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JB Review

Disialic, oligosialic and polysialic acids: distribution, functions and related disease

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Sialic acids (Sia) are involved in many biological activities and frequently exist as monosialyl residues at the non-reducing terminal end of glycoconjugates. Occasionally, polymerized structures in the form of disialic acid (diSia), oligosialic acid (oligoSia) and polysialic acid (polySia) are also found in glycoconjugates. In particular, polySia, which is an evolutionarily conserved epitope from sea urchin to humans, is one of the most biologically important glycotopes in vertebrates. The biological functions of polySia, especially on neural cell adhesion molecules, have been well studied and an in-depth body of knowledge concerning polySia has been accumulated. However, considerably less attention has been paid to glycoproteins containing di- and oligoSia groups. However, advances in analytical methods for detecting oligo/polymerized structures have allowed the identification and characterization of an increasing number of glycoproteins containing di/oligo/polySia chains in nature. In addition, sophisticated genetic techniques have also helped to elucidate the underlying mechanisms of polySia-mediated activities. In this review, recent advances in the study of the chemical properties, distribution and functions of di-, oligo- and polySia residues on glycoproteins are described.

Keywords: disialic acid/oligosialic acid/polysialic acid/polysialyltransferase/sialic acid.

Abbreviations: ALCAM, activated lymphocyte cell adhesion molecule; AMPA-Rs, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors; BDNF, brain-derived neurotrophic factor; CA, Cornet d'Ammon; DG, dentate gyrus; diSia, disialic acid; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DP, degree of polymerization; DRD2, dopamine receptor D2; Endo-*N*, endo-*N*-acylneuraminidase; FAC, frontal affinity chromatography; FGF2, fibroblast growth factor 2; FN_{III}, fibronectin type-III; FSP, fucose–sulphate polymer; GAGs, glycosaminoglycans; GDNF, glial-derived neurotrophic factor; GFR1, GDNF receptor 1; GPI, glycosylphosphatidylinositol; HPLC, high-performance liquid chromatography; HSPG, heparin sulphate proteoglycan; HY, hippocampus; KDN, deaminoneuraminic acid; LTD, long-term depression; LTP, long-term potentiation; ManNAc, *N*-acetylmannosamine; MOE, molecular operating environment; NCAM, neural cell adhesion molecule; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid; NGF, nerve growth factor; NMDA-Rs, *N*-methyl-D-aspartate receptors; NSCL, non-small cell lung; NT-3, neurotrophin-3; OB, olfactory bulb; oligoSia, oligosialic acid; p75NTR, p75 neurotrophin receptor; polySia, polysialic acid; PSGP, polysialoglycoprotein; Sia, sialic acids; SNPs, single-nucleotide polymorphisms; SPR, surface plasmon resonance; ST8SIA, alpha2,8-sialyltransferase; SVZ, subventricular zone; synCAM-1, synaptic cell adhesion molecule 1; Trk, tropomyosin-receptor-kinase.

Sialic acids (Sia) or neuraminic acids comprise a family of 9-carbon carboxylated sugars, named 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acids, which are condensed with pyruvic acid and *N*-acetylmannosamine (ManNAc) or mannose. The presence of Sia were first noticed by Levene and Landsteiner (*1*) in USA and Walz (*2*) in Germany as sugar-like components of purified animal lipids that reacted with Bial's reagent to give a purple colour rather than the typical green product. Subsequent studies by Klenk (*3*), Blix *et al*. (*4*), Blix *et al*. (*4*,*5*), Gottschalk (*6*), Comb and Roseman (*7*) and Yu and Ledeen (*8*) from 1935 to 1970 confirmed the chemical and conformational structures of Sia. Sia were named based on the organ from which they were originally crystalized; sialic acid from the salivary gland and neuraminic acid from neurological organs. To date, more than 50 types of Sia have been characterized and include derivatives of *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc) and deaminoneuraminic acid (KDN; 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid), which are the three major backbones of sialic acid (*9*) (Fig. 1).

Sia are typically present as monosialyl residues at the non-reducing termini of glycan chains on glycoproteins and glycolipids where they function as mediators for ligand–receptor and cell–cell interactions in fertilization, differentiation, immunological and neurological events (*9*). Sia are critical for mammalian development, because mice deficient for GlcNAc 2-epimerase/ManNAc kinase, a key enzyme for the biosynthesis of sialic acid, are embryonic lethal (*10*). Polymerized Sia (and Sia) are also interesting sugars from the viewpoint of evolution, as the distribution of Polymerized Sia (and Sia) is predominantly restricted to Gram-negative bacteria and

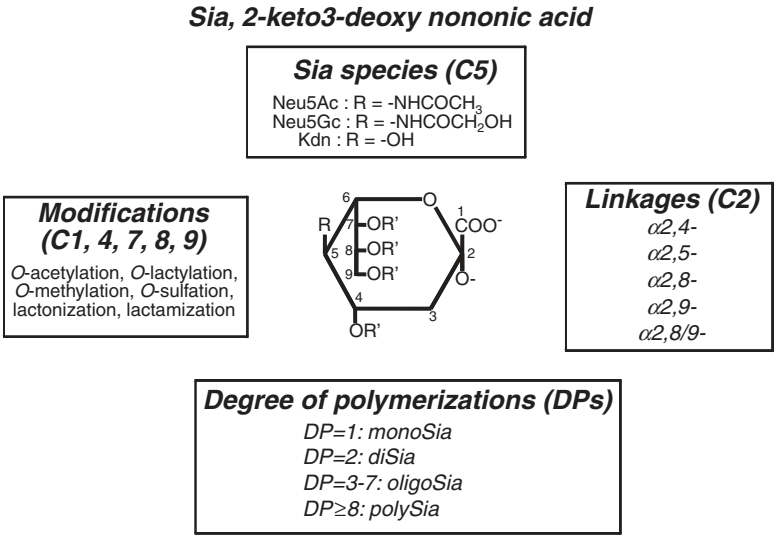

Fig. 1 Diversity in polymerized Sia structure. Sia, 2-keto-3-deoxy nononic acid, has diversity in Sia species at C5 positions (Neu5Ac, Neu5Gc and Kdn), substitutions at C1, 4, 7, 8 and 9 positions, including *O*-acetylation and sulphation. In addition, polymerized Sia structure has diversity in the DPs (DP = 2 (di), 3–7 (oligo), and 8 or greater (poly)) and intersialyl-linkages.

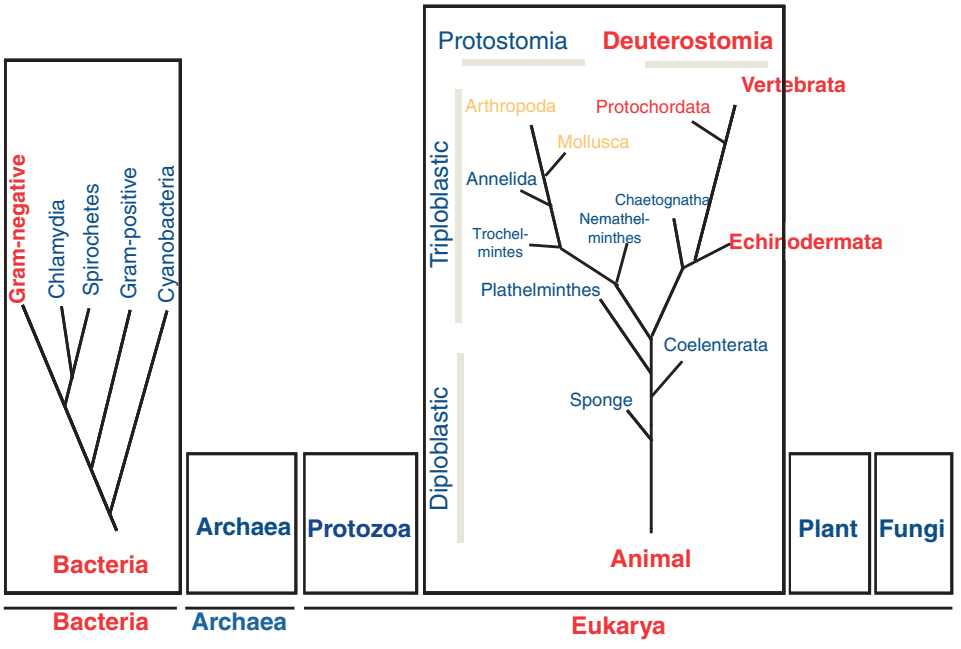

Fig. 2 Distribution of polymerized Sia structure in life. Simple phylogenic tree of living organism with information concerning the presence of polymerized Sia structure based on published reports. Red colour shows that the presence of polymerized Sia structure is confirmed by many reports. Gram-negative bacteria, echinoderms and vertebrate are rich in polymerized Sia structures. Yellow colour indicates ambiguity concerning the presence of polymerized Sia, even though several reports have been published.

deuterostome lineage animals (Fig. 2), although the existence of Polymerized Sia (and Sia) in the protostome lineage has also been proposed (*9*). Another unique aspect of Sia is that, unlike other sugars, Sia often form a homo-oligo/polymer structures, specifically disialic acid (diSia), oligosialic acid (oligoSia) and polysialic acid (polySia) (*11–13*). Therefore, polymerized Sia glycotopes exhibit structural diversity with respect to not only the backbone components (Neu5Ac, Neu5Gc and KDN) and modifications (acetylation, sulphation, methylation, lactylation and lactonization) but also in the type of intersialyl linkage (α2,4, α2,5$O_{glycolyl}$, α2,8, α2,9 and α2,8/9) and degree of polymerization (DP), which ranges from 2 to 400 (Fig. 1) (*13*). Recently developed chemical methods that sensitively detect Sia and oligo/polymerized Sia structures have revealed that di/oligoSia frequently modify glycoproteins and have a large diversity in the DP, ranging from 2 to 400. In addition, an increasing body of knowledge concerning the functions of di/oligo/polySia structures in vertebrate cells has accumulated. In this review, the structure, distribution and functions of di/oligo/polySia are described and discussed.

Definition of Di/Oligo/PolySia and Detection Methods for Oligo/polySia Structure

PolySia was first identified in Gram-negative bacterial polysaccharide (*Escherichia coli* K235) and the neuroinvasive bacterium *Neisseria meningitidis* groups C and B as a structure consisting of an extremely large number of Sia chains (DP > 200) (*14*). The antibodies raised against bacterial polySia were termed anti-polySia antibodies (*15–18*) and are widely available for detection of polySia and isolation of polySia-containing glycoproteins or even cells, such as the purification of neuronal lineage cells from pluripotent mouse ES cells (*19*). However, the antigenic specificity of available antibodies, particularly concerning the DP, is not precisely defined. Therefore, it is important to understand the precise antigenicity of the antibody, particularly concerning components, linkages and DP, before use. According to the antibody specificity and conformational aspect of polymerized Sia structures, we have proposed the following classifications: diSia (DP = 2), oligoSia (DP = 3–7) and polySia (DP ≥ 8) (*11–13*). PolySia can be identified with specific probes, such as the anti-polySia antibodies monoclonal antibody mAb.735 and mAb.12E3, enzymes such as endo-*N*-acylneuraminidase (Endo-N) or by using chemical methods, as described below.

Detection Methods for Polymerized Sia Structure

For the analysis of samples containing relatively high amounts (10–100 μg) of di-, oligo- and polySia structures, a number of conventional methods, including methylation analysis (*20*), NMR (*21*) and mild acid hydrolysis—thin-layer chromatography (*22*) can be applied. However, these approaches are not suitable for samples containing only small amounts of di/oligo/polySia residues (<1 μg), as is often the case. To overcome this limitation, the following highly sensitive chemical and biochemical methods have been successfully used to confirm the ubiquitous occurrence of di/oligo/polySia in a wide variety of glycoproteins and glycolipids at femtomole levels. These improved detection methods have led to the identification of polymerized Sia-modified carrier proteins and have helped identify the specific functions of polySia.

Chemical analyses

Fluorometric C_7/C_9 analysis. When an di/oligo/polymer of α2 → 8-linked *N*-acylneuraminic acid (Neu5Acyl) residues is subjected to periodate oxidation, the non-reducing terminal residue is oxidized to the C_7 analogue of *N*-acylneuraminic acid, C_7(Neu5Ac) (5-acetoamido-3,5-dideoxy-L-*arabino*-2-hepturosonic acid) or C_7(Neu5Gc) (5-hydroxyacetoamido-3,5-dideoxy-L-*arabino*-2-hepturosonic acid), from Neu5Ac or Neu5Gc residues, respectively, whereas the internal residues of Neu5Ac (C_9(Neu5Ac)) or Neu5Gc (C_9(Neu5Gc)) remain unchanged (*14*, *23*). Accordingly, the detection of C_9-compounds among the periodate oxidation products indicates the presence of internal sialyl residues or a polymeric structure composed of α2 → 8-linked *N*-acylneuraminic acid. C_7- and C_9-compounds can be quantitated by fluorometric high-performance liquid chromatography (HPLC) after treatment with the α-keto acid-specific fluorescent labelling reagent 1,2-diamino-4,5-methylenedioxybenzene (DMB) (*23–25*) (Fig. 3A). However, this method has several limitations that warrant mention. First, this method is only applicable for the detection of α2 → 8-linked oligo/polymers of *N*-acylneuraminic acid and cannot be used to determine the DP of polymers with α2 → 9, α2 → 8/α2 → 9-mixed linkages or α2 → 5$O_{glycolyl}$-linkages. Second, the detected C_9-derivatives do not always arise from α2 → 8-linked Neu5Ac, because 8-*O*-substituted Neu5Acyl residues may also yield indistinguishable C_9-derivatives. For this reason, samples are typically saponified by mild alkali treatment prior to periodate oxidation, although a few substituents are not released under these conditions. Third, the molar proportion of C_9- to C_7-derivatives does not directly represent the DP, unless linear polySia chains are being analysed. Thus, this method does not allow determination of the DP for samples containing multiple sialylated chains.

Mild acid hydrolysis—fluorescent HPLC analysis. Our group was the first to report that di/oligo/polymers produced by mild acid hydrolysis of di/oligo/polySia chains can be directly labelled with DMB and analysed by anion-exchange HPLC (*26*) (Fig. 3B). Several anion-exchange chromatography columns can be used to analyse DMB-labelled Sia polymers, such as Mono or Mini Q HR5/5 (0.5 × 5 cm; GE, Uppsala, Sweden), Resource Q (1 ml; GE), CarbopacPA100 (4 × 250 mm; Dionex) and DNApac PA100 (4 × 250 mm; Dionex) columns. DMB labelling is applicable for the detection of various types of oligo/polymers of Sia found in glycoconjugates, which can differ in component Sia species, inter-residual linkages and DP. This analysis can be applied to glycoproteins blotted on PVDF membrane. However, because oligo/polySia easily degrades under mild acidic conditions, it is difficult to accurately determine the DP of oligo/polySia in glycans.

Biochemical probes

Antibody. To study the structure and function of α2,8-linked polySia glycotopes, several 'anti-polySia antibodies' have been developed in the past three decades. Among them, the immunospecificities of only horse polyclonal antibody H.46 (*17*) and mouse monoclonal antibody mAb.735 (*15*) had been determined, whereas the immunospecificity of the majority of other 'anti-polySia antibodies' remained unknown. However, comprehensive examination of the immunospecificity of these 'anti-polySia' antibodies using an ELISA-based method and phosphatidylethanolamine-conjugated oligo/polySia chains as test antigens demonstrated that these 'anti-polySia antibodies' recognized different species of Sia residues and chain lengths (*27*). Thus, a large list of characterized

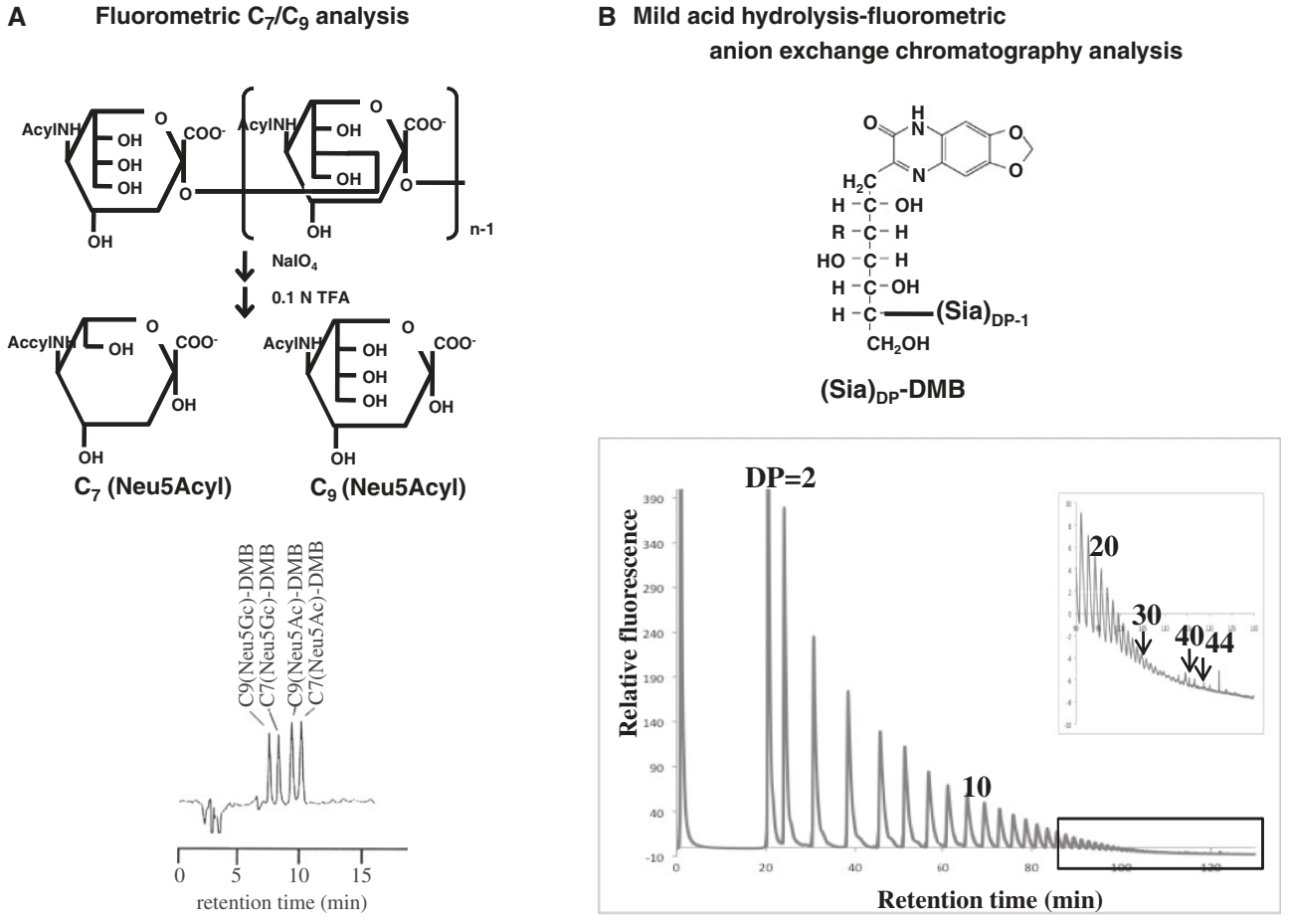

Fig. 3 Chemical methods to detect polymerized Sia structures. (A) Fluorometric C_7/C_9 analysis. A typical elution profile of DMB derivatives of C_7-analogues and authentic sialic acids (C_9) on fluorometric HPLC. Disialyl silalitols, 12.5 ng each of Neu5Acα2 → 8Neu5Acα2 → 8-Neu5Ac-ol and Neu5Gcα2 → 8Neu5Gcα2 → 8-Neu5Gc-ol were subjected to periodate oxidation/reduction/hydrolysis, DMB derivatization and fluorometric HPLC on a TSK-gel ODS-120T column (250 × 4.6 mm i.d.). The column was eluted with methanol/acetonitrile/water (7 : 9 : 84, v/v/v) at 1.0 ml/min at 26°C. Elution profiles were monitored by the measurement of fluorescence (excitation, 373 nm; emission, 448 nm). (**B**) Mild acid hydrolysis-fluorometric anion exchange chromatography analysis. Mini Q anion exchange chromatography of α2 → 8-linked di/oligo/polyNeu5Ac-DMB. α2 → 8-Linked oligo/polyNeu5Ac was labelled with DMB and applied to a mini Q HR5/5 anion exchange column (1 ml, Cl^--form). The column was eluted with 5 mM Tris–HCl (pH 8.0) with a gradient from 0 to 0.3 M NaCl for 75 min and 0.3 M NaCl to 0.4 M NaCl for 120 min after a 15-min wash. The elution was monitored by a fluorescence detector (set at wavelength of 373 nm excitation and 448 nm emission).

antibodies recognizing di-, oligo- and/or polySia structures now exists (Table I), although the immunospecificity of a few antibodies remains to be determined. Interestingly, anti-di/oligo/polySia antibodies can be classified into three groups based on the immunospecificity for chain length and involvement of the non-reducing terminus in antibody recognition. Group I consists of antibodies that recognize chains of α2,8-linked Sia with DP ≥ 8, including fully extended polySia chains with a DP 8–400. Group I antibodies are thought to recognize the helical conformation formed by Sia residues within the internal region of polySia chains, but not the non-reducing terminal residues. Group II antibodies, designated as 'anti-oligo + polySia antibodies', recognize di/oligoSia with a DP 2–7 and also polySia chains. In addition, these antibodies are considered to recognize the distal portion of oligo/polySia chains, including the non-reducing termini. Group III antibodies, designated as 'anti-di/oligoSia antibodies', recognize specific conformations of di- and oligoSia with a DP 2–4, but do not bind to polySia. Group II and III antibodies are useful for detecting and determining di- and oligoSia structures in combination with exo- and endo-sialidase treatment, as described below (Table II). One interesting anti-polySia antibody is IgM^{NOV}, which was identified in the serum of a patient with IgM gammopathy and reacts with both polySia and DNA/polynucleotides (*28*). This property is important because of the helical conformation of polySia, as described below. Another approach that has been used to obtain anti-polySia antibodies is the use of *N*-substituted polySia as an immunizing antigen. For example, Jennings *et al*. (*29*,*30*) substituted the *N*-acetyl group of polySia with *N*-propionyl to obtain an anti-polySia antibody that reacts not only with *N*-propionylated polySia (polyNeu5Pro) but also with *N*-acetylated polySia (polyNeu5Ac) with high affinity. These studies demonstrated that the antigenic specificity of anti-polySia antibodies is intimately related to the conformational state of the di/oligo/polySia.

Table I. Antigenic specificities and class of anti-α2,8-linked diSia/oligoSia/polySia antibodies.

Antibody	Animal origin[a] and immunoglobulin-type[b]	Sia in oligo/polySia for recognition	Specificity on DP
<Group I>Anti-polySia antibody			
H.46	ho, poly, IgM	Neu5Ac	≥8
735	mo, mono, IgG2a	Neu5Ac	≥11
<Group II>Anti-oligo + polySia antibody			
12E3	mo, mono, IgM	Neu5Ac	≥5
5A5	mo, mono, IgM	Neu5Ac	≥3
2-2B	mo, mono, IgM	Neu5Ac	≥4
OL.28	mo, mono, IgM	Neu5Ac	≥4
2-4B	mo, mono, IgM	Neu5Gc	≥2
kdn8kdn	mo, mono, IgM	KDN	≥2
<Group III>Anti-oligoSia antibody			
S2-566	mo, mono, IgM	Neu5Ac	2[c]
1E6	mo, mono, IgM	Neu5Ac	2
A2B5	mo, mono, IgM	Neu5Ac	3
AC1	mo, mono, IgG3	Neu5Gc	2–4

[a]ho, horse; mo, mouse. [b]Poly, polyclonal; mono, monoclonal. [c]Neu5Acα2 → 8Neu5Acα2 → 3Gal (Gal residue is required).

Table II. Reactivity of di−, oligo− and polySia chains towards biochemical probes.

Biochemical probes	diSia (DP = 2)	oligoSia (DP = 3–7)	PolySia (DP ≥ 8)
Group I antibody	−	−	+
Group II antibody	−	+	+
Group III antibody	+	+ or −	−
Endo-sialidase (Endo-*N*)	−	− or +[a]	+
Endosialidase[b]	−	+	+
α2,3,6-Sialidase	−	−	−
α2,3-, α2,6, α2,8-Sialidase	+	+	+

+, reactive or sensitive; −, unreactive or insensitive. [a]+ in the case of DP = 6 and 7. [b]Refers to [32].

Enzymes. Endosialidase can serve as a specific molecular probe to detect and selectively modify α2,8-linked polySia chains (*31–33*). A soluble enzyme derived from bacteriophage K1F, designated Endo-N, catalyzes the depolymerization of polySia chains as follows: $(\rightarrow 8\text{Neu5Acyl}\alpha 2\rightarrow)_n\text{-X} \quad (n \geq 5) \rightarrow (\rightarrow 8\text{Neu5Acyl}\alpha 2\rightarrow)_{2-4} + (\rightarrow 8\text{Neu5Acyl}\alpha 2\rightarrow)_2\text{-X}$ (*31*). Two other types of endosialidases with substrate specificities that differ from Endo-N of bacteriophage K1F have been isolated: Endo-NE (*33*) and a bacteriophage endosialidase (*32*), which require a minimum chain length of DP ≥ 11 and DP ≥ 3, respectively, for cleavage. Exosialidases that cleave specific linkages, for example, α2,3- and α2,6-sialidase and α2,3-, α2,6-, α2,8 and (α2,9)-sialidase have also been identified. As di- and oligoSia (DP = 3–5) structures are not recognized by Endo-N, but are cleavable by exosialidases, it is possible to confirm the length of di-, oligo- and polySia chains by treatment with endo- and exosialidase treatments before and after immunostaining with anti-di/oligo/polySia antibodies (Table II). Finne *et al.* established a specific probe from Endo-NE that lacks enzymatic activity, but retains the ability to bind and detect polySia. Using this probe, they successfully detected polySia-neural cell adhesion molecule (NCAM) (*34*).

Chemical reagents

For the purpose of detection, imaging and targeting, *in vivo* modification of Sia by treating samples with the precursors of Sia biosynthesis is a useful and widely available technique. Reutter and co-workers first demonstrated that the addition of *N*-substituted mannosamine changed cell-surface Sia to an *N*-substituted form, such as ManPro, ManBut and ManPent (*35*). Mahal and Bertozzi (*36*) and Saxon *et al.* (*37*) used ManLev and ManNAz as precursors to modify Sia in a highly selective manner and observed that the incorporation of these unnatural Sia occurred not only on Sia-containing glycoconjugates but also on polySia chains (*38*). In the search for inhibitors of biosynthetic pathway enzymes of polymerized Sia, the acceptor substrate specificity of the enzymes STX/alpha2,8-sialyltransferase 2 (ST8SIA2)/ST8SiaII/siat8b, which play pivotal roles in the biosynthesis of polySia, was found to be more restricted than that of PST/ST8SIA4/ST8SiaIV/siat8d, and in addition, ManBut was identified as a possible inhibitor of ST8SIA2 (*39*).

Conformation of Di/Oligo/Polysia

In 1987, Jennings and colleagues reported a conformational difference between triSia and colominic acid (polySia) by NMR (*21*) and proposed that the unexpectedly large size of the epitope of the anti-polySia antibody H.46 (*16*). ^{1}H- and ^{13}C-NMR spectroscopy and molecular modelling revealed that α2,8-linked polyNeu5Ac structures adopt a helical conformation (*40–42*), which is common conformational feature among α2,8-linked polyNeu5Ac, polyNeu5Gc and other *N*-substituted polySia (*40*, *43*). X-ray crystallographic analysis of anti-polySia mAb.735 also suggested that the helical conformation (six residues per turn, 36 Å pitch) consisted of at least eight Neu5Ac residues (*41*) and was well accommodated by the antigen-binding site of the antibody. It has also been reported that polySia adopts random structures. In contrast, NMR studies of α2,8-linked di- and triSia structures revealed that Neu5Ac residues have different conformations than internal Neu5Ac residues of polySia chains with a DP ≥ 8. It was also demonstrated that the conformation of proximal and distal diSia residues in polySia chains differed from those of internal residues. Together, these results suggest that di- and oligoSia structures have large conformational differences compared with polySia structures, and accordingly, are likely to have distinct functions from those described for polySia glycotopes.

The conformational features of di- and oligoSia structures that differ from α2,8-linked polySia are not well understood and need to be further explored. As accurate molecular modelling techniques are now readily available, we predicted the structures of α2,4-, α2,5-, α2,7- (not reported), α2,8- and α2,9-linked polySia using the molecular operating environment (MOE) molecular modelling program (Chemical Computing Group Inc., Montreal, Canada; Ryoka System, Inc., Tokyo, Japan). The structures were optimized using the energy minimization tools in MOE, and conformational differences among these structures

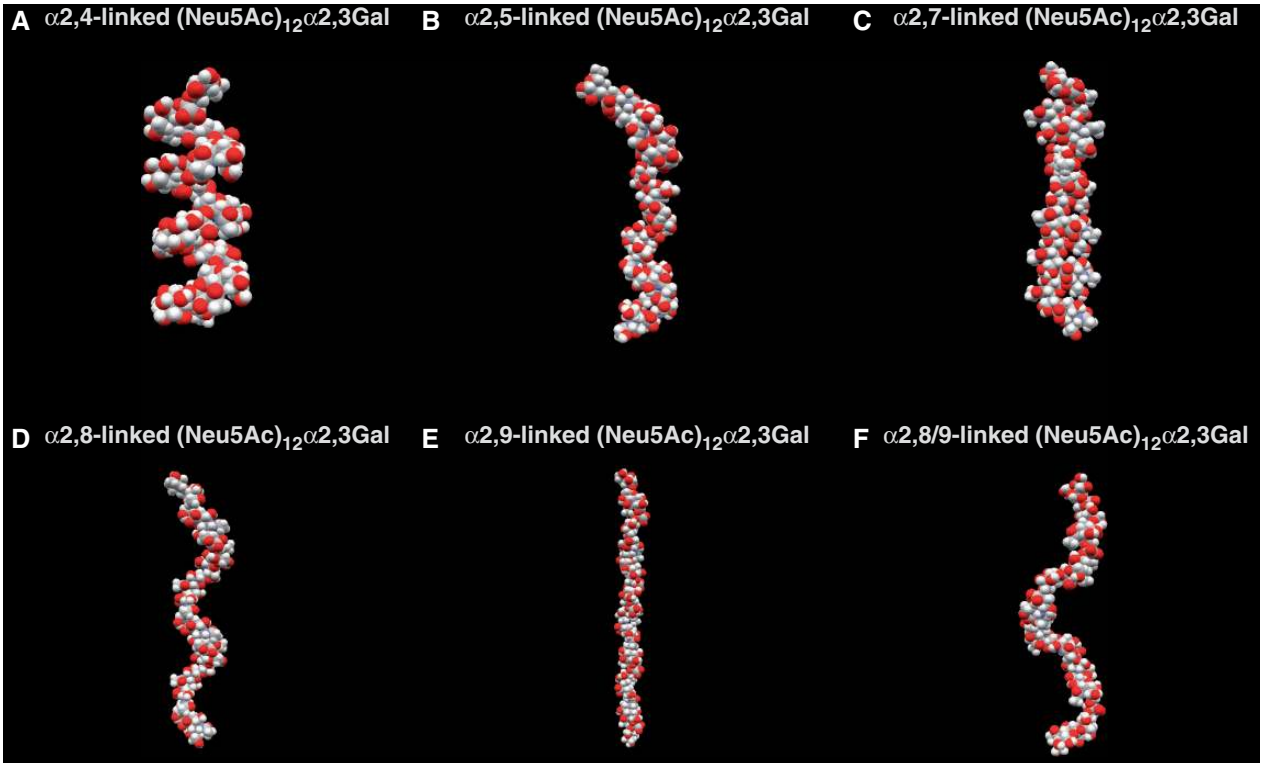

Fig. 4 Molecular modelling of polySia. α2,4- (**A**), α2,5- (**B**), α2,7- (**C**), α2,8- (**D**), α2,9- (**E**) and α2,8/9-linked (**F**) polySia structures (DP = 12) are linked to Gal at the C3-position. Calculated dodecaNeu5Ac-Gal structures are shown as space-filling models. The MOE program was used for the construction and calculation of the energies of polySia under force field, MMF94. The α2,7-linkgae is not found in nature.

(linkages) were clearly observed (Fig. 4). Interestingly, α2,8- and α2,5-linked polySia exist as helical structures, whereas α2,9-linked polySia forms a linear structure. Molecular modelling of mono/di/tri/polySia also suggested that conformational differences exist among these structures (depending on DP) (*13*).

Distribution and Functions of Polysia

Eukaryotes

Mammals. NCAM, which is mainly expressed in the embryonic brain of vertebrates, including fishes, birds, reptiles, amphibians and mammals, is the most well-characterized polysialylated molecule. The specific spacio-temporal expression of polySia has been the focus of numerous studies since the discovery of polySia-NCAM in 1982 (*44*). Based on these investigations, polySia-NCAM was shown to have an α2,8-linked polyNeu5Ac glycotope, which is the same structure found in neuroinvasive determinants derived from pathogenic bacteria, such as *N. meningitidis* group B. To date, 27 isoforms of NCAM generated by RNA splicing have been identified, among which four major isoforms, NCAM-180, -140, and -120 and soluble NCAM, have been characterized. All NCAMs consist of five immunoglobulin-like (Ig) domains with six *N*-glycosylation sites and two fibronectin type-III (FN_{III})-like domains in the extracellular region. NCAM is attached to the transmembrane region via a glycosylphosphatidylinositol (GPI) anchor (NCAM-120) or connected through the membrane to the cytosol and transduces extracellular signals into the cell (NCAM-140 and -180) (Fig. 5C). PolySia chains are linked to the di-, tri- or tetra-antennary *N*-linked glycan chains on immunoglobulin domain-V of NCAM (*45*, *46*).

PolySia is mainly expressed in embryonic brains and is only present at very low levels in adult brains, although the NCAM expression level remains relatively unchanged. PolySia persists in adult brains in distinct regions where neural plasticity, remodelling of neural connections or neural generation are ongoing, such as the hippocampus (HY), subventricular zone (SVZ), thalamus, prefrontal cortex and amygdala.

The biological functions of polySia, particularly in embryonic brains, have been shown to include neural cell migration, axonal guidance, fasciculation, myelination, synapse formation and functional plasticity of the nervous system. The molecular mechanism underling these functions is considered to be the antiadhesive effect of polySia on cell-cell or/and cell-matrix interactions, including not only through the homophilic binding but also the heterophilic binding (*47*). The binding of these counterparts by NCAM affects many downstream signalling pathways, including those that regulate neurite outgrowth, cell migration, fasciculation, axonal guidance and branching and synaptogenesis. PolySia is considered to function as an anti-adhesive molecule because of its bulky polyanionic nature, which imparts a large negative field (Fig. 6A).

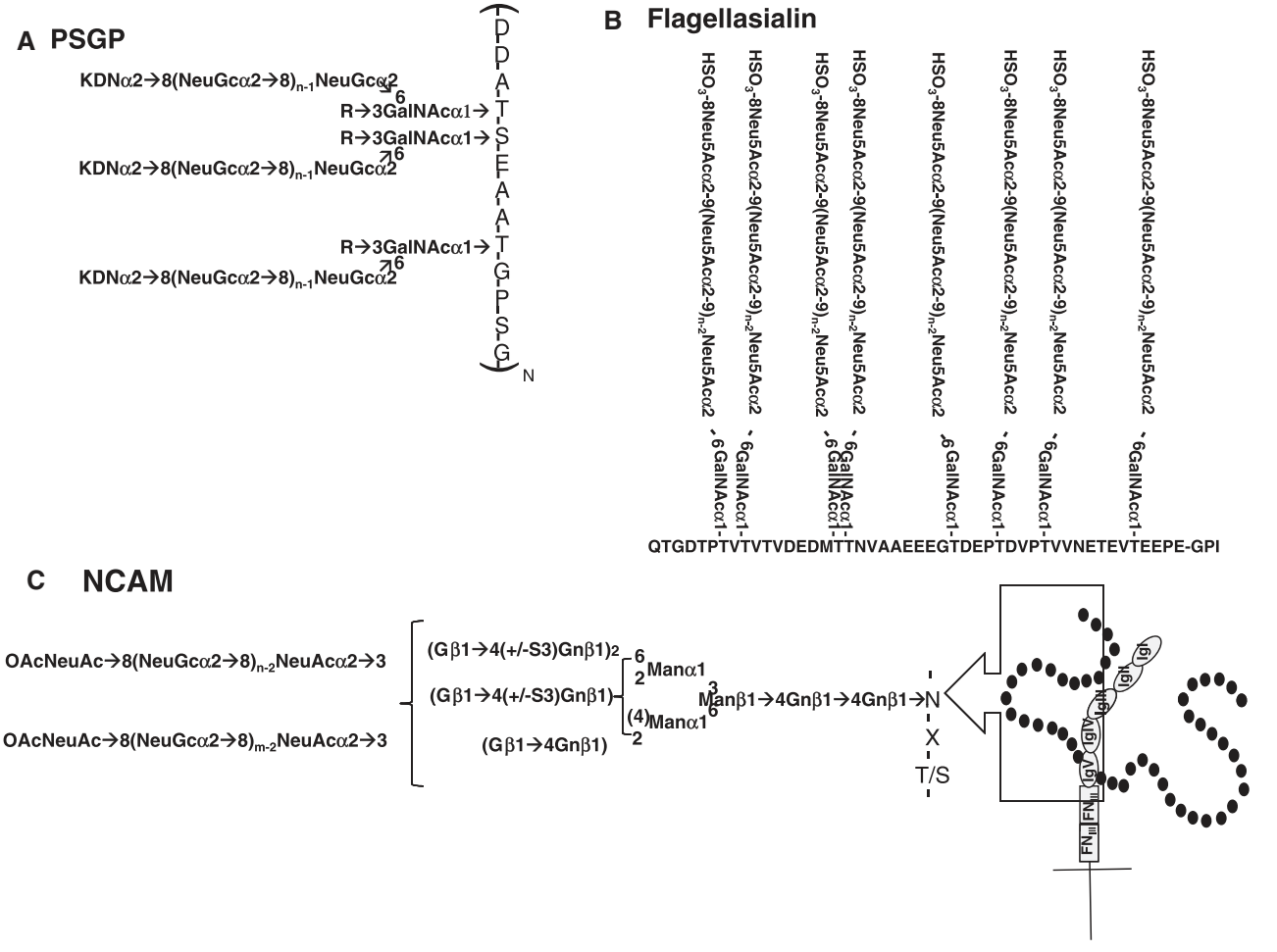

Fig. 5 Examples of polySia-containing glycoproteins. (**A**) Flagellasialin, discovered on the surface of sea urchin surface, is a peripheral (or GPI-) protein composed of α2,9-linked polyNeu5Ac chains capped with 8-*O*-sulphated Neu5Ac at the non-reducing terminal end. The function of polySia on flagellasialin involves the regulation of Ca^{2+} ions. (**B**) PSGP from trout egg is composed of α2,8-linked polyNeu5Gc chains capped with Kdn at the non-reducing terminal end. PSGP is a soluble protein in cortical alveoli and the perivitelline space. The functions of PSGP include anti-bacterial effects, retaining Ca^{2+} ions and regulation of ion balance. (**C**) NCAM discovered in the avian embryonic brain is composed of α2,8-linked polyNeu5Ac chains capped with *O*-acetylation at the non-reducing terminal sialic acid residue. The majority of NCAM is the transmembrane type (NCAM-180, 140 and 120). All NCAM isoforms have five immunoglobulin domains (IgI–IgV) and two FN_{III} domains. On the IgV domains, two of the three *N*-glycosylation sites are polysialylated.

Recently, polySia has been shown to directly bind and regulate the function of a number of soluble bioactive factors (*13*), including neurotrophins (brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and nerve growth factor (NGF)) (*48–51*), growth factors (*52*) and neurotransmitters (dopamine, epinephrine and norepinephrine) (*53*). Thus, polySia also appears to have an attractive field of force that retains specific bioactive factors involved in neural function in intercellular spaces (Fig. 6B) and can therefore regulate the function of these neurologically active molecules. This property clearly indicates that polySia is involved in not only neurogenesis but also in the regulation of neural function. The bioactive molecules that bind to polySia have been well characterized in relation to behaviour and social interaction, as well as schizophrenia and other psychiatric disorders. Interestingly, polySia-impaired mice have profound impairment in movement and social behaviours (*54*). It is also reported that polySia regulate ion channels probably through direct binding to the channels. We describe these new functions of polySia recently reported in detail.

Regulator of neurotrophins—BDNF, NT3 and NGF. BDNF is the most abundant neurotrophin in brain and promotes the growth and development of immature neurons, and survival and functional maintenance of adult neurons and neural plasticity, which is important for memory and learning, through binding to a low-affinity receptor, p75 neurotrophin receptor (p75NTR) and a high-affinity receptor, tropomyosin-receptor-kinase B (TrkB). Biochemically, the direct binding between polySia and BDNF was first demonstrated using gel filtration, horizontal native-PAGE and surface plasmon resonance (SPR) methods (*50–52*). Using these solid-based approaches, it was shown that BDNF dimers directly bind to polySia with a minimum DP 12 or greater. The complex formed between polySia and BDNF is extremely large (~2500 kDa, 14 mol BDNF dimer and 28 mol polySia (mean DP = 43) chains), as calculated from

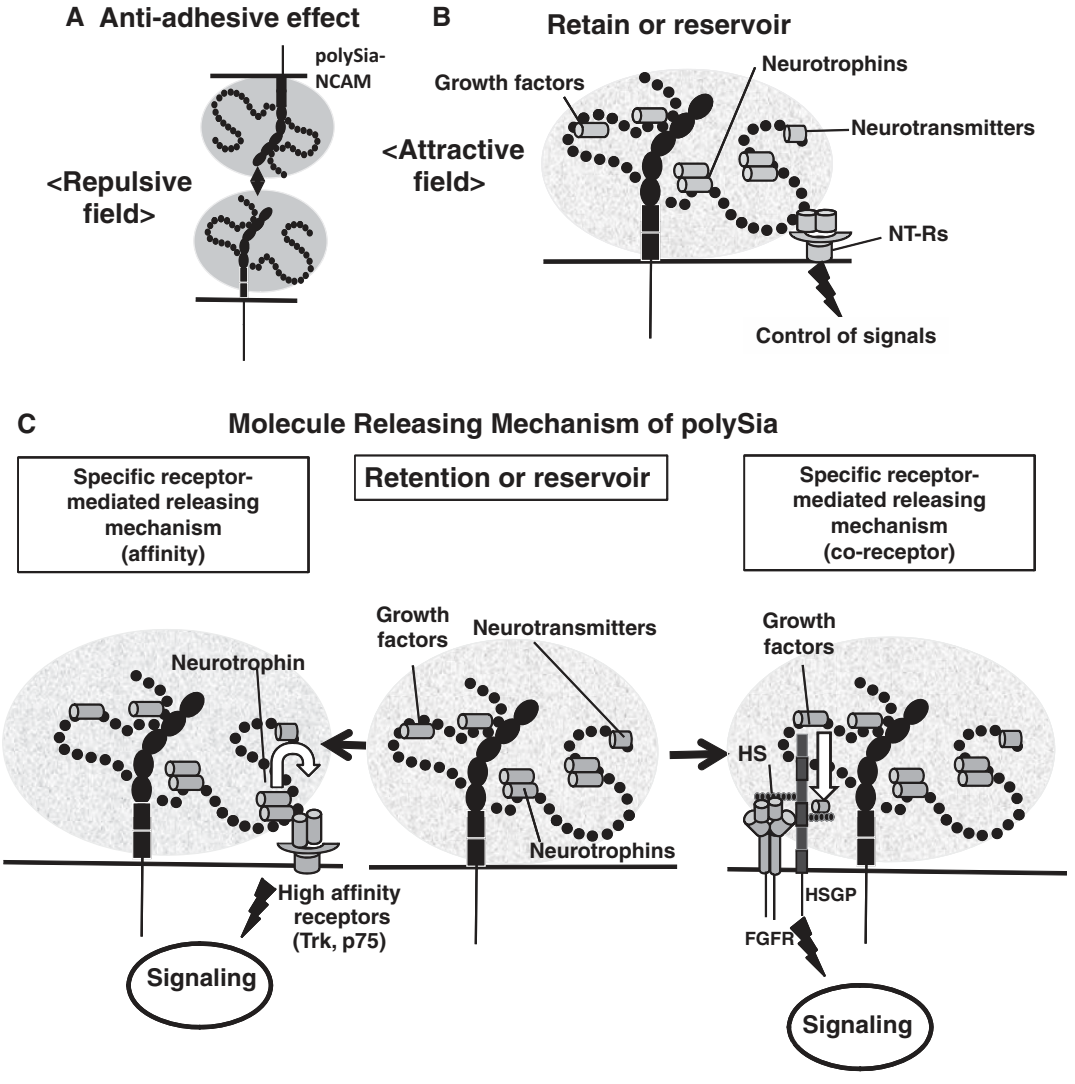

Fig. 6 Functions of polySia. (**A**) Anti-adhesive effect. PolySia-NCAM has repulsive fields on the cell surface to negatively regulate cell–cell interaction due to the large volume of polySia, as shown in grey. PolySia is considered to function only as a negative regulator among molecules. (**B**) New functions of polySia are suggested based on the recent findings of occurrence of various polySia-binding molecules. As an attractive field, polySia on NCAM directly binds to bioactive molecules involved in neural function, such as neurotrophins, neurotransmitters and growth factors. The binding regulates their concentrations outside the cells and signalling modes. (**C**) Proposed mechanism for the retention and release of bioactive molecules. PolySia captures bioactive molecules by direct binding. The retained molecules are released by several ways, as shown in the right and left panels. Left panel shows the specific receptor-mediated mechanism (affinity-mediated model). For example, BDNF in BDNF-polySia complexes migrates to its receptors, TrkB or p75NTR, according to differences of the affinity between BDNF, the receptors, and polySia. Right panel shows the specific receptor-mediated, but co-receptor-mediated mechanism. In the case of FGF2, polySia does not release FGF2 from the FGF2-polySia complex to FGFR. Interestingly, FGF2 in the FGF2-polySia complex can migrate to heparan sulphate (HS) to form FGF2–HS complex, which can bind to FGFR as a ternary complex to enhance FGF signalling. Therefore, polySia regulates FGF2 signalling by passing FGF2 to HS and finally FGFR. This is a hypothetical model for the new function of polySia (retain and release hypothesis).

titration and gel filtration experiments. The neurotrophins NT-3 and NGF also bind polySia, most likely through basic regions in their C-terminal.

Unlike fibroblast growth factor 2 (FGF2)–FGFR–heparan sulphate (HS), BDNF and polySia do not form ternary complexes with BDNF receptors, BDNF after forming a complex with polySia easily migrate towards receptors. The migration can be explained by the differing binding affinities of BDNF. The K_D of BDNF towards polySia, as calculated by SPR, is $\sim 10^{-9}$ M, whereas the K_D of BDNF towards TrkB and p75NTR is 10^{-12} and 10^{-10} M, respectively. Based on these affinities, BDNF in BDNF–polySia complexes would move towards BDNF receptors because BDNF has one to three orders of magnitude stronger affinity towards BDNF receptors than towards polySia (Fig. 6C). With regard to the mechanism by which polySia and BDNF disassociate (releasing mechanism), studies using the microglia cell line Ra2 revealed that cell polysialylation rapidly disappeared after lipopolysaccharide-induced secretion of sialidase secreted into the cell culture medium. Under these conditions, both BDNF and GDNF in complex with polySia are rapidly released by sialidase-mediated degradation of polySia, which represents a novel release mechanism (C. Sato *et al.*, unpublished data). From a biological point of view, polySia and polySia–BDNF complexes were also

shown to increase the proliferation of neuroblastoma cells compared with untreated control cells. Based on these findings, polySia also has the ability to prolong the effects of neurotrophins. Recently, ProBDNF processed extracellularly by tPA/plasmin was shown to be important for memory in the HY (*55*). In this context, it is also important to consider the reservoir function of polySia, because proBDNF and BDNF, but not the pro-domain alone, are capable of binding polySia (K. Matsuoka *et al*., unpublished data). Taken together, the findings from these studies demonstrate that polySia is involved in several neurotrophin-mediated biological functions, including cell growth, neurogenesis and memory.

Regulator of neurotransmitters—catecholamines. The specific binding between polySia and catecholamine neurotransmitters, particularly dopamine, has been demonstrated by frontal affinity chromatography (FAC) analyses of numerous factors, including histamine, acetylcholine, serotonin, catecholamines (dopamine, epinephrine and norepinephrine) and their precursors. Catecholamine appears to specifically bind polySia, because binding is not observed with diSia (DP = 2), and it is speculated that these intermolecular interactions occur between specific structures of polySia and the catechol backbone. As the K_D of dopamine towards polySia changes depending on pH, the specific interaction between these molecules might be fine-tuned by subtle changes of the extracellular pH (*50*). PolySia is also involved in Akt signalling in the human neuroblastoma cell line SK-N-SH through dopamine receptor D2 (DRD2) (*53*). It is also reported that polySia is required for DRD2-mediated plasticity of inhibitory circuits of the rat medial prefrontal cortex (*56*). Together, these results suggest that polySia–NCAM localized on postsynaptic membranes directly interacts with catecholamine neurotransmitters, such as dopamine, and represents a novel function of polySia.

Regulator of growth factors—FGF2. FGF2 is a prototypical member of the FGF family that stimulates the growth of various cell types, from fibroblasts to tumour cells. FGF2 is highly expressed in the brain during earlier stages of development and is involved in brain formation. As recent studies have demonstrated that FGF2 is a potent modulator of proliferation and differentiation of multi-potent neural progenitor cells isolated from the adult SVZ, FGF2 also appears to play a pivotal role in adult neurogenesis (*57*). Due to its importance in both brain development and function, it is not surprising that FGF2 is implicated in psychiatric disorders (*58–63*). FGF2–FGFR signals are enhanced following the formation of ternary complexes with HS on HSPG. However, the relationship between polySia and FGF2 was not identified until the results of several recent biochemical analyses, including gel shift assays, gel filtration and SPR, demonstrated that FGF2 monomers bind polySia directly and form a large complex that does not migrate towards FGFR, even if the receptors are located in close proximity to the complex (*52*). The K_D of FGF2 towards polySia (1.5×10^{-8} M) is smaller than that towards HS (2.8×10^{-8} M). Consistent with these differences in affinity, FGF2–polySia and FGF2–HS complexes display unique physical and biochemical properties. For example, FGF2–polySia binds to HS- or polySia-coated surfaces, whereas HS-polySia does not bind to either of these surfaces, indicating that the polySia-binding regions of FGF2 and HS differ. In addition, FGF2 complexed with polySia cannot migrate towards FGFRs, but does migrate towards HS, and FGF2 can also disassociate from polySia and then bind HS. It was also demonstrated that Erk and Akt signallings are regulated by polySia and HS in polySia- and HS-expressing cells, respectively (*52*). Taken together, these findings with FGF2 show that polySia can be released by in-direct mechanisms that are distinct from those of BDNF (Fig. 6C), as described above, and exhibits binding specificity among complex anionic glycan molecules and bioactive molecules in the brain. This study is the first demonstration of an intimate interaction between polySia and HS.

Regulator of ion channels. Zuber *et al*. (*64*) reported that the α-subunit of Na^+ channels in the adult rat brain is modified with α2,8-linked polyNeu5Ac. In this regard, it is interesting that James and Agnew (*65*) reported the presence of α2,8-linked polySia in voltage sensitive-Na^+ channels in the electric eel (*Electrophorus electricus*). Although the function of polySia on Na^+ channel is unknown, it is reported that polySia plays some roles in regulation of channels. For example, the relationship between polySia–NCAM and memory has been investigated using *in vitro* electrophysiological methods, which demonstrate that polySia on NCAM modulates the activity of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPA-Rs) in immature pyramidal neurons isolated from the CA1 region of the HY (*66*). Specifically, polySia prolongs the open channel time of AMPA-R-mediated currents and alters the bursting pattern of the receptor channels, but does not modify AMPA-R single-channel conductance (*66*). These properties suggest that polySia likely directly interacts with AMPA-R. Several reports have also examined the relationship between polySia and *N*-methyl-D-aspartate receptors (NMDA-Rs). Impaired CA1 long-term potentiation (LTP) in hippocampal slices is rescued by the addition of polySia or polySia–NCAM but not NCAM alone (*67*), and treatment with polySia alone or polySia–NCAM inhibits the activation of GluN2B-containing NMDA-Rs by low micro-molar concentrations of glutamate (*68*). PolySia reduces the open probability, but not the conductance, of NR2B-containing NMDA-Rs in a polySia- and glutamate concentration-dependent manner by inhibiting NR2B subunit-containing NMDA-Rs through the Ras-GRF1-p38 MAPK signalling cascade, which is intimately involved in LTP. These findings suggest that polySia–NCAM is involved in synaptic function in the HY, where it regulates different types of channels in a specific manner.

Consistent with the regulation of Ca^{2+} channels, polySia also has the ability to restore Ca^{2+} ions (*69*).

Miscellaneous. The polysialyltransferases ST8SIA2 and ST8SIA4 are capable of directly synthesizing polySia (*70*, *71*) on them, although polysialylation is not required for their enzymatic activity. CD36 from human milk was reported to be modified with polySia and the state of polySia was developmentally changed (*72*). Recently, neuropilin-2 from human dendritic cells was shown to be modified with α2,8-linked polySia (*73*) and to regulate chemotaxis through binding of CCL21 (*74*). PolySia synthesized by ST8Sia IV on T cells is reported to be involved in haematopoietic development (*75*). Inoue *et al*. (*76*) detected the presence of α2,9-linked polySia in C-1300 mouse neuroblastoma cells (NB41A3) by chemical analyses. Recently, synaptic cell adhesion molecule 1 (synCAM-1; also known as Cadm1 or TSLC1) was found to have polySia on an *N*-linked glycan chain of Ig domain I of NG2-positive cells in the mouse brain, and polySia was demonstrated to inhibit homophilic binding through an anti-adhesive effect (*77*). Although several types of mammalian proteins are modified with polySia, as described above, NCAM is the major and most critical carrier protein, because mice deficient in NCAM have only small amounts of polySia. Notably, however, polySia may still play important functional roles, even in small amounts.

Fish and Vertebrates Other than Mammals

In 1978, an α2,8-linked polyNeu5Gc structure in salmonid fish eggs was discovered (*78*) and represented the first demonstration of polySia in vertebrates. The composition of the polySia-containing carrier glycoprotein was determined (*78–81*) and named polysialoglycoprotein (PSGP). PSGPs are ubiquitously found in *Salmonidae* fish eggs and are the major glycoprotein components of cortical alveoli, which are Golgi-derived secretory organelles found in the peripheral cytoplasm of mature eggs of almost all animal species, including humans. After fertilization, cortical alveoli fuse with the egg plasma membrane and release their contents into the perivitelline space. In cortical alveoli, PSGP is present as a high-molecular-weight form (H-PSGP, ~200 kDa) and co-localizes with a degradative enzyme, PSGPase, which is inactive in the cortical vesicles. PSGPase is only active under low salt concentrations (<50 mM), and therefore remains inactive at the physiologic salt concentration of the cortical vesicles (*82*). After fertilization, H-PSGP is degraded to a low-molecular-weight form (L-PSGP, ~10 kDa), which is the repetitive unit of H-PSGP, through the action of PSGPase upon its activation in the low salt environment of the perivitelline space. The glycan structure of PSGP does not change before or after fertilization. The peptide and polySia structures of the PSGPs derived from eight species of *Salmonidae* fishes, *Salvelinus namaycush* (Lake trout), *Salvelinus fontinalis* (Brook trout), *Salvelinus leucomaenis pluvius* (Japanese common char, Iwana), *Salmo trutta falio* (Brown trout), *Oncorhynchus keta* (Chum salmon), *Oncorhynchus masou ishikawai* (Land-locked cherry salmon, yamame), *Oncorhynchus mykiss* (Rainbow trout) and *Oncorhynchus nerka adonis* (Kokanee salmon), have been well studied. Apo-L-PSGP is a single, tri- or dodecapeptide with the structure (D)DAT*S*XAAT*GPSX (X = E or A, Z = D or S or G, * indicates the position of the *O*-linked polySia chain) (Fig. 5B). Diversity in the polySia structure was first observed in Salmonid fish egg and included α2,8-linked polyNeu5Ac, polyNeu5Gc and polyNeu5Ac/Neu5Gc and its *O*-acetylated form (*83*), with each species displaying a characteristic structure of polySia.

PolySia chains on PSGP are thought to serve two main functions in addition to ionic regulation and the blockage of polyspermy (*80*). PolySia protects the embryo from bacterial invasion (*i.e*. bacterial sialidase versus polySia). Notably, the polysialyl groups of salmonid PSGP are highly modified, including *O*-acetylation of the hydroxyl-groups at C-4, -7 and -9, and the presence of KDN at non-reducing termini. These modifications of PSGP confer resistance to bacterial sialidases. PolySia also functions as a regulator of Ca^{2+} concentration in the perivitelline space during embryogenesis, as polySia on PSGP has been shown to bind Ca^{2+} (*69*).

Echinoderms

Echinoderms are the most primitive organisms to contain Sia. Echinoderms have several notable features: an abundance of Sia, several types of polySia are often found within the same cell, and the presence of oligo/polysialylated gangliosides when compared with the typical mono- or disialylated gangliosides found in vertebrates other than fish. The starfish *Asterias forbesi* was the first echinoderm reported to contain Sia (*84*), and polymerized chains of $\rightarrow$8Neu5Acα2$\rightarrow$ were first detected in a sperm ganglioside derived from the sea urchin *Anthocidaris crassispina* (*85*). In addition, the $\rightarrow 5O_{glycolyl}$Neu5Gcα2$\rightarrow$ chain was first reported in gangliosides of eggs from *Asterias amurensis* and *Asterias rubens* (*86*), and more recently, a $\rightarrow$4Neu5Gcα2$\rightarrow$ chain was found in the gangliosides of the sea cucumber *Holothuria leucospilota* (*87*). The longest DP of an echinodermal ganglioside had been only 6 (*88*); however, a polysialoganglioside composed of as many as 16 residues from *Hemicentrotus pulcherrimus* was reported (*89*).

The sea urchin is the most abundant and widely dispersed echinoderm in which polySia has been studied in great detail. Sea urchin eggs are surrounded by egg jelly, a gelatinous layer that is composed of a fucose–sulphate polymer (FSP) and sialic acid-rich glycoproteins (SGPs) (*90*). The structure of polySia in SGP, designated as polySia-gp, was characterized as $(\rightarrow 5O_{glycolyl}$Neu5Gcα2$\rightarrow)_n$, with *n* ranging from 4 to ~40 (*91*). An oligomerized structure of 8-*O*-sulphated $(\rightarrow 5O_{glycolyl}$Neu5Gcα2$\rightarrow)_n$ is also found on the sperm receptor on the egg cell surface, although the DP was only 2–3 (*92*). SGP in sea urchin egg jelly plays a role in the sperm acrosome reaction, which is an important process that must occur before a sperm

cells bind to an egg and involve a change of the intracellular pH $[pH]_i$ and Ca^{2+} concentration $[Ca^{2+}]_i$. The $(\rightarrow 5O_{glycolyl}Neu5Gc\alpha2\rightarrow)_n$-containing glycan chain from SGP upregulates the $[pH]_i$ of sperm although the $[Ca^{2+}]_i$ does not change, indicating that this polySia structure is involved in the acrosome reaction through a different mechanism than that of FSP (*92*).

Interestingly, a different type of polySia, 8-*O*-sulphated $(\rightarrow 9Neu5Ac\alpha2\rightarrow)_n$ structure ($DP_{avr.}$ of 15) is also present in sperm of the sea urchin, *H. pulcherrimus* (*93*, *94*). The carrier protein of this new type of polySia structure was cloned and designated as flagellasialin (*94*), which is a highly *O*-linked polysialylated cell-surface glycoprotein that displays 8-*O*-sulphated $(\rightarrow 9Neu5Ac\alpha2\rightarrow)_n$ residues on the cell surface and lacks a cytosolic region (Fig. 5A). This protein was recently shown to be GPI anchored and is an ancestor of CD52 of vertebrates. Upon treatment of sea urchin sperm with antibodies 4F7 and 3G9, which recognize internal $(\rightarrow 9Neu5Ac\alpha2\rightarrow)_n$ (*93*) and terminal (8-*O*-sulphated Neu5Ac$\alpha2\rightarrow9$) (*95*) structures of the polySia chain, respectively, sperm motility was only inhibited by 4F7. In addition, measurement of sperm $[Ca^{2+}]_i$ with and without antibodies demonstrated that 4F7, but not 3G9, led to increased $[Ca^{2+}]_i$, which resulted in the impairment of sperm motility (*94*). The regulation of Ca^{2+} appears to be dependent on the binding of α2,9-linked polySia to Ca^{2+} transporters, suNCKX (K^+-dependent Na^+/Ca^{2+} exchanger) and suPMCA (Ca^{2+} ATPase), which are involved in regulating the influx and efflux of Ca^{2+} in sperm (*96*). Collectively, these data suggest that the internal structure of this unique polySia chain is important for the regulation of intracellular Ca^{2+} concentration and that 8-*O*-sulphation might protect polySia chains from degradation, because *O*-sulphate groups on Sia are stable in alkaline conditions, such as seawater, in contrast to *O*-acetylation, which is sensitive to such conditions. We observed the presence of $(\rightarrow 8Neu5Ac\alpha2\rightarrow)_n$ on not only glycoproteins but also on glycolipids with the same sperm cells, and it is interesting that different types of polySia are present in the same cell. Several species of sea urchin, including *Strongylocentrotus purpuratus*, *Strongylocentrotus intermedius*, *Strongylocentrotus undus*, *A. crassispina*, *Pseudocentrotus depressus* and *Clypeaster japonicus*, contain the $(\rightarrow 8Neu5Ac\alpha9\rightarrow)_n$ structure on flagellasialin, although the molecular weight of this protein varies among the species, likely due to variation in the DP of the polySia chain.

Prokaryotes

Bacteria. PolySia was first identified in the Gram-negative bacterium *E. coli* K-235 and was designated as colominic acid (*97*). After determination of the composition of the *E. coli* K-235 polysaccharide capsule, the structure of polySia was reported as α2,8-linked polyNeu5Ac with a DP >200 (*14*, *98*). Later, polysaccharides isolated from *N. meningitidis* groups B and C were also shown to contain α2,8-linked polyNeu5Ac and α2,9-linked polyNeu5Ac, respectively (*99*–*101*). PolySia from *E. coli* K1 and *N. meningitidis* group B was reported to be neuroinvasive determinant (*100*). The *O*-acetylation of polySia residues on capsular polysaccharide chain was also reported in *E. coli* K1 and was demonstrated to increase the immunogenicity and invasiveness of cells into host neurons (*102*, *103*). In *Legionella pneumophili*, α2,4-linked homopolymer of 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-nononic acid within polysaccharides chains was reported (*104*), and *E. coli* K-92 strain was shown to contain alternately linked α2,8- and α2,9-linked polySia (*105*). The Gram-negative and pathogenic bacteria *Pasteurella haemolytica* and *Moraxella nonliquefaciens* have α2,8-linked polyNeu5Ac residues (*106*). Notably, several uncharacterized bacteria may also have polySia chains as the presence of nonulosonic acid has been reported (*107*).

In neuroinvasive bacteria, particularly within the *Neisseria* group, polySia appears to be involved in the invasion of host cells and functions to protect bacteria from the host innate immune system (*106*, *108*). PolySia is also associated with the difficulties of producing vaccines against these neuroinvasive organisms because polyNeu5Ac is also present in the brains of humans and rodents, as described below. It is noteworthy that Neu5Gc has not been found in bacteria.

Distribution and Functions of Di/OligoSia

The distribution of α2,8-linked diNeu5Ac on glycoproteins in the brain and other tissues derived from rat was first reported by Finne *et al.* in 1977 (*20*, *109*). Several diSia-containing glycoproteins have since been detected in mammals. For example, α2,8-linked diNeu5Ac and diNeu5Gc structures were shown to be linked to a GalNAc residue on chromogranins, which are a class of related acidic glycoproteins located in chromaffin granules in the bovine adrenal medulla (*110*). The glycopeptides human erythrocyte glycophorin and umbilical cord erythrocyte Band 3 were also found to contain α2,8-linked diNeu5Ac residues on *O*- and *N*-linked glycan chains, respectively (*111*, *112*). In 1985, Fukuda *et al.* (*113*) demonstrated the presence of the α2,9-linked diNeu5Ac structure on lactosaminoglycan in human teratocarcinoma cells (PA1) by methylation analysis and fast atom bombardment-mass spectroscopy.

The presence of α2,8-linked Neu5Gc-bearing glycoproteins was also detected in the rat thymus using chemical and immunochemical methods with the newly developed anti-oligo/polyNeu5Gc antibody 2-4B (*114*). Notably, a 100-kDa glycoprotein on the rat T-cell surface was shown to contain the AC1 epitope, which is an α2,8-linked diNeu5Gc structure (*115*), and was thought to be activated lymphocyte cell adhesion molecule (ALCAM). These findings led us to conclude that di- and oligoSia structures occur in glycoproteins far more frequently than previously recognized.

As presented in Table III, an extremely large number of glycoproteins in mammals are modified with diSia and oligoSia. Although a few of these structures have been determined, many remain unknown. We have screened several cultured mammalian cell lines, including human myelocytic leukaemia cells (HL-60), human teratocarcinoma cells (PA1), mouse

Table III. Distribution of di/oligo/polySia in glycoconjugates.

Structure	DP	Occurrence	Carrier protein	Type
$(\rightarrow 5O_{glycolyl}$/Neu5Gcα2 $\rightarrow)_n$	2–40	Sea urchin egg jelly	PolySia-gp	*O*
	2–3	Sea urchin egg	Sperm receptor	*O*
	2–3	Starfish gonad	Glycolipid (Gl)	
$(\rightarrow 8$Neu5Acα2 $\rightarrow)_n$	<200	*N. meningitidis* Gp. B *E. coli* K1 *Pasteurella haemolytica* *Moraxella nonlinquefacies*	Capsular Polysaccharide (Cp)	–
	2–25	Lake trout egg	PSGP	*O*
	~100	Vertebrates embryonic brain, tumours	N-CAM	*N*
	≥11	Eel, Rat brain	Na^+-channel	*N*
	≥4	Human tumour, rat tumour	n.d.	*O*
	≥11	Fruit fly (*Drosophila*)	n.d.	n.d.
	≥11	Cicada	n.d.	n.d.
	≥11	Several cell lines	Polysialyltransferase	*N*
	2–18	Human milk	CD36	*O*
	≥11	Human dendritic cells	Neuropilin-2	*O*
	≥11	Mouse brain NG2 cells	SynCAM	*N*
	5–7	Human melanoma cells, fibroblast cells, leukaemia cells	Integrin α5	n.d.
	2	Ovarian fluid of rainbow trout	Glycoprotein (Gp)	*N*
	2	Rat tissues	Gp	n.d.
	2–5	Pig brain	Gp	*N*
	2	Bovine adrenal medulla	Chromogranin	*O*
	2	Human erythrocyte	Band-3	*N*
	2	Human erythrocyte	Glycophorin	*O*
	2	Bovine serum	Fetuin, adipoQ, α_2-macroglobulin	n.d.
	2	Human-cultured cell: HL60, PA1	Gp	n.d.
	2	Mouse-cultured cell: 3T3-L1, Neuro2A, C2C12	Gp	n.d.
	2	Rat brain	IgLON family	*N*
	2	Rat serum	Vitronectin	n.d.
	2–16	Sea urchin	Gl	–
	2–4	Several vertebrate cells and tissues	Gl	–
	2–4	Zebrafish	Gl/Gp	*N*, *O*
$(\rightarrow 8$Neu5Gcα2 $\rightarrow)_n$	2–25	Rainbow trout egg	PSGP	*O*
	2	Bovine adrenal medulla	Chromogranin	*O*
	2	Bovine serum	α_2-Macroglobulin, fetuin	*N*
	2	Rat thymus, mouse T cell	100 kDa-gp (ALCAM?)	O?
	2	Mouse-cultured cells: 3T3-L1, Neuro2A, C2C12	n.d.	n.d.
	2	Mouse serum	plasminogen, Ig	n.d.
	2–4	Zebrafish	Gl/Gp	*N*, *O*
$(\rightarrow 8$Neu5Ac/Neu5Gcα2 $\rightarrow)_n$	2–25	Brown trout egg, Iwana egg	PSGP	*O*
$(\rightarrow 8$KDNα2 $\rightarrow)_n$	2–7	Rainbow trout ovarian fluid	KDN-gp	*O*
	2–7	Rat kidney	Megalin	*O*
	2–7	Rat various organ	Ceruloplasmin	*O*
$(\rightarrow 9$Neu5Acα2 $\rightarrow)_n$	<200	*N. meningitidis* Gp. C	Cp	–
	2	Human teratocarcinoma	n.d.	*N*
	2–20	Sea urchin sperm	Flagellasialin	*O*
	2–30	Mouse neuroblastoma	n.d.	n.d
$(\rightarrow 8/9$Neu5Acα2 $\rightarrow)_n$	<200	Bos^-12, *E. coli* K92	Cp	–
$(\rightarrow 4$Neu5Acα2 $\rightarrow)_n$	2	*N. meningitidis* Gp. Y	Cp	–
$(\rightarrow 4$Neu5Gcα2 $\rightarrow)_n$	2–4	Sea cucumber	Gl	–
$(\rightarrow 4$Legα2 $\rightarrow)_n$	n.d.	*Legionella pneumophilli*	Cp	–

neuroblastoma cells (Neuro2A), mouse myoblasts (C2C12) and mouse preadipocytes (3T3-L1), for the di/oligoSia glycotope before and after cell differentiation. The findings from these various mammalian cell types clearly demonstrate that di/oligosialylation changes during differentiation (*11*), although the biological relevance of these changes remain unknown. DiNeu5Ac and triNeu5Ac occur on different glycoproteins in the mouse brain (*116*, *117*), and the expression of these Sia residues matches that of ST8SIA3 during neurogenesis. The high-molecular-weight band of triSia-containing glycoprotein was shown to increase in an age-dependent manner, indicating that trisialylation might be related to ageing. DiSia-containing glycoproteins are also present in mouse serum (Table II) (*118*). Interestingly, a 32-kDa glycoprotein that was demonstrated to be a carbonic anhydrase II and to lack Sia was shown to cross-react with anti-diSia antibodies, suggesting that such cross-reactivity might be the source of autoimmune antibodies that occasionally cause neurodegenerative diseases (*119*).

In the ovarian fluid of rainbow trout, *N*-linked glycoproteins contain an abundance of α2,8-linked diNeu5Ac residues (*120*), and a large amount of

mono to oligoKDN (DP = 1–7) linked to mucin (KDN-gp) has also been detected (*121*, *122*), although the specific function of these modifications remains unknown. Guérardel and colleagues (*123*) examined the di/oligosialylation state of zebrafish embryos during development and found that the amount of oligoSia on glycoproteins decreased during embryogenesis, whereas the oligosialylation of glycolipids was dramatically upregulated, indicating that oligoSia, including its DP and composition (Neu5Ac–Neu5Ac, Neu5Ac–Neu5Gc, Neu5Gc–Neu5Ac and Neu5Gc-Neu5Gc) may play a role in embryogenesis (*123*). Recently, morpholino-knockdown of ST8Sia III in zebrafish appeared to lead to anomalous somite morphologies (*124*), indicating that di/oligosialylation is involved in somite development in zebrafish.

Integrins on human melanoma cells, fibroblasts and leukaemia cells are also modified with oligoSia (*125*). The significance of this oligoSia modification was examined by a pull-down assay with fibronectin-Sepharose before and after the linkage-specific sialidase digestion of integrin, demonstrating that the deletion of oligoSia on the integrin molecule inhibits adhesion with fibronectin. Notably, colominic acid (average DP = 15) did not inhibit the integrin–fibronectin interaction. In addition, the susceptibility of integrin to an antibody recognizing the fibronectin-binding domain decreased after the removal of oligoSia. Together, these results indicate that oligoSia may help human integrins maintain a suitable conformation for forming strong associations with fibronectin.

Binding Molecules of Di/Oligo/PolySia

Oligo/polysialyltransferases, Endo-N and anti-di/oligo/polySia antibodies are considered to be binding molecules for di/oligo/polySia. In addition, it is well known that many bacteria and viruses contain haemagglutinin that is capable of binding to Sia residues on host cells. Some of these haemagglutinins, such as those of Sendai virus, specifically bind to α2,8-linkages (*126*).

The Sia-recognizing molecules that are present on animal cells consist of a family of lectins, known as Sia-binding immunoglobulin-like lectins (siglecs) (*127*). Siglec-1 to -15 are present on red blood cells and neuronal cells. Siglecs-1, -5, -7, -10 and -11 are reported to have affinity towards α2,8-linkages (*128*). In particular, siglec-7 and -11 bind to α2,8-linked diSia and oligoSia with high affinity (*129–131*). Although the natural ligands of siglecs are disialylated gangliosides such as GD3, the di/oligoSia-containing glycoproteins described in this review, including yet unidentified di- and oligoSia-containing glycoproteins or bacterial determinants that have or mimic di/oligoSia, are also likely candidates for siglec ligands. The bacterium *Campylobacter jejuni* has a diSia epitope that is reported to bind to siglec-7 (*132*).

A number of neurotrophic factors, such as BDNF, NT-3 and NGF, and the growth factor FGF2 bind to polySia (*48*, *52*). Very recently, histone H1 secreted from human neuronal cells was demonstrated to bind polySia and regulate cell activity (*133*). The cytokine CCL21 is also reported to bind to polySia; however, we could not detect an interaction between polySia and CCL21 using SPR and $(GlcNAc)_3$ as a negative control, although HS binding to CCL21 was observed.

Recently, we also demonstrated by FAC that several types of small molecules, such as neurotransmitters, particularly the catecholamine dopamine, bind to polySia, but not to diSia (*50*, *53*), as described above. X-ray crystallography, NMR and tomography are important tools for investigating the structural basis for the interaction between di/oligo/polySia and biologically active molecules. Understanding the molecular mechanisms of the interaction between Sia-modified proteins and their target molecules, including the detachment and release mechanisms, is important for understanding polySia function. The use of new techniques, such as ITC, may be necessary to analyse weak-binding interactions, because some are not stable or static.

Diseases

PolySia is associated with a number of diseases, including various types of cancer. NCAM is thought to be the main carrier protein of polySia in cancer cells, although some cells do not express NCAM protein (*134*). The majority of polysialylated NCAM is expressed in embryos and normal cells in adult tissues do not typically display polySia on the cell surface; however, some cancer cells express polySia. Thus, polySia is recognized as an oncodevelopmental antigen. For example, neuroblastomas (*135*, *136*), Wilms' tumours (*137*), medulloblastomas (*138*), pheochromocytomas (*139*), medullary thyroid carcinomas (*140*), non-small cell lung (NSCL) carcinomas (*141*, *142*), pituitary adenomas (*138*) and breast cancer (*134*) are shown to re-express polySia on cell surface. In NSCL carcinoma cells, tumour progression is related with the expression of polySia and the levels of its biosynthesizing enzyme, ST8SIA2 (*142*, *143*). As polysialylation has an anti-adhesive effect on cell–cell interactions, it is likely involved in the detachment and metastasis of cancer cells.

Schizophrenia is a psychiatric disorder with multiple factors contributing to pathogenesis. Interestingly, some reports suggest that polySia is involved in schizophrenia and other related psychiatric disorders. For example, the number of polySia–NCAM immunostained cells derived from the HY of schizophrenic brains is decreased compared with that of normal brains (*144*). Chromosome 15q26, which is the genomic region where the gene encoding ST8SIA2 localizes, is related to schizophrenia and bipolar disorders among the population of Eastern Quebec (*145*). Recently, it was also shown that a relationship exists between single-nucleotide polymorphisms (SNPs) in the promoter region of ST8SIA2 and schizophrenia by genome-wide studies among Japanese (*146*) and Chinese-Han (*147*) populations. The ST8SIA2 gene is also reported to be a generalized susceptibility marker for psychotic and mood disorders on chromosome 15q25-26 (*148*) and is associated with an increased risk of mental illness, such as autism (*149*). Interestingly, the mutation of synCAM,

Table IV. Phenotypes of polySia-impaired mice.

Mouse type	Wild Type	NCAM$^{-/-}$	ST8SIA2$^{-/-}$	ST8SIA4$^{-/-}$	ST8SIA2$^{-/-}$ ST8SIA4$^{-/-}$
References		**152,156–158,56**	**154,155,56**	**160,155,56**	**162,163,155**
		Biochemical aspects			
Amount of ST8SIA2	100	100	0	100	0
Amount of ST8SIA4	100	100	100	0	0
Amount of polySia	100	Negligible	50	95	0
		Lethality			
	No	No	No	No	Yes (<8w)
		Brain morphology			
Size	Normal	↓	Normal	Normal	↓
OB	Normal	Abnormal	Normal	Normal	Abnormal
HY (mossy fiber)	Normal	Abnormal	Abnormal	Normal	Abnormal
		Electrophysiology			
LTP and LTD in CA1	Normal	↓	Normal	↓	?
LTP in CA3 and DG	Normal	↓	Normal	Normal	?
		Memory, learning and behaviour			
Spacial learning	Normal	↓	Normal	↓	?
Contextual fear conditioning	Normal	↓	↓	Normal	?
Cued fear conditioning	Normal	↓	↓	Normal	?
Locomotion (OF)	Normal	↑	↑	↑	?
Social interaction	Normal	↓	↓	↓	?

which is another substrate for ST8SIA2, is also related with autism spectrum disorders (*150*). Biochemical-based studies using SNP-7 (Glu141Lys) in the coding region of ST8SIA2 reported from a schizophrenic patient have shown that the *in vitro* and *in vivo* enzymatic activity of ST8SIA2 with SNP-7 decreases dramatically, and that the polySia products are also impaired with respect to quantity and quality (*53*). Considering that polySia functions as a regulator of biologically active molecules, such as BDNF, FGF2 and dopamine, which are intimately involved in brain function (*48*), polySia–NCAM synthesized by mutated ST8SIA2 likely plays a role in the development of schizophrenia (*51*, *53*).

Anatomically, the volume of olfactory bulbs (OBs) derived from schizophrenic brains is reduced (*151*), which is a similar phenotype to that of NCAM-KO mice (*152*). The functional impairment and disturbed organization of the HY are also involved in the etiology of schizophrenia (*153*). In this regard, it is interesting that the loss of ST8SIA2 or NCAM results in the misguidance of infrapyramidal mossy fibres and formation of ectopic synapses in the HY (*154*). In addition, several characteristic properties, such as brain structure, neural plasticity and various morphological, cognitive and emotional deficits related to schizophrenia have been observed in ST8SIA2 single KO mice (*54*, *155*). Very recently, NCAM-KO mice were demonstrated to be useful for studying specific endophenotypes related to schizophrenia, although these mice do not display typical schizophrenia-like phenotypes (*156*). Although these results highlight the importance of polySia in psychiatric disorder, biochemical studies that examine the underlying molecular mechanism between behaviour or anatomical phenotypes and polySia are needed.

Phenotypes of PolySia-Impaired Mice

To understand the function of polySia structure at an animal level, several approaches have been performed using specific probes for polySia, such as Endo-N and anti-polySia antibodies and gene-targeting techniques. The phenotypes of polySia-impaired mice were summarized in Table IV.

NCAM$^{-/-}$ mice was first established to understand the function of polySia and NCAM (*152*) because NCAM is the major carrier of polySia in brain. Almost all polySia disappeared in NCAM$^{-/-}$ mice. NCAM$^{-/-}$ mice have several morphological changes for example, reduced OB size due to disturbed migration from SVZ, disturbed mossy fibre architecture (*152*), and a reduced amygdalo-hippocampal theta synchronization during fear memory retrieval (*156*). NCAM$^{-/-}$ mice show some behavioural changes such as impairment of spacial learning (*152*), locomotion (*156*) and social interactions (*156*). Cognitive functions of NCAM$^{-/-}$ mice and conditional NCAM-deficient mice (forebrain specific) such as contextual fear conditioning and cued fear conditioning are also impaired especially under stress (*157*). Interestingly, expression of D2-receptor and sensitivity of dopamine are upregulated in cells derived from NCAM$^{-/-}$ mice (*158*). It

should be noted that NCAM is not the only substrate of polysialyltransferase as described above.

The other strategy to understand the polySia function more clearly is to establish polysialyltransferase-deficient mice. As polySia can be biosynthesized by two polysialyltransferases, ST8SIA2 and ST8SIA4 (*159*), single polysialyltransferase-deficient mice, such as ST8SIA2$^{-/-}$ mice (*154*) or ST8SIA4$^{-/-}$ mice (*160*), contain a large amount of remaining polySia in brain (*158*). Interestingly, the change of polySia staining and the phenotypes are different among them. ST8SIA2$^{-/-}$ mice were first established by Angata *et al.* (*154*) and well characterized. PolySia staining greatly decreased at OB and cerebral cortex. In HY, polySia deficit in the dentate gyrus (DG) (inner rim of the granular layer where newborn precursors from the subgranular layer first acquire polySia-staining) was observed (*154*). ST8SIA2$^{-/-}$ mice show the misguidance of infrapyramidal mossy fibres and the formation of ectopic synapses in the HY CA3 region. ST8SIA2$^{-/-}$ mice exhibit higher exploratory drive and reduce behavioural responses to Pavlovian fear conditioning. In addition, ST8SIA2$^{-/-}$ mice show impairment of social interaction (*54*). ST8SIA4$^{-/-}$ mice were first established by Eckhardt and Cremer and surprisingly, the polySia amounts slightly decreased. The precursor migration and mossy fibre organization were normal. However, the expression of polySia in CA1 region of Ammon's horn was down-regulated and LTP and long-term depression (LTD) in CA1 were also impaired (*160*). ST8SIA4$^{-/-}$ mice display a decreased motivation in social interaction (*54*).

It was unexpected that large amounts of polySia remain in ST8SIA2$^{-/-}$ (55%) and ST8SIA4$^{-/-}$ (95%) (*161*). Therefore, it was necessary to establish ST8SIA2 and ST8SIA4 double KO mice to remove polySia completely (*162*, *163*). ST8SIA2$^{-/-}$/ST8SIA4$^{-/-}$ mice show severe phenotypes and die within 8 weeks. The major phenotypes are hypoplasia of corticospinal tract, size reduction of internal capsule, hypoplasia of mammillothalamic tract, high incidence of hydrocephalus, growth retardation and precocious death. These functions are considered to be NCAM-specific. Other phenotypes such as small OBs and rostral migratory stream expansion, and delamination of mossy fibres are considered to be polySia-specific function. Interestingly, in NCAM, ST8SIA2 and ST8SIA4 triple-KO mice, the severe phenotype of the DKO mice is rescued, suggesting that an uncontrolled type of NCAM-mediated cell adhesion is followed by increased signal transduction events (*155*). In NCAM$^{-/-}$/ST8SIA2$^{-/-}$/ST8SIA4$^{-/-}$ mice, improved signalling through increased cell–cell interactions in the polySia-deficient brain is likely to result from the reduced levels of cell adhesion molecules resulting from the NCAM deficiency. Thus, the reduction of NCAM leads to the recovery of normal physiological interactions and to the rescue of the severe phenotype of polySia-deleted mice.

Perspective

Based on the current status of polySia research, the following are interesting topics to pursue in future polySia studies. First, polySia has long been recognized as a negative regulator of cell–cell adhesion. This characteristic of polySia is important for neurogenesis during embryogenesis, as well as neuroplasticity. In addition to anti-adhesive and ion regulation functions, we recently proposed and demonstrated that of polySia serves as a reservoir for components involved in the maintenance of neural activity and growth of brain cells, including particular groups of neurotrophic factors, growth factors and neurotransmitters. In particular, the interaction of polySia with small molecules other than calcium ions (*69*) had not been examined in detail before our study (*48*–*53*). Numerous other interactions between polySia and biologically active molecules, such as histone H1 (*133*) and CCL21 (*74*), are expected to be revealed, which will shed light on the potential roles of polySia in neural, immunological and reprogramming phenomena.

Second, the reservoir function of polySia for growth factors, morphogens and cytokines is also exemplified by glycosaminoglycans (GAGs), such as HS, keratan sulphate and chondroitin sulphate, which are another group of acidic polymers. However, polySia and GAGs exhibit different binding properties to neurotrophic and growth factors with respect to strength, stoichiometry and range of binding counterparts (*52*). The fact that polySia and GAGs share a similar reservoir function is interesting because it indicates that polySia can function in roles though to be typically performed by GAGs. This functional mimicry is most likely due to the molecular mimicry of GAGs by polySia. As described above, antibodies against polySia occasionally cross-react with polynucleotides, which are a different group of polyanions than polySia or GAGs. Thus, polySia may share mimetic conformations with these polyanionic compounds, such as steric distribution of carboxyl anions along the helical chain. This may explain why anti-polySia antibodies sometimes detect the polySia epitope in organisms that would not be expected to express even monomeric Sia (*164*, *165*). An alternative possibility is that the polySia epitope is synthesized by unknown mechanisms in those organisms. However, we have encountered molecular mimicry of diSia by carbonic anhydrase lacking carbohydrates (*119*). Of course, the detection of polySia by methods other than immunochemical detection, such as chemical assays, needs to be confirmed before a definitive conclusion can be reached. The molecular mimicry of di/oligo/polySia structure in various cell types is an interesting phenomenon that warrants further attention.

Third, many important questions concerning the biosynthesis of di/oligo/polySia remain to be resolved. For example, how are the expression and disappearance of polySia regulated at the transcriptional, translational and protein levels? Which sialyltransferases are responsible for the synthesis of glycosidic linkages other than the α2,8-linkage? Although chain length is known to be biologically important, as we first demonstrated that a DP of at least 12 is required for polySia to act as a reservoir for BDNF (*48*), whereas a DP of 17 is needed to bind FGF2, the regulation of diSia,

oligoSia and polySia chain length is not well understood. The answers to these, and many other questions, will supply the necessary insights for understanding polySia biosynthesis.

Fourth, although polySia has been relatively well studied, greater focus on di- and oligoSia glycoproteins is expected to provide in-depth knowledge concerning the function of these interesting glycoproteins. After we identified a large group of diSia/oligoSia-containing glycoproteins (*11–13*), a growing number of studies on di- and oligoSia structures have demonstrated that the biological functions of di- and oligoSia are clearly distinct from those of polySia, although many details remain unknown.

In conclusion, oligo/polymerized Sia is a distinct, unusual, carbohydrate structure with respect to its size, properties and functions compared with carbohydrates that are commonly present on cell surfaces. For this reason, the study of diSia/oligoSia/polySia-containing glycoproteins, including the structures of oligomerized Sia, is expected to continuously reveal interesting findings, as long as we view these molecules from a distance, as well as close range.

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Conflict of Interest

None declared.

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