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Activation of phospholipase C pathways by a synthetic chondroitin sulfate-E tetrasaccharide promotes neurite outgrowth of dopaminergic neurons

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Abstract

In dopaminergic neurons, chondroitin sulfate (CS) proteoglycans play important roles in neuronal development and regeneration. However, due to the complexity and heterogeneity of CS, the precise structure of CS with biological activity and the molecular mechanisms underlying its influence on dopaminergic neurons are poorly understood. In this study, we investigated the ability of synthetic CS oligosaccharides and natural polysaccharides to promote the neurite outgrowth of mesencephalic dopaminergic neurons and the signaling pathways activated by CS. CS-E polysaccharide, but not CS-A, -C or -D polysaccharide, facilitated the neurite outgrowth of dopaminergic neurons at CS concentrations within the physiological range. The stimulatory effect of CS-E polysaccharide on neurite outgrowth was completely abolished by its digestion into disaccharide units with chondroitinase ABC. Similarly to CS-E polysaccharide, a synthetic tetrasaccharide displaying only the CS-E sulfation motif stimulated the neurite outgrowth of dopaminergic neurons, whereas a CS-E disaccharide or unsulfated tetrasaccharide had no effect. Analysis of the molecular mechanisms revealed that the action of the CS-E tetrasaccharide was mediated through midkine-pleiotrophin/protein tyrosine phosphatase ζ and brain-derived neurotrophic factor/tyrosine kinase B receptor pathways, followed by activation of the two intracellular phospholipase C (PLC) signaling cascades: PLC/protein kinase C and PLC/inositol 1,4,5-triphosphate/inositol 1,4,5-triphosphate receptor signaling leading to intracellular Ca^{2+} concentration-dependent activation of Ca^{2+}/calmodulin-dependent kinase II and calcineurin. These results indicate that a specific sulfation motif, in particular the CS-E tetrasaccharide unit, represents a key structural determinant for activation of midkine, pleiotrophin and brain-derived neurotrophic factor-mediated signaling, and is required for the neuritogenic activity of CS in dopaminergic neurons.

Keywords: chondroitin sulfate, dopaminergic neurons, neurite outgrowth, sulfation sequence, synthetic oligosaccharide, tetrasaccharide.

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In the CNS, chondroitin sulfate (CS) proteoglycans are an important component of the cell surface and extracellular matrix (ECM) (Properzi and Fawcett 2004) and play an essential role in neuronal development and regeneration. CS shows a particular spatiotemporal expression in the CNS during development and after injury (Fernaud-Espinosa *et al.* 1996; Charvet *et al.* 1998a; Carulli *et al.* 2005) and modulates various cellular events including cell adhesion, cell migration, neurite outgrowth, neuronal polarity, synaptic plasticity and regeneration (Bovolenta and Fernaud-Espinosa 2000; Carulli *et al.* 2005). CS is generally considered to inhibit the migration and neurite outgrowth of neuronal cells. After injury in the CNS, CS is strongly expressed in the scar tissue of injury sites and restricts axon regeneration (Davies *et al.* 1999). *In vitro* studies using cultured cerebellar granule neurons also demonstrated that CS inhibits neurite outgrowth (Sivasankaran *et al.* 2004). In contrast to these findings, several studies demonstrated the ability of CS to promote the outgrowth of embryonic hippocampal neurons (Clement *et al.* 1998, 1999; Tully *et al.* 2004) and of mesencephalic

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Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; BDNF, brain-derived neurotrophic factor; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; CHase, chondroitinase ABC; CS, chondroitin sulfate; DAG, diacylglycerol; ECM, extracellular matrix; FGF-2, basic fibroblast growth factor; GalNAc, *N*-acetylgalactosamine; GlcA, D-glucuronic acid; IP_3, inositol 1,4,5-triphosphate; NFAT, nuclear factor of activated T-cells; PKC, protein kinase C; PLC, phospholipase C; PLO, poly-L-ornithine; PTN, pleiotrophin; PTPζ, protein tyrosine phosphatase ζ; TBS, Tris-buffered saline; TH, tyrosine hydroxylase; TrkB, tyrosine kinase B receptor.

dopaminergic neurons (Lafont *et al.* 1992; Faissner *et al.* 1994). However, the mechanism underlying the neuritogenic activity of CS is not well understood.

The dopaminergic system plays a central role in the regulation of motor and cognitive functions (Nestler *et al.* 2001). Abnormalities in the dopaminergic system induce various neuropsychiatric disorders including Parkinson's disease, schizophrenia, and attention deficit/hyperactivity disorder. Functional recovery of dopaminergic neurons and their axonal regeneration are hopeful strategies against the neuronal damage, and may be modulated by ECM and cell surface molecules including CS. Lafont *et al.* (1992) reported that CS purified from astrocyte-conditioned medium enhances the neurite outgrowth of dopaminergic neurons. In another report, CS expressed in the wounded striatum was shown to increase the attachment of embryonic dopaminergic neurons on the surface of striatal tissues and stimulate the neurite outgrowth of dopaminergic neurons (Gates *et al.* 1996). In addition, during development, neurocan, a major CS proteoglycan in the CNS, is involved in the formation of the connections of dopaminergic neurons from the substantia nigra to the striatum (Charvet *et al.* 1998b). Thus, in dopaminergic neurons, CS plays important roles in neuronal development and regeneration, and may be useful for the treatment of neurodegenerative disorders such as Parkinson's disease. Molecular mechanisms that underlie the CS-induced growth of dopaminergic neurons need to be identified for the development of therapeutic agents.

CS proteoglycans are macromolecules consisting of a protein core and glycosaminoglycan sidechains of CS (Bandtlow and Zimmermann 2000). Glycosaminoglycans of CS are sulfated polysaccharides composed of the repeating disaccharide unit, *N*-acetylgalactosamine (GalNAc) and D-glucuronic acid (GlcA) residues. CS is divided into at least four subclasses, known as CS-A, CS-C, CS-D and CS-E, on the basis of the sulfation patterns of their major disaccharide units (Sugahara *et al.* 2003; Properzi and Fawcett 2004). CS chains display multiple diverse sulfation patterns that are tightly regulated *in vivo* (Kitagawa *et al.* 1997; Plaas *et al.* 1998). The sulfation patterns are thought to determine the functional interaction of CS with growth factors, cell surface receptors and other components of the ECM (Sugahara *et al.* 2003; Gama *et al.* 2006). However, the complexity and heterogeneity of CS sulfation and a lack of molecular tools has hampered efforts to identify the precise structural motif of CS with biological activity. Furthermore, a molecular-level understanding of interactive proteins and pathways activated by CS has been lacking.

To develop new tools for studying CS structure-function relationships, we chemically synthesized CS oligosaccharides with specific sulfation sequences (Gama *et al.* 2006). By utilizing these well-defined synthetic CS oligosaccharides, we could analyze the sulfation sequence and chain length of CS required for the induction of neurite outgrowth of mesencephalic dopaminergic neurons. Our studies identified a CS-E tetrasaccharide as the minimal essential structure and demonstrated that the synthetic CS-E tetrasaccharide interacts with midkine, pleiotrophin (PTN) and brain-derived neurotrophic factor (BDNF) to activate phospholipase C (PLC) signaling pathways in dopaminergic neurons. These findings provide insight into the molecular basis of CS activity and information relevant to the prevention and treatment of neurodegenerative disorders.

Materials and methods

Reagents

CS-A polysaccharide from whale cartilage, CS-C and CS-D polysaccharides from shark cartilage, CS-E polysaccharide from squid cartilage, protease-free chondroitinase ABC lyase (EC 4.2.2.4) from *Proteus vulgaris* (CHase), monoclonal mouse anti-CS-A antibody (2H6) and monoclonal mouse anti-CS-D antibody (MO225) were purchased from Seikagaku (Tokyo, Japan). Synthetic CS oligosaccharides such as CS-E disaccharide, CS-E tetrasaccharide and unsulfated tetrasaccharide were synthesized as described (Tully *et al.* 2004). Monoclonal antibodies against CS-C (5D2-1D2) (Gama *et al.* 2006) and CS-E (2D11-2A10) (Tully *et al.* 2006) were generated against synthetic CS-C and CS-E tetrasaccharides, respectively. Other reagents were obtained from the following sources: polyclonal rabbit anti-tyrosine hydroxylase (TH) antibody from Pel-Freeze (Rogers, AK, USA); polyclonal sheep anti-TH antibody from Chemicon (Temecula, CA, USA); polyclonal goat anti-midkine antibody, polyclonal goat anti-PTN antibody, polyclonal rabbit anti-BDNF antibody, polyclonal rabbit anti-protein tyrosine phosphatase ζ (PTPζ) antibody and polyclonal goat anti-tyrosine kinase B receptor (TrkB) antibody from Santa Cruz (Santa Cruz, CA, USA); polyclonal goat anti-basic fibroblast growth factor (FGF-2) antibody from R&D Systems (Minneapolis, MN, USA); FITC-conjugated anti-rabbit IgG, FITC-conjugated anti-sheep IgG, rhodamine red-conjugated anti-goat IgG and rhodamine red-conjugated anti-mouse IgG from Jackson ImmunoResearch (Baltimore, PA, USA); Dulbecco's modified eagle medium, N2 supplement and fetal bovine serum from Invitrogen (Grand Island, NY, USA); minimal eagle's medium, poly-L-ornithine (PLO), deoxyribonuclease I, soy bean trypsin inhibitor type II, bovine serum albumin, bisindolylmaleimide II, xestospongin C, BAPTA-AM and H89 from Sigma (St. Louis, MO, USA); U73122 from Tocris Cookson (Bristol, UK); cyclosporin A from Alexis Biochemicals (San Diego, CA, USA); KN62 from Seikagaku.

Coating coverslips with CS

Glass coverslips for cell culture were coated as described by Clement *et al.* (1998) with some modifications. Briefly, the glass coverslips were pre-coated with 0.1 mg/mL PLO for 2 h at 35°C with 5% CO_2, washed three times with double distilled water and then coated with CS (CS-A, -C, -D or -E) polysaccharide, CHase-digested CS-E polysaccharide or synthetic CS oligosaccharide overnight at 35°C with 5% CO_2. CS-E polysaccharide at 16 and 50 μg/mL was digested with CHase (10 and 100 mU/mL) at 37°C for 2 h. The glass coverslips were washed three times with Ca^{2+}/

Mg^{2+}-free phosphate-buffered saline and flooded with minimal eagle's medium supplemented with the N2 mixture.

The amounts of CS polysaccharides and CS-E tetra- and disaccharides, immobilized to PLO-coated glass coverslips, were monitored by immunofluoresence using anti-CS antibodies; anti-CS-A antibody (2H6), anti-CS-C antibody (5D2-1D2), anti-CS-D antibody (MO225) and anti-CS-E antibody (2D11-2A10), and rhodamine red-conjugated secondary antibody. The immobilized CS was quantified by densitometry using Image J 1.36b software (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/). The binding of CS-E polysaccharide to PLO-coated glass coverslips was linear at concentrations from 1 to 16 μg/mL, and reached a plateau at 50 μg/mL. The binding of CS-A, -C and -D polysaccharides was similar to that of CS-E polysaccharide. We recently reported that approximately 86% of the polysaccharide heparin, a highly related glycosaminoglycan, binds to poly-L-lysine-coated slides regardless of its sulfation pattern (Shipp and Hsieh-Wilson 2007). Based on this assumption, the estimated amount of CS polysaccharides immobilized to PLO-coated glass coverslips (2 cm^2) is 1.03 and 3.22 $\mu g/cm^2$ when coated with 150 μL of CS solutions at concentrations of 16 and 50 μg/mL, respectively. Fluorescence intensities of CS-E tetra- and disaccharides at 50 μg/mL were 53% and 30% of that of CS-E polysaccharide at the same concentration, respectively, likely due to the lower binding affinity of the anti-CS-E antibody for CS-E tetra- and disaccharides.

Primary mesencephalic neuronal culture

Primary mesencephalic dopaminergic neurons were prepared as described (Hida *et al.* 2003). Staged pregnant Sprague–Dawley rats were obtained from CLEA Japan, Inc. (Tokyo, Japan). Ventral mesencephalic tissues of rats at embryonic day (E) 15 were isolated under a dissecting microscope. The tissues were incubated in 0.25% trypsin in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline for 20 min at 37°C, and treated with deoxyribonuclease I (50 μg/mL) and soy bean trypsin inhibitor type II (50 μg/mL) for 5 min at 37°C. The tissues were then triturated to yield a suspension of single cells in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Cells were plated on glass coverslips coated with various substrates as described above at the density of 1.0×10^5 cells/cm^2, and were cultured at 35°C with 5% CO_2 for 5 days. Coating of coverslips with CS-A, -C, -D or -E polysaccharide at 16 and 50 μg/mL did not affect the number of TH-positive dopaminergic neurons and the protein levels of TH analyzed by western blot (data not shown).

Treatment of mesencephalic neurons with antibodies

Mesencephalic cells were cultured on glass coverslips coated with CS-E tetrasaccharide (16 μg/mL) for 2 days. The culture medium was switched to that containing the antibody specific for midkine (4 μg/mL), PTN (2 μg/mL), BDNF (1 μg/mL), FGF-2 (4 μg/mL), PTPζ (2 μg/mL) or TrkB (1 μg/mL) (Gama *et al.* 2006). These cells were cultured for 3 days in the presence of either antibody.

Pharmacological inhibition of intracellular signaling molecules

To analyze the intracellular signaling cascades, mesencephalic cells were cultured on glass coverslips coated with CS-E tetrasaccharide and CS-E polysaccharide for 2 days, and then the medium was switched to that containing U73122 (PLC inhibitor; 10 nmol/L), bisindolymaleimide II [protein kinase C (PKC) inhibitor; 10 nmol/L], xestospongin C [inositol 1,4,5-triphosphate (IP_3) receptor inhibitor; 1 μmol/L], BAPTA-AM (intracellular calcium chelator; 5 nmol/L), KN62 [Ca^{2+}/calmodulin-dependent kinase II (CaMKII) inhibitor; 1 μmol/L], cyclosporin A (calcineurin inhibitor; 1 μmol/L) or H89 (protein kinase A inhibitor; 2 μmol/L). These cells were cultured for 3 days in the presence of various inhibitors.

Immunocytochemistry of dopaminergic neurons

After 5 days in culture, mesencephalic cells on glass coverslips were washed with Tris-buffered saline (TBS) and fixed with 4% paraformaldehyde in TBS for 2 h at 23°C. Cells were permeabilized with TBS containing 1% bovine serum albumin and 0.2% Triton X-100 for 1 h at 23°C, and then incubated with rabbit anti-TH antibody (1 : 1000 dilution) at 4°C overnight. After washing with TBS (three times for 5 min), the cell samples were incubated with FITC-conjugated secondary antibody (1 : 800 dilution) for 3 h at 20°C. The cells were mounted on glass slides using Vectashield (Vector, Burlingame, CA, USA) for fluorescent microscopic examination.

To visualize the expression of PTPζ and TrkB in dopaminergic neurons, cells were incubated with rabbit anti-TH antibody (1 : 1000 dilution), sheep anti-TH antibody (1 : 500 dilution), rabbit anti-PTPζ antibody (1 : 100 dilution) and goat anti-TrkB antibody (1:100 dilution), followed by the incubation with FITC-conjugated secondary antibody for TH and rhodamine red-conjugated secondary antibody for PTPζ or TrkB. The cells were mounted in Vectashield and examined with a confocal microscope (LSM 5 PASCAL) using a 63X oil-immersed objective (Zeiss, Oberkochen, Germany).

Quantification of neurite outgrowth

The immunostained cells on each coverslip were scanned by fluorescence microscopy equipped with high-resolution digital camera. The neurite length from the neuronal perikarya of immunostained TH-positive neurons was measured with Neurolucida 2000 (MicroBrightField Inc., Williston, VT, USA). The neurite length is expressed as the total length of neurite from perikarya in each neuron. In each experiment, we measured at least ~50 cells and repeated the same experiments two or three times. The results were expressed as the mean ± SEM, and statistical analysis was carried out using one-way ANOVA followed by Scheffe's *post hoc* test.

Results

CS-E polysaccharide stimulates the neurite outgrowth of dopaminergic neurons

To evaluate the structure-function relationships of CS in promoting the outgrowth of dopaminergic neurons, rat mesencephalic dopaminergic neurons were cultured on glass coverslips pre-coated with PLO and then coated with CS-A, -C, -D or -E polysaccharide. Coating of coverslips with CS-E polysaccharide significantly increased the total neurite length of dopaminergic neurons, compared to coating with PLO alone. The total neurite length (mean ± SEM) of dopaminergic neurons cultured on PLO alone, CS-E polysaccharide at

16 μg/mL, and CS-E polysaccharide at 50 μg/mL was 325.8 ± 12.6, 400.3 ± 14.6 and 429.4 ± 18.1 μm, respectively. Neither CS-A, -C nor -D polysaccharide affected the neurite outgrowth of dopaminergic neurons (Fig. 1a and b). CS-E polysaccharide increased the neurite length of dopaminergic neurons maximally at concentrations of 16 and 50 μg/mL, with a half-maximal effect at ~10 μg/mL (Fig. 1c).

CS-E polysaccharide also increased the length of the longest neurite. The length of the longest neurite (mean ± SEM) of dopaminergic neurons cultured on PLO alone, CS-E polysaccharide at 16 μg/mL, and CS-E polysaccharide at 50 μg/mL was 161.2 ± 6.78, 195.0 ± 9.84 and 202.3 ± 9.88 μm, respectively. Neither CS-A, -C nor –D polysaccharide affected the length of the longest neurite. The number of primary neurites per neuron was 1.82 ± 0.42 under control conditions (PLO alone), and was not affected by either CS-A, -C, -D or –E polysaccharide (data not shown). These results suggest that CS-E mainly promotes the outgrowth of axon-like neurites (Bao *et al.* 2005).

CHase is known to digest CS-E polysaccharide into disaccharide units with an unsaturated uronic acid moiety at the non-reducing end (Nadanaka *et al.* 1998). The stimulatory

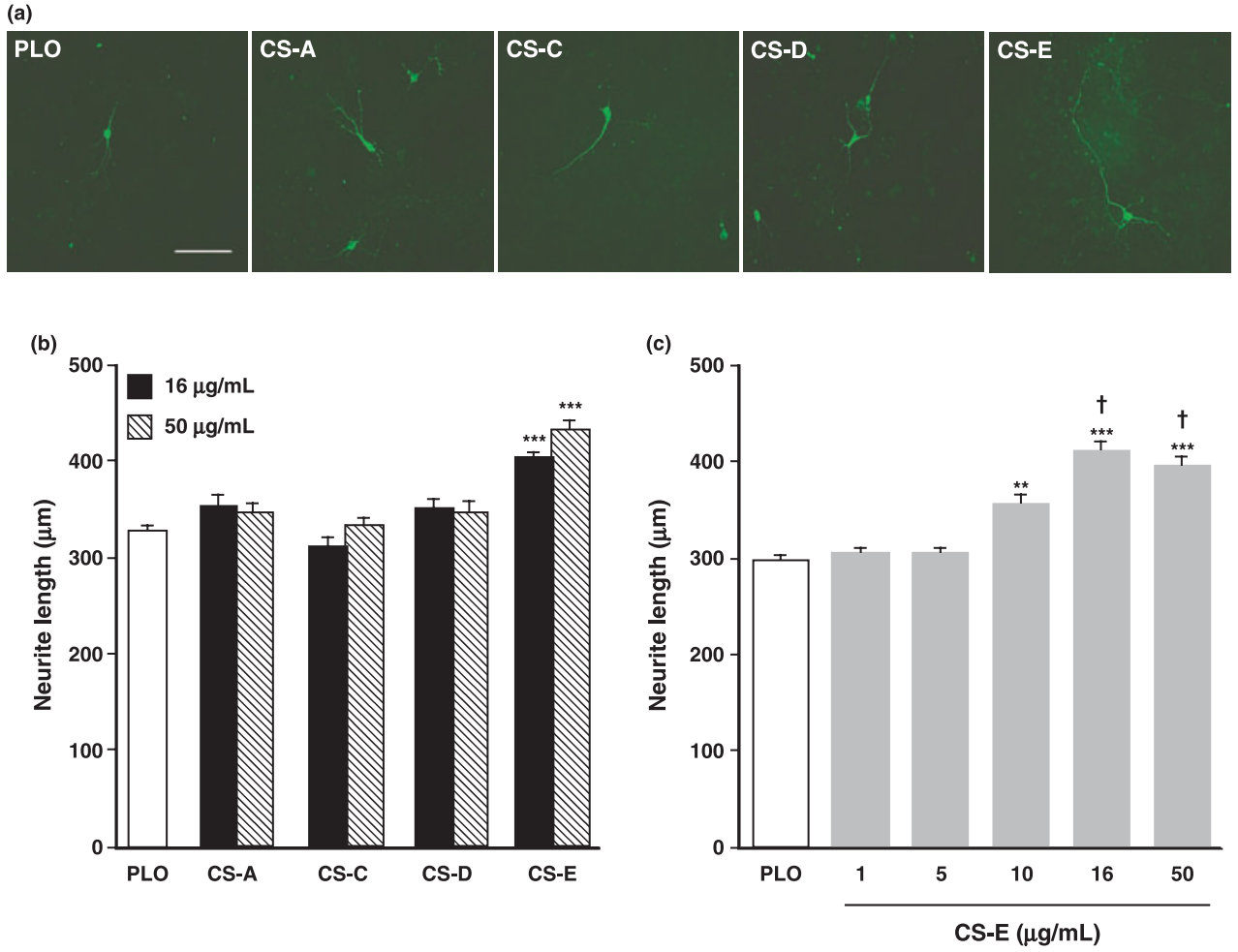

Fig. 1 Neurite outgrowth of tyrosine hydroxylase (TH)-positive neurons cultured on different chondroitin sulfate (CS) polysaccharides. Mesencephalic cells were cultured for 5 days on glass coverslips coated with poly-L-ornithine (PLO) alone (control) or CS (CS-A, -C, -D or -E) polysaccharide. The cells were immunostained with an anti-TH antibody and were scanned by fluorescence microscopy. (a) Representative morphologies of TH-positive, dopaminergic neurons cultured on coverslips coated with PLO alone or CS (CS-A, -C, -D or -E) polysaccharide (16 μg/mL). Scale bar, 100 μm. (b) Quantitative analysis of effects of CS (CS-A, -C, -D or -E) polysaccharide at 16 and 50 μg/mL on the neurite outgrowth of TH-positive neurons. The neurite length of TH-positive neurons was measured with Neurolucide 2000. The neurite length is expressed as total length of neurite from perikarya. Data represent mean ± SEM of 152–180 cells obtained from three independent experiments. ***$p < 0.001$ compared with PLO alone. (c) Dose-dependent effect of CS-E polysaccharide on the neurite outgrowth of TH-positive neurons. Mesencephalic cells were cultured on glass coverslips coated with PLO alone or with various concentrations of CS-E polysaccharide (1, 5, 10, 16 and 50 μg/mL). Data represent mean ± SEM of 100–124 cells obtained from two independent experiments. **$p < 0.01$, ***$p < 0.001$ compared with PLO alone; $^{\dagger}p < 0.05$ compared with CS-E polysaccharide at 10 μg/mL.

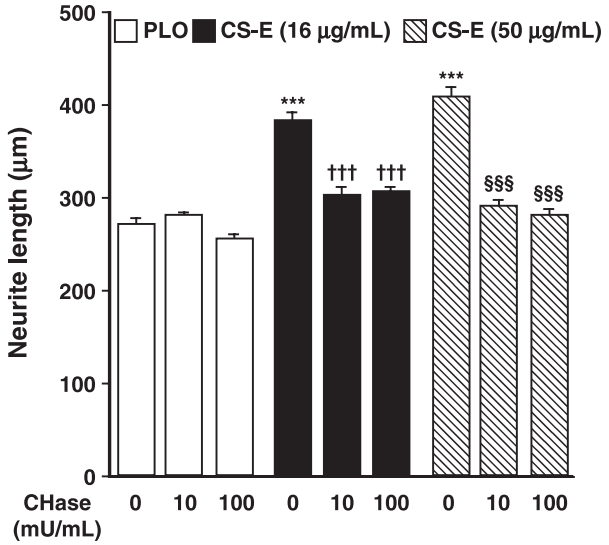

Fig. 2 Effect of digestion of chondroitin sulfate (CS)-E polysaccharide with chondroitinase ABC (CHase) on the bioactivity of CS-E polysaccharide to promote the neurite outgrowth. Mesencephalic cells were cultured for 5 days on glass coverslips coated with poly-L-ornithine (PLO) alone, CHase (10 and 100 mU/mL) or CS-E polysaccharide (16 and 50 μg/mL), undigested and digested with CHase (10 and 100 mU/mL). The neurite outgrowth of TH-positive neurons was analyzed as described in Fig. 1. Data represent mean ± SEM of 141–165 cells obtained from three independent experiments. ***$p < 0.001$ compared with PLO alone; $^{\dagger\dagger\dagger}p < 0.001$ compared with CS-E polysaccharide at 16 μg/mL; $^{\S\S\S}p < 0.001$ compared with CS-E polysaccharide at 50 μg/mL.

effect of CS-E polysaccharide (16 and 50 μg/mL) on the neurite outgrowth was completely abolished by digestion of CS-E polysaccharide with CHase (10 and 100 mU/mL) before coating on coverslips (Fig. 2). Coating of coverslips with CHase (10 and 100 mU/mL) *per se* did not affect the neurite outgrowth of dopaminergic neurons. These results suggest that the CS-E sulfation motif is the critical determinant for the biological activity of CS in dopaminergic neurons.

CS-E tetrasaccharide is the minimal structural motif of CS for the biological activity in dopaminergic neurons

CS-E polysaccharide purified from squid cartilage is enriched in the CS-E sulfation motif, but the content of CS-E motif is less than 60% (Bao *et al.* 2004). As such, CS-E polysaccharide is a heterogeneous mixture of various sulfation patterns, and the biological activity of CS-E polysaccharide might be due to other sulfation motifs. To establish that the CS-E sulfation motif was indeed responsible for the neurite outgrowth-promoting ability of CS, we utilized a synthetic CS tetrasaccharide that displayed two repeating CS-E motifs (Fig. 3a) (Tully *et al.* 2004). By utilizing the structurally defined, synthetic CS-E, the structural sequence of CS-E was evaluated for its biological activity in the absence of other contaminating motifs. The

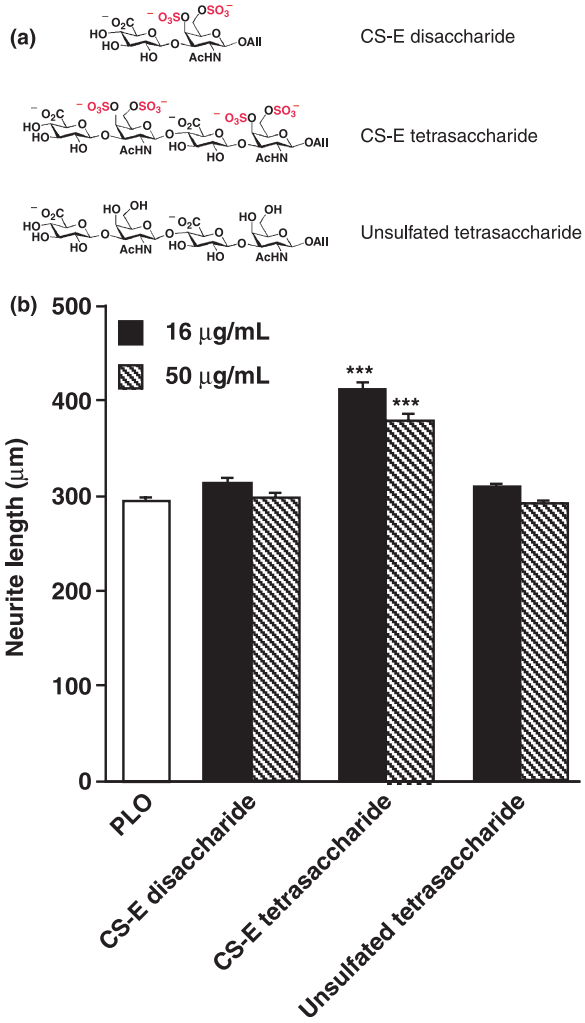

Fig. 3 Neurite outgrowth of tyrosine hydroxylase (TH)-positive neurons cultured on synthetic chondroitin sulfate (CS) oligosaccharides. (a) Structures of CS-E disaccharide, CS-E tetrasaccharide and unsulfated tetrasaccharide. Ac, acetyl; All, allyl. (b) Quantitative analysis of effects of synthetic CS oligosaccharides on the neurite outgrowth of TH-positive neurons. Mesencephalic cells were cultured for 5 days on glass coverslips coated with PLO alone, CS-E disaccharide (16 and 50 μg/mL), CS-E tetrasaccharide (16 and 50 μg/mL) or unsulfated tetrasaccharide (16 and 50 μg/mL). The neurite outgrowth of TH-positive neurons was analyzed as described in Fig. 1. Data represent mean ± SEM of 89–135 cells obtained from two independent experiments. ***$p < 0.001$ compared with PLO alone.

synthetic CS-E tetrasaccharide, coated on coverslips at 16 and 50 μg/mL, stimulated the neurite outgrowth of dopaminergic neurons, similarly to CS-E polysaccharide (Fig. 3b). In contrast, neither a CS-E disaccharide nor an unsulfated tetrasaccharide affected the neurite outgrowth of dopaminergic neurons. We confirmed that the lack of activity for the CS-E disaccharide was not due to low coating efficiency on coverslips using an anti-CS-E antibody (Tully *et al.* 2006). The fluorescence intensity of the

CS-E disaccharide (50 μg/mL) immobilized to PLO-coated glass coverslips was 60% of that of the CS-E tetrasaccharide, indicating that the CS-E disaccharide adheres well to the coverslips. The reduced fluorescence intensity is likely due to the lower affinity of the antibody for the CS-E disaccharide relative to the tetrasaccharide (Tully *et al.* 2006). Importantly, the CS-E disaccharide at 50 μg/mL showed similar fluorescence intensity to the CS-E tetrasaccharide at 16 μg/mL (data not shown), yet the disaccharide did not exhibit neurite outgrowth-promoting activity. Together, these results indicate that a CS-E tetrasaccharide represents the minimal structural motif for the biological activity of CS in dopaminergic neurons.

CS-E tetrasaccharide requires activation of midkine-PTN/PTPζ and BDNF/TrkB signaling pathways for the neurite outgrowth of dopaminergic neurons

To elucidate the molecular mechanisms by which CS-E tetrasaccharide induces the neurite outgrowth of dopaminergic neurons, the stimulatory effect of CS-E tetrasaccharide (16 μg/mL) was examined in the presence of blocking antibodies against either midkine, PTN, BDNF or FGF-2 (Gama *et al.* 2006). Treatment of dopaminergic neurons with each antibody did not affect the neurite outgrowth under control conditions (PLO alone) (Fig. 4a). The neurite outgrowth promoted by CS-E tetrasaccharide was completely abolished by antibodies against midkine, PTN and BDNF, but not by an antibody against FGF-2.

As midkine and PTN bind to the cell surface receptor, PTPζ, and BDNF binds to TrkB, we analyzed the effects of function-blocking antibodies that recognize the extracellular domains of PTPζ and TrkB on CS-E tetrasaccharide-induced neurite outgrowth (Gama *et al.* 2006). Treatment with either antibody did not affect the neurite outgrowth under control conditions (PLO alone) (Fig. 4a). In contrast, the neurite outgrowth stimulated by CS-E tetrasaccharide was completely abolished by PTPζ and TrkB antibodies. Immunofluorescence analysis revealed the expression of PTPζ and TrkB (Hoover *et al.* 2007) in dopaminergic neurons at cell bodies, neurites and growth cones (Fig. 4b). In immunoblotting analysis of PTPζ, a band with a relative molecular mass of ~250 kDa, which corresponded to the short form of PTPζ (Sakurai *et al.* 1996; Garwood *et al.* 1999), was detected. These results suggest that the ability of CS-E tetrasaccharide to promote the neurite outgrowth of dopaminergic neurons is mediated through activation of midkine-PTN/PTPζ and BDNF/TrkB signaling pathways.

Role of PLC-mediated and intracellular Ca^{2+} concentration-dependent signaling in the neurite outgrowth of dopaminergic neurons induced by CS-E tetrasaccharide

We next examined the intracellular mechanisms of CS-E tetrasaccharide-induced neurite outgrowth of dopaminergic

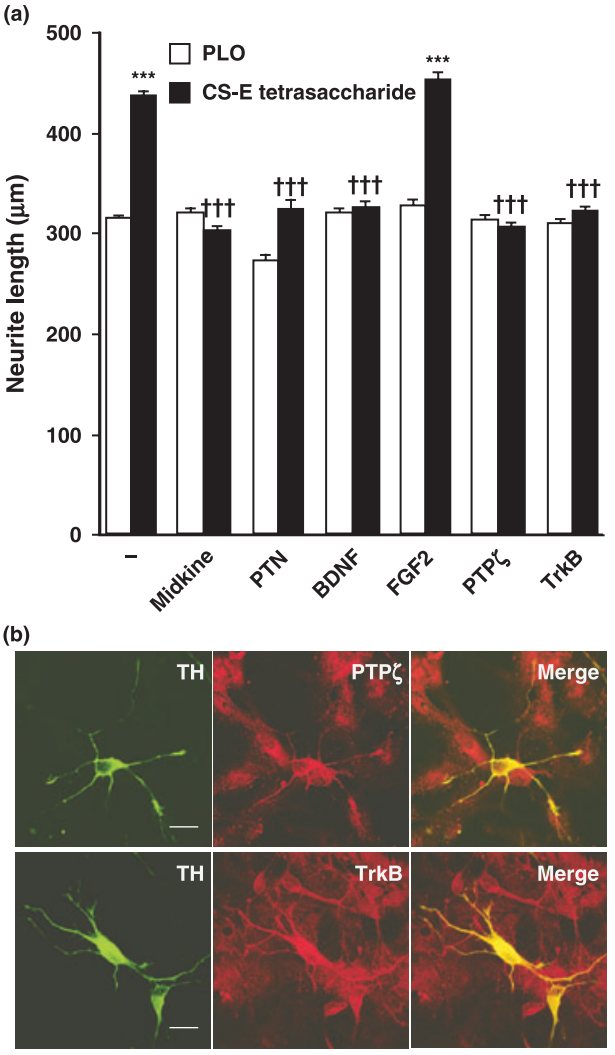

Fig. 4 Effect of antibodies for midkine- pleiotrophin (PTN)/protein tyrosine phosphatase ζ (PTPζ) and brain-derived neurotrophic factor (BDNF)/tyrosine kinase B receptor (TrkB) signaling on chondroitin sulfate (CS)-E tetrasaccharide-induced neurite outgrowth of tyrosine hydroxylase (TH)-positive neurons. (a) Mesencephalic cells were cultured for 2 days on glass coverslips coated with poly-L-ornithine (PLO) alone or CS-E tetrasaccharide (16 μg/mL). The culture medium was switched to that containing either antibody specific for midkine (4 μg/mL), PTN (2 μg/mL), BDNF (1 μg/mL), basic fibroblast growth factor (FGF-2) (4 μg/mL), PTPζ (2 μg/mL) or TrkB (1 μg/mL). These cells were cultured for 3 days in the presence of either antibody. The neurite outgrowth of TH-positive neurons was analyzed as described in Fig. 1. Data represent mean ± SEM of 114–140 cells obtained from two independent experiments. $^{***}p < 0.001$ compared with PLO alone; $^{†††}p < 0.001$ compared with CS-E tetrasaccharide. (b) Photomicrographs of mesencephalic cells after immunostaining with anti-TH, -PTPζ and -TrkB antibodies. Fluorescent images were collected at 63X magnification by confocal laser scanning microscopy. Scale bar, 20 μm.

neurons. It has been reported that PLC is involved in the neurite outgrowth mediated by extracellular matrices, neurotrophic factors and neural cell adhesion molecules in

various types of neurons (Ming *et al.* 1999; Kolkova *et al.* 2000; Leshchyns'ka *et al.* 2003). Therefore, we examined whether PLC is involved in the neurite outgrowth induced by CS-E tetrasaccharide. Treatment of dopaminergic neurons with a PLC inhibitor, U73122 (10 nmol/L), did not affect the neurite outgrowth under control conditions (PLO alone), but completely abolished the stimulatory effect of CS-E tetrasaccharide on the neurite outgrowth (Fig. 5a). As activation of PLC subsequently stimulates PKC and IP_3 receptors (Berridge 1998), the effect of inhibitors of PKC and IP_3 receptors was examined. The neurite outgrowth stimulated by CS-E tetrasaccharide was completely abolished by a PKC inhibitor, bisindolylmaleimide II (10 nmol/L), and by an IP_3 receptor inhibitor, xestospongin C (1 μmol/L) (Fig. 5a). Neither inhibitor affected the neurite outgrowth under control conditions (PLO alone).

As IP_3 receptors are involved in the neurite outgrowth induced by CS-E tetrasaccharide, it is possible that the CS-E tetrasaccharide increases intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and activates Ca^{2+}-dependent molecules such as CaMKII and calcineurin. Treatment with an intracellular calcium chelator, BAPTA-AM (5 nmol/L), did not affect the neurite outgrowth under control conditions (PLO alone), but completely abolished CS-E tetrasaccharide-induced neurite outgrowth (Fig. 5b), indicating the essential role of $[Ca^{2+}]_i$ in the action of CS-E. Treatment with a CaMKII inhibitor, KN62 (500 nmol/L), and a calcineurin inhibitor, cyclosporin A (1 μmol/L), did not affect the neurite outgrowth under control conditions, but abolished the CS-E tetrasaccharide-induced neurite outgrowth (Fig. 5b). In agreement with results obtained using the CS-E tetrasaccharide, the stimulatory effect of CS-E polysaccharide (16 μg/mL) on the neurite outgrowth was abolished by U73122 (10 nmol/L), bisindolylmaleimide II (10 nmol/L), xestospongin C (1 μmol/L), KN62 (500 nmol/L) and cyclosporin A (1 μmol/L), but not by a protein kinase A inhibitor, H89 (2 μmol/L) (data not shown). Taken together, these results suggest that the CS-E sulfation motif promotes the neurite outgrowth of dopaminergic neurons via activation of PLC-mediated signaling pathways involving PKC and IP_3 receptors (Fig. 6). Action of IP_3 receptors is likely mediated through subsequent activation of $[Ca^{2+}]_i$-dependent CaMKII and calcineurin signaling.

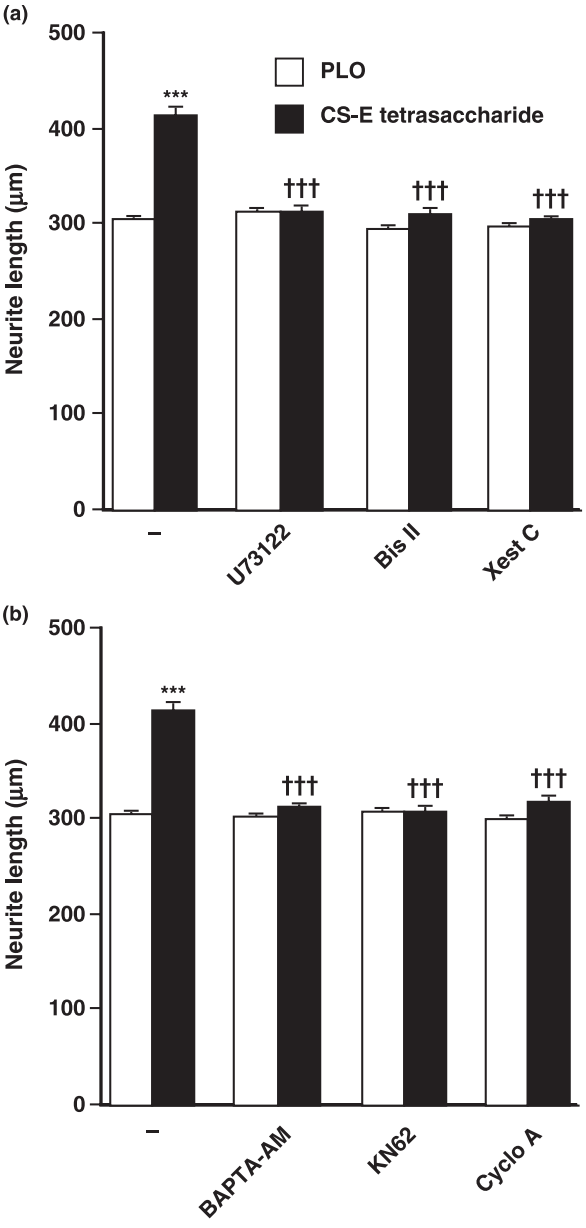

Fig. 5 Effect of inhibitors for (a) phospholipase C-mediated and (b) $[Ca^{2+}]_i$-dependent signaling on chondroitin sulfate (CS)-E tetrasaccharide-induced neurite outgrowth of tyrosine hydroxylase-positive neurons. Mesencephalic cells were cultured for 2 days on glass coverslips coated with poly-L-ornithine (PLO) alone or CS-E tetrasaccharide (16 μg/mL). The culture medium was switched to that containing U73122 (phospholipase C inhibitor; 10 nmol/L), bisindolylmaleimide II (Bis II) (PKC inhibitor; 10 nmol/L), xestospongin C (Xest C) (IP_3 receptor inhibitor; 1 μmol/L), BAPTA-AM (intracellular Ca^{2+} chelator; 5 nmol/L), KN62 (CaMKII inhibitor; 500 nmol/L) or cyclosporin A (Cyclo A) (calcineurin inhibitor; 1 μmol/L). These cells were cultured for 3 days in the presence of either inhibitor. The neurite outgrowth of tyrosine hydroxylase-positive neurons was analyzed as described in Fig. 1. Data represent mean ± SEM of 90–109 cells obtained from two independent experiments. ***$p < 0.001$ compared with PLO alone; $^{†††}p < 0.001$ compared with CS-E tetrasaccharide.

Discussion

In this study, we found that CS-E polysaccharide, but not CS-A, CS-C or CS-D polysaccharide, facilitates the neurite outgrowth of dopaminergic neurons. Using synthetic CS oligosaccharides as tools, we demonstrated that the CS-E sulfation motif has the unique ability to promote the neurite outgrowth of dopaminergic neurons, and that a CS-E tetrasaccharide is the minimal essential structure for the biological activity of CS-E in dopaminergic neurons. We

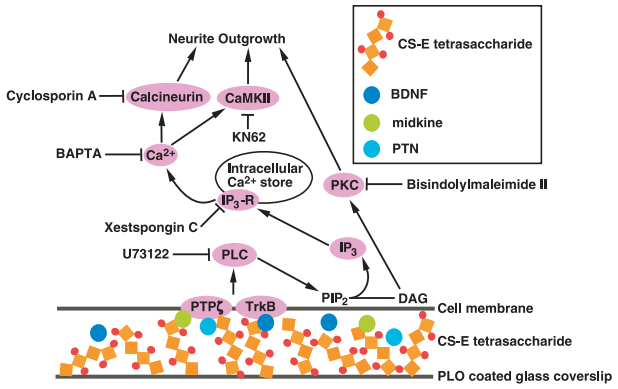

Fig. 6 Model for signaling pathways involved in chondroitin sulfate (CS)-E tetrasaccharide-induced neurite outgrowth of dopaminergic neurons. CS-E tetrasaccharides selectively interact with midkine, pleiotrophin (PTN) and brain-derived neurotrophic factor (BDNF), recruit them to the cell surface, and modulate the midkine-PTN/protein tyrosine phosphatase ζ (PTPζ) and BDNF/tyrosine kinase B receptor (TrkB) signaling pathways, leading to activation of phospholipase C (PLC) signaling. PLC activated by PTPζ and TrkB stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and the generation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). The generated DAG subsequently activates protein kinase C (PKC). At the same time, the generated IP_3 activates IP_3 receptors and increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), resulting in activation of Ca^{2+}-dependent signaling molecules including Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and calcineurin. Activation of intracellular PLC/DAG/PKC and PLC/IP_3/IP_3 receptor/$[Ca^{2+}]_i$ signaling induces the neurite outgrowth of dopaminergic neurons. Thus, CS-E tetrasaccharide promotes the neurite outgrowth of dopaminergic neurons by activation of midkine-PTN/PTPζ and BDNF/TrkB signaling pathways, coupled with the PLC signaling cascades.

further analyzed the molecular mechanisms and showed that the action of CS-E is mediated through midkine-PTN/PTPζ and BDNF/TrkB signaling pathways, leading to activation of intracellular PLC/diacylglycerol (DAG)/PKC and PLC/IP_3/IP_3 receptor/$[Ca^{2+}]_i$ signaling (Fig. 6).

The biological activity of CS is defined by the sulfation pattern of repeating disaccharide units, consisting of GlcA and GalNAc (Ueoka *et al.* 2000). The disaccharide units of CS-A and CS-C are monosulfated in the 4- and 6-positions of GalNAc residue, respectively. The disaccharide units of CS-D and CS-E are disulfated. CS-D is sulfated in the 2-position of GlcA and the 6-position of GalNAc, and CS-E is sulfated in the 4- and 6-position of GalNAc. In this study, we demonstrated that the CS-E motif, but not the CS-A, CS-C or CS-D motif, has the ability to stimulate the neurite outgrowth of dopaminergic neurons. In addition, digestion of CS-E polysaccharide with CHase, yielding disaccharides with an unsaturated uronic acid moiety at the non-reducing end, abolished the effect of CS-E polysaccharide. These results indicate that the CS-E motif with disulfation in the 4- and 6-positions of the GalNAc is critical for its biological activity. Furthermore, a synthetic CS-E tetrasaccharide facilitated the neurite outgrowth, but a synthetic CS-E disaccharide did not affect the neurite outgrowth of dopaminergic neurons. Thus, the CS-E tetrasaccharide motif is the minimum structure for CS-E to induce its biological activity in dopaminergic neurons.

In previous reports, CS-D as well as CS-E polysaccharides was shown to promote neurite outgrowth of embryonic hippocampal neurons (Nadanaka *et al.* 1998; Clement *et al.* 1999). However, in this study, CS-D polysaccharide was not able to promote the outgrowth of dopaminergic neurons. The proportion of the CS-D sulfation motif in CS-D polysaccharide is as low as 20% (Maeda *et al.* 2006). In contrast, CS-E polysaccharide contains more than 60% of CS-E motif. The low content of CS-D motif is not the cause for the lack of neuritogenic activity of CS-D polysaccharide in dopaminergic neurons, because CS-D polysaccharide at 50 μg/mL, three times higher concentration of CS-E polysaccharide (16 μg/mL) with a maximum effect, had no effect. Although our results support the notion that CS-D is not stimulatory toward dopaminergic neurons, we cannot rule out the possibility that the CS-D preparation used in this study lacks the correct serial arrangement of CS-D in combination with other sulfation motifs. Future studies will address the functional role of CS-D using pure, synthetic oligosaccharides in a variety of neuronal cell types.

CS-E has been shown to bind to heparin-binding growth factors, such as midkine and PTN, which participate in the development and repair of neural and other tissues (Ueoka *et al.* 2000; Deepa *et al.* 2002; Muramatsu 2002). We recently reported that the CS-E tetrasaccharide directly interacts with midkine and BDNF *in vitro* using carbohydrate microarrays and that it promotes the neurite outgrowth of cultured hippocampal neurons by modulating midkine/PTPζ and BDNF/TrkB pathways (Gama *et al.* 2006). In this study, we demonstrated that the CS-E tetrasaccharide utilizes midkine-PTN/PTPζ and BDNF/TrkB signaling pathways to induce the neurite outgrowth of dopaminergic neurons. Immunoblockade of either the midkine-PTN/PTPζ or the BDNF/TrkB signaling pathway abolished CS-E tetrasaccharide-induced neurite outgrowth, suggesting that the two pathways interact synergistically to elicit neuritogenic activity. Interestingly, previous reports have suggested that the length of oligosaccharide required for binding to midkine (≥22-mer) (Kaneda *et al.* 1996) and PTN (≥18-mer) (Maeda *et al.* 2006) is longer than a tetrasaccharide. It is possible that the synthesized, pure CS-E tetrasaccharide has an enhanced ability to bind with midkine, PTN and BDNF. In addition, CS-E tetrasaccharide on coverslips may form oligomers and thus bind with higher affinity to proteins. Together, our results indicate that the interaction of CS-E tetrasaccharide with midkine, PTN and BDNF is critical for its action in dopaminergic neurons as well as hippocampal neurons.

CS concentrations are estimated to be at least 60 μmol/L in the brain and may exist at 5- to 10-fold higher local concentrations at the cell surface and in the ECM (Herndon *et al.* 1999). In addition, the CS-E sulfation motif was detected in appreciable proportions in developing rat brain (Ueoka *et al.* 2000; Shuo *et al.* 2004) and was identified on the proteoglycans, appican, an isoform of the amyloid precursor protein that exhibits neurotrophic activity, syndecan-1 and -4, neuroglycan C, neurocan and phosphacan (Tsuchida *et al.* 2001; Deepa *et al.* 2004; Shuo *et al.* 2004). The estimated size of CS-E polysaccharide is 70 kDa (Ueoka *et al.* 2000), and therefore CS-E polysaccharide at the two concentrations used in our assays (16 and 50 μg/mL) corresponds to 0.21 and 0.67 μmol/L, respectively. In case of the CS-E tetrasaccharide, the molar concentrations (14 and 42 μmol/L) are higher due to the small molecular weight of the tetrasaccharide (1,131), but remain within the physiological range. As the proportion of CS-E motif in the CS-E polysaccharide is ~60%, one CS-E polysaccharide molecule is estimated to contain ~37 CS-E tetrasaccharide epitopes maximally. For these reasons, the concentrations of CS-E polysaccharide and CS-E tetrasaccharide used in this study are physiologically relevant and are in the range expected for selective binding to midkine, PTN and BDNF (Gama *et al.* 2006).

BDNF is known to promote the development of dopaminergic neurons *in vitro* (Hyman *et al.* 1991, 1994) and their survival after injury with neurotoxins in adult rodents (Altar *et al.* 1994; Frim *et al.* 1994). In BDNF null mutant mice, dopaminergic neurons have fewer dendrites in the substantia nigra during early postnatal development (Baker *et al.* 2005), confirming the role of BDNF in the development of dopaminergic neurons. However, the role of midkine and PTN in the development of dopaminergic neurons is not known, although midkine and PTN are reported to promote survival and/or differentiation of dopaminergic neurons *in vitro* (Kikuchi *et al.* 1993; Hida *et al.* 2003; Mourlevat *et al.* 2005). Interestingly, CS sulfated in 4- or 6-position of the GalNAc (CS-A or CS-C, respectively) is expressed in and along the developing medial forebrain bundle, an axonal tract of nigrostriatal pathway of dopaminergic neurons, and in the striatum in a spatio-temporal manner (Charvet *et al.* 1998a). As developmental data on CS-E is not available in the medial forebrain bundle and striatum, it will be extremely important to analyze the expression pattern of CS-E during prenatal and postnatal development. One exciting possibility is that CS-E might function as an axonal guidance cue for developing dopaminergic neurons as well as injured dopaminergic neurons by interacting with midkine, PTN and BDNF.

The intracellular mechanism by which CS acts on dopaminergic neurons was unknown. Using the CS-E tetrasaccharide and pharmacological inhibitors, we showed that PLC signaling pathways coupled with two key signaling molecules, PKC and IP_3, play a central role for the activation of dopaminergic neurons by CS-E. PLC signaling is involved in midkine-induced cell migration (Qi *et al.* 2001) and TrkB signaling (Huang and Reichardt 2003), supporting the role of PLC in the action of CS-E. It has been reported that midkine/PTPζ signaling involves Src (Qi *et al.* 2001; Muramatsu 2002), and that the activation of a Src pathway results in activation of PLC (Rhee 2001). On the other hand, binding of PTN or midkine to PTPζ has been shown to inactivate the catalytic activity of PTPζ and increase tyrosine phosphorylation of its substrates (Meng *et al.* 2000; Kawachi *et al.* 2001). However, it is currently unknown whether inactivation of PTPζ results in activation of PLC.

Activated PLC cleaves phosphatidylinositol 4,5-bisphosphate into DAG and IP_3 (Rebecchi and Pentyala 2000). DAG stimulates PKC, whereas IP_3 stimulates the release of Ca^{2+} from internal stores by activating IP_3 receptors. In this study, the stimulatory effect of CS-E tetrasaccharide on the neurite outgrowth was abolished by a PKC inhibitor. Choe *et al.* (2002) reported that activation of PKC, especially the PKCα isoform, induces the cytoskeletal rearrangement of filamentous actin and β-tubulin via the extracellular signal-regulated kinase pathway, leading to the neurite outgrowth in GT1 hypothalamic neurons. In addition, plasma membrane-associated PKC substrates such as GAP-43, CAP-23 and MARCKS are known to regulate the dynamics of the actin cytoskeleton by interacting with phosphatidylinositol 4,5-bisphosphate (Laux *et al.* 2000). Taken together, the PLC/PKC signaling cascade likely plays an important role in CS-E-induced neurite outgrowth of dopaminergic neurons.

IP_3-induced Ca^{2+} release from internal stores is reported as a key regulatory factor of neurite outgrowth (Takei *et al.* 1998). The increase in $[Ca^{2+}]_i$ activate numerous target proteins and cellular machinery, and thereby regulate cytoskeletal elements and membrane dynamics (Henley and Poo 2004). In this study, we found that CS-E tetrasaccharide-induced neurite outgrowth of dopaminergic neurons is mediated through IP_3 receptors and $[Ca^{2+}]_i$-dependent activation of CaMKII and calcineurin as well as PKC. In agreement with our results, the inhibition of CaMKII was reported to attenuate the Ca^{2+}-dependent axon outgrowth (Kuhn *et al.* 1998; Tang and Kalil 2005), whereas over-expression of CaMKII was reported to promote the neurite outgrowth (Goshima *et al.* 1993). The role of calcineurin in the neurite outgrowth is still controversial (Henley and Poo 2004). Neurotrophins and netrins were reported to stimulate the neurite outgrowth by activating calcineurin/nuclear factor of activated T-cells (NFAT) signaling in dorsal root ganglion neurons (Graef *et al.* 2003). It is possible that calcineurin, activated by the increased $[Ca^{2+}]_i$ in response to CS-E, dephosphorylates NFAT and induces the nuclear translocation of NFAT (Crabtree 2001), resulting in activation of genes required for the neurite outgrowth of dopaminergic neurons. Target effector proteins of CaMKII, calcineurin and PKC, required

for the CS-E-induced neurite outgrowth of dopaminergic neurons, will be identified in future studies.

In summary, we have demonstrated that the specific sulfation motif of CS-E enables CS to promote the neurite outgrowth of dopaminergic neurons, and we define a CS-E tetrasaccharide as a minimum structural motif for CS activity. Furthermore, our studies show that the action of CS-E is mediated through midkine-PTN/PTPζ and BDNF/TrkB signaling pathways, leading to activation of PLC/DAG/PKC and PLC/IP_3/IP_3 receptor/$[Ca^{2+}]_i$ signaling. Identification of the precise structural motif of biologically active CS and the molecular mechanisms utilized by CS-E should provide useful information relevant to the treatment of degenerative disorders of dopaminergic neurons such as Parkinson's disease.

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