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A characteristic chondroitin sulfate trisaccharide unit with a sulfated fucose branch exhibits neurite outgrowth-promoting activity: Novel biological roles of fucosylated chondroitin sulfates isolated from the sea cucumber *Apostichopus japonicus*

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ABSTRACT

Chondroitin sulfate (CS) is a class of sulfated glycosaminoglycan (GAG) chains that consist of repeating disaccharide unit composed of glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc). CS chains are found throughout the pericellular and extracellular spaces and contribute to the formation of functional microenvironments for numerous biological events. However, their structure-function relations remain to be fully characterized. Here, a fucosylated CS (FCS) was isolated from the body wall of the sea cucumber *Apostichopus japonicus*. Its promotional effects on neurite outgrowth were assessed by using isolated polysaccharides and the chemically synthesized FCS trisaccharide β-D-GalNAc(4,6-*O*-disulfate) (1−4)[α-L-fucose (2,4-*O*-disulfate) (1−3)]-β-D-GlcA. FCS polysaccharides contained the E-type disaccharide unit GlcA-GalNAc(4,6-*O*-disulfate) as a CS major backbone structure and carried distinct sulfated fucose branches. Despite their relatively lower abundance of E unit, FCS polysaccharides exhibited neurite outgrowth-promoting activity comparable to squid cartilage-derived CS-E polysaccharides, which are characterized by their predominant E units, suggesting potential roles of the fucose branch in neurite outgrowth. Indeed, the chemically synthesized FCS trisaccharide was as effective as CS-E tetrasaccharide in stimulating neurite elongation *in vitro*. In conclusion, FCS trisaccharide units with 2,4-*O*-disulfated fucose branches may provide new insights into understanding the structure-function relations of CS chains.

1. Introduction

Chondroitin sulfate (CS), a class of sulfated glycosaminoglycans (GAGs), is ubiquitously distributed in the peri/extracellular matrices in the form of CS proteoglycans (PGs), in which one or more CS polysaccharide chains are covalently linked to one of a panel of core proteins. CSPGs fine-tune the local microenvironmental niche to support a variety of cellular events, including cell-cell and cell-matrix interactions, cell proliferation, morphogenesis, and neurite outgrowth [1–4]. The functional divergence of CSPGs is well known to be associated with the structural characteristics of CS moieties [1–14]. Although CS has a simple, linear polysaccharide backbone that consists of repetitive disaccharide units of glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc), structural variations can be generated via several types of enzymatic modifications, including sulfation [1,3,15,16]. Therefore, glycobiological approaches focusing on the structure-function relationships of CS moieties are essential to understand the multifunctionality of CSPGs.

Unlike in vertebrates, CS chains derived from marine invertebrates, including ascidians, sea cucumbers, and sea urchins, show unique structural features [17,18]. In particular, sea cucumbers contain distinct CS analogs, fucosylated CS (FCS) chains, in their body wall. The central core of FCS is composed of a conventional CS disaccharide unit, as found in vertebrates, but also has

Abbreviations: BDNF, brain-derived growth factor; CNS, central nervous system; CS, chondroitin sulfate; FCS, fucosylated CS; GAG, glycosaminoglycan; GlcA, glucuronic acid; GalNAc, *N*-acetylgalactosamine; PLO, poly-L-ornithine; PG, proteoglycan.

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branches of sulfated α-fucose residues that are linked to the 3-*O*-positions of GlcA residues embedded in the CS backbone [19–21]. FCS reportedly has wide-ranging biological activities, such as anti-coagulant [22,23], anti-thrombotic [23,24], anti-virus [25], and anti-cancer [26] effects. Since the anti-coagulant/anti-thrombotic activities of FCS disappear after removal of the sulfated fucose branches by mild acid hydrolysis [22,23], at a minimum these two actions of FCS are apparently dependent mainly on the degree of sulfation and the position of sulfate in the fucose branches, and/or on the distribution of branches along the CS backbone. In contrast, in addition to the fucose branches, the backbone structure of FCS may also affect the other biological functions of FCS.

Notably, FCS polysaccharides derived from several species of sea cucumber contain CS backbones with non-negligible amounts of the disulfated disaccharide E unit [GlcA-GalNAc(4,6-*O*-disulfate)] [21,27–29]. Among typical CS subtypes, CS-E polysaccharides, a representative oversulfated CS subtype with predominant E units, are well known to exhibit strong neurite outgrowth-promoting activity toward primary hippocampal neurons *in vitro* [2,3,7,30,31]. Typical CS chains found in mammalian tissues are generally considered as major axon growth inhibitory molecules in the injured adult central nervous system (CNS); consequently, the apparently paradoxical ability of CS-E is of special interest for therapeutic applications for nerve regeneration after adult CNS injury. FCS may therefore be an ideal CS analog for determining the relationship between the structures of CS-related polysaccharides and their biological effects, especially on promoting neurite outgrowth. In the present study, we isolated FCS from the body wall of the sea cucumber *Apostichopus japonicus*. This FCS has a high content of E unit in the polysaccharide backbone and bears characteristic fucose branches. Comparative analyses of CS-E and FCS polymers, and their respective (chemically synthesized) minimal functional oligosaccharide units, demonstrated the functional equivalence of the E-type CS disaccharide unit and a 2,4-*O*-disulfated fucose branch found in *A. japonicus* FCS preparation in promoting neurite outgrowth of primary hippocampal neurons. Our findings provide additional insights into understanding the basis for CS-E-mediated neurite outgrowth.

2. Materials and methods

2.1. Materials

The sugars CS-C from shark cartilage, CS-E from squid cartilage, standard unsaturated CS disaccharides, and the enzyme *Proteus vulgaris* chondroitinase ABC (EC 4.2.2.4) were purchased from Seikagaku Corp. (Tokyo, Japan). The CS-C disaccharide GalNAc(6-*O*-sulfate)-GlcA-*O*-*p*-methoxyphenyl (CS-C-di) [32], two CS-E oligosaccharides [32] [(GalNAc(4,6-*O*-disulfate)-GlcA-*O*-*p*-methoxyphenyl) (corresponding to the E disaccharide unit, CS-E-di) and GalNAc(4,6-*O*-disulfate)-GlcA-GalNAc(4,6-*O*-disulfate)-GlcA-*O*-*p*-methoxyphenyl (CS-E tetrasaccharide, CS-E-tetra)], and the FCS trisaccharide (FCS-tri) β-D-GalNAc (4,6-*O*-disulfate) (1–4)[α-L-Fuc (2,4-*O*-disulfate) (1–3)]-β-D-GlcA-*O*-*p*-methoxyphenyl [33], were previously chemically synthesized.

2.2. Extraction and purification of FCS from the sea cucumber *A. japonicus*

A suspension of freeze-dried body wall of sea cucumber (20.56 g) was treated in 160 ml of boiling water for 10 min, followed by the addition of 0.5 M borate buffer (200 ml), pH 7.0. The suspension was then incubated with protease N Amano G (1.46 g ×3 every 3 days) at 55 °C for 9 days total. The incubated suspension was boiled for 15 min and filtered through Celite. The filtrate was subjected to ultrafiltration using a Pellicon Biomax PXB008A50 module (Merck Millipore, Darmstadt, Germany), and subsequent precipitation with 80% ethanol containing 1.25% NaOAc afforded crude glycans, which were collected by centrifugation at 0 °C and dried *in vacuo*. One portion of crude glycans (3.02 g) was diluted with a small amount of 0.15 M LiCl/0.05 M acetic acid, pH 4.0 and applied to a column (ϕ4.4 × 35 cm) of DEAE-cellulose. The column was washed stepwise with 750 ml of buffer (per step) containing 0.15, 0.5, 1.0, and 2.0 M LiCl. The 2.0 M LiCl fractions containing glycans were subjected to ultrafiltration as above, gel permeation (LH-20, H_2O, ϕ4.6 × 34 cm), and ultrafiltration. The solution was freeze-dried to give FCS (1.18 g).

2.3. Mild acid hydrolysis of FCS

FCS (23.2 mg) in 1 M formic acid was kept at 80 °C for 15 h. Volatiles were removed under reduced pressure and the residue was subjected to gel permeation column chromatography (LH-20, 1% AcOH, ϕ1.3 × 86 cm) to give de-fucosylated polysaccharide (4.4 mg).

2.4. Disaccharide composition of FCS

The CS polysaccharide fraction released by mild acid hydrolysis of the purified FCS fraction was digested with chondroitinase ABC at 37 °C for 2 h. The digests were derivatized with fluorophore 2-aminobenzamide (2-AB) and then analyzed by anion-exchange HPLC using an amino-bound silica PA-03 column (YMC, Kyoto, Japan) [34]. The resultant disaccharides were identified and quantified by comparison with authentic unsaturated CS disaccharides.

2.5. In vitro neurite outgrowth promotion assay

Primary cultures of hippocampal neurons were prepared as described previously [7] with some modifications. Briefly, 8-well chamber slides were precoated with poly-L-ornithine (PLO) (Sigma), 1.5 μg/ml) and then overlaid with individual CS polymers or CS oligosaccharides at the concentrations indicated in the respective figure legends. Pregnant wild-type (C57BL/6) mice were euthanized at 16 days postcoitum and the fetuses were quickly dissected. Hippocampal tissue blocks collected from the embryos were dissociated with 0.25% trypsin and 0.05% DNase. The single hippocampal cells were resuspended with Neurobasal™ medium (Thermo Fisher Scientific) containing B27 supplement (1 × ; Thermo Fisher), 5 mM GlutaMAX™ I (Thermo Fisher), 0.1% ovalbumin, and penicillin-streptomycin (1 ×), plated at a cell density of 10,000-12,000 cells/cm^2 in each well precoated with a defined CS substrate, and then maintained for 24 h at 37 °C in the presence of 5% CO_2. Endogenous influences were neutralized by adding an anti-brain-derived neurotrophic factor (BDNF) antibody (1 μg/ml, Santa Cruz Biotechnology) or an isotype-matched control immunoglobulin (IgG) 2 h after plating [35].

The cultured cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS containing 3% bovine serum albumin (BSA), then immunostained with Cy3-conjugated, anti-β-tubulin antibody (Sigma Aldrich, 1:2000) in PBS containing 3% BSA followed by nuclear counterstaining with Hoechst 33342. For morphometric analysis, clearly isolated neurons with at least one neurite longer than the cell body diameter were chosen at random. For each test condition, the length of the longest neurite of at least 50 neurons was measured using morphological analysis software (FLVFS-LS, Flovel, Japan).

All animal procedures were approved by the Kobe Pharmaceutical University Committee on Animal Research and Ethics. All experiments were conducted in accordance with the institutional

ethical guidelines for animal experiments.

2.6. Statistical analysis

All values are shown as mean ± standard error of the mean (S.E.M.). The data were evaluated using one-way analysis of variance (ANOVA) using the SPSS statistics program (IBM). *P*-values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Structural features of FCS preparations isolated from the sea cucumber A. japonicus

The structural uniqueness of the fucose branches of FCS preparations isolated from sea cucumber has led to significant effort to characterize these branches, whereas their CS backbone architectures have received relatively less attention. We independently isolated an FCS preparation from the body wall of the representative Japanese sea cucumber *A. japonicus*, and this FCS was previously described as “fucose-branched CS-E” [21,36]. Consistent with prior research, the intact FCS preparation was resistant to enzymatic digestion with a bacterial CS-degrading enzyme, chondroitinase ABC (ChABC) (data not shown), likely due primarily to the presence of fucose branches linked to the 3-*O*-position of GlcA residues [19,20]. Indeed, when subjected to mild acid hydrolysis to release the fucose branches, the FCS preparation became susceptible to eliminative cleavage by ChABC. To determine the precise disaccharide composition of the CS backbone, the resultant CS disaccharides were derivatized with fluorophore 2-aminobenzamide (2-AB) and analyzed by high-performance liquid chromatography (Table 1). Of note, the major disaccharide unit in the FCS preparation was disulfated E unit [GlcA-GalNAc(4,6-*O*-disulfate)] (48%), with small proportions of other units, including the monosulfated units C [GlcA-GalNAc(6-*O*-sulfate)] (28%) and A [GlcA-GalNAc(4-*O*-sulfate)] (12%), and the non-sulfated unit O [GlcA-GalNAc] (12%). Although the proportion of E unit in the FCS preparation was approximately 20% less than that of a conventional CS-E preparation derived from squid cartilage (68%) (Table 1), the proportion of E unit was nonetheless considerable, allowing us to use our FCS preparation as “fucose-branched CS-E” to investigate its involvement in CS-E-mediated biological events.

3.2. Effect of the FCS preparation on neurite outgrowth in vitro

Highly sulfated CS preparations containing CS-E are well known to promote neurite outgrowth of primary hippocampal neurons [2,3,7,30,31]. To clarify whether the FCS preparation also exhibits such ability, we used a previously established method to assess CS-mediated neurite outgrowth [2,3,7,30,31]. Mouse embryonic day (E) 16 hippocampal neurons were cultured at low cell density on defined substrata precoated with poly-L-ornithine (PLO) and subsequently with CS polysaccharides (CS-C, CS-E, or FCS), because PLO enhances not only neuronal cell adhesion, but also coating with negatively charged CS. After 24 h incubation with each substrate, the neurons were fixed and visualized by immunofluorescence staining (Fig. 1A). CS-C, a shark cartilage-derived CS preparation rich in C unit (Table 1), was used as a control CS polymer because C unit is the second most predominant unit in the FCS preparation. Consistent with previous observations [2,3,7], CS-C was a poorly permissive substrate for neurite outgrowth. In contrast, most neurons cultured on substratra precoated with CS-E or FCS tended to have a single prominent long neurite (Fig. 1A). Thus, the length of the longest neurite of randomly selected neurons was measured for quantitative evaluation of neurite outgrowth promotion by each CS preparation (Fig. 1B). The neurite outgrowth-promoting activity of the FCS preparation was significantly higher than that of the PLO control and comparable to that of CS-E, despite the lower proportion of E unit in FCS. This suggested that fucose branches in the FCS preparation have a complementary role in “CS-E backbone”-mediated neurite outgrowth promotion.

3.3. Validation of the neurite outgrowth-promoting potential of FCS oligosaccharide

Studies with chemically synthesized CS oligosaccharides indicate that the CS-E tetrasaccharide comprising a tandem-repeat sequence of E units is the minimal structural determinant for CS-E-dependent neurite outgrowth [35,37]. Therefore, given that fucose branches in FCS preparation are functionally involved in CS-E backbone-dependent neurite outgrowth, an FCS trisaccharide unit comprising a single E unit with a (sulfated) fucose branch was also expected to stimulate neurite outgrowth. The most direct approach to test this hypothesis is to use structurally defined chemical compounds corresponding to this putative functional unit of FCS and assess their biological activities. The branched fucose residues in sea cucumber-derived FCS polysaccharides can be sulfated at the 2-*O*-, 3-*O*-, and/or 4-*O*-positions [22]. Indeed, the 2,4-*O*-disulfated fucose residue is the predominant branched structure in *A. japonicus* FCS preparations [29]. The chemical synthesis of a representative FCS trisaccharide unit (FCS-tri), β-D-GalNAc (4,6-*O*-disulfate) (1–4)[α-L-Fuc (2,4-*O*-disulfate) (1–3)]-β-D-GlcA, has been achieved previously [33]. We therefore examined the ability of FCS-tri to modulate neurite outgrowth. Intriguingly, FCS-tri-precoated substrate enhanced neurite outgrowth in a concentration-dependent fashion, with maximum outgrowth observed by coating with 15 μg/ml FCS-tri (Fig. 2A). An undesirable inhibitory effect was observed with our culture conditions when higher concentrations (above 20 μg/ml) of FCS-tri were used, likely due to the anti-adhesive nature of highly charged compounds. The following assays using CS oligosaccharides were therefore conducted with substrata precoated with 15 μg/ml of the corresponding chemical compounds.

To further evaluate the structural importance of FCS-tri on

Table 1
Disaccharide composition of the CS backbone of *A. japonicus* FCS preparation.

Composition	*A. japonicus* FCS	Shark cartilage CS-C[a] mol%	Squid cartilage CS-E[a]
GlcA-GalNAc	12	0.8	6.2
GlcA-GalNAc(6-*O*-sulfate)	28	78	8.2
GlcA-GalNAc(4-*O*-sulfate)	12	13	17
GlcA (2-*O*-sulfate)-GalNAc(6-*O*-sulfate)	ND[b]	8.0	ND
GlcA-GalNAc(4,6-*O*-disulfate)	48	0.5	68

[a] Disaccharide compositions of commercial CS preparations are described in Ref. [7].
[b] ND, not detected.

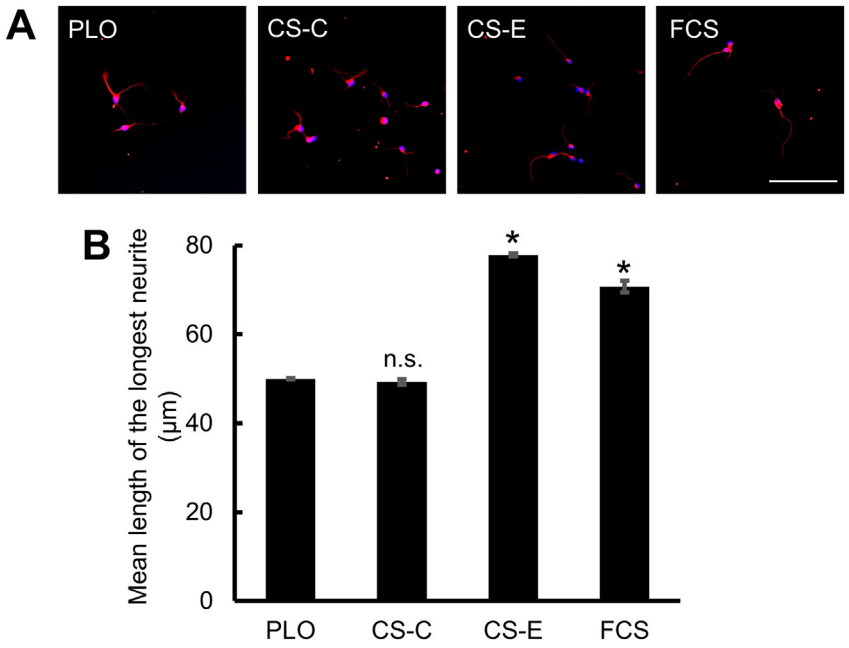

Fig. 1. *A. japonicus* FCS preparation exhibits neurite outgrowth-promoting activity *in vitro*. (A) E16 hippocampal cells (10,000 cells/cm^2) were grown for 24 h on various substrata precoated with PLO, then subsequently coated with the CS polysaccharides (5 μg/ml) CS-C or CS-E, or with *A. japonicus* FCS preparation. The neurons were fixed and visualized with anti-β-tubulin antibody (*red*) and Hoechst 33342 (*blue*). Scale bar, 100 μm. (B) Mean length of the longest neurite of each of >50 randomly selected neurons for each condition was measured (n = 3; *, $p < 0.01$; n. s. not significant; versus PLO-control). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

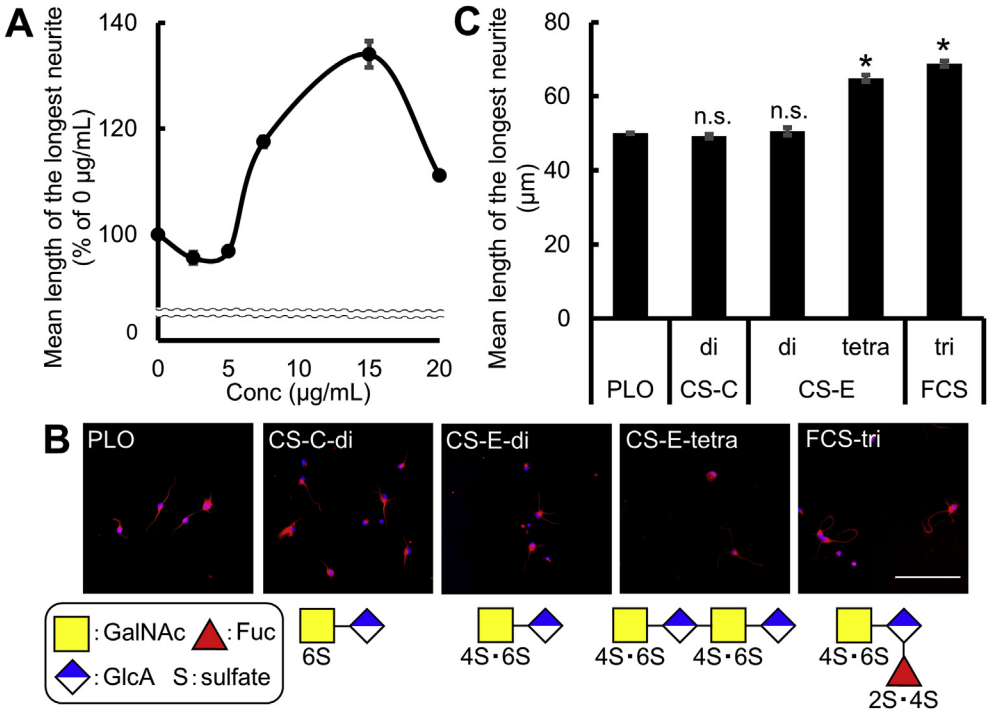

Fig. 2. Neurite outgrowth-promoting abilities of chemically synthesized CS oligosaccharides containing FCS trisaccharide (FCS-tri). (A) Neurite outgrowth-promotion assay was conducted using culture substrates precoated with PLO (0 μg/ml) and subsequently with different doses (2.5, 5.0, 7.5, 15, or 20 μg/ml) of FCS-tri (n = 3). (B,C) E16 hippocampal cells (10,000 cells/cm^2) were cultured for 24 h on the PLO surface, or on substrata further coated with one of the following CS oligosaccharides (15 μg/ml): CS-C disaccharide (CS-C-di), CS-E disaccharide (CS-E-di), CS-E tetrasaccharide (CS-E-tetra), or FCS-tri. The subsequent assay was conducted as described in the legend to Fig. 1. Scale bar in (B), 100 μm. (C) Mean length of the longest neurite per neuron cultured under each condition (n = 3; *, $p < 0.01$; n. s., not significant; versus PLO).

neurite outgrowth, the promotional potentials of three additional CS oligosaccharides, CS-C disaccharide (CS-C-di) [GalNAc(6-*O*-sulfate)-GlcA], CS-E disaccharide (CS-E-di) [GalNAc(4,6-*O*-disulfate)-GlcA], and CS-E tetrasaccharide (CS-E-tetra) [GalNAc(4,6-*O*-disulfate)-GlcA-GalNAc(4,6-*O*-disulfate)-GlcA], all of which were chemically synthesized [32], were assessed and compared with

that of FCS-tri (Fig. 2B and C). As expected, CS-E-tetra significantly enhanced neurite outgrowth compared with the PLO control, further indicating that a tetrasaccharide structure is essential for CS-E-mediated neurite outgrowth. More importantly, the promotional level exhibited by CS-E-tetra was comparable to that of FCS-tri, whereas neither CS-C-di nor CS-E-di had a significant effect on neurite extension. These findings suggested that sulfated fucose branches are critical components for FCS-mediated neurite outgrowth. These results also implied that sulfated fucose residues may compensate for the relatively low occurrence of the minimal functional unit, i.e., the CS-E tetrasaccharide sequence in the FCS backbone, by coupling with a single E unit to form the characteristic FCS trisaccharide unit.

3.4. Involvement of the BDNF signaling pathway in FCS-mediated neurite outgrowth

Since CS-E binds to several humoral factors, including brain-derived growth factor (BDNF), CS-E-mediated neurite outgrowth must be exerted through activation of BDNF signaling inputs to primary neurons [35,38]. In this aspect, CS-E can act as a coreceptor and/or reservoir for neuritogenic factors [3,4]. We examined whether neurite outgrowth stimulated by FCS oligo/polysaccharides is functionally linked to their structural backbone, i.e., CS-E, by assessing the respective neurite outgrowth abilities of CS-E and FCS oligo/polysaccharides in the presence of a neutralizing antibody against BDNF. An isotype-matched control IgG showed no significant inhibition toward all substrata, whereas application of the anti-BDNF antibody significantly suppressed the neurite outgrowth-promoting effects of the CS-E and FCS oligo/polysaccharides (Fig. 3A and B). These findings indicate that neurite outgrowth on substrata precoated with FCS oligo/polysaccharides is also likely mediated via positive regulation of the BDNF signaling pathway. Therefore, FCS-mediated neurite outgrowth may be fundamentally CS-E backbone-dependent, supporting the aforementioned compensatory role of fucose branches in FCS preparation.

In summary, we attempted to identify the functional domain structure required for the neurite outgrowth-promoting potential of *A. japonicus* FCS preparation. FCS preparation can be considered as a fucosylated CS-E due to the high content of E unit in the CS backbone. Using chemically synthesized CS compounds, we found that the 2,4-*O*-disulfated fucose branch is a critical component of the minimal determinant for FCS-mediated neurite extension. Furthermore, an FCS trisaccharide (a disulfated fucose-branched E unit) is functionally comparable to a neuritogenic CS-E tetrasaccharide. Interestingly, although FCS polysaccharides with 2,4-*O*-disulfated fucose branches reportedly possess anti-coagulant/anti-thrombotic activities [27,28], the corresponding FCS trisaccharide used in the present study exhibited no such activity (unpublished observation). Therefore, such a characteristic FCS trisaccharide unit can be a lead compound for developing CS-based regenerative drugs for therapeutic intervention for adult CNS injury, with potentially fewer side effects than current treatments. Our results also demonstrated the potential utility of FCS as a benchmark CS analog for deciphering the functional domain structures required for their respective CS-dependent biological activities. Therefore, comprehensive analyses of structure-function correlations of CS chains using additional CS- and/or FCS-derived chemical compounds are needed to better understand the molecular mechanisms underlying CS-mediated biological functions, including neuroregulatory roles.

Declaration of interest

The authors declare that they have no conflicts of interest with the contents of this article.

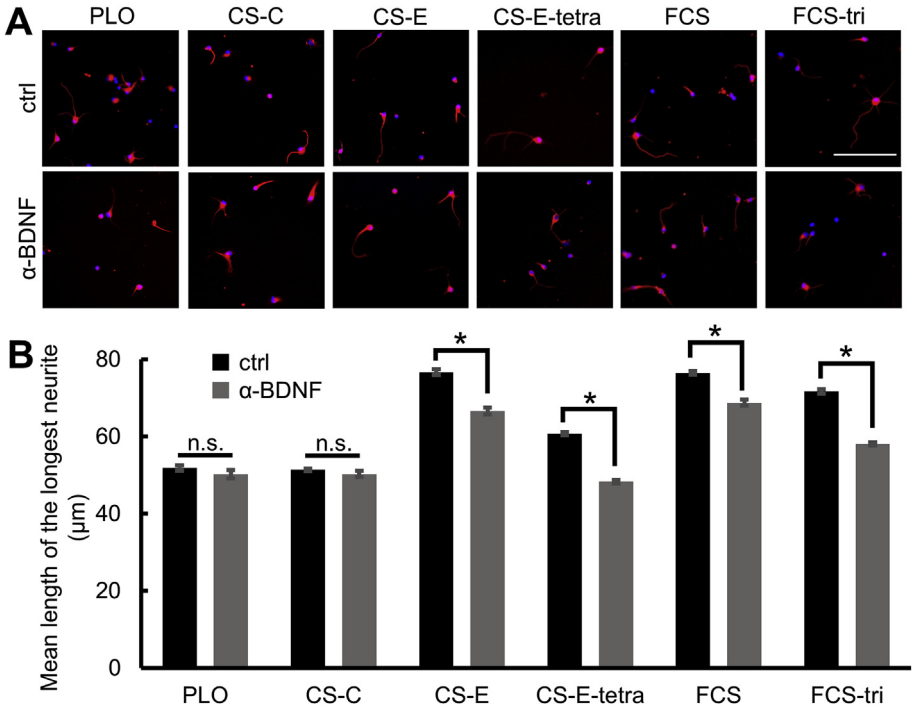

Fig. 3. Effects of anti-BDNF antibody on CS-E- and FCS-dependent neurite outgrowth promotion. (A,B) E16 hippocampal cells (12,000 cells/cm^2) were seeded on defined CS oligo- (15 μg/ml) or polysaccharides (5 μg/ml). Two hours after plating, anti-BDNF antibody (α-BDNF, 1 μg/ml) or an isotype-matched control IgG (ctrl, 1 μg/ml) was added to the culture medium. Subsequent procedures were as described in the legend to Fig. 1. Scale bar in (A), 100 μm. (B) Mean length of the longest neurite per neuron cultured under each condition (n = 5; *, $p < 0.01$; n. s., not significant; versus ctrl).

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