Novel Regulation of Fibroblast Growth Factor 2 (FGF2)-mediated Cell Growth by Polysialic Acid

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Background: Polysialic acid (polySia) binds neurological factors, BDNF and dopamine.

Results: PolySia specifically binds FGF2 and inhibits the cell growth facilitated by signaling through a ternary complex of FGF2, FGFR, and heparan sulfate (HS).

Conclusion: PolySia regulates the FGF2-mediated cell growth differently from HS.

Significance: A new function of polySia to regulate the action of neurological factors is established.

Polysialic acid (polySia) is a unique polysaccharide that modifies neural cell adhesion molecule (NCAM) spatiotemporally. Recently, we demonstrated that polySia functions as a reservoir for several neurotrophic factors and neurotransmitters. Here, we showed the direct interaction between polySia and fibroblast growth factor-2 (FGF2) by native-PAGE, gel filtration, and surface plasmon resonance. The minimum chain length of polySia required for the interaction with FGF2 was 17. Compared with heparan sulfate, a well known glycosaminoglycan capable of forming a complex with FGF2, polySia formed a larger complex with distinct properties in facilitating oligomerization of FGF2, as well as in binding to FGF receptors. In polySia-NCAM-expressing NIH-3T3 cells, which were established by transfecting cells with either of the plasmids for the expression of the polysialyltransferases ST8SiaII/STX and ST8SiaIV/PST that can polysialylate NCAM, FGF2-stimulated cell growth, but not cell survival, was inhibited. Taken together, these results suggest that polySia-NCAM might be involved in the regulation of FGF2-FGFR receptor signaling through the direct binding of FGF2 in a manner distinct from heparan sulfate.

Although NCAM expression levels remain unchanged (1, 5, 6), in adult brains, polysialylated NCAM (polySia-NCAM) only persists in distinct regions such as the hippocampus (7, 8) and the olfactory system (9) where neurogenesis is ongoing. The modification of NCAM with polySia has an anti-adhesive effect on cell-cell/extracellular matrix interactions due to the bulky polyamionic nature of polySia (1, 5, 6). Through its anti-adhesive effect, polySia is considered to be involved in neural cell migration, axonal guidance, fasciculation, myelination, synapse formation, and functional plasticity of the nervous system, in which homophilic binding of NCAM, as well as heterophilic binding with other cell adhesion molecules, occurs in a tissue-and stage-specific manner (5, 6). However, the function of polySia in both embryonic and adult brains is not completely understood, because neural development is not drastically impaired in NCAM-deficient mice (10) or polySia-reduced mice (11, 12), although learning and memory that are associated with long term potentiation and long term depression in the hippocampus and behaviors such as a fear-motion were influenced (10–12). Recently, we demonstrated that polySia functions as a reservoir for several neurotrophins, such as a brain-derived neurotrophic factor (BDNF), neurotrophin-3, and nerve growth factor (NGF) (13, 14), and is also capable of capturing catecholamine neurotransmitters, including dopamine, that regulate Akt signaling through dopamine receptors (15). Another group has also reported that polySia is involved in CCL21-related T cell migration (16, 17). Based on the evidence accumulated to date, we hypothesize that polySia regulates the action of neurological factors, such as neurotrophin, neurotransmitters, cytokines, and growth factors, via the capturing of these molecules to form specific complexes.

FGF2 is a prototypical FGF that stimulates the growth of various cell types, from fibroblasts to tumor cells (18), and was identified in bovine pituitary gland as a factor with the potential to induce the proliferation of fibroblast cells (19). FGF2 is synthesized as a 155-amino acid precursor and is subsequently processed into a mature form consisting of 146-amino acid residues (17.5 kDa). In its mature form, FGF2 is a soluble protein with a pI of 9.6. Interestingly, although it lacks a signal peptide sequence, FGF2 is localized to the extracellular space. A large amount of FGF2 is expressed in the brain during earlier stages.

Polysialic acid (polySia) is a polymerized structure of sialic acid with a degree of polymerization (DP) ranging from 8 to 400 (1–4) and is mainly found as a post-translational modification of neural cell adhesion molecule (NCAM) in vertebrates (1). PolySia is expressed in embryonic brains during post- and neonatal development and mostly disappears in adult brains,
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EXPERIMENTAL PROCEDURES

Materials—Human recombinant FGFs were purchased from PeproTech, Inc. (Rocky Hill, NJ). Colominic acid (DPav 15) was obtained from Wako Pure Chemicals (Osaka, Japan) and was used as polySia unless stated otherwise. Recombinant human FGRF1-Fc chimeras were obtained from R & D Systems (Minneapolis, MN). Rabbit anti-FGF2 antibodies and anti-His6 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p44/42 MAPK, phospho-p44/42 MAPK, Akt, and phospho-Akt antibodies were purchased from Cell Signaling (Danvers, MA). Enhanced chemiluminescent reagents, Sephacryl S-500 and S-300, Sephadex G-25, and molecular weight markers for gel filtration (thyroglobulin (670 kDa), ferritin (440 kDa), catalase (230 kDa), and bovine serum albumin (BSA) (67 kDa)) were purchased from GE Healthcare. Polyvinylidene difluoride (PVDF) membrane (Immobilon P) was a product of Millipore (Bedford, MA). Prestained molecular weight markers were purchased from Bio-Rad. HS and other glycosaminoglycans were purchased from Seikagaku Co. (Tokyo, Japan). α2,9-PolySia was prepared as described previously (36).

Preparation of Oligo/PolySia—Mild acid hydrolyses of colominic acid (1 mg) were separated on a Jasco HPLC system equipped with a Mono Q HR5/5 (0.5 × 5 cm) anion-exchange column. Samples were loaded on the column and eluted with 20 mM Tris-HCl (pH 8.0), followed by a NaCl gradient (0–20 min, 0 M; 20–60 min, 0–0.3 M; 60–100 min, 0.3–0.45 M; 100–110 min, 0.45–1 M; and 110–120 min, 1 M) in 20 mM Tris-HCl (pH 8.0). The flow rate was 500 μl/min and fractions were monitored with a UV detector (UV, Jasco Corp., Japan) at a wavelength of 210 nm. α2→8-Linked homo-oligo/polyNeu5Ac fractions (DP 2–24) were pooled and desalted on a Sephadex G-25 column (1.2 × 65 cm, water).

Horizontal Native- and SDS-PAGE—FGF2 (100 ng) in 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl (TBS) was incubated with or without polySia (0–2 μg), Neu5Ac (2 μg), other sialic acid samples (500 ng), or glycosaminoglycans (0–2 μg) at room temperature for 2 h. The final incubation volume was 10 μl. Half of the samples were subjected to horizontal or vertical native-PAGE (13, 14) or SDS-PAGE and blotted onto PVDF membranes. All experiments were performed in duplicate to quintuplicate.

Preparation of Biotinylated Glycans—To prepare biotinylated glycans, chitotriose (GlcnAc)3 (2 mg/ml), polySia (10 mg/ml), or HS (10 mg/ml) in 50 mM sodium acetate buffer (pH 5.5) were mixed with biotin-LC-hydrazide (5 mM final concentration) dissolved in dimethyl sulfoxide (DMSO). After incubation at 50 °C for 2 h, NaBH3CN in methanol (22.4 μM final concentration) was added to the reaction mixture. The biotinylated glycans were then applied to a Sephadex G-25 column (1.2 × 60 cm) and eluted with water to remove free biotin (36).

Immmobilization of Biotinylated Glycans on a Gold Sensor Chip—For SPR measurements, the gold sensor surface was washed once with acetone and allowed to air dry, and the sensor chip was then immersed in 10 μM 4,4′-dithiodibutyric acid in ethanol to form a self-assembly membrane (SAM) on the gold surface. After gentle shaking for 30 min at room temperature, the sensor surface was washed three times with ethanol and allowed to dry. The chip was then placed in a solution of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride and N-hydroxysuccinimide (1:9 mixture of 130 μM 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride in water and 144 μM N-hydroxysuccinimide in 1,4-dioxane) at room temperature for 30 min with gentle shaking to activate the SAM on the gold surface. After adding 10 ml of water to the solution, the chip was further incubated for 5 min at room temperature, and the gold surface was then washed once with water. The gold chip containing surface-activated SAM was placed on the sensor chip support using the sensor chip assembly unit and was...
then set in a Biacore 2000 or 3000 instruments (GE Healthcare). After priming the system with water for 7 min, a 0.1 mg/ml streptavidin solution was loaded twice, each time for 7 min at a flow rate of 10 µl/min. The immobilized streptavidin was monitored by the resonance unit (RU) value, which typically reaches 490–580 RU for streptavidin. To destroy the excess activated groups, 1 mM ethanolamine was injected into the system for 7 min. After washing with 0.01 HEPES, pH 7.4, containing 0.15 M NaCl, 3 mM EDTA, and 0.0005% Surfactant P20 (HBS-EP), the target biotinylated glycan (0.1 mg/ml in HBS-EP) was injected into the system to allow immobilization on the gold surface. Binding of the captured glycans was monitored based on the observed RU values, which typically reach ~30 RU for (GlcNAc)₃, 120 RU for polySia, and 120 RU for HS.

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**Immobilization of FGF2 on a CM5 Sensor Chip**—To immobilize FGF2 for SPR analysis, a research grade CM5 chip was first set in a Biacore 2000. After washing the surface of the chip with 40% glycerol, the sensor chip surface was activated with a mixture of 400 mM 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride and 100 mM N-hydroxysuccinimide for 7 min at a flow rate of 10 µl/min. Immediately after activation, a FGF2 solution (5 ng/µl) in sodium acetate buffer (pH 5.0) was added onto the chip surface. After the RU reached an appropriate value, 1 mM ethanolamine was flowed over the chip surface for 7 min to destroy activated residues (37).

**Biacore Analysis**—The interactions between FGF2 and several glycans were measured using a Biacore 2000 or 3000. For the analysis of the interactions of immobilized glycans with FGF2, varying concentrations of FGF2 (0–220 nM), FGF2-polySia complexes (1:7), or HS-FGF2 complexes (1:7) in HBS-EP were injected over the glycan-immobilized sensor chip surfaces at a flow rate of 20 µl/min. For the analysis of the interactions between immobilized FGF2 and glycans, varying concentrations of polySia (0–80 µM) and HS (0–36 µM) in HBS-EP were injected over the glycan-immobilized sensor chip surfaces 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.

**Gel Filtration of FGF2- and PolySia Complexes**—FGF2-polySia complexes (final volume, 50 µl) and incubation with FGF2 (2 µg) and polySia (400 µg) at room temperature for 2 h were subjected to Sephacryl S-300 chromatography (0.58 × 28 cm, TBS). FGF2 (2 µg) and polySia (400 µg) or HS (400 µg) with or without FGF2 (2 µg) at room temperature for 2 h were subjected to separation using the same column. The eluents were analyzed by SDS-PAGE and Western blotting using anti-FGF2 antibody for FGF2 and anti-human IgG + M + A for Fc site of FGF2.

**Cross-linking**—FGF2 (40 ng) was incubated with or without polySia (2 µg), HS (2 µg), or Neu5Ac (2 µg) (8 µl total volume) at room temperature for 2 h, and proteins were then cross-linked by adding 1 µl of 2.5 mM disuccinimidyl suberate (DSS, Pierce) to the sample, which was further incubated at room temperature for 30 min. After stopping the reaction by adding 1 µl of 0.5 M Tris-HCl (pH 7.5) to the sample, FGF2 was analyzed by SDS-PAGE and Western blotting with anti-FGF2 antibodies.

Establishing Stable Cell Lines—Stable cell lines were collected with collagenase I (Amano Enzyme, Japan) and washed with phosphate-buffered saline (PBS). The cells were incubated with monoclonal antibody (mAb) 735 (2 µg/ml), mAb 12E3 (10 µg/ml), or anti-HS antibody (clone F58-10E4, Seikagaku Co., Japan) (10 µg/ml) at 4 °C for 30 min and then washed twice with PBS. The cells were then incubated with Alexa-labeled anti-mouse IgG or IgM (2 µg/ml) at 4 °C for 30 min. After washing cells with PBS twice, cell surface staining was measured using a flow cytometer (Galilio, Beckman Coulter), and the collected data were analyzed with Karza software (Beckman Coulter).

**RT-PCR**—Total RNA was prepared from NIH-3T3 cells using TRIzol (Invitrogen). Random-primed cDNA (~50 ng) and specific primers were used for the PCR (supplemental Table 1).

**Effect of FGF2-PolySia Complex Formation on Cell Growth and Survival—**NIH-3T3 cells stably expressing STX or PST were cultured in DMEM supplemented with 0.5 mg/ml streptomycin sulfate, 100 units/ml penicillin G, and 10% FBS in a 5% CO₂ and 95% air-humidified atmosphere at 37 °C. For monitoring cell survival, the cells (3 × 10⁴) were transferred to a 96-well plate and incubated overnight in DMEM containing 10% serum. After washing cells with PBS, cells were grown in serum-free DMEM in the presence or absence of FGF2 (50 ng/ml) for 1 and 2 days. Ten µl of CCK-8 (Dojindo, Japan) was then added to each of the wells, and the cells were further incubated for 3 h, and the absorbance at 450 nm was measured to quantitate cell numbers. For evaluating cell growth, the cells (5 × 10⁵) were plated into 96-well plates and incubated overnight with DMEM containing 10% serum. After washing cells with PBS, cells were grown in DMEM containing 2% serum in the presence or absence of FGF2 (50 ng/ml). The cell numbers at days 1, 3, and 5 were measured as described above. For Western blot analysis of p42/44 and Akt signaling, cells incubated with FGF2 (50 ng/ml) for 0, 10, 30, 60, and 120 min were collected using lysis buffer (50 mM Tris-HCl (pH 8.0) containing 1% Triton X-100, 1 mM PMSF, 1 µg/ml of aprotinin, leupeptin, and pepstatin, 5 mM EDTA, 10 mM sodium pyrophosphate, 25 mM glycercophosphate, 10 mM NaF, 1 mM Na₃VO₄) by a cell scraper. Cell homogenates were stored on ice for 1 h and centrifuged at 10,000 × g for 15 min, and the resulting supernatant was used for Western blot analysis with anti-signaling antibodies, rabbit.
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polyclonal anti-FGF2 (0.2 μg/ml), anti-FGFR (0.2 μg/ml), or anti-p44/42 MAPK antibodies (1:1000 dilution), anti-phospho-p44/42 MAPK antibodies (1:1000 dilution), anti-Akt antibodies (1:1000 dilution), or anti-phospho-Akt antibodies (1:1000 dilution) and incubated overnight at 4 °C. As the secondary antibody, either peroxidase-conjugated anti-rabbit IgG antibodies (1:4000 dilution) or anti-goat IgG antibodies (1:2000 dilution) were used at 37 °C for 60 min, and color development was performed as described previously (28).

Data Analysis—All values are expressed as the mean ± S.D.

RESULTS

To demonstrate direct binding between polySia and FGF2, we first performed horizontal native-PAGE and Western blotting analyses (Fig. 1A). As expected, FGF2 (pl 9.6) alone migrated toward the cathode region (Fig. 1A, FGF2, none). FGF2 preincubated with polySia migrated toward the anode region (Fig. 1A, polySia), whereas the FGF2 preincubated with Neu5Ac migrated toward the cathode region (Fig. 1A, Neu5Ac), like FGF2 alone. These results suggest that FGF2 binds to polySia directly, as was demonstrated for BDNF (13). It was previously demonstrated that basic proteins, such as lysozyme and trypsin, do not always bind polySia when subjected to horizontal native-PAGE analysis (13). As FGF2 has been shown to bind HS specifically, the binding between FGF2 and HS (Fig. 1A, HS) and several glycosaminoglycans was also confirmed with this method (supplemental Fig. 1). Not all examined polyanionic chains bound to FGF2, as demonstrated by the lack of binding by HA (supplemental Fig. 1). Taken together, these findings suggested that FGF2 specifically binds to polySia, similar to HS and other glycosaminoglycans.

We next evaluated the effects of salt concentration (0–1.0 M NaCl) and divalent cations (0.33–3.3 mM CaCl2 and 0.9–9 mM MgCl2) on polySia binding using horizontal native-PAGE analysis, as described previously (13). Almost no effects on the binding of polySia to FGF2 were observed under high NaCl conditions (~1.0 M) or in the presence of divalent cations (data not shown), whereas the binding between BDNF and polySia was inhibited at high salt concentrations (>0.3 M) and in the presence of Ca2+ (3.3 mM) or Mg2+ (9 mM) cations (13).

We previously demonstrated that polySia is structurally diverse with regard to the sialic acid components, linkages, and DP (2) and that the minimum DP requirement of polySia for interaction with BDNF is 12 (13). Therefore, we examined if the structural diversity of polySia affects the binding of FGF2. To evaluate the relevance of polySia DP to the FGF2 binding, we first prepared a series of α2,8-linked oligo/polyNeu5Ac molecules by the anion-exchange chromatography of colomicinic acid (13), and we then analyzed the polySia group with DP in the ranges of 1–10, 11–20, 21–30, and 31–40. As shown in Fig. 1B (left panel), polySia with a DP of 1–10 did not bind to FGF2. PolySia with a DP of 11–20 started to bind to FGF2. Almost all polySia chains with DP of ≥21 bound to FGF2. To more accurately determine the DP of polySia required for binding, we further purified polySia with defined DPs between 11 and 20. For each isolated polyNeu5Ac (20 ≥ DP ≥ 11), we assessed binding to FGF2 using horizontal native-PAGE. We observed that FGF2 migrated toward the cathode region in the presence of polyNeu5Ac (DP <16), similar to FGF2 alone, whereas FGF2 began to migrate toward the anode region when mixed with polyNeu5Ac with a DP of 17 (Fig. 1B, right panel). These results clearly indicated that polySia requires a chain length (DP) of at least 17 Neu5Ac residues to bind FGF2.

To further characterize the binding between FGF2 and polySia or HS, the effects of the amount of polySia and HS on binding affinities were measured. The EC50 values, which represent the concentrations of polyanions required to titrate 50 ng (0.56
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FIGURE 2. Titration of polySia and HS toward FGF2. A, left panel, FGF2 (100 ng) was incubated with increasing amounts of polySia (0–500 nM) in TBS at 37 °C for 2 h. Right panel, amount of FGF2 migrating to the anode region was densitometrically measured. B, left panel, FGF2 (100 ng) was incubated with increasing amounts of HS (0–570 nM) in TBS at 37 °C for 2 h. Right panel, amount of FGF2 migrating to the anode region was densitometrically measured. The half-titrated concentration represents the EC50 value, as indicated by arrow.

A. polySia- or HS-immobilized surface

B. FGF2-immobilized surface

FIGURE 3. SPR measurement of the direct binding between FGF2 and polyanionic glycans. A, FGF2 (1.8–170 nM) was individually injected over polySia-immobilized (left panel) or HS-immobilized (right panel) sensor chips. B, polySia (5–80 μM) (left panel) or HS (12–180 nM) (right panel) was individually injected over a FGF2-immobilized sensor chip. The bars indicate the sample injection period, and the end of the bars indicate the beginning of the dissociation phase initiated with running buffer. Values of the vertical axis are expressed in response units (RU).

μM) of FGF2 and indicate the relative affinities of polyanions (13), were calculated in three independent titration experiments (Fig. 2, A and B). The EC50 values of polySia and HS were estimated to be 6.24 × 10^{-8} ± 1.15 × 10^{-8} M and 1.36 × 10^{-7} ± 0.32 × 10^{-7} M, respectively. The EC50 of polySia toward FGF2 was 2-fold lower than that of HS under native-PAGE conditions. We further determined the dissociation constants of polySia or HS and FGF2 by SPR. After immobilization of biotinylated polySia or HS to streptavidin that was conjugated with 4,4′-dithiodibutyric acid on the SAM on the gold surface, which was performed in the absence of carboxymethyl dextran that sometimes becomes one of the problems for nonspecific binding with basic proteins (37), we measured the interaction between FGF2 and polySia or HS using FGF2 as an analyte (Fig. 3A and Table 1). To observe specific interactions, we used a (GlcNAc)3-biotin as a control glycan and subtracted the control sensorgram from the polySia or HS sensorgrams. As estimated by SPR, the $K_d$ value between FGF2 and polySia was determined to be 1.47 × 10^{-8} ± 6.97 × 10^{-9} M and that between FGF2 and HS was 2.81 × 10^{-9} ± 4.01 × 10^{-9} M. The $K_d$ value of polySia calculated with the gold sensor chip was 2-fold greater than that of HS. We also measured the interaction between FGF2 and the polySia or HS vice versa (Fig. 3B and Table 1). Under this configuration, the $K_d$ values of polySia and HS were 4.81 × 10^{-6} and 3.16 × 10^{-9} M, respectively. Taken together, these results suggested that the amino groups of FGF2 that were used to immobilize onto the surface of the sensor chip were critical for the interaction with polySia, and the interaction mode between polySia and FGF2 and between HS and FGF2 differed from each other.

To confirm the complex formation between FGF2 and polySia and to estimate the molecular size of the polySia-FGF2 complex, we performed gel filtration chromatography analysis. FGF2 (17.5 kDa) exists as a monomer under physiological conditions and elutes with an apparent size of 17.5 kDa on gel filtration chromatography. In this study, FGF2 alone eluted at less than 67 kDa on Sephacryl S-300 chromatography, corresponding to fractions >24 (Fig. 4A, FGF2); however, after incubation with polySia, FGF2 eluted in fractions 14–17 (Fig. 4A, FGF2/PolySia), indicating that FGF2 forms a complex with polySia. Complex formation was also observed after incubation with HS (Fig. 4A, FGF2/HS), although the complex between HS and FGF2 displayed a more disperse $M_r$ distribution, and the complex elution peak (peak fraction 18) was markedly smaller than that of polySia-FGF2 complexes (peak fraction 15). To more accurately estimate the $M_r$ of the FGF2-polySia complex, gel filtration was performed on a Sephacryl S-500 gel because the FGF2-polySia complex was too large (>670 kDa) to analyze using Sephacryl S-300. Nearly all of the complexes eluted at a size greater than 670 kDa, and the elution position of the complex was first observed in fraction 17, which corresponded to an estimated $M_r$ of 5000 (Fig. 4B), according to the calibration curve of the relationship between the elution position and the $M_r$ markers.
To gain insight into the structure of the FGF2-polySia complex, we cross-linked FGF2 before and after incubation with polySia. In the absence or presence of the cross-linking reagent, FGF2 was exclusively observed as a monomer (17.5 kDa) on SDS-PAGE, consistent with previous reports that the majority of FGF2 exists as monomers (38). After incubation with polySia, most FGF2 remained as monomers (Fig. 4, polySia), demonstrating that FGF2 monomers bind to polySia to form large complexes (>670 and <5000 kDa). In contrast, after incubation with HS, oligomerized FGFs were observed. This result is consistent with previously reported results (38). These data suggest that polySia-FGF2 complexes are completely different from those formed between HS and FGF2.

To investigate the differences between FGF2-polySia and FGF2-HS complexes, we performed vertical native-PAGE analysis. As shown in Fig. 5A, complexes formed between polySia and FGF2 could not enter the separating gel (left lane, IB: FGF2); however, those between HS and FGF2 clearly migrated into the separating gel (right lane, IB: FGF2) like other glycosaminoglycans (supplemental Fig. 2). In contrast, the complexes formed between both BDNF and polySia and BDNF and HS migrated into the separating gel (supplemental Fig. 3). These data suggest that complexes formed between polySia and FGF2 are different from those of other complexes. To evaluate the stability of FGF2 complexes in the presence of other polyanionic glycans, competition experiments were performed. As shown in Fig. 5B, the FGF2-HS complex was stable even in the presence of large amounts of polySia (left lane). In contrast, FGF2-polySia complex was changed to FGF2-HS complex in the presence of HS (Fig. 5B, right lane). These results indicate that at least two forms of FGF2 and polyanionic glycan complexes exist and that HS-FGF2 complex formation is practically irreversible. To confirm this, we investigated the possibility of the presence of different binding sites to polySia than HS on FGF2 using SPR. We immobilized polySia or HS onto sensor chips, as described above, and analyzed binding using FGF2-polySia and FGF2-HS complexes as analytes. As shown in Fig. 5C, FGF2-polySia complexes clearly bound to both polySia and HS-modified sensor chips; however, FGF2-HS complexes could not bind to either sensor chip. In addition, it was confirmed that polySia and HS did not bind to chips with either

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**TABLE 1**

Kinetic parameters for the interaction between FGF-2 and polySia or HS

| Immobilized molecule | Analyte molecule | $K_a$ (M$^{-1}$) | $K_d$ (M) | $K_D$ (M)$^{-1}$ |
|----------------------|------------------|----------------|--------|----------------|
| PolySia              | FGF-2            | $2.1 \times 10^5$ | $3.60 \times 10^{-3}$ | $1.47 \times 10^{-8}$ |
|                      |                  | (6.871 x10^4)    | (2.07 x10^{-3})    | (6.97 x10^{-9})    |
| HS                   | FGF-2            | $2.24 \times 10^2$ | $1.16 \times 10^{-3}$ | $2.81 \times 10^{-8}$ |
|                      |                  | (1.45 x10^2)     | (6.56 x10^{-4})    | (4.01 x10^{-8})    |
| FGF-2                | polySia          | $1.05 \times 10^4$ | $4.80 \times 10^{-4}$ | $4.81 \times 10^{-6}$ |
|                      |                  | (1.93 x10^4)     | (5.95 x10^{-5})    | (1.31 x10^{-6})    |
| FGF-2                | HS               | $2.18 \times 10^2$ | $6.86 \times 10^{-4}$ | $3.16 \times 10^{-8}$ |
| PolySia              | FGF-1            | ND              | ND     | ND             |
| HS                   | FGF-1            | $6.78 \times 10^3$ | $1.49 \times 10^{-2}$ | $2.19 \times 10^{-8}$ |

ND means not detected.
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A. FGF2-polySia
   FGF2-HS
   stacking
   separating
   IB: FGF2
   SDS-PAGE
   66k
   440k
   232k
   140k
   60k
   +HS +polySia
   FGF2-polySia
   FGF2-HS
   stacking
   separating
   IB: FGF2
   SDS-PAGE
   66k
   440k
   232k
   140k
   60k
   C. polySia-immobilized surface
      FGF2
      FGF2-polySia
      FGF2-HS
      HS-immobilized surface
      FGF2
      FGF2-polySia
      FGF2-HS

FIGURE 5. Analysis of the complex formation between FGF2 and polySia. A, vertical native-PAGE of FGF2. FGF2 (100 ng) incubated with polySia (1 μg) (polySia) or HS (1 μg) (HS) at 37 °C for 2 h in TBS was subjected to vertical native-PAGE. FGF2 was then blotted onto a PVDF membrane and visualized as described under “Experimental Procedures.” IB, immunoblot. B, competitive complex formation between polySia and HS. FGF2 (100 ng) was incubated with polySia (+ polySia) or heparan sulfate (+ HS) at 37 °C for 2 h in TBS as described in A. To each incubation mixture was added HS (1 μg) or polySia (1 μg); vertical native-PAGE or SDS-PAGE was then performed, and FGF2 was then blotted onto a PVDF membrane and visualized as above. C, SPR measurement of the complex binding between FGF2-polySia and HS-polySia on polySia- or HS-immobilized surfaces. PolySia and HS were directly immobilized onto the gold surface as shown in Fig. 3A, and the complexes formed between FGF2 and polySia (FGF2-polySia) and FGF2 and HS (FGF2-HS) were analyzed. FGF-2 alone is also indicated (FGF2).

polyanionic glycan-modified surface (data not shown). Taken together, these data indicate that the binding sites of FGF2 toward polySia and HS are different and that after forming a complex with HS, FGF2 has an altered conformation that prevents it from binding to polySia or HS.

FGF2 is well known to form a ternary complex with HS and FGFR to enhance signaling after dimerization of FGF. Therefore, it is important to determine whether polySia affects the binding between FGF2 and FGFR. Here, we focused on the migration of FGF2-polySia complexes toward FGFR by analyzing complex formation using gel filtration in the presence and absence of FGFR. A recombinant Fc chimera of the extracellular domain (amino acids 1–374) of human FGFR-1c (190 kDa) was used as a test receptor, and the resulting ligand-receptor complexes were analyzed by Sephacryl S-300 chromatography (Fig. 6A). FGF2 preincubated with polySia eluted from fraction 14 (~2000 kDa) (Fig. 6A, FGF2 + polySia, IB: FGF2), although FGF2 existed as a dimer (360 kDa) and eluted in fractions 15–20 (Fig. 6A, FGF2, IB: Ig(FGFR)). FGF2 preincubated with FGF2 co-eluted with FGF2 in fractions 17–19 (Fig. 6A, FGF2 + FGFR, IB: FGF2), showing that FGF2 binds to FGF2. We next determined if FGF2 binds to FGFR as a FGF2-polySia complex. FGF2 was preincubated with polySia to form FGF2-polySia complexes, which were then incubated with FGF2 and subjected to Sephacryl S-300 chromatography and Western blotting with anti-FGF2 and anti-FGFR antibodies. The resulting elution profiles were almost identical to those observed for both FGF2-polySia complexes and FGF2 alone (Fig. 6A (FGF2 + polySia) + FGFR, IB: FGF2 (left panel) and Ig(FGFR) (right panel)). Thus, no obvious ternary complex was observed between polySia, FGF2, and FGFR.

From the gel filtration analyses, it was suggested that the FGF2 that is involved in forming FGF2-polySia complexes does not migrate toward FGFR, even though BDNF as part of BDNF-polySia complexes was shown to migrate toward TrkB (13). To further evaluate the FGF2 migration possibility toward receptors from FGF2-polySia complexes, we performed a binding assay. Protein A immobilized in 96-well plastic plates were coated with FGFR-Fc in the identical direction and then incubated with polySia or HS, followed by FGF2. The FGF2 in the supernatant that did not bind to FGFR was collected and analyzed by Western blotting. As shown in Fig. 6B, the amount of free FGF2 increased in the presence of polySia in a dose-dependent manner (left panel, polySia). In contrast, nearly all of the FGF2 bound to FGFR in the presence of HS at all examined concentrations (left panel, HS). In addition, the amount of free FGF2 was not influenced by either polySia or HS when FGF2 was first allowed to bind FGFR (Fig. 6B, right panel). These data clearly showed that the FGF2, which formed a complex with polySia, could not migrate toward FGFR.

To gain insight into the biological function of the FGF2-polySia complex, we analyzed cell survival and growth of NIH-3T3 fibroblast cells that endogenously expressed nonpolysialylated NCAM and FGFR1 and are affected by FGF2 addition (35). As NIH-3T3 cells cannot synthesize polySia on NCAM due to a deficiency in the polysialyltransferases, STX and PST, we transfected cells with plasmids encoding STX and PST and established cell lines that stably expressed polySia, which were
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A.

**Sephacryl S-300 chromatography**

![Sephacryl S-300 chromatography](image)

**FIGURE 6.** Complex formation between the FGFR and FGF2 in the presence and absence of polySia. A, FGF2 (2 μg) was incubated with polySia (400 μg) (FGF2 + polySia) or FGF2 (FGF2 + FGFR). In addition, after complex formation between 2 μg of FGF2 and 400 μg of polySia, mixtures were further incubated with FGF2 (FGF2 + polySia + FGFR). Mixtures were then subjected to the Sephacryl S-300 chromatography (0.58 × 28 cm, eluted with TBS), and eluted fractions were collected. Eluted FGF2 or Fc-tagged FGF2 was analyzed by Western blotting using anti-FGF2 antibodies (left panel, IB: FGF2) or anti-Fc antibodies (right panel, IB: IgG). The elution of thyroglobulin (670 kDa), ferritin (440 kDa), catalase (230 kDa), and BSA (67 kDa) is indicated. IB: immunoblot. B, migration of FGF2 toward FGFR in the presence and absence of polySia or HS. Left panel, wells of a 96-well plate immobilized with protein A were incubated with FGF2-Fc. After blocking with 1% BSA, the wells were incubated with polySia or HS in indicated concentrations at 37 °C for 1 h, and FGF2 was then added. After 1 h, the supernatants were collected. Eluted FGF2 or Fc-tagged FGFR was analyzed by Western blotting using anti-FGF2 antibodies. Right panel, plastic wells were incubated with FGF2-Fc. After blocking with 1% BSA, the wells were incubated with FGF2 at 37 °C for 1 h, and polySia or HS was then added, and the plates were further incubated at 37 °C for 4 h. The supernatants were then collected, and the unbound FGF2 was analyzed by SDS-PAGE Western blotting with anti-FGF2 antibodies.

B.

**Incubation with polySia or HS followed by FGF2**

![Incubation with polySia or HS followed by FGF2](image)

**FIGURE 6.** Complex formation between the FGFR and FGF2 in the presence and absence of polySia. A, FGF2 (2 μg) was incubated with polySia (400 μg) (FGF2 + polySia) or FGF2 (FGF2 + FGFR). In addition, after complex formation between 2 μg of FGF2 and 400 μg of polySia, mixtures were further incubated with FGF2 (FGF2 + polySia + FGFR). Mixtures were then subjected to the Sephacryl S-300 chromatography (0.58 × 28 cm, eluted with TBS), and eluted fractions were collected. Eluted FGF2 or Fc-tagged FGF2 was analyzed by Western blotting using anti-FGF2 antibodies (left panel, IB: FGF2) or anti-Fc antibodies (right panel, IB: IgG). The elution of thyroglobulin (670 kDa), ferritin (440 kDa), catalase (230 kDa), and BSA (67 kDa) is indicated. IB: immunoblot. B, migration of FGF2 toward FGFR in the presence and absence of polySia or HS. Left panel, wells of a 96-well plate immobilized with protein A were incubated with FGF2-Fc. After blocking with 1% BSA, the wells were incubated with polySia or HS in indicated concentrations at 37 °C for 1 h, and FGF2 was then added. After 1 h, the supernatants were collected. Eluted FGF2 or Fc-tagged FGFR was analyzed by Western blotting using anti-FGF2 antibodies. Right panel, plastic wells were incubated with FGF2-Fc. After blocking with 1% BSA, the wells were incubated with FGF2 at 37 °C for 1 h, and polySia or HS was then added, and the plates were further incubated at 37 °C for 4 h. The supernatants were then collected, and the unbound FGF2 was analyzed by SDS-PAGE Western blotting with anti-FGF2 antibodies.

named N3-STX and N3-PST, respectively. We confirmed by RT-PCR that N3-STX and N3-PST cells expressed STX and PST mRNAs, respectively (supplemental Fig. 4A, upper panel), and we also confirmed the stationary expression of FGFR (supplemental Fig. 4A, lower panel). To confirm the polysialylation state on the cell surface of the STX- and PST-transfected cells, flow cytometric analysis with mAb 735 (DP ≥ 11) and mAb 12E3 (DP ≥ 5) (39) was performed. As shown in supplemental Fig. 4B, no polySia staining with either mAb 735 or mAb 12E3 with mock-transfected cells (N3-Mock) was observed. After STX or PST gene transfection, mAb 735 and mAb 12E3 staining increased, showing that polySia was expressed on the cell surface of these cells. It was also observed that mAb 735-staining of STX-transfected cells was stronger than that of the PST-transfected, although mAb 12E3 staining was nearly identical for the two transfectants. All of the observed staining disappeared following endo-N-acetylneuraminidase treatment (data not shown). We also confirmed the expression of the cell surface HS by flow cytometric analysis with ST3 cells clones (N3-Mock, N3-STX, and N3-PST) using anti-HS antibody (supplemental Fig. 4C).

Using the two stable cell lines, we analyzed cell survival and growth in the presence and absence of FGF2. Although cell survival was not affected by the expression of polySia on NIH-3T3 cells in the presence or absence of FGF2 (Fig. 7A), cell growth was dramatically decreased in STX-transfected cells in the absence of FGF2 (Fig. 7B). A similar result was also observed with the cells transfected with PST (Fig. 7B). These results clearly showed that expression of polySia affected cell proliferation. In addition, in the presence of FGF2, the enhancement of cell proliferation by FGF2 was inhibited in N3-STX and N3-PST cells, showing that polySia biosynthesized by STX and PST affected FGF2 signaling. Finally, we analyzed MEK and Akt signaling, which is affected by FGF2 through FGFR during cell proliferation, in N3-STX, N3-PST, and N3-Mock cells. A significant change in the Akt signal was not observed in any of the three cell types, although the initial level appeared slightly up-regulated in the two transfectant cell lines compared with that of N3-Mock cells. Phosphorylated p44/42 was transiently enhanced after FGF2 addition in the N3-STX and N3-PST cells, but the signal relatively rapidly diminished. In contrast, the signal gradually up-regulated and was sustained for 2 h in N3-Mock cells. Taken together, these data clearly showed that FGF2 signaling was modulated by polySia.

To further understand effects of the surface polySia on cell proliferation in the presence and absence of the HS expression, we established four types of NIH-3T3 cell clones, polySia−HS+N+, polySia−HS+K+, polySia−HS−K+, and polySia−HS−K+(−) cells (supplemental Fig. 5). We analyzed cell growth of these cells in the presence and absence of FGF2 (Fig. 8). In the absence of the surface polySia, only HS-positive cells greatly proliferated in an FGF2-dependent manner (polySia−HS+N+ versus polySia−HS−K+(−)), suggesting that HS plays a critical role in the FGF2-dependent proliferation. In the presence of the surface polySia, cells proliferated slightly better than polySia-negative cells in the absence of FGF2, and this was independent of the HS expression (polySia−HS+N− versus polySia−HS−K+(−)).
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A. survival (0%)

B. growth (2%)

C. FGF2 signaling by polySia-expressing cells.

** DISCUSSION **

In this study, we demonstrated that polySia specifically and directly binds to the growth factor FGF2 by native-PAGE, gel filtration, and SPR. It has been well documented that in the NCAM protein the FGL peptide derived from the FN3 motif directly binds to FGFR and regulates signaling (33). The dissociation constants of the FGL peptide and several Ig domains of NCAM toward FGFR are reported to be \(1–10 \, \text{M} \) (33, 40).

polySia\(^{+}\) HS\(^{K^{D}}\)(−) versus polySia\(^{-}\) HS\(^{K^{D}}\)(−)). Thus, polySia appears to have an inhibitory effect on cell proliferation, as in the case with the results in Fig. 7. Notably, the inhibitory effect of polySia was prominent for the HS-positive cells even in the presence of FGF2 (polySia\(^{+}\) HS\(^{+}\) (+) versus polySia\(^{-}\) HS\(^{+}\) (+)). These results suggest that the cell surface polySia has the ability to inhibit the FGF2-dependent HS-mediated cell proliferation.
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The regulation of cell proliferation through NCAM-FGFR interaction was also demonstrated using NIH-3T3 cells (35). However, all of these studies to date have focused on nonpolysialylated NCAM, and the possibility that the post-translational modification of NCAM with polySia is directly involved in FGF2 signaling has not been taken into account.

Our new finding of polySia interaction with FGF2, but not with FGFR, suggests that polySia regulates FGF-FGFR signaling through controlling the concentration of FGF2 molecules in the extracellular space. As HS is also a major player in FGF2 signaling through complex formation with FGF2 and FGFR, we compared the binding features of polySia toward FGF2 with that of HS. Although the binding affinity calculated by native-PAGE and SPR was almost the same order of magnitude between polySia and HS, the complexes formed between polySia-FGF2 and FGF2-HS were markedly different based on several results. First, by horizontal native-PAGE, we determined that the minimum DP of polySia required for FGF2 binding is 17 (Fig. 1C), whereas in the case of HS, the minimum DP is around 6 (30), which are much shorter chains. Therefore, it appears that an unusually long polySia chain is required for FGF2 binding. Second, by SPR analyses, we performed two experiments to understand the binding features of the two complex types. Using glycan-immobilized chips, the \( K_d \) values between FGF2 and polySia and between FGF2 and HS were nearly identical (Fig. 3A and Table 1), a finding that was consistent with that obtained in the titration curves by horizontal native-PAGE (Fig. 2, A and B). Interestingly, using an FGF2-immobilized sensor chip, the \( K_d \) value toward polySia was increased by 3 orders, although that toward HS was similar compared with that obtained using the HS-immobilized sensor chip (Fig. 3B and Table 1). This finding indicates that Lys and/or Arg residues on the FGF2 molecule surface, but not in the binding pocket of HS, are important for polySia to bind to FGF2. Third, the complex size of polySia and FGF2 was extremely large compared with that of HS and FGF2 (Figs. 4 and 5A). Fourth, after FGF2-HS complex formation, the complex could no longer bind to both HS and polySia; however, the FGF2-polySia complex retained binding affinity for both polySia and HS (Fig. 5B). This finding is consistent with the results of a previous study showing that complex formation with HS caused FGF2 to change its conformation and promoted di/oligomerization (38). Our present data suggest that the binding site(s) of FGF2 to polySia are completely different from that to HS. Based on the well-known Hajihosseini model (FGFR1-FGF2-heparin (decasaccharide), Protein Data Bank code 1FQ9) (29), HS binds to FGF2 through a basic amino acid cluster, consisting of residues Arg-120, Lys-125, Lys-129, and Lys-135, and promotes formation of a symmetric 2:2:2 FGF-FGFR ternary signaling complex. This model also suggests that there are other basic pockets on the FGF2 surface, such as “Arg-33, Arg-39, Arg-44, and Arg-81” and “Arg-60, Arg-97, Arg-107, Arg-109, and Arg-110”, which might participate in the binding of polySia.

The binding affinity of polySia toward FGF2 was shown to be almost the same order as that of HS based on native-PAGE and SPR. For the analyses, we used HS (\( M_r \), 11,000; sulfation, 5–6%, as determined by gel filtration) and polySia (mean DP of 43, \( M_r \), 13,000, as determined by anion-exchange chromatography). In addition, we also measured the binding affinity of heparin (\( M_r \), 9800; sulfation, 10.5–14.5%, as determined by gel filtration), whose sulfation rate is higher than that of HS; however, as the \( K_d \) of heparin calculated by native-PAGE was higher than that of HS, HS was used in all other experiments. Although the binding affinities of HS toward FGF2 have been reported in several SPR studies (31, 32), they all used carboxymethyl dextran-based chips, which may have reduced the accuracy of the results due to the tendency of FGF2 to exhibit nonspecific binding. In this study, we used a SAM on an gold surface and immobilized the polyanionic glycans. In addition, we subtracted the \((\text{GlcNAc})_3\) sensorgram to minimize the effects of nonspecific binding. Using this approach, we obtained a \( K_d \) value of 1.5 × 10^{-8} \( M \) for the binding of polySia and FGF2. This strength of binding is relevant for biological effects because the \( K_d \) value of biologically active HS is higher than that of polySia.

It is interesting that FGF2 making a complex with polySia might migrate toward HS, but the opposite did not occur (Fig. 5C). To explain this difference, the conformational change of FGF2 after forming a complex with HS is considered to be the primary cause. The conformational change of FGF2, as indicated by the increase of polyclonal antibody reactivity, was observed only after incubation with HS (Fig. 1A) and might promote di/oligomerization of HS (Fig. 4C). In addition, FGF2 making a complex with polySia did not migrate onto FGFR (Fig. 6). In the case of BDNF-polySia complexes, the migration of BDNF toward TrkB or p75NTR is likely dependent on differences of the dissociation constant. It is noteworthy that the complex between polySia and FGF2 differed from that between BDNF and polySia.

Based on our present results, we hypothesize a two-complex model that encompasses the retaining and releasing properties of polySia (Fig. 9). PolySia can retain neurologically active molecules such as BDNF and FGF2; however, polySia displays these molecules differently. In the case of BDNF, BDNF making com-

![FIGURE 8. Relative cell growth ratio.](image-url)
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PolySia migrates toward high affinity receptors, with the degree of migration depending on the affinity of the two molecules. For example, 50 and 25% of BDNF was shown to migrate toward TrkB and p75NTR, respectively (13). In contrast, FGF2 making complex with polySia-NCAM does not migrate toward FGFR, although FGF2 migrates toward HS easily. Therefore, polySia may regulate the concentration of FGF2 differently and HS might be the final regulator of signal transduction of FGF2 into the cell (Fig. 8). Related to this point, the spatiotemporal expression of polySia and HS is interesting, particularly where neurogenesis is ongoing, as polySia and HS are more highly expressed. More detailed knowledge on co-expression and separate expression of polySia and HS on the same cells would be necessary. It is interesting that polyanionic chains such as polySia and HS display different binding features toward the same molecule. Not only combinations involving polySia and HS, but also other combinations between polyanionic chains and/or sulfated epitopes containing glycoprotein, such as HNK-1, might also be involved in the regulation of growth factors and neurotrophic factors.

To determine the biological implications of the polySia-FGF2 complex, we used NIH-3T3 cells that express nonpolysialylated NCAM (35) due to the lack of the polysialyltransferases STX and PST. NIH-3T3 cells also express FGFR and respond to FGF2 (33). Using this cell line, we analyzed the effects of polySia on cell survival and growth after transfection with plasmids for the expression of STX and PST genes responsible for the biosynthesis of polySia. In the presence of FGF2, polySia biosynthesized by STX and PST affected FGF2 signaling and inhibited cell growth. It might be related to the low path filter in signaling reported recently (49) because the signals via p42/44 in the polySia-expressing cells were transient but observed. Notably, there are other autocrine polySia-binding growth factor(s) secreted by NIH-3T3, and polySia biosynthesized by STX and PST affects cell growth via that secreted factor(s) likely by restoring these molecules and passing them toward high affinity receptors such as BDNF and TrkB, as recently reported by our group (13). It would be interesting to identify these factors. In previous studies, polySia promotes cell growth and migration, affecting FGFR signaling in neuroblastsoma cells and other cell models (31, 33, 51). These results suggest the positive effects on cell growth, and contrasted with our results in NIH-3T3 cells. To understand the underlying mechanisms for these different effects of polySia, many subjects will have to be solved, such as surface distribution of polySia, FGF, and FGFR on each cell type, structural features of polySia, amount and chain length, that may affect FGF2-polySia complex formation in each cell and involvement of other signaling than FGFR signaling in observed phenomena. These studies are underway in our laboratory. PolySia is also expressed on numerous tumor cells (41–45) and is considered to be involved in metastasis via its anti-adhesive effect. Tumor cells with polySia are occasionally reported to re-express STX genes (40), and polySia on tumor cell surfaces might affect tumor cell growth via growth factors such as FGF2.

FGF2 is a multifunctional molecule that is widely distributed in the mammalian CNS and exerts multiple trophic actions on neurons and glial cells during CNS development, injury, and disease. FGF2 plays critical roles in not only embryonic but also in adult brains, through the promotion of neuronal survival and growth, and is also involved in a number of psychiatric disorders. Schizophrenia, bipolar disorder, and major depressive disorder are conditions with mixed genetic and environmental etiologies and are associated with significant morbidity worldwide. These disorders are associated with hippocampal abnormalities. Reduced hippocampal volume is seen in depression and psychotic bipolar disorder, although in schizophrenia reduced hippocampal neuronal size with altered shape and reduced olfactory bulbs are often observed. FGF2 functions as a differentiation factor for hippocampal neurons and is also involved in fear-conditioned activities (26). Furthermore, FGF2 is shown to exert anti-depressant-like effects, similar to BDNF (25). Recently, Arai et al. (46) revealed an association between the promoter region of STX and schizophrenia by genome-wide analyses. We previously demonstrated that STX from schizophrenic patients has impaired biosynthetic activities and also adversely affects the structure of polySia (15). Such abnormality of the polySia structure impaired the binding toward

FIGURE 9. Proposed mechanism of the new function of polySia. A, PolySia is considered to be a negative regulator of cell-cell adhesion via an anti-adhesive effect. B, new functions of polySia are proposed based on the recent findings of binding molecules. PolySia can bind to neurological active factors, such as BDNF (13), neurotransmitters (37), and FGF2 (this study), resulting in a unique special microenvironment (Retain state). For releasing bound molecules, two pathways are proposed. One involves direct migration from the complexes toward high affinity receptors (e.g. BDNF and TrkB) (13). The other pathway is indirect migration toward receptors (e.g. FGF2 and FGFR). In the latter case, other adaptor molecules, such as HS (e.g. HS proteoglycan), might be required for migration from polySia to the receptors or another mechanism such as the degradation of polySia (50) might occur.
BDNF and dopamine that are deeply involved in the development of schizophrenia (15). FGFR binding of impaired polySia biosynthesized in such brains might be expected in schizophrenic brains. The expression of polySia is reported to change depending on drug usage (47) and alcohol exposure (48). The quality and quantity of polySia might be well regulated on cell surfaces, and the concentration of polySia-binding molecules may be finely tuned to activate or inactivate cells through receptors spatiotemporally, likely in conjunction with other regulatory molecules, such as HS.

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