

Direct binding of polysialic acid to a brain-derived neurotrophic factor depends on the degree of polymerization

Yukihiro Kanato, Ken Kitajima, and Chihiro Sato¹

Graduate School of Bioagricultural Sciences and Bioscience and
Biotechnology Center, Nagoya University, Nagoya 464-8601, Japan

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Polysialic acid (polySia) is the homopolymer of sialic acid and negatively regulates neuronal cell–cell and cell–extracellular matrix interactions through steric and repulsive hindrance due to its bulky polyanionic structure. Whether polySia also functions as a positive regulator in the nervous system through binding to specific ligands is not known. In the present study, we demonstrated that a brain-derived neurotrophic factor (BDNF) dimer binds directly to polySia to form a large complex with an M_r greater than 2000 kDa under physiologic conditions. Although somewhat affected by the linkage and type of sialic acid components in the polySia, the complex formation is highly dependent on the polySia chain length. The minimum degree of polymerization required for the complex formation is 12. This is the first study to demonstrate the biologic significance of the degree of polySia polymerization in eukaryotes. Similar large polySia complexes form with other neurotrophic factors such as nerve growth factor, neurotrophin-3, and neurotrophin-4. Furthermore, the BDNF, after making a complex with polySia, can bind to the BDNF receptors, TrkB and p75NTR. The complex formation of BDNF with polySia upregulates growth or/and survival of neuroblastoma cells. These findings suggest that polySia functions as a reservoir of BDNF and other neurotrophic factors and may serve to regulate their local concentrations on the cell surface.

Keywords: brain-derived neurotrophic factor/NCAM/p75NTR/polysialic acid/TrkB

Introduction

Polysialic acid (polySia) is a polymerized structure of sialic acid with a degree of polymerization (DP) ranging from 8 to 400 (Troy 1996; Sato and Kitajima 1999; Nakata and Troy 2005). The most common structure of polySia is the Neu5Ac polymer whose interresidual linkage is $\alpha 2 \rightarrow 8$. There are six proteins so far identified as polysialylated glycoproteins in vertebrates, polysialoglycoprotein in fish eggs (Inoue and Iwasaki 1978; Sato et al. 1993), neural cell adhesion molecule (NCAM) in brain (Finne 1982; Troy 1996), sodium channel in electroplax (James and Agnew 1987) and rat brain (Zuber et al. 1992),

CD36 in human milk (Yabe et al. 2003), and neuropilin-2 in human lymphocytes (Curreli et al. 2007). Among these, NCAM modified with a polySia chain is well studied in the nervous system (Troy 1996; Bonfanti 2006; Rutishauser 2008). PolySia is expressed in embryonic brains during neural differentiation and mostly disappears in adult brain, although the NCAM expression level remains unchanged (Troy 1996; Bonfanti 2006; Rutishauser 2008). In adult brains, polysialylated NCAM persists in distinct regions such as hippocampus (Seki and Arai 1991), hypothalamic nuclei (Theodosios et al. 1991; Seki and Arai 1993), and olfactory system (Miragell et al. 1988; Seki and Arai 1991; Bonfanti and Theodosios 1994) where neurogenesis is ongoing. PolySia on NCAM has antiadhesive effects on the cell–cell/extracellular matrix interaction due to its bulky polyanionic nature (Troy 1996; Bonfanti 2006; Rutishauser 2008). It is 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 of other CAMs occur in a tissue- and stage-specific manner (Bonfanti 2006; Rutishauser 2008). Recent phenotype analyses of mice deficient in NCAM and the responsible enzymes for polysialylation (ST8Sia II/STX and/or ST8Sia IV/PST) confirmed the importance of polySia in development, long-term potentiation (LTP) in the hippocampus CA3 region, long-term depression and LTP in the hippocampus CA1 region, guidance of mossy fibers and synapse formation, spatial learning, circadian rhythm, and various behaviors (Cremer et al. 1994; Eckhardt et al. 2000; Angata et al. 2004; Weinhold et al. 2005). Among studies exploring the function of polySia-NCAM, Muller et al. demonstrated in NCAM-knockout mice that brain-derived neurotrophic factor (BDNF) restores LTP in polySia-NCAM-deficient hippocampus and suggested that polySia-NCAM interacts with BDNF (Muller et al. 2000), although they provided no direct evidence of this interaction.

Neurotrophic factors promote neuronal survival and differentiation during development (Sofroniew et al. 2001). They have vital roles in the functional maintenance of neurons in normal homeostasis and in neuronal regeneration. Nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) are structurally and functionally related and comprise a family of neurotrophic factors called neurotrophins (Barde et al. 1982; Brade 1994; Sofroniew et al. 2001). Neurotrophins have a number of shared characteristics, such as molecular size, isoelectric point, and primary structure (~50% identity). They exist in solution as noncovalently bound dimers (Sofroniew et al. 2001). The neurotrophins interact with two types of receptors on the cell surface, the low affinity neurotrophin receptor p75NTR and the high affinity protein-kinase receptors of tropomyosin-related kinase (Trk) (Huang and Reichardt 2003); NGF preferentially binds TrkA, BDNF and NT4/5 bind TrkB, and NT3 binds TrkC (and TrkA to a lesser extent). Of these neurotrophins,

¹To whom correspondence should be addressed: Tel: +81-52-789-4295; Fax: +81-52-789-5228; e-mail: chi@agr.nagoya-u.ac.jp

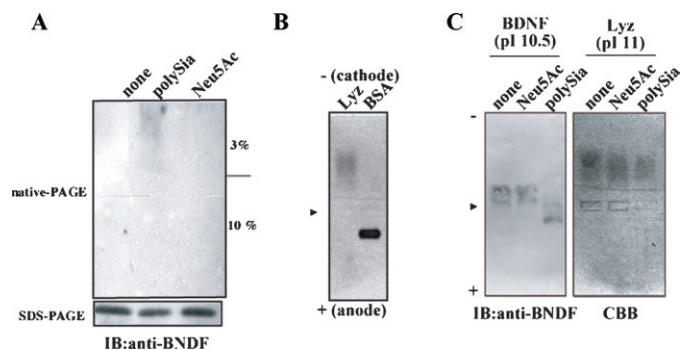


Fig. 1. Analysis of the complex formation between BDNF and polySia by native PAGE. **(A)** conventional native PAGE. BDNF (100 ng) incubated with polySia (*polySia*) or Neu5Ac (*Neu5Ac*) or without sialic acid (*none*) at 37°C for 2 h in TBS was subjected to conventional native PAGE or SDS-PAGE. BDNF was then blotted onto the PVDF membrane and visualized as described in *Material and methods*. **(B)** Horizontal native PAGE of typical acidic protein (bovine serum albumin, *BSA*) and basic protein (lysozyme, *Lyz*). Proteins (1 μ g) were run on horizontal native PAGE and visualized by Coomassie brilliant blue staining. **(C)** Horizontal native PAGE of BDNF. Left panel: the same BDNF samples (100 ng) as described in panel A were loaded on the gel (arrowhead) and electrophoresed. After blotting onto the PVDF membrane, BDNF was visualized with anti-BDNF antibodies as described in *Material and methods*. Right panel: lysozyme (1 μ g) was incubated with 20 μ g of polySia (*polySia*) or Neu5Ac (*Neu5Ac*) or without sialic acid (*none*) at 37°C for 2 h in TBS, subjected to horizontal native PAGE and the protein was visualized by Coomassie brilliant blue staining. Arrowhead, origin; –, cathode; +, anode.

BDNF is the most abundant in brain. BDNF promotes the growth and development of immature neurons and enhances the survival and functional maintenance of adult neurons (Barde 1994; Barde et al. 1982; Sofroniew et al. 2001). BDNF levels are correlated with several disorders, including depression, epilepsy, bipolar disorder, and Parkinson's and Alzheimer's disease (Buckley et al. 2007; Kozisek et al. 2008).

Recently, we demonstrated that polySia has structural diversity in Sia component type, linkage, and DP (Sato and Kitajima 1999; Sato 2004; Sato et al. 2000). In brain especially, not only α 2,8-linked polyNeu5Ac but also α 2,8-linked di/oligoSia structures are present on glycoproteins (Sato et al. 2000). Recently developed sensitive chemical methods for detecting oligo/polySia (Sato et al. 1998, 1999, 2000) allow for the determination of the DP of polysialylated NCAM (Inoue S and Inoue Y 2001; Galuska et al. 2006, 2008); however, the biologic relevance of polySia-NCAM DP remains unknown. We are interested in the biologic significance of the sialic acid DP on glycoproteins and investigating the specific binding counterparts of di/oligo/polySia in nature (Sato and Kitajima 2004). We hypothesized that polySia directly binds to neurotrophins depending on the DP and serves as a reservoir of neurotrophins for their efficient supply to the neurotrophin receptor. To test this hypothesis, we used various methods to examine whether direct binding of polySia and BDNF occurs under physiologic conditions. We also characterized the BDNF–polySia complex in terms of its binding to TrkB and p75NTR.

Results

To demonstrate binding between polySia and BDNF, we first performed conventional native PAGE (Figure 1). BDNF mi-

grated toward the cathode region because of its basic isoelectric point ($pI = 10.5$) and did not enter the gel (Figure 1A, *none*). After preincubation with polySia, BDNF migrated into the gel (Figure 1A, *polySia*). Preincubation with Neu5Ac did not affect BDNF migration toward the cathode region (Figure 1A, *Neu5Ac*). The amount of BDNF used in these experiments was the same as that used in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)/Western blotting (Figure 1A, lower panel). These results indicate that BDNF directly binds to polySia and migrates into the gel as a negatively charged complex with polySia. To confirm these results, we performed horizontal native PAGE (Figure 1B). Lysozyme ($pI = 11$) and BSA ($pI = 4.7$) migrated toward the cathode (Figure 1B, *BSA*), respectively. As expected, BDNF ($pI = 10.5$) migrated toward the cathode region (Figure 1C, BDNF, *none*). BDNF preincubated with polySia migrated toward the anode region (Figure 1C, BDNF, *polySia*), while the BDNF preincubated with Neu5Ac migrated toward the cathode region, like BDNF alone (Figure 1C, BDNF, *Neu5Ac*). These results suggest that BDNF specifically binds to polySia, but not to Neu5Ac. In contrast, the migration behaviors of lysozyme did not change even after incubation with polySia or Neu5Ac (Figure 1C, *Lyz*, lane *polySia* or *Neu5Ac*). We also tested another basic protein, trypsin ($pI = 10$), and this protein did not migrate toward cathode region (Supplementary data 1). These results indicate that basic proteins do not always bind to polySia. Thus, these findings suggest that BDNF specifically binds to polySia to form an anionic complex.

To further confirm the complex formation of BDNF with polySia, we performed gel chromatography analysis. BDNF (13.5 kDa) exists as a dimer under physiologic conditions and elutes around 27 kDa on gel chromatography (Rao and Finkbeiner 2007). In the present study, BDNF eluted at around 27 kDa on Bio-gel P-100 chromatography (Figure 2A). After incubation with polySia, BDNF eluted in the void volume on Bio-gel P-100 chromatography, indicating that BDNF forms a complex with polySia. To estimate the molecular weight of the BDNF–polySia complex, gel filtration on a Sephacryl S-500 gel, instead of a Bio-gel P-100 gel, was performed because the BDNF–polySia complex was too large (> 100 kDa) to analyze on the Bio-gel P-100. Almost all of the complex eluted at an M_r greater than 670 kDa and the complex size, based on the peak fraction, was estimated to be around 2000 kDa, according to the calibration curve of the relationship between the elution position and the M_r (Figure 2B). A supermolecular complex that eluted close to the void volume was also formed. These results indicate that the BDNF–polySia complex is extremely large, consistent with the results that the BDNF–polySia complex did not enter the separating gel on conventional native PAGE. To gain insight into the BDNF–polySia complex structure, we cross-linked BDNF before or after incubation with polySia. Without the cross-linking reagent, BDNF was exclusively observed as a monomer (13.5 kDa) on SDS–PAGE (Figure 2C, DSS(–)), while, with the cross-linking reagent, BDNF was observed as a dimer (Figure 2C, *none*), consistent with previous reports that the majority of BDNF exists as a dimer (Sofroniew et al. 2001; Rao and Finkbeiner 2007). After incubation with Neu5Ac and polySia, most BDNF still exists as a dimer (Figure 2C, *Neu5Ac* and *polySia*). These results demonstrate that the BDNF dimer binds to polySia to form large complexes (M_r greater than 670 kDa, around 2000 kDa). We performed

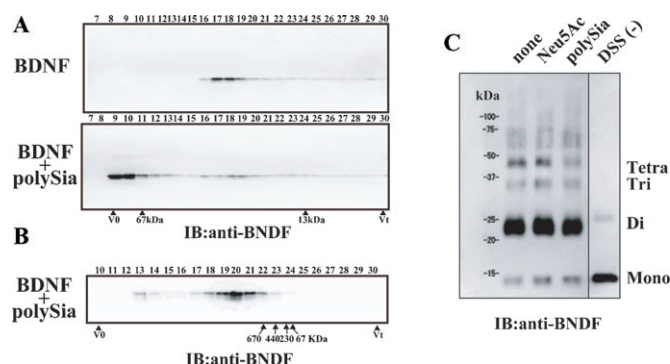


Fig. 2. Analysis of the complex formation between BDNF and polySia by gel filtration. (A) Bio-gel P-100 chromatography of BDNF and BDNF complexed with polySia. BDNF (2 μ g) incubated without (BDNF) or with polySia (400 μ g as sialic acid) (BDNF + polySia) was subjected to Bio-gel P-100 chromatography (0.58 \times 28 cm, TBS). The collected samples were electrophoresed and analyzed by Western blotting using anti-BDNF antibodies. The elution of ovalbumin (67 kDa) and cytochrome C (13 kDa) is indicated. (B) Sephacryl S-500 chromatography of BDNF complexed with polySia. BDNF (2 μ g) incubated with polySia (400 μ g as sialic acid) (BDNF + polySia) was subjected to Sephacryl S-500 chromatography (0.58 \times 28 cm, TBS). The samples collected were electrophoresed and analyzed by Western blotting using anti-BDNF antibodies. The elution of thyroglobulin (670 kDa), ferritin (440 kDa), and catalase (230 kDa) is indicated. (C) Cross-linking of BDNF. BDNF without (*none*) or with polySia (*polySia*) or Neu5Ac (*Neu5Ac*) was incubated at room temperature for 2 h and cross-linked by adding DSS to the sample. Cross-linked sample or noncross-linked samples (DSS (-)) were analyzed by SDS-PAGE and Western blotting with anti-BDNF antibodies.

rechromatography of the BDNF–polySia complex and showed that the complex continued to elute at the same fraction numbers (data not shown), thus indicating that the complex was stable once formed.

To characterize the complex formation between BDNF and polySia, the effect of the amount of polySia, salt concentration, and divalent cations were analyzed using horizontal native PAGE analysis (Figure 3). We first incubated BDNF (100 ng) with polySia (0–1000 ng) and analyzed the complex formation (Figure 3A, left panel). The cationic behavior of BDNF was neutralized with increased amounts of polySia, with almost all of the BDNF–polySia complex migrating toward the anode at polySia levels of 125 ng or greater. From the titration curve constructed by measuring the amount of BDNF remain in the cathode region densitometrically, 50% of the BDNF–polySia complex formation occurred with 50 ng of polySia with 100 ng of BDNF (Figure 3, right panel). We then examined the effect of the NaCl concentration (0.1–1.0 M) (Figure 3B). The NaCl concentration affected the complex formation between BDNF and polySia. Under physiologic conditions (0.1–0.2 M NaCl), all BDNF migrated toward the anode; at ≥ 0.3 M NaCl, some BDNF remained in the cathode region, similar to BDNF alone, and the amount of BDNF remaining in the cathode region increased depending on the NaCl concentration. These results suggest that electrostatic interactions are involved in the formation of the BDNF–polySia complex. PolySia interacts with calcium ions (Shimoda et al. 1994). Therefore, we then analyzed the effects of divalent cations on BDNF–polySia binding. Under physiologic conditions (0.9 mM Ca^{2+} and/or 0.33 mM Mg^{2+}), complex formation occurred (Figure 3C). At concentrations 10 times higher than physiologic concentrations (9 mM Ca^{2+} ,

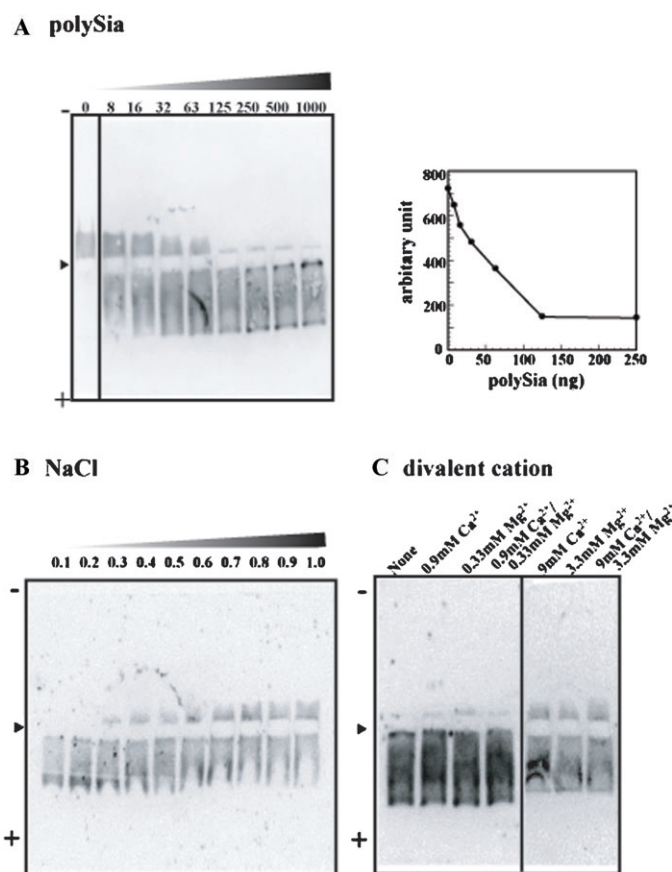


Fig. 3. Complex formation between BDNF and polySia under different conditions. (A) Left panel: amount of polySia. BDNF (100 ng) was incubated with polySia (0–1000 ng) in TBS at 37°C for 2 h. Right panel: the amount of BDNF remaining in the cathode region was densitometrically measured. (B) Concentration of NaCl. BDNF (100 ng) was incubated with polySia (100 ng) in TBS containing 0–1.0 M NaCl at 37°C for 2 h. (C) Divalent cation requirement. BDNF (100 ng) was incubated with polySia (100 ng) in TBS at 37°C for 2 h in the absence (*none*) or presence of 0.9 mM CaCl_2 (0.9 mM Ca^{2+}), 0.33 mM MgCl_2 (0.33 mM Mg^{2+}), 0.9 mM CaCl_2 + 0.33 mM MgCl_2 (0.9 mM Ca^{2+} /0.33 mM Mg^{2+}), 9 mM CaCl_2 (9 mM Ca^{2+}), 3.3 mM MgCl_2 (3.3 mM Mg^{2+}), and 9 mM CaCl_2 + 3.3 mM MgCl_2 (9 mM Ca^{2+} /3.3 mM Mg^{2+}). All samples were loaded onto horizontal native PAGE and analyzed as described in *Material and methods*. Arrowhead, origin; –, cathode; +, anode.

3.3 mM Mg^{2+}), BDNF–polySia complex formation was inhibited and the inhibition was enhanced by coincubation with both of these cations (9 mM Ca^{2+} and 3.3 mM Mg^{2+}). These results suggest that divalent cations are not necessary for BDNF–polySia complex formation.

We previously demonstrated that polySia is structurally diverse with regard to the Sia components, linkages, and DP (Sato and Kitajima 1999, 2000; Sato 2004; Sato et al. 2000); however, the biologic relevance of polySia diversity is unknown. Therefore, we examined how the structural diversity of polySia affects the formation of the BDNF–polySia complex. First, to determine the relevance of polySia DP in the formation of the BDNF–polySia complex, we prepared a series of α 2,8-linked oligo/polyNeu5Ac with defined DPs by anion-exchange chromatography of colominic acid (Figure 4A). With each isolated oligo/polyNeu5Ac, we assessed complex formation of BDNF using horizontal native PAGE: oligoNeu5Ac (DP < 8), BDNF

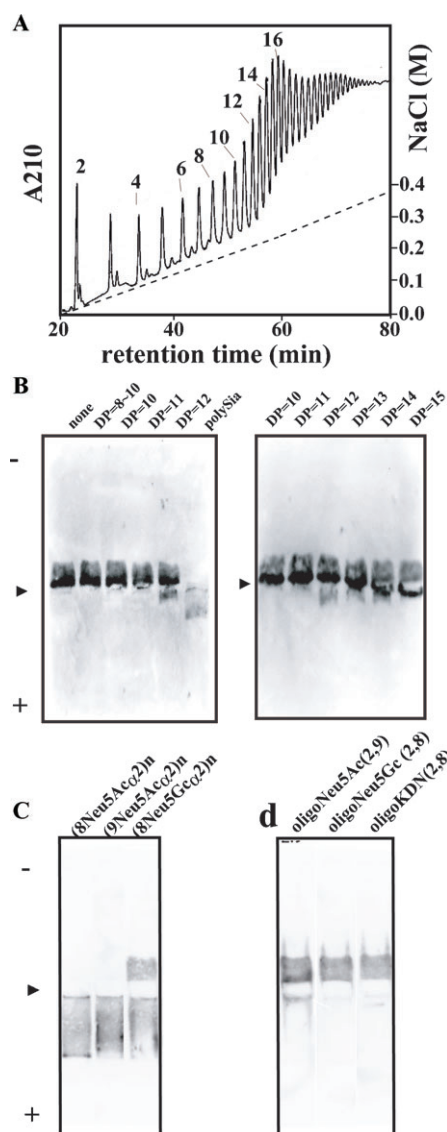


Fig. 4. Degree of polymerization required for complex formation between BDNF and polySia. (A) Purification of polySia according to the degree of polymerization (DP). Mild acid hydrolysate of colominic acid (1 mg) was purified according to the DP using monoQ anion-exchange chromatography. Oligo/polySia eluted was monitored with a UV detector. NaCl (dotted line) gradient and DP (number) are presented. (B) Left Panel: BDNF (100 ng) was incubated without (none) or with polySia (DP = 8–10 (mixture of 8, 9, 10), DP = 10, DP = 11, DP = 12, or polySia (colominic acid) in TBS at 37°C for 2 h. Right Panel: BDNF (100 ng) was incubated with polySia (DP = 10, DP = 11, DP = 12, DP = 13, DP = 14, DP = 15) in TBS at 37°C for 2 h. Samples were analyzed by horizontal native PAGE. (C) Component and linkage differences of polySia. BDNF (100 ng) was incubated with 1 μg of α2,8-linked polyNeu5Ac from colominic acid ((8Neu5Acα2)n), α2,9-linked polyNeu5Ac from *Neisseria meningitidis* group C ((9Neu5Acα2)n), α2,8-linked polyNeu5Gc from rainbow trout polysialoglycoprotein (PSGP) ((8Neu5Gcα2)n). (D) Component and linkage differences of oligoSia. α2,9-Linked oligoNeu5Ac from mild acid hydrolysates of α2,9-linked polyNeu5Ac, α2,8-linked oligoKDN from rainbow trout ovarian fluid (oligoKDN), and α2,8-linked oligoNeu5Gc from mild acid hydrolysates from rainbow trout PSGP (oligoNeu5Gc) in TBS at 37°C for 2 h. All samples were loaded onto horizontal native PAGE and analyzed as described in *Material and methods*. Arrowhead, origin; –, cathode; +, anode.

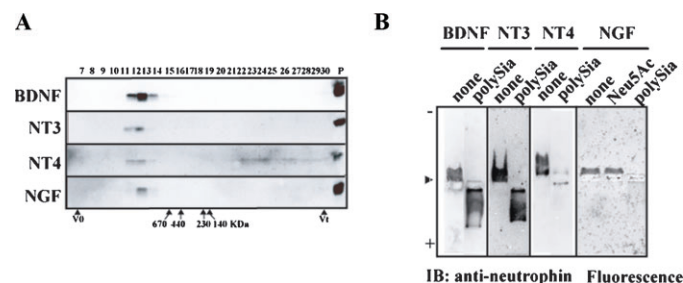


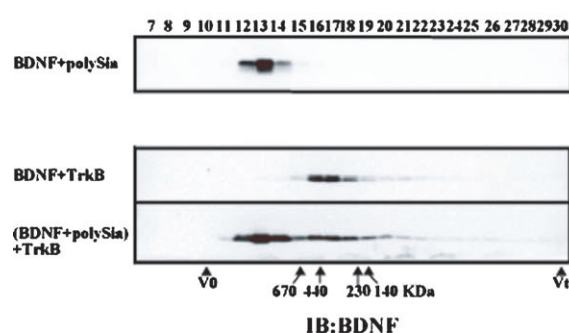
Fig. 5. Complex formation between neurotrophins and polySia. (A) Gel filtration of the complex between neurotrophins and polySia. Neurotrophins (2 μg; BDNF, NGF, NT-3, NT-4) were incubated with 400 μg polySia at 37°C for 2 h and subjected to Sephacryl S-300 chromatography (0.58 × 28 cm, eluted with TBS). The samples collected were electrophoresed and analyzed by Western blotting using anti-neurotrophin (anti-BDNF antibodies, anti-NT-3 antibodies, anti-NT-4 antibodies or anti-NGF antibodies). The elution of thyroglobulin (670 kDa), ferritin (440 kDa), catalase (230 kDa), and lactate dehydrogenase (140 kDa) are indicated. (B) Horizontal native PAGE of the neurotrophin-polySia complex. Neurotrophins (100 ng; BDNF, NT-3, NT-4, NGF) were incubated with 2 μg of polySia at 37°C for 2 h and subjected to horizontal native PAGE. Migration of the proteins was analyzed by the specific antibodies after blotting onto PVDF membrane. In the case of NGF, protein was visualized with fluorescent dye staining using the imaging analyzer (Ex 532 nm, Em 580 nm). Arrowhead, origin; –, cathode; +, anode.

migrated toward the cathode region, similar to BDNF alone (data not shown); polyNeu5Ac with DP = 8–11, BDNF did not migrate toward the anode region (Figure 4B); polyNeu5Ac (DP ≥ 12), BDNF migrated toward the anode region (Figure 4B, DP = 12–15). These results indicate that polySia requires a chain length (DP) of at least 12 Neu5Ac residues to bind with BDNF. This is the first demonstration of the biologic impact of polySia DP in animal. We then evaluated how the type and linkage of Sia residues affect BDNF–polySia complex formation. In these experiments, 100 ng of BDNF and 1 μg of each polySia compound were mixed. Based on the results of the horizontal native PAGE analysis, not only α2,8-linked polyNeu5Ac but also α2,9-linked polyNeu5Ac formed the BDNF–polySia complex (Figure 4C, (8Neu5Acα2)n, (9Neu5Acα2)n). For α2,8-linked oligo/polyNeu5Gc with DP = 2–25, half of the BDNF migrated toward the anode region, indicating complex formation (Figure 4C, (8Neu5Gcα2)n).

For oligoSia with shorter DPs, BDNF preincubated with α2,9-linked oligoNeu5Ac with DP = 2–10, α2,8-linked oligoNeu5Gc with DP = 2–10 or α2,8-linked oligoKDN with DP = 2–7 migrated to the anode region (Figure 4D, oligoNeu5Ac (2,9), oligoNeu5Gc(2,8), oligoKDN(2,8)), indicating that oligoSia did not bind to BDNF. These results shown in Figure 4C and D suggest that the formation of the BDNF–polySia complex does not depend on the Sia type or linkage, although we cannot deny the possibility that the avidity of various polySia molecules for BDNF differs.

In addition to BDNF, there are three other types of neurotrophins in the brain, NGF, NT-3, and NT-4. Because the primary and steric structures of these neurotrophins are similar to each other, we presumed that these neurotrophins also bind to polySia to form large complexes. We thus analyzed the complex formation of polySia with these neurotrophins using Sephacryl S-300 chromatography and horizontal native PAGE. Consistent with the results on Sephacryl S-500 chromatography

A TrkB



B p75NTR

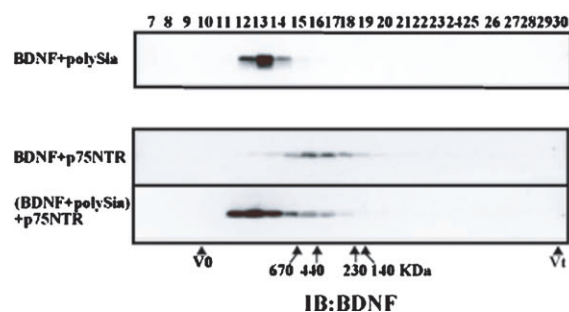


Fig. 6. Complex formation between neurotrophin receptors and BDNF in the presence or absence of polySia. BDNF (2 μ g) was incubated with polySia (400 μ g) (BDNF + polySia), TrkB (BDNF + TrkB), or p75NTR (BDNF + p75NTR). In addition, after complex formation between 2 μ g BDNF and 400 μ g polySia, samples were further incubated with TrkB ([BDNF + polySia] + TrkB) or p75NTR ([BDNF + polySia] + p75NTR). Samples were subjected to the Sephacryl S-300 chromatography (0.58 \times 28 cm, eluted with TBS) and proteins were collected. Eluted BDNF or His-tagged neurotrophin receptor (TrkB or/and p75NTR) was analyzed by the Western blotting using anti-BDNF antibodies (left pane, IB: BDNF) or anti-His antibodies (right panel, IB: His). The elution of thyroglobulin (670 kDa), ferritin (440 kDa), catalase (230 kDa), and lactate dehydrogenase (140 kDa) is indicated. P indicates His-tagged TrkB or p75NTR as positive control. (A) TrkB. (B) p75NTR.

(Figure 2B), BDNF preincubated with polySia eluted at an M_r greater than 670 kDa (Figure 5A). Likewise, NGF, NT-3, and NT-4 also eluted at an M_r greater than 670 kDa (Figure 5A), confirming that these neurotrophins bind to polySia to form large complexes. On horizontal native PAGE (Figure 5B), NGF, NT-3, and NT-4 migrated toward the anode region (Figure 5B), indicating the formation of a complex between these molecules and polySia. All of these data suggest that polySia binds to neurotrophins and may therefore function to form a neurotrophin reservoir in the brain.

Neurotrophins are involved in neural cell survival, axonal growth, synaptic plasticity, and neurotransmission via binding to their specific receptors. Therefore, it is important to know if polySia affects the binding between the neurotrophins and their receptors. We focused on the relationship between polySia, BDNF, and the BDNF receptors, TrkB and p75NTR. The recombinant His-tagged Fc-chimera of the extracellular domain (aa 1–430) of human TrkB (140 kDa) and the recombinant His-tagged Fc-chimera of the extracellular domain (aa 1–210) of human p75NTR (100 kDa) were used as test receptors. The ligand–receptor complexes were analyzed by Sephacryl S-300 chromatography (Figure 6). BDNF preincubated with polySia eluted from fraction 12 (M_r ~2000 kDa) on Sephacryl S-300 chromatography (Figure 6A, BDNF + polySia, IB: BDNF). TrkB existed as a dimer (280 kDa) and eluted at fractions 15–17 (Figure 6A, TrkB, IB: His). TrkB preincubated with BDNF coeluted with BDNF at fractions 16–18 because BDNF was

also detected at fractions 16–18 (Figure 6A, BDNF + TrkB, IB: BDNF), indicating that TrkB binds to BDNF. Coincubation with polySia did not affect the elution profile of the TrkB receptor (Figure 6A, polySia + TrkB, IB: His). We then determined if BDNF binds to TrkB as a BDNF–polySia complex. BDNF preincubated with polySia to form the BDNF–polySia complex was incubated with the TrkB receptor and subjected to Sephacryl S-300 chromatography. BDNF was detected not only as a BDNF–polySia complex at fractions 12–14, but also as a BDNF–TrkB complex at fractions 16–18 (Figure 6A, (BDNF + polySia) + TrkB versus BDNF + polySia versus BDNF + TrkB, IB: BDNF). TrkB was only detected as the BDNF–TrkB complex at fractions 16–18 (Figure 6A, (BDNF + polySia) + TrkB versus polySia + TrkB versus BDNF + TrkB, IB: His). Thus, no ternary complex was detected. When estimated by the color density of immunostaining of BDNF at fractions 12–14, the amount of the BDNF–polySia complex decreased by 50% after incubation with the TrkB receptor. These results indicate that the BDNF making complex with polySia can bind to the TrkB receptor. Similar results were obtained with recombinant p75NTR (Figure 6B). p75NTR bound to BDNF because BDNF (27 kDa) was detected at fractions 15–18 after incubation with p75NTR (100 kDa) (Figure 6B, BDNF + p75NTR, IB: BDNF). In addition, when the BDNF–polySia complex was incubated with the p75NTR receptor, no ternary complex was observed. Instead, some BDNF making complex with polySia moved to the BDNF–p75NTR complex fraction (fractions 15–18)

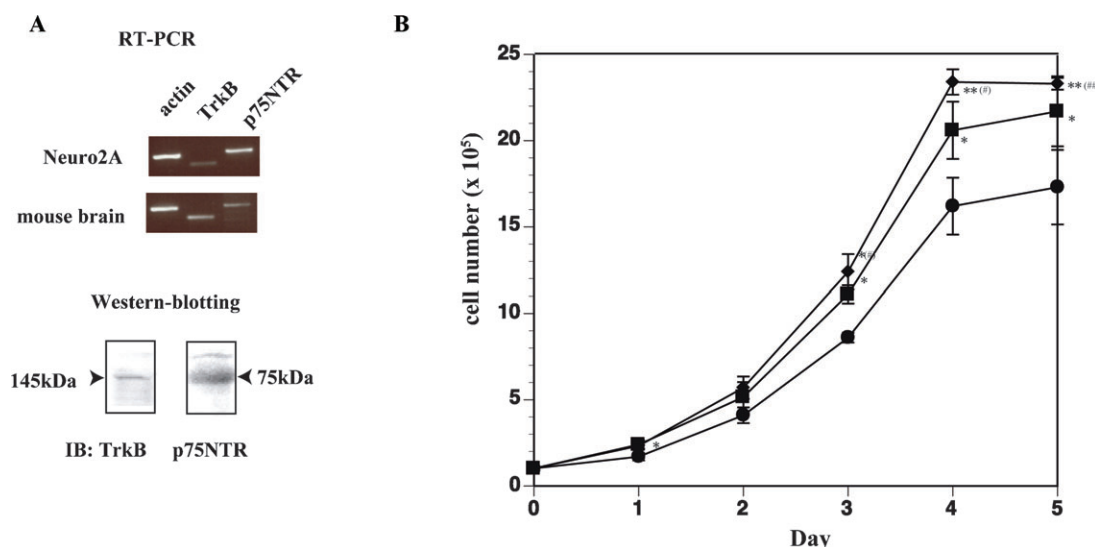


Fig. 7. Effect of BDNF-polySia complex formation on growth and survival of neuroblastoma cells. **(A)** Expression of BDNF receptors on the cells. RT-PCR was performed as described under *Material and methods*. Aliquots of the PCR products amplified at 30 cycles were analyzed for the expression of the mRNAs for TrkB, p75NTR, and actin on 2% agarose gel electrophoresis. As a positive control, adult mouse brain was used (upper panel, RT-PCR). Homogenates were subjected to Western blot analysis for the protein expression of TrkB and p75NTR as described in *Material and methods* (lower panel, Western blotting). **(B)** Growth curve of the cells. Cells (2×10^5) were plated on to the 6-well plate and incubated for 24 h. BDNF (20 ng/mL), or BDNF-polySia complex (20 ng/mL BDNF mixed with 5 μ g/mL polySia) was added and incubated for 5 days. The cell number was counted at 1–5 days after the addition of BDNF or the BDNF-polySia complex. Closed circle, PBS; closed square, BDNF; and closed diamond, BDNF-polySia complex. Cells were counted in three wells. Data are expressed as mean \pm SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$ (compared with control), # $P < 0.05$, ## $P < 0.01$ (compared with BDNF incubation). A typical result from three independent experiments is shown.

(Figure 5B, (BDNF + polySia) + p75NTR, IB: BDNF), and the amount of the BDNF-polySia complex decreased by 72% after incubation with p75NTR. These results demonstrate that the BDNF making complex with polySia can bind to its receptors, such as TrkB and p75NTR, depending on its affinity toward receptors.

To seek to elucidate the biological significance of the BDNF-polySia complex formation, we focused on cell number during cell growth stages because BDNF is known to affect growth and survival of neural cells through its receptors. First, we analyzed the expression of the BDNF receptors, TrkB and p75NTR, in a neuroblastoma cell line by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. The messages and proteins of these molecules were detected (Figure 7A). Then, we asked if cell growth and survival was affected when the neuroblastoma cells were cultured in the absence or presence of the BDNF or BDNF-polySia complex (Figure 7B). BDNF had the increasing effect of cell number during growing and confluent stages, when the cell number was compared with that in the absence of BDNF (Figure 7B, closed square versus closed circle). The BDNF-polySia complex increased the cell number by 15% compared with BDNF only (Figure 7B, closed diamond versus closed square). These results indicate that the BDNF-polySia complex enhances the cell growth and/or survival more greatly than BDNF.

Discussion

The present study clearly demonstrated that polySia directly binds to BDNF and that BDNF-binding is a novel feature of polySia function. Based on the results from gel filtrations

(Figure 2), polySia binds to BDNF to form a large, anionic complex. BDNF exists and functions as a dimer (27 kDa) under physiologic conditions (Brade et al. 1982; Barde 1994; Sofroniew et al. 2001; Huang and Reichardt 2003; Rao and Finkbeiner 2007). The present cross-linking study also indicates that BDNF exists as a dimer, but not as a multimer, in the BDNF-polySia complex. The BDNF-polySia complex is large enough (around 2000 kDa) not to enter the separating gel on conventional native PAGE (Figure 1A) and is excluded on Bio-gel P-100 chromatography. The M_r of the polySia sample (colominic acid) averaged 60 kDa, as estimated by the elution position on Sephacryl S-100 chromatography (Supplementary data 2), while the average DP of the same sample was estimated to be 43 (Molecular weight 12,500) by anion-exchange HPLC (Supplementary data 3). The titration experiment of the BDNF-polySia complex formation with varying amounts of polySia indicates that the complex contains 1.9 pmol of BDNF dimer (50 ng) and 4.0 pmol of polySia (50 ng). This means that 1 mol of BDNF dimer is complexed with about 2 mol of polySia with a DP of 43, indicating that the M_r of the BDNF and polySia complex is 147 kDa as estimated from the gel filtration (60 kDa for polySia (2 mol) and 27 kDa for BDNF dimer (1 mol)). However, the complex eluted at around 2000 kDa (about 14 times larger than that of estimated size), suggesting that association between complexes might occur. It is interesting to note that polySia forms the filament bundle network structure under atomic force microscope (Toikka et al. 1998) and such a network might work in vivo for storing neurotrophins.

The BDNF-polySia complex is formed under physiologic conditions in terms of pH, NaCl, and divalent cations. Among these, divalent cations have an important role in the function of polySia in cell adhesion, signal transduction, and channel

actions (Rafuse and Landmesser 1996; Vaithianathan et al. 2004; Miyata et al. 2006). For the divalent cations to inhibit the BDNF–polySia complex formation, 10 times higher concentrations than the physiological ones are necessary (Figure 3C). Such high concentrations might be locally possible, e.g., near ion channels, indicating that polySia changes the binding molecule depending on the environmental conditions.

PolySia has a structural diversity in its DP, interresidual linkage (α 2,8-, α 2,9-), and Sia component type (Neu5Ac, Neu5Gc, KDN). BDNF forms a complex with α 2,8-linked polyNeu5Ac with DP ≥ 12 (Figure 4). The findings of the present study clarify that polySia DP ≥ 12 for BDNF binding in neural systems is biologically relevant. BDNF binds to not only α 2,8- as well as α 2,9-linked polySia to form a large complex. Both α 2,8- and α 2,9-linked polyNeu5Ac take helical conformations, although the pitches of the helices are different (Yamasaki 1992). Interestingly, they can form helical structures only when they are polymers longer than 10 mer. Considering that BDNF requires a 12 mer of α 2,8-linked Neu5Ac, polySia might take a helical structure for docking with BDNF. Further structural elucidation of the BDNF–polySia complex is underway in our laboratory. In addition, BDNF binds to α 2,8-linked polyNeu5Gc, although the binding is weaker compared to polyNeu5Ac. This suggests that oxidation of the methyl group on the acetyl group may reduce the binding ability of BDNF. Alternatively, the weaker binding might be due to the smaller DP of oligo/polyNeu5Gc (DP = 2–25, average 6) than that of α 2,8-linked polyNeu5Ac (DP = 2–100, average 43).

BDNF binds to the cell surface neurotrophin receptors, TrkB and p75NTR, to exhibit its functions (Barde 1994; Sofroniew et al. 2001). The present study indicates that BDNF does not form a ternary complex with polySia and the receptor (Figure 6). BDNF is usually associated with polySia on the cell surface, and once the BDNF receptors are present, BDNF may be supplied from the BDNF–polySia complex. Indeed, the transfer of BDNF from the BDNF–polySia (DP = 43) complex to TrkB and p75NTR occurs when polySia and the receptors are present in equimolar concentrations (Figure 6). The apparent K_d value of the BDNF–polySia (DP = 43) complex from native PAGE was roughly 400 nM as estimated from the titration experiment, and this value is not comparable with those of BDNF–TrkB and BDNF–p75NTR, 0.01 nM and 1 nM, respectively (Ebendal 1992). These differences in the K_d by two to four orders between polySia and BDNF receptors result in the transfer of BDNF from the polySia to the receptors: 50% and 30% of the BDNF making complex with polySia moved to BDNF receptors, TrkB and p75NTR, respectively (Figure 6).

BDNF is a neurotrophin involved in the survival of a wide range of neuronal cells, the modulation of dopamine, GABAergic and serotonergic receptors, and the regulation of synaptic transmission and plasticity in adult synapses and is widespread in adult brain, including cerebral cortex, hippocampus, basal forebrain, striatum, hypothalamus, brainstem, and cerebellum (Barde 1994; Sofroniew et al. 2001). PolySia is also reported to be present in adult brain, for example, in hippocampus, hypothalamus, etc. (Seki and Arai 1991, 1993; Theodosios et al. 1991; Bonfanti and Theodosios 1994; Bonfanti 2006), regions in which BDNF is also detected; in these areas of the adult brain, BDNF might be present in the form of a BDNF–polySia complex.

BDNF levels are correlated with several disease states, such as depression, epilepsy, bipolar disorder, Parkinson's and

Alzheimer's disease (Huang and Reichardt 2003). Of these, the relation between BDNF and schizophrenia and Alzheimer disease is well studied (Huang and Reichardt 2003; Kozisek et al. 2008). Interestingly, decreased polySia immunostaining and intense polySia immunostaining are observed in brain sections derived from patients with schizophrenia (Barbeau et al. 1995) and Alzheimer's disease (Mikkonen et al. 1999), respectively, as compared with sections from normal brains. The lower expression of polySia with short DPs or higher expression of polySia with large DPs in these diseases may allow the polySia to release or strongly trap BDNF, respectively, resulting in undesirable BDNF concentrations around the BDNF receptors. In this regard, a recent report by Arai et al. (2006) on the association between polymorphisms in the promoter region of the sialyltransferase 8B (SIAT8B, STX/ST8SiaII) gene and schizophrenia is noteworthy.

The complex formation of BDNF with polySia reflects the upregulation of growth and/or survival of the neuroblastoma cells (Figure 7B). Thus, the BDNF–polySia complex formation may be related with regulation of lifetime and the local concentration of BDNF on the cell surface. PolySia binds not only BDNF but also other neurotrophins such as NGF, NT-3, and NT-4, forming a large complex, although the binding affinity to these neurotrophins differs from that of BDNF (Figure 5). These neurotrophins are expressed and function in a time- and space-dependent manner, as is the case with polySia expression (Troy 1996; Bonfanti 2006; Rutishauser 2008). Therefore, polySia may function to produce a reservoir of these neurotrophins on the neural cell surface and as a regulator of the local concentration of neurotrophins by condensing them and inhibiting their diffusion. Nonneuronal tissues, such as natural killer cells and natural killer T-cells, also express polySia, but the function of polySia in these cells is unclear (Husmann et al. 1989; Curreli et al. 2007). PolySia might function as a reservoir of cytokines in these tissues. Like polySia, heparan sulfate proteoglycans and chondroitin sulfate proteoglycans are polyanionic molecules that are also present in brain and bind growth factors (Schwarz and Domowicz 2004). In our preliminary experiments (Kanato, Kitajima and Sato to be published elsewhere), such biologically active glycosaminoglycans also bound to BDNF and other neurotrophins with different EC50 values, although chondroitin and hyaluronic acid did not bind to BDNF. Therefore, not all polyanions bind to neurotrophins, suggesting that particular structures in polyanions might be required for neurotrophin binding. Combined expression of the neurotrophins with the reservoir glycans, polySia and proteoglycans such as heparan sulfate and chondroitin sulfate, that are regulated in a spatiotemporal manner, might allow for the fine-tuning of brain functions such as neural plasticity.

Material and methods

Materials

BDNF and NGF were purchased from PeproTech Inc. (Rocky Hill, NJ). NT-3 and NT-4 were purchased from MBL (Nagoya, Japan). Colominic acid was obtained from Wako (Osaka, JAPAN). α 2,8-Linked oligo/polyNeu5Gc (DP = 2–25) was prepared from polysialoglycoproteins derived from rainbow trout eggs (Sato et al. 1993). α 2,8-Linked oligo KDN (DP = 2–7) was prepared from KDN-glycoprotein from rainbow trout ovarian

fluid (Sato et al. 1993). Oligo/polymers of $\alpha 2 \rightarrow 9$ -linked Neu5Ac from *Neisseria meningitidis* group C were prepared as previously described (Bundle et al. 1974). Recombinant human TrkB/Fc chimera and p75NTR/Fc chimera containing (His)₆-tag were obtained from RSD Systems (Minneapolis, MN). Rabbit anti-BDNF, anti-NT-3, anti-NT-4, anti-NGF antibodies, and anti-His antibodies, rabbit anti-TrkB and goat anti-p75NTR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence reagents, Mono Q HR 5/5, Sephacryl S-500, S-300, Sephadex G-25, and molecular weight markers for gel filtration (thyroglobulin (670 kDa), ferritin (440 kDa), catalase (230 kDa), lactate dehydrogenase (140 kDa), bovine serum albumin (BSA; 67 kDa), and cytochrome C (12 kDa)) were purchased from General Electric Company (Piscataway, NJ). Polyvinylidene difluoride (PVDF) membrane (Immobilon P) was a product of Millipore (Bedford, MA). Prestained molecular weight markers, Bio-gel P-100, and Flamingo gel stain were purchased from Bio-Rad (Hercules, CA).

Preparation of oligo/polySia

Mild acid hydrolysates of colominic acid (1 mg) were subjected to a Mono Q HR5/5 (0.5 × 5 cm) anion-exchange column and separated on a JASCO HPLC system. The sample was loaded on a column and eluted with 20 mM Tris-HCl (pH 8.0), followed by 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; 110–120 min, 1 M) in 20 mM Tris-HCl (pH 8.0). The flow rate was 500 μ L/min and fractions were monitored with a UV detector (UV, JASCO, Japan) at a wavelength of 210 nm. $\alpha 2 \rightarrow 8$ -Linked homooligo/polyNeu5Ac fractions (DP 2–16) were pooled and desalted on a Sephadex G-25 column (1.2 × 65 cm, ddw).

Native PAGE and SDS-PAGE

BDNF (200 ng) in 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl (TBS) were incubated with or without polySia (2 μ g as sialic acid) or Neu5Ac (2 μ g as sialic acid) at 37°C for 2 h. The final incubation volume was 10 μ L. Half of the samples were subjected to native PAGE (3% stacking gel, 10% separating gel) or SDS-PAGE (3% stacking gel, 10% separating gel) and blotted onto PVDF membranes.

Horizontal native PAGE

Lysozyme (1 μ g) and BSA (2 μ g) were loaded onto the horizontal native gel (4.5% polyacrylamide gel) and electrophoresed (Lutz et al. 1994; Kunou et al. 2000). The gel was visualized by Coomassie brilliant blue staining. BDNF (100 ng) in 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl or 0.1–1.0 M NaCl with or without cations (0.33 mM or 3.3 mM CaCl₂ and/or 0.9 mM or 9 mM MgCl₂) was incubated with or without colominic acid (0–20 μ g as sialic acid), Neu5Ac (2 μ g or 20 μ g as sialic acid), or other sialic acid samples (1 μ g as sialic acid) at room temperature for 0–2 h. Other neurotrophins (NT-3, NT-4, and NGF) (100 ng) or trypsin (150 ng) were also incubated with colominic acid (2 μ g). Samples were subjected to horizontal native PAGE as described above and proteins were blotted onto PVDF membranes. In the case of NGF and trypsin, proteins were visualized with Flamingo gel stain on Typhoon

9400 (Ex 532 nm, Em 580 nm). All experiments were done in duplicate to quintuplicate.

Immunostaining

PVDF membranes were blocked with phosphate-buffered saline (PBS) or 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl (TBS) containing 0.05% Tween 20 and 1% BSA or 1% skim milk at 25°C for 1 h. The membrane was incubated overnight with the primary antibody, rabbit polyclonal anti-BDNF antibodies (0.2 μ g/mL), anti-NT-3 antibodies (0.2 μ g/mL), anti-NT-4 antibodies (0.2 μ g/mL), anti-TrkB antibodies (0.2 μ g/mL), anti-p75NTR antibodies (0.2 μ g/mL), anti-His antibodies (0.2 μ g/mL) at 4°C. As the secondary antibody, peroxidase-conjugated anti-rabbit IgG antibodies (1/4000 diluted) or anti-goat antibodies (1/2000 dilutions) were used at 37°C for 60 min and the color development was performed as previously described (Sato et al. 2000).

Cross-linking

BDNF (40 ng) was incubated with or without polySia (2 μ g) or Neu5Ac (2 μ g) (8 μ L of total volume) at 37°C for 2 h and proteins were cross-linked by adding 1 μ L of 2.5 mM disuccinimidyl suberate (DSS; Pierce Chemical, Rockford, IL) to the sample and incubated at room temperature for 30 min. After stopping the reaction by adding 1 μ L of 0.5 M Tris-HCl (pH 7.5) to the sample, BDNF was analyzed by SDS-PAGE and Western blotting with anti-BDNF antibodies.

Gel-filtration of the BDNF and polySia complex

The BDNF-polySia complex (final volume, 50 μ L) before and after incubation with BDNF (2 μ g) and polySia (400 μ g) at room temperature for 2 h was subjected to the Bio-gel P-100 chromatography (0.58 × 28 cm, TBS) or Sephacryl S-500 chromatography (0.58 × 28 cm, TBS). The eluents were analyzed by SDS-PAGE and Western blot analysis using anti-BDNF antibody.

Gel-filtration of BDNF and polySia complex with neurotrophin receptors

BDNF, NT-3, or NT-4 (2 μ g) and polySia (400 μ g) with or without neurotrophin receptors (TrkB and/or p75NTR) (2 μ g) at 37°C for 2 h were subjected to Sephacryl S-300 chromatography (0.58 × 28 cm, TBS). The eluents were analyzed by SDS-PAGE and Western blot analysis using anti-BDNF antibody for BDNF and anti-His antibody for TrkB or p75NTR.

Effect of BDNF-polySia complex formation on cell growth and survival

Murine neuroblastoma Neuro2A cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 0.5 mg/mL of streptomycin sulfate, 100 units/mL of penicillin G, and 10% fetal bovine serum in a 5% CO₂ and 95% air humidified atmosphere at 37°C (Evangelopoulos et al. 2004). Cells (2 × 10⁵) were plated on to the 6-well plate and incubated for 24 h. To the wells, the recombinant human BDNF (20 ng/mL) (mouse and human BDNF share the identical amino acid sequence), colominic acid (5 μ g/mL) or the BDNF-polySia complex (20 ng/mL and 5 μ g/mL, respectively) was then added.

The cell number was counted at 1–5 days after the addition of the BDNF–polySia complex.

RT-PCR

The following primers for mouse proteins were used: TrkB (accession number X17647, nucleotides 722–830), 5'-ATGAAACAAGCCACACACAG-3' and 5'-TCTTGATCTTCTCTACAAG-3'; p75NTR (accession number AF105292, nucleotides 781–944), 5'-GCTGTGGTTGTGGGCCTTGT-3' and 5'-TGCAGGCTCTGGCTGTCCAC-3'. Total RNA was prepared from Neuro2A cells and mouse brain (Balb/c, 8-week female, SLC Co., Japan) using TRIZOL (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Random-primed cDNA (~50 ng) was used as a template for PCR as described previously (Sato et al. 2001).

Data analysis

All values are expressed as means \pm SEM. Analysis of variance with Student's *t*-test was used to determine significant differences in the control and treated groups.

Supplementary Data

Supplementary data for this article is available online at <http://glycob.oxfordjournals.org>.

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Conflict of interest statement

None declared.

Abbreviations

BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; DP, degree of polymerization; DSS, disuccinimidyl suberate; LTP, long-term potentiation; NCAM, neural cell adhesion molecule; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; polySia, polysialic acid; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; Sia, sialic acid; Trk, tropomyosin-related kinase.

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