PTPs interaction of PTPs with TrkB is enhanced by phosphorylation of TrkB significantly increased after the application of BDNF. Stimulation of BDNF-treated cortical neurons with CSPGs also significantly increased the amount of PTPs co-immunoprecipitated with TrkB, indicating that PTPs interacts with TrkB in the cortical neurons (Fig. 2A). The amount of PTPs 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 PTPs 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. 2B). These results suggest that the interaction of PTPs with TrkB is enhanced by phosphorylation of TrkB. Because CSPG stimulation tended to decrease the binding of PTPs with TrkB in the BDNF-treated neurons, phosphorylation levels of TrkB may be a key determinant of the binding of TrkB with PTPs.
CSPGs Down-regulate Spine Formation by Targeting TrkB

**FIGURE 2. PTPα interacts with TrkB.** A, co-immunoprecipitation of PTPα with TrkB in lysates of mouse cortical neurons. Cortical neurons at 10–14 DIV were preincubated with vehicle or 2 μg/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 PTPα and total TrkB. Control experiments were carried out in parallel by excluding the antibody for immunoprecipitation. B, amount of co-immunoprecipitated PTPα 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-unstimulated 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-unstimulated neurons.

We next examined whether these effects of CSPGs on dendritic spine density were mediated by PTPα. Neurons were transfected with pSUPER gfp.neo-PTPα 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 PTPα demonstrated that the PTPα immunoreactivities in GFP-positive neurons were lower in pSUPER gfp.neo-PTPα-transfected neurons than in pSUPER gfp.neo or pSUPER gfp.neo-PTPα-transfected neurons (Fig. 3C). Thus, we successfully achieved shRNA-mediated knockdown of PTPα. In the neurons where PTPα was knocked down, we observed that BDNF increased spine density even after CSPGs stimulation (Fig. 3D), as observed in the absence of CSPG stimulation. We also investigated the effects of overexpression of wild-type or catalytically inactive PTPα on dendritic spine formation. The spine density of neurons transfected with wild-type PTPα did not significantly differ from that of control neurons, suggesting that PTPα was expressed at an adequate level in the control neurons. Neurons transfected with catalytically inactive PTPα had higher density of dendritic spine than the control neurons after stimulation with CSPGs and BDNF (Fig. 3E). This result is consistent with the observation using PTPα knocked down neurons. It has been shown that catalytically inactive PTPα acts as a dominant negative mutant (14). Therefore, PTPα 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 spine formation on BDNF-treated neurons (Fig. 4, A and B). These results indicate that p75NTR is required for 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. 4C). This result indicates that CSPGs does not stimulate p75NTR directly.

**DISCUSSION**

Here, we showed that CSPGs dephosphorylate TrkB via PTPα 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 in cortical 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. 4C). This result indicates that CSPGs does not stimulate p75NTR directly.
CSPGs Down-regulate Spine Formation by Targeting TrkB

or TrkC (12). This discrepancy might be due to molecules that facilitate binding of PTPα/H9268 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 PTPα (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...
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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