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Structural and functional impairments of polysialic acid (polySia)-neural cell adhesion molecule (NCAM) synthesized by a mutated polysialyltransferase of a schizophrenic patient*

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Abstract: Polysialic acid (polySia) is a homopolymer of sialic acid with a degree of polymerization (DP) of 8–400. When present on neural cell adhesion molecule (NCAM), polySia has anti-adhesive effects on cell–cell interactions owing to its bulky polyanionic nature, and is involved in the regulation of neurogenesis and neuronal functions. Recently, we demonstrated that polySia functions not only as an anti-cell adhesion molecule, but also as a reservoir scaffold for brain-derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF2), which are biologically active molecules in neurogenesis. To understand the significance of polySia structure in the reservoir function, we focused on polySia-NCAM biosynthesized by mutated polysialyltransferase (ST8SiaII or STX) that was reported in a schizophrenia patient. The polySia-NCAM biosynthesized by mutant ST8SiaII/STX contained less polySia with shorter chain length and exhibited impaired reservoir function for BDNF and FGF2 as compared with that synthesized by wild-type (wt) ST8SiaII/STX. Our findings suggest that the quantity and quality of polySia on NCAM are important for normal neuronal functioning.

Keywords: brain-derived neurotrophic factor (BDNF); fibroblast growth factor (FGF); neural cell adhesion molecule (NCAM); polysialic acid; polysialyltransferase; schizophrenia; sialic acid.

INTRODUCTION

Polysialic acid (polySia) is a linear polymer of sialic acid with a degree of polymerization (DP) ranging from 8 to 400 [1–3] and is found mainly on neural cell adhesion molecule (NCAM) in the brains of vertebrates [1]. PolySia is present in embryonic brains during neonatal and postneonatal development and mostly disappears in the adult brain, although NCAM expression levels remain relatively unchanged [1,4,5]. In adult brains, polySia-NCAM persists in distinct regions, such as the hippocampus [6,7] and olfactory system [8], where neurogenesis is ongoing. When present on NCAM, polySia has anti-adhesive effects on cell–cell interactions owing to its bulky polyanionic nature [1,4,5] and as such, it is considered to be involved in neural cell migration, axonal guidance, fasciculation, myelination, synapse formation, and functional plasticity of the nervous system [4,5]. However, the functions

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of polySia in not only embryonic brains, but also in adult brains are not completely understood. In both NCAM-deficient [9] and polySia-reduction mice [10,11], although neural development is not dramatically impaired, learning and memory due to impairment of long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus, and behaviors such as fear motion are markedly influenced [9–11].

Recently, we reported that polySia serves as a reservoir for neurotrophins, including brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and nerve growth factor (NGF) [12,13]. We also demonstrated that polySia captures the catecholamine neurotransmitter dopamine (DA) and regulates Akt signal through DA receptors [14]. More recently, we showed that fibroblast growth factor 2 (FGF2) specifically binds to polySia in a different manner than to heparan sulfate [15]. Based on these observations, we hypothesized that polySia regulates biologically active molecules, particularly neurotrophins, neurotransmitters, and growth factors, through the capture of these molecules among polySia chains, thereby indirectly regulating cell activities.

Schizophrenia is a severe psychiatric disorder that affects approximately 1 % of the population worldwide. To date, several causative genes that are associated with an increased risk of developing schizophrenia, including those encoding disrupted-in-schizophrenia 1 (DISC1) [16], Neuregulin 1 [17], catechol-*o*-methyltransferase (COMT) [18], DA receptors [19], and BDNF [20] have been identified [21], however, the overall mechanism leading to schizophrenia remains unclear. Schizophrenia is intimately related to neurodevelopmental and neurodegenerative disorders involving synapse disconnection and disorder [22,23]. Notably, these abnormalities occur at a very restricted stage during brain development. A number of reports have examined the relationships between polySia expression and schizophrenia [24–27]. For example, a decrease in polySia-NCAM immunoreactivity was observed in schizophrenic hippocampi [24], and schizophrenia patients often have low olfactory volume [25]. Impairment of hippocampal functions and disturbance of its anatomical organization are also involved in the etiology of schizophrenia [17]. Interestingly, the chromosome (15q26) that encodes the polysialyltransferase ST8SiaII/STX, which is responsible for the synthesis of polySia, was reported to be a common susceptibility region for both schizophrenia and bipolar disorder in a genome scan of Eastern Quebec families [26]. Recently, Arai et al. [27] also revealed an association between polymorphisms in the promoter region of the stx gene and schizophrenia in the Japanese population. However, structural features of the polySia-NCAM in any case of the schizophrenia patients have not clarified yet.

To gain further insight into the relationship between psychiatric disorders such as schizophrenia and the polysialylation state of NCAM [14], here, we focused on a single nucleotide polymorphism (SNP) of the stx gene, SNP7 (421G>A) on exon 4, which has been identified in a schizophrenia patient [27]. We analyzed the enzymatic activity of the mutated STX toward membrane-bound and secreted forms of NCAM, and chemically evaluated the polySia-NCAM biosynthesized by the mutated ST8SiaII/STX. In addition, we investigated the ability of polySia-NCAM to bind BDNF and FGF2, using a surface plasmon resonance (SPR)-based method. This is the first application of the SPR method to analyze interaction of the polySia in polySia-NCAM with BDNF and FGF2, although polySia glycan was tested for the binding to BDNF [30] or FGF2 [14].

MATERIALS AND METHODS

Materials

Recombinant FGF and BDNF were purchased from PeproTech, Inc. (Rocky Hill, NJ). Colominic acid was obtained from Wako (Osaka, Japan). Enhanced chemiluminescent reagents, DEAE-Sephadex A-25, and the Mini Q anion exchange column were purchased from General Electric Co. (Piscataway, NJ). Polyvinylidene difluoride (PVDF) membrane (Immobilon P) was a product of Millipore (Bedford, MA). Pre-stained molecular weight marker was purchased from Bio-Rad (Hercules, CA).

Flow cytometry of Neuro2A cells transfected with the stx gene

Stable Neuro2A cell lines expressing wild-type (wt) or mutant ST8SiaII/STX (g421a) were generated by transfecting cells with either pBudCE4.1-stx(wt)-pst or pBudCE4.1-stx(g421a)-pst using Lipofectamine (Invitrogen), as directed by the manufacturer, and then selecting cells with Zeocin. The stable cell lines, named N2A-stx (wt) and N2A-stx (g421a), respectively, were cultured, collected at growing stage, and washed with phosphate-buffered saline (PBS) containing 5 mM EDTA and 0.1 % bovine serum albumin (BSA) (PBE). The cells were incubated with monoclonal antibody (mAb) 12E3 (10 μg/ml) at 4 °C for 1 h and then washed twice with PBE. The cells were then incubated with Alexa-labeled anti-mouse IgM (2 μg/ml) at 4 °C for 1 h. After washing cells twice with PBE, cell-surface staining was measured using a Gallios flow cytometer (Beckman Coulter), and the collected data were analyzed using Karza software (Beckman Coulter).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from Neuro2A stable cell lines using TRIZOL (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Random-primed cDNA (~50 ng) and specific primers for stx (5'-CTCGTGGTCTTCCTCATCTT-3' and 5'-GCCGACAGTCAGTTTCAATG-3') were used for the RT-PCR.

Purification of polySia-NCAM-Fc

A Chinese hamster ovary (CHO) cell line stably secreting NCAM-Fc was transfected with either pcDNA-stx (wt) or pcDNA-stx (g421a) [14] using Lipofectamine. Stable cell lines secreting polySia-NCAM were established by culturing the transfected cells in the presence of G418. PolySia-NCAM-Fc secreted into the culture medium was purified by first loading culture supernatant on a DEAE-Sephadex A-25 column, which was then washed with 10 mM phosphate buffer containing 0.2 M NaCl. PolySia-NCAM-Fc was eluted with 10 mM phosphate buffer containing 0.6 M NaCl, as described previously [14].

Characterization of polySia-NCAM-Fc

PolySia-NCAM-Fc purified as described above was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/Western blotting using anti-polySia (12E3) (1 μg/ml) and anti-NCAM (H300) (0.2 μg/ml) antibodies at 4 °C. As the secondary antibody, either peroxidase-conjugated anti-rabbit IgG antibodies (1/4000 dilution) or anti-mouse IgG+M antibodies (1/5000 dilution) was applied to the membranes, incubated for 60 min at 37 °C, and then color development was performed using standard reagents. The polysialylation state was also analyzed chemically by fluorescent C_7/C_9 analysis [28] and mild acid hydrolysis-anion-exchange chromatography analysis [29], using the same preparation for Western blotting.

Biacore analysis

For SPR measurements, a Au sensor chip containing surface-activated self-assembled monolayer (SAM) was placed on the sensor chip support using the sensor chip assembly unit, and was then set in a Biacore 3000 instrument [30]. After priming the system with water for 7 min, a 0.1 mg/ml protein A solution was loaded twice, each time for 7 min at a flow rate of 10 μl/min. Immobilized streptavidin was monitored by measuring the resonance unit (RU) value, which typically reached 1300–1850 RU for protein A. To destroy excess activated groups, 1 mM ethanolamine was injected into the system for 7 min. After washing with HBS-EP (0.01 M HEPES pH 7.4 containing 0.15 M NaCl, 3 mM EDTA, and

0.0005 % Surfactant P20), purified polySia-NCAM-Fc (0.1 mg/ml in 500 mM HBS-EP) was injected into the system to allow immobilization on the Au surface. Immobilization of the polySia-NCAM-Fc was monitored based on the observed RU values, which typically reached approximately 850–1300 RU. NCAM-Fc derived from a mock transfectant was used as a negative control [30].

For analysis of the interactions between immobilized polySia-NCAM-Fc and the two neuroactive molecules, varying concentrations of BDNF (0–37.0 nM) or FGF2 (0–56.8 nM) in HBS-EP were injected over the polySia-NCAM-immobilized sensor chip surface at a flow rate of 20 µl/min. After 120 s, HBS-EP was flowed over the sensor surface to monitor the dissociation phase. Following 180 s of dissociation, the sensor surface was fully regenerated by the injection of 10 µl of 3 M NaCl. All values are expressed as the mean ± SD.

RESULTS

Cell surface polysialylation in N2A-stx (wt) and N2A-stx (g421a)

We first established two cell lines stably expressing wt ST8SiaII/STX and mutant ST8SiaII/STX (g421a), N2A-stx (wt) and N2A-stx (g421a), respectively, to evaluate the levels of cell surface expressed polySia biosynthesized by the two ST8SiaII/STXs. As Neuro2A cells produce NCAM, but not ST8SiaII/STX [12], they do not express polySia on the cell surface. The expression of the stx gene was confirmed by RT-PCR analysis, which showed that the N2A-stx (wt) and N2A-stx (g421a) cell lines produced similar amounts of stx mRNA (Fig. 1A).

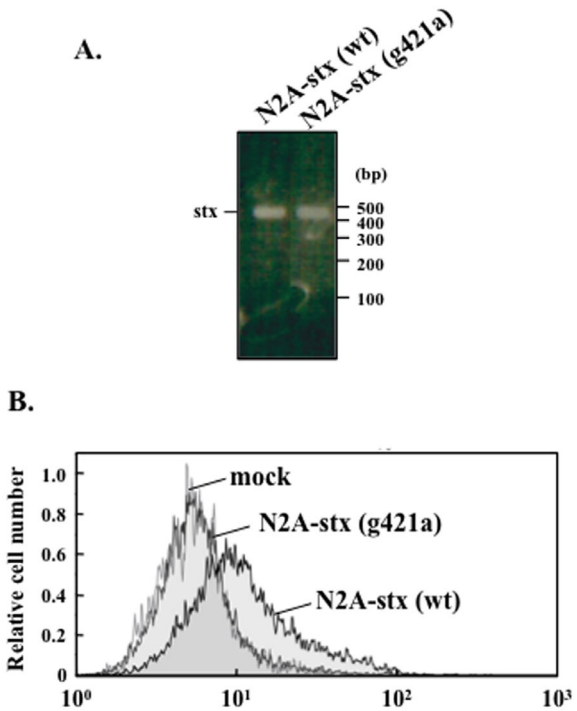

Fig. 1 PolySia expression on Neuro2A cell lines, N2A-stx (wt) and N2A-stx (g421a), stably expressing wt and mutant ST8SiaII/STX (g421a), respectively. (A) RT-PCR of the stx gene in N2A-stx (wt) and N2A-stx (g421a) cells. The stx gene was amplified with specific primers and cDNAs derived from these cells as a template, and analyzed by 1 % agarose gel electophoresis followed by visualization with ethidium bromide. (B) Flow cytometric analysis of Neuro2A stable cell lines expressing polySia on the cell surface using anti-polySia antibody (12E3). A mock-transfected cell line was used as a negative control.

The two cell lines were then analyzed for the levels of cell surface expressed polySia by flow cytometry with an anti-polySia mAb (12E3). The polySia derived from wt ST8SiaII/STX showed strong cell-surface expression, while that derived from mutant ST8SiaII/STX (g421a) was dramatically decreased on the surface of N2A-stx (g421a) cells (Fig. 1B). These results indicated that the enzymatic activity of the mutant ST8SiaII/STX (g421a) in Neuro2A cells toward intact NCAM was lower than that of wt ST8SiaII/STX.

Characterization of polySia-NCAM biosynthesized by N2A-stx (wt) and N2A-stx (g421a) cells

To evaluate the ability of polySia to bind BDNF and FGF2, we established cell lines that secreted polySia-NCAM by transfecting CHO cells constitutively secreting NCAM-Fc with plasmids encoding full-length ST8SiaII/STX (wt) or mutant ST8SiaII/STX (g421a) gene. NCAM-Fc secreted from the two transfected cell lines was purified and analyzed for their polysialylation state using anti-polySia (12E3) and anti-NCAM antibodies (H300) (Fig. 2A). The relative amount of polySia was expressed as the ratio of the immunostaining intensity by anti-polySia to that of anti-NCAM (Fig. 2B). This ratio also reflects the enzymatic activity of the ST8SiaII/STX enzyme. As shown in Fig. 2A, NCAM was polysialylated by both wt ST8SiaII/STX and mutant ST8SiaII/STX (g421a), however, polysialylation of NCAM was significantly reduced in CHO cells expressing mutant ST8SiaII/STX (g421a) (52 %, $p = 0.0075$) compared to those expressing wt ST8SiaII/STX.

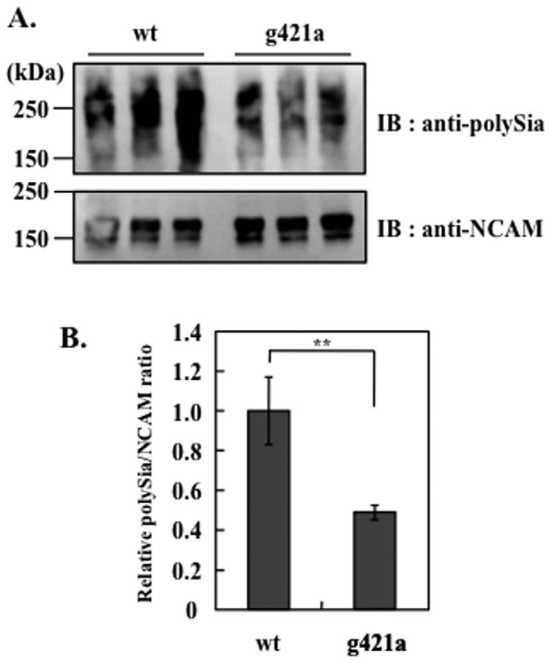

Fig. 2 PolySia formation on soluble NCAM secreted from CHO cells stably expressing wt ST8SiaII/STX (*wt*) or mutant ST8SiaII/STX (*g421a*). (A) polySia-NCAM-Fc purified from culture medium of CHO cells stably expressing NCAM-Fc and either wt or mutant ST8SiaII/STX. Purified polySia-NCAM-Fc was subjected to Western blotting with anti-polySia (12E3) and anti-NCAM (H300) antibodies. (B) The relative intensity of polySia to NCAM immunostaining (*polySia/NCAM*) was densitometrically determined. The data are expressed as the mean ± SD, with three replicates per experiment. The relative intensity for wt ST8SiaII/STX was set to 100 %. ** indicates significance at $p = 0.0075$.

The polySia-NCAM-Fc secreted from cells expressing either wt or mutant ST8SiaII/STX (g421a) was analyzed for the quality and quantity of polySia by chemical methods, including fluorometric C_7/C_9 analysis [28] (Table 1) and mild acid hydrolysis/anion-exchange chromatography [29] (Fig. 3). Fluorometric C_7/C_9 analysis involves the periodate oxidation, reduction, hydrolysis, and fluorescent labeling of polySia-NCAM, followed by fluorometric high-performance liquid chromatography (HPLC). In this analysis, non-reducing terminal sialic acid residues give C_7-Neu5Ac or C_7, whereas internal α2-8-linked polySia residues remain unchanged as C_9-Neu5Ac or C_9. Thus, the $(C_7+C_9)/C_7$ ratio represents an approximate estimation of the chain length of polySia on NCAM [28]. The results of the analysis are summarized in Table 1. The $(C_7+C_9)/C_7$ ratio of polySia-NCAM synthesized by mutant ST8SiaII/STX (g421a) was 1.41, which was dramatically lower than that of wt ST8SiaII/STX (16.0), suggesting that the chain length of polySia synthesized by the mutant enzyme was shorter and/or lower, respectively, than that by wt ST8SiaII/STX. In addition, as the amount of C_9 directly reflects the quantity of polySia, the lower value for the mutant ST8SiaII/STX indicates that the amount level of polySia was decreased on NCAM-Fc compared to that for wt ST8SiaII/STX.

Table 1 Fluorometric C_7/C_9 analysis of polySia-NCAM derived from wt ST8SiaII/STX- or mutant ST8SiaII/STX (g421a)-expressing cells.

PolySia-NCAM derived from	C_7 (Neu5Ac) (mol/mol protein)	C_9 (Neu5Ac) (mol/mol protein)	$(C_7+C_9)/C_7$
wt ST8SiaII/STX	2.70 ± 0.0424	40.5 ± 0.181	16.0
mutant ST8SiaII/STX (g421a)	21.6 ± 0.129	8.88 ± 0.115	1.41

The purified polySia-NCAM-Fc was further analyzed by mild acid hydrolysis/anion-exchange chromatography after DMB derivatization (Fig. 3). Using this method, it is possible to estimate the chain length and amount of oligo/polySia on NCAM based on peak number and intensity, respectively. However, as mild acid hydrolysis easily destroys polySia structure [12], polySia with DP = x is observed as polySia with DP = 1~x in the chromatogram, as was shown using an authentic sample of colominic acid, whose average DP has been confirmed as 43 (Fig. 3A). In the analysis of polySia-NCAM derived from wt ST8SiaII/STX, mono-, di-, oligo-, and polySia chains with DP 1-42 were observed, with DP = 14 being the major product. In contrast, oligo/polySia chains with a DP ranging from 1 to 33 were observed on polySia-NCAM derived from mutant ST8SiaII/STX (g421a), and it was confirmed that the total amount of polySia was lower than that found on NCAM from cells expressing wt ST8SiaII/STX. These results indicate that the mutant ST8SiaII/STX synthesized shorter polySia chains in lower quantity than wt ST8SiaII/STX.

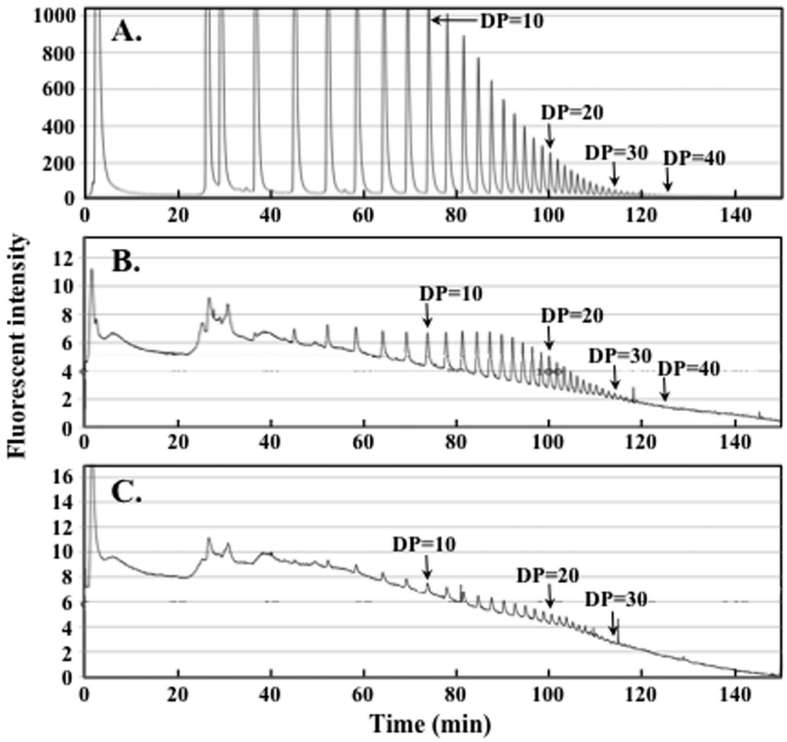

Fig. 3 Structural characterization of polySia on soluble NCAM by the fluorometric anion-exchange chromatography of mild acid hydrolysate. The purified polySia-NCAM-Fc secreted from the CHO cells stably expressing wt or mutant ST8SiaII/STX (g421a) were subjected to mild acid hydrolysis. Released oligo/polySia were derivatized with DMB and loaded onto a mini Q anion-exchange column, which was eluted with 5 mM Tris-HCl (pH 8.0), followed by a NaCl gradient (0–15 min, 0 M; 15–100 min → 0.3 M; and 100–145 min, 0.3 → 0.4 M) in 5 mM Tris-HCl (pH 8.0) at a flow rate was 0.5 ml/min. The elution profile was monitored with a fluorescence detector by excitation at 373 nm and emission at 448 nm. (A) colominic acid as authentic polySia. (B) polySia-NCAM-Fc derived from wt ST8SiaII/STX-expressing cells. C. polySia-NCAM-Fc derived from mutant ST8SiaII/STX (g421a)-expressing cells. DP = degree of polymerization.

Recently, we demonstrated that polySia functions as a reservoir of neurotrophic and growth factors, including BDNF and FGF2, which are involved in psychiatric disorders and memory [12–15]. Here, we examined whether the BDNF- and FGF2-retaining activities of polySia on NCAM were affected by mutant ST8SiaII/STX. To evaluate the interaction between BDNF or FGF2 and synthesized polySia, the binding between polySia-NCAM and these factors was measured by SPR. To confirm the specificity of the interaction, NCAM-Fc purified from a stable CHO cell line transfected with mock vector was used as a negative control, and the control sensorgram was subtracted from that of polySia-NCAM-Fc. As shown in Fig. 4 and Table 2, a specific interaction of BDNF and FGF2 with polySia-NCAM derived from both wt and mutant ST8SiaII/STX could be detected. As estimated from the SPR sensorgrams, the K_d values between BDNF and polySia-NCAM derived from wt ST8SiaII/STX and mutant ST8SiaII/STX (g421a) were found to be 1.18×10^{-9} M and 5.66×10^{-9} M, respectively, representing a five-fold increase for mutant ST8SiaII/STX (Table 2). Compared with polySia-NCAM derived from wt ST8SiaII/STX, the interaction between BDNF and polySia-NCAM derived from mutant ST8SiaII/STX decreased to 70 % (Fig. 4A). A similar decrease (75 %) in the interaction between polySia-NCAM-Fc derived from mutant ST8SiaII/STX and FGF2 was also observed

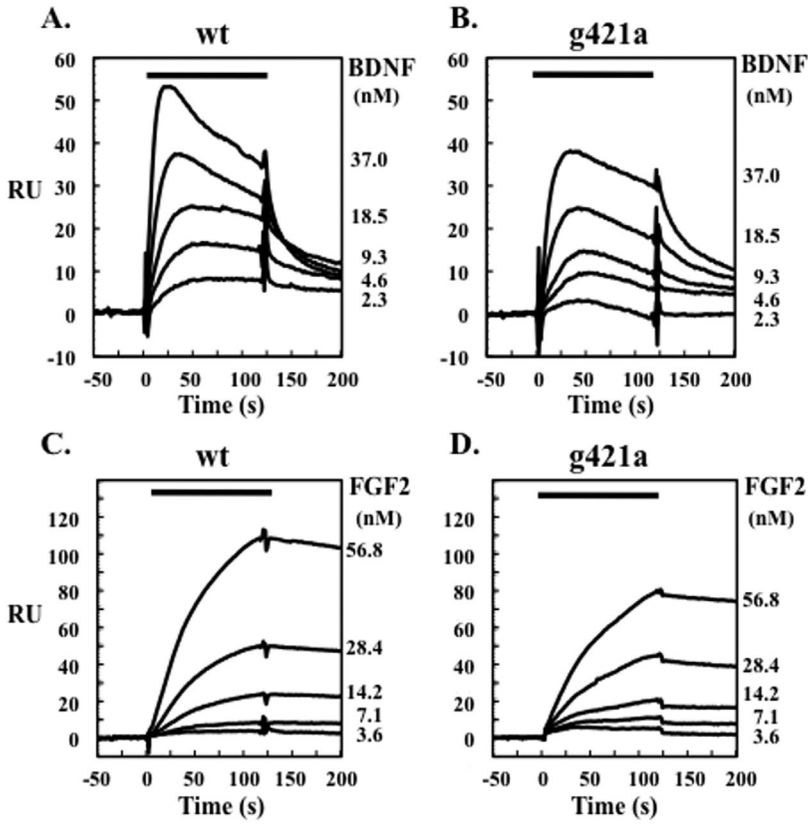

Fig. 4 Binding properties of polySia-NCAM with biologically active molecules on SPR measurement. PolySia-NCAM-Fc purified from secretion of the CHO cells stably expressing wt ST8SiaII/STX (*wt*, A and C) or mutant ST8SiaII/STX (*g421a*, B and D) was immobilized on a sensor chip. (A,B) BDNF was injected over the polySia-NCAM-Fc at 0~37.0 nM. (C,D) FGF2 was injected over the polySia-NCAM-Fc at 0~56.8 nM. Bars indicate the sample injection period, and the end of the bars indicate the beginning of the dissociation phase initiated with running buffer. RU = response unit.

Table 2 Summary of kinetics parameters for the interaction of BDNF and FGF2 with polySia-NCAM derived from wt ST8SiaII/STX- or mutant ST8SiaII/STX (g421a)-expressing cells.

Immobilized polySia-NCAM derived from	Analyte	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_d (M)
wt ST8SiaII/STX	BDNF	1.07×10^6 ($\pm 1.84 \times 10^6$)	9.47×10^{-4} ($\pm 1.12 \times 10^5$)	1.18×10^{-9} ($\pm 4.13 \times 10^{-10}$)
mutant ST8SiaII/STX (g421a)	BDNF	1.00×10^6 ($\pm 1.30 \times 10^5$)	5.69×10^{-3} ($\pm 1.18 \times 10^{-3}$)	5.66×10^{-9} ($\pm 4.10 \times 10^{-10}$)
wt ST8SiaII/STX	FGF2	1.12×10^5 ($\pm 1.12 \times 10^5$)	5.63×10^{-4} ($\pm 1.11 \times 10^{-4}$)	5.28×10^{-9} ($\pm 1.97 \times 10^{-9}$)
mutant ST8SiaII/STX (g421a)	FGF2	9.68×10^4 ($\pm 2.71 \times 10^4$)	6.06×10^{-4} ($\pm 2.20 \times 10^{-4}$)	6.82×10^{-9} ($\pm 3.71 \times 10^{-9}$)

(Fig. 4B). However, the K_d value of the interaction was almost the same as that of polySia-NCAM-Fc derived from wt ST8SiaII/STX (6.82×10^{-9} M vs. 5.28×10^{-9} M, respectively, Table 2).

DISCUSSION

PolySia (Fig. 5A) is a unique polyanionic glycan found on NCAM with a well-characterized function and tightly regulated spacio-temporal regulation in the brain. Due to its extremely large exclusive volume (Fig. 5B), polySia is considered to have anti-adhesive effects on cell–cell interactions [1]. Recently, we demonstrated that polySia specifically captures biologically active molecules, including BDNF, neurotransmitters (DA), and growth factor (FGF2), and modifies their functions through regulation of their physiologically available concentrations (Fig. 5C) [31]. In addition, we have shown that the structure of polySia is important for capturing neuroactive molecules, for example, polySia with DP 12 [12] and DP 17 [15] is required for binding to BDNF and FGF2, respectively, and that the in vitro biosynthetic activity of mutated ST8SiaII/STX reported in a schizophrenic patient was lower than that of wt [15]. Therefore, it is important to understand the relationship between ST8SiaII/STX and the structure and function of polySia.

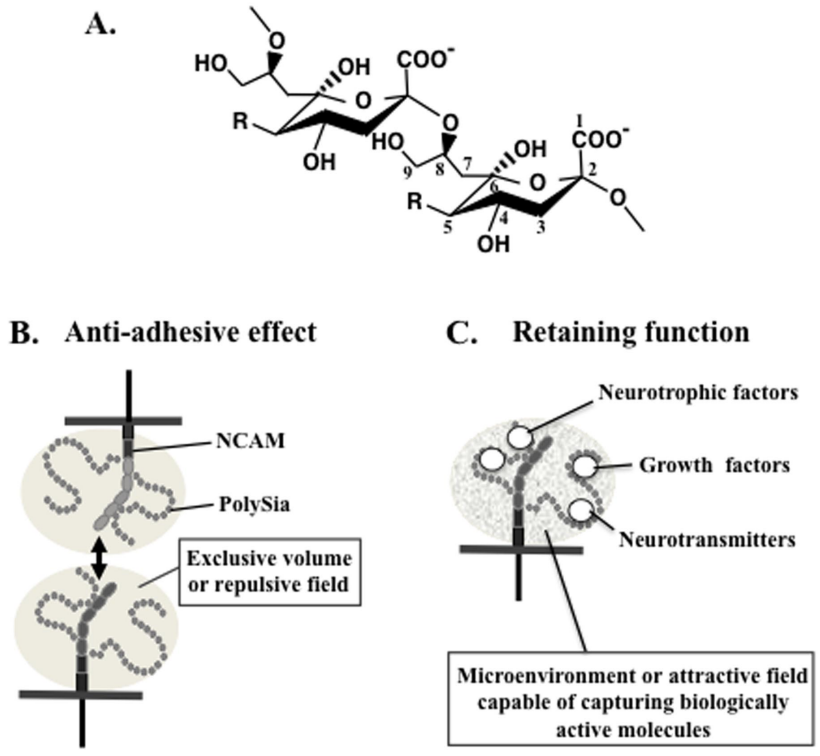

Fig. 5 Structure and proposed mechanism underlying the function of polySia. (A) Structure of α2 → 8-linked polySia. (B) Anti-adhesive effect of polySia. PolySia on NCAM has been proposed to function as a negative regulator of cell–cell adhesion via anti-adhesive effects. (C) Retaining function of polySia. PolySia can capture biologically active molecules, such as neurotransmitters, BDNF, and FGF2, and displays these molecules within the cell-surface microenvironment formed by polySia on NCAM.

Here, we focused on the SNP-7 mutation (g421a) of ST8SiaII/STX and clearly showed that the mutation affects the expression of polySia on NCAM of the Neuro2A cell surface (Fig. 1B). The point

mutation of g421a results in a change of residue 141 from glutamic acid (E) to lysine (K), indicating that 141E in ST8SiaII/STX is important for polySia biosynthetic activity in vivo. As the 141E residue is located close to the sialyl motif L, which is important for ST8SiaII/STX activity through binding to CMP-Sia [32], the observed reduction of activity might be due to the impaired binding of ST8SiaII/STX to substrate. However, it is noteworthy that plasmids used to transfect Neuro2A cells expressed the tandemly connected polysialyltransferases ST8SiaII/STX and ST8SiaIV/PST, either of which is alone able to synthesize polySia on NCAM. Therefore, the marked reduction of polySia on the Neuro2A cell surface in cells expressing mutant ST8SiaII/STX was interesting because wt ST8SiaIV/PST was also present. We speculate that ST8SiaII/STX may regulate the synthesis of polySia by ST8SiaIV/PST via direct binding. The 141E residue in ST8SiaII/STX may be involved in binding to ST8SiaIV/PST to form an active form of presumptive polysialyltransferase complex.

PolySia-NCAM-Fc purified from transfected CHO cells was analyzed for the amount of polySia therein by flow cytometry using mAb 12E3, which recognizes oligo/polySia (DP ≥ 5) containing a non-reducing terminal end. The analysis suggested that the amount of polySia biosynthesized by ST8SiaII/STX (wt) was higher than that by ST8SiaII/STX (g421a), a result that is consistent with that of the cell-surface staining (Fig. 1B). The polySia level was also evaluated by fluorometric C_7/C_9 [28] and mild acid hydrolysis/anion-exchange chromatography analyses [29] to evaluate the amount of internal sialic acid and average DP of the polySia chains, respectively. Based on the comparative fluorometric C_7/C_9 analysis, the amount of C_9 derived from ST8SiaII/STX (g421a) was 22 % of the amount generated by wt ST8SiaII/STX, while the average DP, as indicated by the $(C_7+C_9)/C_7$ ratio, was also dramatically decreased for the mutant ST8SiaII/STX. These results are consistent with the flow cytometry analysis using mAb 12E3 (Fig. 1B). Based on the mild acid hydrolysis-anion-exchange chromatography, the amount of oligo/polySia obtained from polySia-NCAM derived from mutant ST8SiaII/STX (g421a) (DP = 5~31) dramatically decreased compared with that from polySia-NCAM derived from wt ST8SiaII/STX (DP = 5–42). It is also noteworthy that a longer DP (up to 42) was detected from polySia-NCAM derived from wt ST8SiaII/STX, although the maximum DP detected in this assay was 31 with polySia-NCAM derived from mutant ST8SiaII/STX. Based on these analyses, polySia-NCAM derived from mutant ST8SiaII/STX was clearly impaired in the amount and quality of polySia. To make a structure–function relationship clearer, it is important to determine the detailed structures of polySia-NCAM derived from wt and mutant ST8SiaII/STX, including the number of polySia-modified antennae and DP of polySia per antennae. To date, however, the overall structure of polySia-NCAM remains unknown because of a lack of a suitable evaluation method.

Finally, we evaluated the newly described function of polySia, namely, the binding to BDNF and FGF2, which are important in brain development and functioning. As a negative control in this experiment, we used NCAM-Fc derived from mock-transfected CHO cells that stably secreted NCAM-Fc; therefore, we were only able to evaluate the effects of polySia structure derived from wt and mutant ST8SiaII/STX. The binding of polySia-NCAM-Fc derived from mutant enzyme toward BDNF and FGF2 were significantly decreased depending on the degree of impairment of polySia, i.e., due to smaller amount and/or low quality of polySia, as shown in the chemical analyses. The structure of polySia on NCAM influenced the affinity of this molecule toward BDNF and FGF2 based on the SPR analysis, with K_d values in the order of 10^{-9}. Notably, differences in binding properties to BDNF and FGF2 were detected, as the K_d value of mutant ST8SiaII/STX-synthesized polySia-NCAM was higher for BDNF compared to that synthesized by wt ST8SiaII/STX, whereas no obvious changes between polySia-NCAMs derived from wt and mutant ST8SiaII/STX were observed for FGF2. In addition, the K_d values between polySia and BDNF and between polySia and FGF2 were previously estimated to be 6.40×10^{-9} M and 1.47×10^{-8} M, respectively [12,15,30], indicating that polySia-NCAM has a high affinity for these neurologically active molecules, particularly BDNF. We speculate that the high affinity results from fine structural requirements of polySia or the underling structure of NCAM, as described above.

Polysialylation has been shown to occur in other glycoprotein synCAM-1 in mouse brain [33]. synCAM1 functions as an inducer of synapse formation by its homophilic binding [34], and polySia on synCAM1 negatively regulates its binding [33]. As the synCAM1 is also polysialylated by ST8SiaII/STX and ST8SiaIV/PST [33,35], it would be interesting to see if the synCAM1 polysialylation is impaired by the mutated ST8SiaII/STX.

The expression of polySia is reported to change depending on drug usage [36] and alcohol exposure [37]. In addition, an imbalance of polySia expression has also been reported in brain diseases other than schizophrenia, such as Alzheimer's [38] and Parkinson's diseases [39]. Based on our present results, the quality and quantity of polySia are clearly important for mediating not only its anti-adhesive effects (Fig. 5B), but also its retaining function (Fig. 5C).The quantity and quality of polySia expressed on cell surfaces might be well regulated by polysialyltransferases, ST8SiaII/STX, and ST8SiaIV/PST, although the potential mechanisms are unknown and should be resolved in future studies. In addition, it is necessary to experimentally demonstrate the molecule-releasing mechanism of polySia, which we have revealed here in part [13–15,31].

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