

Polysialylation and disease

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ABSTRACT

Polysialic acid (polySia, PSA) is a unique constituent of the glycocalyx on the surface of bacterial and vertebrate cells. In vertebrates, its biosynthesis is highly regulated, not only in quantity and quality, but also in time and location, which allows polySia to be involved in various important biological phenomena. Therefore, impairments in the expression and structure of polySia sometimes relate to diseases, such as schizophrenia, bipolar disorder, and cancer. Some bacteria express polySia as a tool for protecting themselves from the host immune system during invasion. PolySia is proven to be a biosafe material; polySia, as well as polySia-recognizing molecules, are key therapeutic agents. This review first comprehensive outlines the occurrence, features, biosynthesis, and functions of polySia and subsequently focuses on the related diseases.

1. Introduction

Glycosylation is one of the important posttranslational modifications of proteins. In humans, more than 50% of proteins are post-translationally modified with glycans (Stanley, 2017). This provides them with physicochemical and biological stability, and with additional and/or specific functions that the original proteins did not have. Glycoproteins are usually present on the cell surface and their glycans sometimes extend from the cell membrane to form an ~500 nm-thick layer, called the glycocalyx (Marki et al., 2015). Due to the location, the glycocalyx protects cells and become the first groups of molecules through which the cells communicate with other cells and sense the external environment. Therefore, it is essential to understand the functions of glycans in the recognition of molecules, during cell-cell and cell-extracellular molecule interactions. With respect to disease, it is particularly important to understand the pathophysiological mechanisms in which impaired expression and structures of glycans are involved, because such mechanistic analyses have not been satisfactorily conducted so far. In addition, the utilization of glycans as therapeutic targets sometimes offers a powerful tool for diagnosis and treatment. Polysialic acid (polySia, PSA), a unique homopolymer of sialic acid, is one of the most attractive glycans for study. It is known to be highly

related to particular diseases that are increasingly problematic in the contemporary world: mental and neurodegenerative disorders and cancers. Although much remains to be clarified in the understanding of molecular mechanisms of polySia function and impairment, we will try to sum up the current state of knowledge of the structures, biological functions, and pathophysiological significance of polySia, in a comprehensive way.

2. Structure of polySia

Sialic acids (Sias), or neuraminic acids, are the general names of a family of 3-deoxy-9-carbon carboxylated sugars, called 2-keto-3-deoxy-D-glycero-D-galacto-nulosonic acids (Angata and Varki, 2002; Sato and Kitajima, 2013a). Sias were named based on the organ from which they were originally crystallized; sialic acid from the salivary gland and neuraminic acid from neurological organs. To date, more than 50 types of Sia have been characterized. They include O-acetylated, O-sulfated, O-lactylated, O-methylated, lactonized, and lactamized derivatives of N-acetylneuraminic acid (Neu5Ac), N-glycolyneuraminic 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 (Fig. 1A) (Angata and Varki, 2002). Sias are usually

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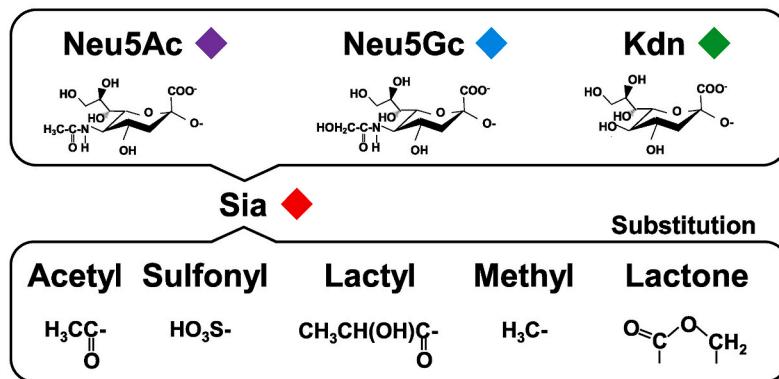
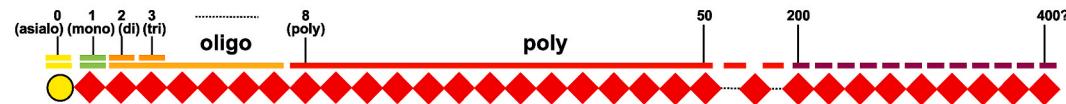
A Components of Sia**B Intersialyl linkage****C Degree of polymerization (DP)**

Fig. 1. Structures of sialic acid and polysialic acid. A. Structures of sialic acids (Sias). Sia is a collective name and usually illustrated as a red diamond based on the symbol nomenclature for Glycans (<https://www.ncbi.nlm.nih.gov/glycans/snfg.html>). The Sia species contain two components. One is a basic structure, which is Neu5Ac (purple diamond), Neu5Gc (blue diamond), or Kdn (green diamond). The other is the substituted group on hydroxyl group of the basic structure, including acetyl, sulfonyl, lactyl, methyl, and lactone groups. B. Intersialyl linkages of polymerized Sia. α 2,8-, α 2,9-, α 2,8/9-, α 2,4-, and α 2,5-O-glycolyl-linkages are known. C. The degree of polymerization (DP) of polymerized Sia structure. Mono, DP = 1; di, DP = 2; tri, DP = 3; oligo, DP = 2–7; and poly, DP = 8 or greater. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

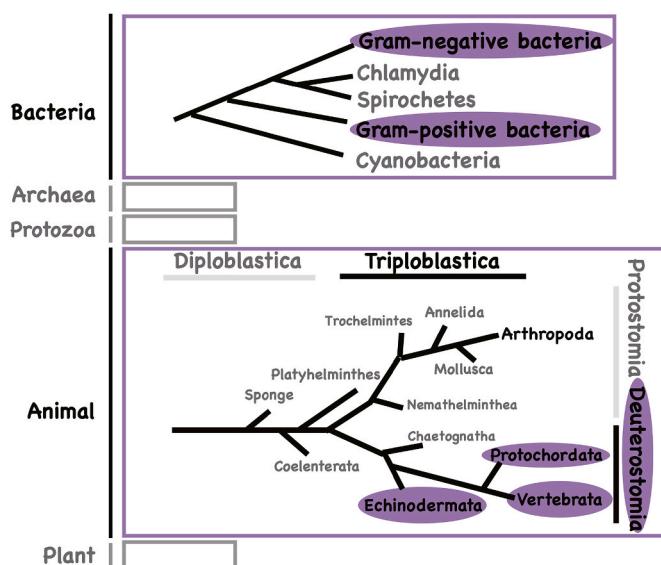


Fig. 2. Distribution of polySia in nature. The organisms that are reported to express polySia are shown in purple. The organisms that are reported to express Sia are shown in black character. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

condensed with pyruvic acid and *N*-acetylmannosamine (ManNAc) or mannose (Man). Therefore, the biosynthetic pathways are divided into two major streams: Neu5Ac and Kdn. Neu5Gc is synthesized from an activated form of Neu5Ac, CMP-Neu5Ac, via hydroxylation of an

N-acetyl residue at the C5 position of Neu5Ac, by CMP-Neu5Ac hydroxylase (CMAH). Sias are typically present as monosialyl residues, at the non-reducing termini of glycan chains of glycoproteins and glycolipids, where they function as mediators of ligand-receptor and cell-cell interactions in fertilization, differentiation, and in immunological and neurological events (Angata and Varki, 2002). Sias are considered to be essential to mammalian development, because mice deficient for GlcNAc 2-epimerase/ManNAc kinase, a key enzyme for the biosynthesis of Neu5Ac, are embryonic lethal (Schwarzkopf et al., 2002). In addition, in vertebrate cells, the study of Sia-specific lectins, sialic acid-binding immunoglobulin-like lectins, Siglecs (Macaulay et al., 2014), also demonstrates the importance of Sia. Sias are interesting carbohydrate epitopes because their occurrence is predominantly restricted to some bacteria and deuterostome lineage animals (Fig. 2A), although the presence of Sias in specific protostome lineages (such as some insects) has also been proposed (Angata and Varki, 2002). The importance of Sias is well documented in several reviews (Angata and Varki, 2002) and this article focuses on the polymerized Sia structure.

The most unique aspect of Sias, among other carbohydrate epitopes, is that they often form a homo-oligo/polymer structure, specifically disialic acid (diSia) (DP = 2), oligosialic acid (oligoSia) (DP = 3–7), and polysialic acid (polySia) (DP \geq 8) (Sato, 2004; Sato, 2013; Sato and Kitajima, 1999, 2013a). Therefore, polymerized Sia glycotopes exhibit structural diversity with respect to not only the Sia components of polySia (Neu5Ac, Neu5Gc, and KDN) and their modified forms (acetylation, sulfation, methylation, lactylation, and lactonization), but also the type of intersialyl linkage (α 2,4, α 2,5-Oglycolyl (α 2,11), α 2,8, α 2,9, and α 2,8/9) (Fig. 1B) and degree of polymerization (DP, 2 to 400) (Fig. 1C) (Sato and Kitajima, 2013a). The presence of α 2,8-linked polySia structures can usually be confirmed by two chemical methods, fluorometric

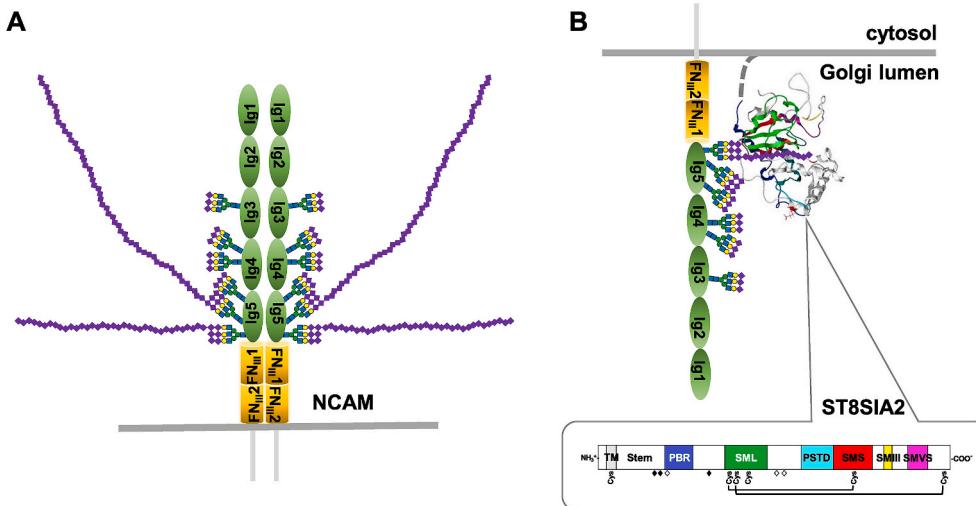


Fig. 3. Structure of polySia-NCAM and the polySia-synthesizing enzyme, ST8SIA2. A. Structure of polySia on polySia-NCAM. NCAM usually has 6 N-linked glycans and polySias are present on the 5th and 6th N-glycosylation sites in the Ig5 domain of NCAM. The di, tri, and tetra antennary N-linked glycans are reported. B. Schematic structure of a polysialyltransferase (polyST), ST8SIA2. ST8SIA2 consists of 375 amino acids and has several domains common with most sialyltransferases, such as sialyl motif large (SML; green), sialyl motif small (SMS; red), sialyl motif III (SMIII; yellow), and sialyl motif very small (SMVS; pink). PolySTs have common domains, a polybasic region (PBR; dark blue), and a polysialyltransferase domain (PSTD; light blue), which are important for NCAM-specific polysialylation. Cys indicates cysteine residues that are important for making disulfide bonds. Purple diamond symbol indicates Neu5Ac. Diamonds indicate N-glycans and white diamonds indicate N-glycans that are known to be autopolsialylated. Molecular modeling of ST8SIA2 protein was performed by MOE molecular modeling software using the crystal structure of human ST8SIA3 (5BO9) as a template. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

C_7/C_9 analysis (Sato et al., 1998) or mild acid hydrolysis-fluorometric anion exchange chromatography (MH-FAEC) (Sato et al., 1999), or by an immunochemical method using specific probes, for example, anti-polySia antibodies (Sato and Kitajima, 2013a) in combination with a specific oligo/polySia degrading enzyme, such as an endo-N-acetylcyclenaminidase (Endo-N) (Hallenbeck et al., 1987). The conformation of polySia is different from that of oligoSia, based on NMR spectroscopy (Brisson et al., 1992; Evans et al., 1995; Michon et al., 1987; Yamasaki and Bacon, 1991) and the specificities of anti-polySia antibodies (Sato and Kitajima, 2013a). The unique conformational feature of polySia, a helical conformation of α 2,8-linked polyNeu5Ac, explains the unexpectedly large size of the epitope of the anti-polyNeu5Ac antibody H.46 (Jennings et al., 1985). Not only polyNeu5Ac but also polyNeu5Gc, and other N-substituted polySias show the helical conformation (Baumann et al., 1993; Brisson et al., 1992). X-ray crystallographic analysis of anti-polySia monoclonal antibody 735 also demonstrates that the helical conformation of polyNeu5Ac (6 residues per turn, 36 Å pitch) consist of at least eight Neu5Ac residues and is well accommodated by the antigen-binding site of the antibody (Evans et al., 1995). Later, Nagae showed that the triSia structure among 8 Sia residues is the epitope of an scFv of 735 by crystallization with poly(Neu5Ac)₈ (Nagae et al., 2013). Not only helical structures but also random structures are considered to be present in polySias. The Neu5Aca2,8Neu5Ac epitope, at the terminal end of polySia, has a different conformation to internal Neu5Ac residues of polySia (DP \geq 8). These results suggest that di/oligoSia structures have large conformational differences compared to polySia structures, even if the same epitope is present inside the structure. Accordingly, they are likely to have different specific functions from those listed for polySia glycotopes. Using accurate molecular modeling techniques, DP-dependent conformational differences are also suggested from the predicted structures of α 2,4-, α 2,5-, α 2,7- (not reported in nature), α 2,8-, α 2,9-, and α 2,8/9-linked polySia and molecular modeling of mono-/di/tri/polySia (Sato, 2013).

3. Occurrence of polySia structure

PolySia was discovered in the filtrate of a medium used to culture *E. coli* K-235 (Barry and Goebel, 1957) and it was named colominic acid, although commercially available colominic acid is nowadays derived from *E. coli* K1. The structure of colominic acid was shown to be an α 2,8-linked polyNeu5Ac (McGuire and Binkley, 1964) and later, the filtrate containing polySia was shown to be derived from capsular polysaccharides of *E. coli*. The DP was at least 200 Neu5Ac residues (Rohr and Troy, 1980). PolySias were also isolated from *Neisseria meningitidis* group B and group C and were shown to be α 2,8-linked polyNeu5Ac and α 2,9-linked polyNeu5Ac, respectively (Bhattacharjee, 1975; Troy, 1979). *E. coli* K-92 strain was shown to contain alternately linked α 2,8- and α 2,9-linked polySia (Egan et al., 1977). Other gram-negative, pathogenic bacteria, *Pasteurella haemolytica* and *Moraxella nonliquefaciens*, also have α 2,8-linked polyNeu5Ac residues (Adlam et al., 1987). Crystallized *Mannheimia haemolytica* polysialyltransferase (polyST) is the only polyST to be conformationally analyzed (Lizak et al., 2017). Many uncharacterized bacteria may also have polySia, inferred from the presence of the responsible gene.

Animals from a deuterostome lineage, from echinoderms to humans, have been shown to have polySia (Sato and Kitajima, 2013a). Interestingly, the richest and most diverse examples are found in the echinoderms, such as sea urchins (Sato and Kitajima, 2013a). The tissues of these animals are almost entirely derived from gonadal tissues. So far, α 2,5-O-glycolyl-linked-(α 2,11-linked) polyNeu5Gc, α 2,8-linked polyNeu5Ac, α 2,9-linked polyNeu5Ac, and α 2,4-linked oligoSia have been demonstrated in echinoderms (Sato and Kitajima, 2013a).

In vertebrates, including fishes, birds, reptiles, amphibians, and mammals, the most fully characterized polysialylated protein is a neural cell adhesion molecule (NCAM), which is mainly expressed in the brain. In 1982, α 2,8-linked polyNeu5Ac was discovered on NCAM (Finne, 1982), and since then a lot of results have accumulated. Based on the immunoreactivity of polySia from NCAM-deficient mice (Cremer et al., 1994) and hierarchical specificity of the polyST, the majority of polySia

(~90%) is linked to NCAM, although other minor carrier proteins are shown to be present as polySia carriers, such as CADM1 (Galuska et al., 2010) and NRP2 (Curreli et al., 2007), when NCAM as the dominant substrate for polySTs is absent. NCAM protein has four major isoforms: NCAM-180, -140, and -120, and sNCAM (soluble NCAM) (Sato and Kitajima, 2019). The extracellular region of all NCAMs consists of five immunoglobulin-like (Ig) domains with six *N*-glycosylation sites and two fibronectin type-III-like (FN_{III})-domains. PolySia chains are shown to be linked to the two di-, tri-, or tetra-antennary *N*-linked glycan chains on Ig5 of NCAM (Fig. 3A) (Kudo et al., 1996; Liedtke et al., 2001). NCAM is attached to the transmembrane region via a glycosylphosphatidylinositol (GPI) anchor (NCAM-120) or connected through the membrane to the cytosol (NCAM-140 and NCAM-180). Spectrin, PKC, actin, and FAK are associated with NCAM-140 and NCAM-180, via the cytosol, and they are involved in signal transduction. The soluble form is considered to be secreted into the extracellular space, due to presence of the mRNA variant or shedding by MMP-10 or -17.

The expression of polySia on NCAM in vertebrates, especially in mammals, is mainly restricted to brain tissues. All NCAMs are modified with polySia in the embryonic brain. In the mouse, the expression of polySia starts at around the middle of the embryonic stage, increases until just before birth (E20/P0), decreases soon after birth, then drastically decreases from P10 to 3 weeks, and mostly disappears by 8 weeks after birth (Schnaar et al., 2014). In humans, the rate of decrease of polySia is lower than that of the mouse, with polySia persisting until the age of 12. It is considered that the human brain is somehow plastic until around 12 years of age (adolescent period) and polySia is considered as one of the markers of plasticity. The level of polySia remains low from age 12 to at least age 80. However, in adult brains, a significant amount of polySia still remains in restricted brain regions (Bonfanti, 2006, 1992; Rutishauser, 2008). Two plastic areas are well known for polySia expression. One is the olfactory bulb (OB) system, which consists of the precursor cells of interneurons. Their origin is the subventricular zone (SVZ) of the lateral ventricle. Interneurons migrate in the rostral migratory stream (RMS) to the OB (Miragall et al., 1988). The other area is the hippocampus (HP), especially the subgranular zone (SGZ) of the dentate gyrus (DG), which includes the granule cell layer (GCL) and mossy fibers (MF) in the HP (Seki and Arai, 1991b). These two areas are characteristic for their neurogenesis and neural plasticity in adults. Close observation shows that polySia-NCAM is also present in other important areas which are related to physiological plasticity, such as the hypothalamus (Hypo), especially at the supraoptic nucleus and suprachiasmatic nucleus (SCN), and in the thalamus (periventricular, paratenial and anteroventral-anterodorsal nuclei). In the cortex, polySia expression is reported to be observed in layer II of the piriform cortex (Seki and Arai, 1991a) and, with high sensitivity, it was observed in all layers of the PFC. Of the six layers, III and IV showed a higher intensity in an examination of the rostromedial region of the frontal superior gyrus of the dorsolateral PFC (Brodmann area 9) (Varea et al., 2007). PolySia-NCAM expression has been well observed in the amygdala (AMG) (Nacher et al., 2002), substantia nigra (SNA) (Aaron and Chesselet, 1989), and pons (Bonfanti et al., 1992). In the spinal cord, polySia is shown to express in sensory neurons, but not in motor neurons. In the peripheral nervous system, polySia expression is observed in the visual system (Bonfanti et al., 1992). Other important polySia-expressing cells are NK cells in the blood system (Lanier et al., 1989).

4. Biosynthesis of polySia structure

The biosynthetic pathways of polysialylation in bacteria and eukaryotes are completely different, although polyST can synthesize polySia using CMP-Sia. The *E. coli* system was well examined by Troy F. A. II (Rohr and Troy, 1980). The principles of the pathway are as follows: (i) CMP-Neu5Ac + P-C₅₅(undecaprenylphosphate) → Neu5-Ac-P-C₅₅ + CMP; (ii) n(Neu5Ac-P-C₅₅) → (8Neu5Ac_n-P-C₅₅) + (n-1)

(P-C₅₅) (n = 100–200); (iii) (8Neu5Ac_n-P-C₅₅) + endogenous acceptor → (8Neu5Ac_n-acceptor + P-C₅₅). Reaction (iii) is unique to bacteria because of the polySia-transfer *en bloc* to an acceptor substrate, followed by the translocation of polySia across the membrane, in order to display polySia on the outside of the bacterial cell. The responsible enzyme is a CMP-Sia:poly- α 2,8-sialosylsialyltransferase (polyST), and this enzyme forms a complex with other biosynthetic enzymes. A gene cluster of polySia synthesis-related genes are located in the kps (capsular polysaccharide) gene cluster. The same system is observed in *Neisseria meningitidis*.

The biosynthetic reaction for the synthesis of α 2,8-linked oligo/polymerized Sia, catalyzed by CMP-Sia:ST8 α -sialoside α 2,8-sialyltransferase (ST8SIA) in vertebrates, is as follows: (8Sia α 2)_n-glycoconjugates + CMP-Sia → (8Sia α 2)_{n+1}-glycoconjugates + CMP (Harduin-Lepers et al., 2005; Tsuji, 1996). ST8SIA 1 to 6 have been demonstrated to be present in humans, and ST8SIA2 and 4 are two polysialyltransferases (polySTs) responsible for the NCAM polysialylation (Harduin-Lepers et al., 2008). The sequence homology is around 60% and the domains of the two enzymes are the same. They have type II transmembrane topology and localize in the Golgi apparatus (Harduin-Lepers et al., 2005; Tsuji, 1996) (Fig. 3B). ST8SIAs consist of a short cytoplasmic region, the transmembrane (TM) region, a stem region and a catalytic domain. Within the catalytic domains, there are 4 conserved motifs: the sialyl motif large (SML), SM-small (SMS), motif III (MIII), and SM-very short (SMVS) (Fig. 3B) (Datta and Paulson, 1995; Datta et al., 1998; Harduin-Lepers et al., 2005; Jeanneau et al., 2004; Tsuji, 1996). A catalytic domain has a special conformation via disulfide bonds between Cys residues (Cys in SM-L and COOH terminal domain, Cys in SM-L and SM-S) (Angata et al., 2001). Among ST8SIA1 to 6 cloned from rodents and humans, enzymes involved in the biosynthesis of oligo/polySia are ST8SIA2, ST8SIA4, and ST8SIA3 (Harduin-Lepers et al., 2008), although ST8SIA3 never synthesizes the polySia structure (Kitajima et al., 2015). Interestingly, ST8SIA2 and ST8SIA4 have other special motifs: a polybasic region (PBR) and a polysialyltransferase domain (PSTD), which are important for NCAM polysialylation. In particular, PBR is required for binding to the acidic patch of the first FN_{III}1 of NCAM, to synthesize polySia on the 5th and 6th *N*-glycosylation sites of *N*-glycans, on the Ig5 domain of NCAM (Colley et al., 2014). Vertebrate polyST has yet to be successfully crystallized, with the exception of oligoST (ST8Sia3) (Volkers et al., 2015).

5. Physical functions of polySia

The biological functions of polySia are well demonstrated: neural cell migration, axonal guidance, fasciculation, myelination, synapse formation, functional plasticity of the nervous system, learning and memory, and circadian rhythm (Bonfanti, 2006; Schnaar et al., 2014). Using polySia-expressing cells, tissues, and gene-targeting mice, interesting polySia-related biological functions have been shown. However, the molecular mechanisms underlying these functions are still not well characterized. It is important to understand the features of polySia in a pure system before using complex systems because polySia itself and the polySia-area are highly complex.

5.1. Formation of repulsive field

The most important physical feature of polySia is its anti-adhesive effect. α 2,8-linked polyNeu5Ac was demonstrated to inhibit cell-cell and cell-extracellular matrix (ECM) interactions via polySia's large volume, due to the hydration effect of the long, negatively charged linear polymer of Sias (Yang et al., 1994). The effect on the cell surface was measured by estimating the intercellular distance, by electron microscopy, before and after endo-N treatment. The distance of 10–15 nm was reduced by Endo-N, indicating that a 10–15 nm intercellular space was maintained by the presence of polySia (Yang et al., 1992). To understand the steric effects of polySia on polySia-NCAM, direct

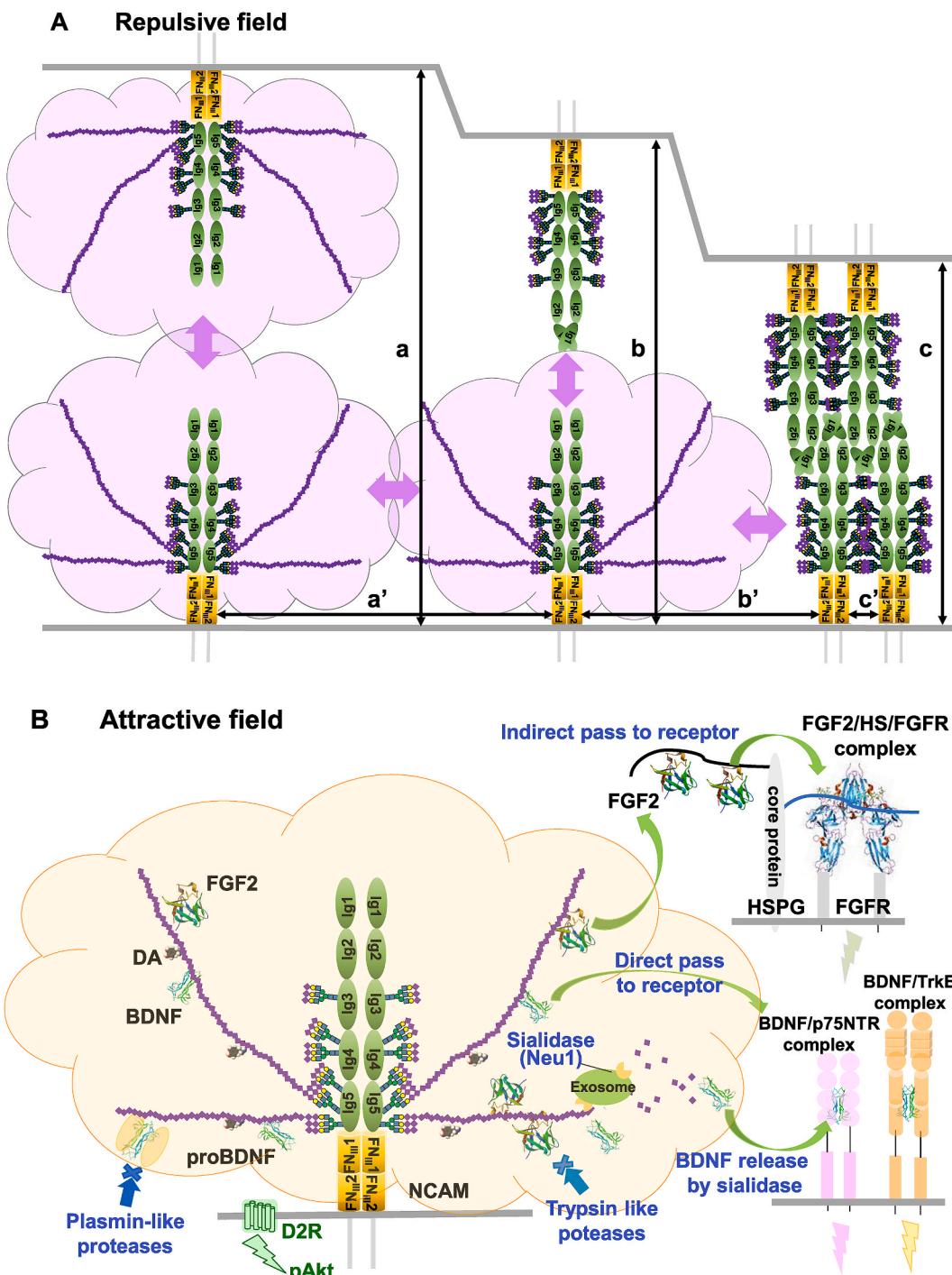


Fig. 4. Formation of Functional fields by polySia. A. Formation of a repulsive field. Repulsive fields of polySia on NCAM, indicated as light purple shadow. The physicochemical properties of polySia produce repulsive fields. Through the repulsive field, polySia on the cell surface regulates cell-cell *trans* interactions negatively and regulates cell-cell interspace distance. In addition, polySia-NCAM regulates the space between molecules on the same cell surface (*cis*-interaction). Therefore, the *trans*-interspace (a, b, c) and *cis*-interspace (a', b', c') are regulated by polySia. B. Formation of an attractive field. Attractive fields of polySia on NCAM, indicated as yellow shadow. Biologically active molecules, including neurotransmitters, such as dopamine (DA), norepinephrine, and epinephrine; growth factors, such as FGF2; and neurotrophic factors, such as BDNF, NT-3, and proBDNF, are shown to bind directly to polySia on NCAM. BDNF in polySia-NCAM migrates to TrkB directly if the receptors come in proximity. In addition, BDNF can be released by sialidase action, therefore, the supply of BDNF is achieved towards cells nearby. FGF2 in polySia-NCAM can only migrate towards heparan sulfate, HS. On the HS, FGF2 form dimers and make an HS-FGF2-FGFR ternary complex. The proBDNF and FGF2 are protected from protease action by polySia binding. DA activates Akt signal via binding to D2 dopamine receptor (D2R) and the presence of polySia changes the Akt signaling. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Comparison of complex.

Neurologically active molecules	Acidic glycans	$K_D^{a)}$ (M)	Complex size ^{b)} (kDa)	Stoichiometry ^{c)}	Migration from complex to glycan ^{d)}	Migration from complex to receptor ^{e)}
BDNF	polySia	6.4×10^{-9}	2000 (dimer)	0.46	n.d. ^{f)}	Yes
	HS	1.4×10^{-9}	670 (dimer)	3.1	n.d. ^{f)}	Yes
FGF2	polySia	1.5×10^{-8}	5000 (monomer)	4.5	Yes	No
	HS	2.8×10^{-8}	440 (dimer)	4.0	No	No

^a Determined by SPR based methods. The sensor was immobilized with glycan and analyte was neurologically active molecule.^b Determined by gel filtration. The oligomerization state of neurologically active molecule was determined by cross-linking experiments.^c mol of molecule/mol of glycan.^d Migration of the neurologically active molecule in the polySia (HS) complex towards HS(polySia).^e Migration of the neurologically active molecule (BDNF or FGF2) in the polySia (HS) complex towards TrkB or FGFR.^f n.d., not determined.

measurements, light scattering studies, fluorescence correlation spectroscopy, and surface force apparatus-based measurements were performed. It was demonstrated that the attachment of polySia onto NCAM doubles its hydrodynamic radius, and that the magnitude of repulsion also depends on the amount of polySia on the membrane (Rutishauser, 2008). NCAM usually interacts with other NCAM molecules, homophilically, but heterophilically with other CAMs (L1, Tag-1), with receptors such as FGF receptor (FGFR), with glial cell line derived neurotrophic factor family receptor alpha 1 (GFR1), and with ECM (collagen, heparan sulfate proteoglycan (HSPG), and chondroitin sulfate proteoglycan CSPG) (Gascon et al., 2007). Therefore, polySia-NCAMs inhibit cell-cell or/and cell-ECM interactions, via their repulsive field (Fig. 4A). The counterparts of NCAM, including trans-NCAMs, are involved in a variety of biological signaling, including cell migration, fasciculation, axonal guidance and branching, and synaptogenesis. Therefore, polySia is considered to function by adjusting the cell-cell interface, to regulate the strength of signaling. Recently, the repulsive force of polySia, as part of polySia-NCAM, was visualized using surface plasmon resonance (SPR) (Mori et al., 2017). Based on these results, the repulsive field of polySia was only observed in polySia synthesized by ST8SIA2, but not by ST8SIA4. Interestingly, polySia-NCAM was shown to bind to polySia-NCAM synthesized by ST8SIA4, indicating that the repulsive field is regulated by the polySia structure synthesized by polySTs.

5.2. Formation of attractive field

As described above, polySia shows an extremely large hydration effect and its physical properties provide it with a large exclusion volume to inhibit interactions mediated by polySia-NCAM. Until 2008, polySia was considered to function only as an anti-adhesive molecule (Rutishauser, 2008), and the only unique function of polySia was to display a repulsive field in trans- or cis-interactions. Therefore, although polySia expression was observed in the context of many biological activities, it was always considered that its activity was due to anti-adhesive properties. However, in 2008, polySia was clearly proven to specifically bind to a relatively small and neurologically/biologically active molecule: brain-derived neurotrophic factor, BDNF (Kanato et al., 2008). Since then, polySia has been demonstrated to bind to various neurologically/biologically important molecules and there is an attractive field around polySia-expressing regions (Fig. 4B) (Sato, 2013; Sato et al., 2016; Sato and Kitajima, 2013b) (Sato and Kitajima, 2013a, 2019). The molecular binding properties of polySia were investigated by horizontal native-PAGE, gel filtration, SPR measurements, ELISA, and frontal affinity chromatography. Since then, the paradigm of how polySia functions has changed. PolySia displays two different types of functional field at the same time, regulating the intercellular space and regulating

neurologically important molecules. It is time to reconsider the real functions of polySia in cell migration, neurogenesis, axon guidance, fasciculation, cell proliferation, learning and memory, and social interactions that have been shown to be related to neurologically active molecules, such as neurotrophins, growth factors, and neurotransmitters. All of these polySia-related biological functions have previously been considered to be due to its anti-adhesive effect. Now, following the discovery of new molecular binding properties, polySia can be evaluated biochemically and functionally, as described in the sections on disease.

5.2.1. Neurotrophic factors

Neurotrophic factors (NTs) are involved in neural cell survival and neural development. They function by binding to specific receptors (Trk and p75NTR) in the central nervous system (CNS) and peripheral nervous system (PNS). NTs are classified into three groups: the neurotrophin family, transforming growth factor (TGF) family, and ciliary neurotrophic factor (CNTF) family (Sato, 2017). Among these, the relationship between the neurotrophin family and polySia has been well studied. BDNF is the first molecule shown to bind to polySia directly, by several investigative methods, such as horizontal native-PAGE, gel filtration (Kanato et al., 2008), and SPR-based measurement (Ono et al., 2012). The binding of polySia to BDNF dimers is dependent on the DP, and the minimum DP required for BDNF-binding is 12 (Kanato et al., 2008). This was the first report of the biological significance of DP of polySia, although the concept had been reported previously (Sato, 2004). BDNF-polySia binding is specific and occurs not because the protein is basic, but because polySia has a specific array of negative charges (Sato and Kitajima, 2019). Interestingly, the binding properties of BDNF to polySia and GAGs are completely different, although the K_D s are very similar (i.e., 6.4×10^{-9} M (polySia-BDNF) and 1.4×10^{-9} M (HS-BDNF)). The DPs of polySia and HS, the binding sites of BDNF, and the complex formation with BDNF are all different. The properties of these interactions are shown in Table 1. BDNF in the BDNF-polySia complex (10^{-9} M) can easily migrate toward its receptors, TrkB (10^{-12} M toward BDNF) or p75NTR (10^{-10} M, toward BDNF), probably depending on the K_D values between molecules (Kanato et al., 2008). PolySia was shown to serve as a reservoir of BDNF molecules for enabling their efficient supply to TrkB and p75^{NTR}, which are located close to the polySia-NCAM complex. The release of BDNF from the polySia-BDNF complex was also demonstrated, using a microglial cell line (Sumida et al., 2015) which contains polySia. This was shown to hold BDNF, based on the observation that BDNF was released by Endo-N treatment. After activation of microglial cells, using LPS, the release of BDNF within the culture supernatant was observed, due to the secretion of sialidase Neu1 on the exosome (Sumida et al., 2015). Another function of polySia is the regulation of the production of BDNF via a protecting protease (Hane et al., 2015). PolySia not only binds to mature

BDNF, but also to proBDNF ($K_D = 1.3 \times 10^{-9}$ M). However, it does not bind to the pro-domain at all. Interestingly, the cleavage of proBDNF by plasmin (to produce mBDNF) was inhibited in the complex with polySia, but not in the complex with HS (Hane et al., 2015). This phenomenon shows that the reservoir function of polySia may be involved in the regulation of BDNF/proBDNF activities in hippocampal cells, via protecting the molecule from external protease. Notably, in mice that cannot convert proBDNF to BDNF effectively, a low concentration of BDNF was observed in the hippocampus as a result, and they showed phenotypes associated with "depression" (Koshimizu et al., 2009).

The underlying molecular mechanisms of several polySia-related biological functions remain unknown. However, the link between biochemical analysis, physiological analysis, and animal analysis is complete in the case of LTP (long term potentiation) regulation in CA1, during learning and memory. In 2000, BDNF was shown to rescue the impaired LTP in the CA1 region of the HP, derived from NCAM-KO mice (Muller et al., 2000). It was also shown that LTP, normally observed in the CA1 region of the HP derived from wild-type mice, was inhibited after the addition of colominic acid or polySia-NCAM, although the mechanism of the action remained unknown until 2008. The demonstration of direct binding between BDNF and polySia provided proof of the phenomenon (Kanato et al., 2008). In addition, polySia was demonstrated to be a good scaffold material for the supply of BDNF.

5.2.2. Growth factors

FGF is a family of growth factors which stimulate the growth of various cell types (Itoh, 2007) by binding to receptors (FGFRs). FGF2 is a member of the FGF family, and is expressed in the brain during early stages of development, and is involved in brain formation. FGF2 promotes the cell growth and survival of fetal and postnatal cells in various areas of the brain (Gage et al., 1995) (Walicke, 1988) (Matsuda et al., 1990) (Vicario-Abejón et al., 1995) (Aoyagi et al., 1994) (Mudò et al., 2009). FGF2 is involved in the differentiation of embryonic hippocampal cells (Gage et al., 1995), the acceleration of axonal branching (Aoyagi et al., 1994), and the proliferation and differentiation of multipotent neural progenitor cells isolated from the adult SVZ (Mudò et al., 2009). FGF2 is also related to fear memory, social interaction (Graham and Richardson, 2011), and mental disorders (Fumagalli et al., 2005; Gaughran et al., 2006; Graham and Richardson, 2010; Perez et al., 2009; Turner et al., 2008, 2009). The interactions among FGF2, FGFR and heparan sulfate (HS) are well studied. HS on HS-proteoglycan (HSPG) binds to FGF2, and FGF2 in HS changes its conformation. After ternary complex formation among HS-FGF2-FGFR, the proliferative signal from FGFR is enhanced. Although the polySia is a polyanionic glycan in the brain, like HS, no study has been carried out on the possible involvement of polySia in FGF2 signaling, via direct binding to FGF2, because it was considered solely as an anti-adhesive molecule. The direct binding between FGF2 and polySia was confirmed in 2012 and it was also shown that polySia is involved in FGF signaling via direct binding to FGF2 (Ono et al., 2012). FGF2 monomers directly bind to polySia, with the minimum DP requirement of 17. A comparison of the interactions between acidic glycans and FGF2 are shown in Table 1. Like BDNF binding, the affinities of the acidic glycans towards FGF2 are very similar, and the K_D is around 10^{-8} M. However, the features of formation of the complex are completely different, indicating that the bindings have specificity. The most prominent feature is that FGF2 in the FGF2-polySia complex does not migrate toward FGFR from the complex, but instead migrates to HS (Ono et al., 2012). In addition, FGF2-polySia binding is largely related to the correct conformation of FGF2, because FGF2 after several hours of pre-incubation at 37 °C, no longer binds to polySia, although it can bind to HS after an even longer incubation (Hane et al., 2015). Because of this property, polySia is more suitable as FGF2 reservoir than HS. It is suggested that, at first, FGF2 is captured by polySia and then it is handed from polySia to HS, followed by formation of the ternary complex with FGF2-HS and FGFR for signaling. This is confirmed by an experiment using polySia(+)/HS(+), polySia(+)/HS(knock down), polySia(-)/HS

(+), and polySia(-)/HS(knock down) cells (Ono et al., 2012). It is noted that polySia-negative cells showed a sustained signal for p42/44 (Erk) phosphorylation and the polySia-positive cells showed a transient but strong peak signal, indicating that polySia affects the FGF-dependent Erk signaling pathway. This unique feature can be explained by a low-path filter effect of signaling, in which a sustained signal enhances the downstream signal, while a transient one reduces the downstream signal (Fujita et al., 2010). The interaction between NCAM and FGFR had been demonstrated. The K_D of FGFR and NCAM was reported to be approximately 1–10 μM (Kiselyov et al., 2005), and this interaction could influence FGF/FGFR signaling (Francavilla et al., 2007). NCAM is modified by polySia, in the embryonic brain, and at restricted regions in the adult brain. Once NCAM polysialylation occurs, because polySia strongly binds to FGF2 ($\sim 10^{-8}$ M), then a different proliferative signal is given. So, polySia changes the proliferative signal by regulation of FGF2 and FGFR, via attractive and (probably) repulsive fields. It is important to consider the contribution of the microenvironment caused by polySia-NCAM, NCAM, HS, and FGFR all expressing on the same cell surface because neural stem cells that show proliferative character in OB and HP have polySia on their cell surfaces. Furthermore, it is known that the K_D between polySia and FGF2 is 1.5×10^{-8} M, while that between polySia-NCAM and FGF2 is 8.8×10^{-10} M, and the dissociation rates of FGF from polySia and from polySia-NCAM are completely different. In addition, the properties of the interaction between FGF2 and polySia-NCAM are different, depending on whether they are synthesized by ST8SIA2 or ST8SIA4. All these data strongly indicate that the polySia moiety, in polySia-NCAM, shows a much higher affinity to FGF2 than does free polySia, and is highly regulated by genetic factors, such as ST8SIA2 and ST8SIA4. Therefore, it is important to comprehensively understand FGF signaling. Non-polySia-NCAM (homophilic and heterophilic interaction in cis and trans modes), polySia-NCAM (anti-adhesive and FGF2-binding properties), FGFR (NCAM, HS, and FGF2 binding properties) and FGF2 (polySia, FGF2, FGFR binding properties) should each be carefully considered. In a schizophrenia patient, the impairment of binding properties of polySia structures biosynthesized by ST8SIA2 is demonstrated and discussed below.

5.2.3. Neurotransmitters and ions

It is difficult to understand the interaction between polySia and small molecules (Molecular weight, 50–300), such as neurotransmitters and ions; the interaction was first measured using frontal affinity chromatography (FAC) analyses (Isomura et al., 2011). As estimated by FAC analyses of numerous neurotransmitters in neural systems, including histamine, acetylcholine, serotonin, catecholamines (dopamine (DA), epinephrine, and norepinephrine), and their precursors, polySia can bind directly to catecholamines, with high affinity. The K_D of DA toward polySia is estimated at $\sim 10^{-5}$ M. K_D changes sensitively, depending on the extracellular pH of the solution (Sato et al., 2010). Since no binding of polySia toward positively charged molecules, such as histamine, takes place and no binding of diSia toward DA was observed, interactions must occur between specific structures of polySia and the catechol backbone. In human neuroblastoma cells, polySia is involved in Akt signaling via dopamine receptor D2 (D₂R) (Isomura et al., 2011). Strong relationships between polySia-NCAM and DA/DR are indicated by reports that polySia is required for D₂R-mediated plasticity involving inhibitory circuits of the rat medial prefrontal cortex (Castillo-Gómez et al., 2011), that polySia is present on dopaminergic neurons, such as mesencephalic dopaminergic cells, and that polySia-NCAM is involved in target-induced morphological differentiation of arcuate dopaminergic neurons (Loudes et al., 1997).

PolySia was shown to modify voltage-sensitive Na⁺ channels in the electric eel (*Electrophorus electricus*) (James and Agnew, 1987) and an α-subunit of Na⁺ channels in the adult rat brain (Zuber et al., 1992). Although the effect of polySia on Na⁺ channels is still unknown, the polyanionic nature of polySia may be involved in the retention of Na⁺ or polySia might directly interact with the channel. There is no evidence

that polySia binds to Na^+ , but there is evidence that polySia binds directly to Ca^{2+} (Shimoda et al., 1994). Ca^{2+} is one of the most important ions for the regulation of neural and biological activities, inside and outside of cells. PolySia can both bind to Ca^{2+} . Colominic acid ($\text{DP}_{\text{av}} = 24$ and 15) but not oligoSia ($\text{DP} = 5$) bind to Ca^{2+} with an affinity of 1.4×10^4 , 1.5×10^4 , and $0.065 \times 10^4 (\text{M}^{-1})$, with n (number of binding site) = 0.3, 0.3, and 0.2, respectively, as estimated by equilibrium dialysis (Shimoda et al., 1994). Binding to Ca^{2+} was inhibited by the same amount of Mn^{2+} but not by Na^+ , although, in the presence of 0.11 M NaCl (physiological conditions), binding to Ca^{2+} was not observed. It is noteworthy that a neurotransmitter binding to polySia has been observed in physiological conditions. Both neurotransmitters and Ca^{2+} are very important to learning and memory, and polySia is involved in both of these particles and in ion-channels, as described below.

5.2.4. Cytokines

Chemokine (C-C motif) ligand 21 and 19 belong to the CC chemokine family and are involved in T cell accumulation. The binding of polySia to CCL21 was demonstrated by ELISA (Rey-Gallardo et al., 2011) (Rey-Gallardo et al., 2010). NRP-2 mediated chemotaxis of mature dendritic cells was driven by the C-terminal region of CCL21 through CCR7 (Rey-Gallardo et al., 2011) (Rey-Gallardo et al., 2010). Confirmation of the direct binding of polySia and CCL21 might be worthy of further investigation because of the high binding affinity of CCL21 to HS. CCR7 was shown to have polySia (Kiermaier et al., 2016).

5.2.5. Transcription regulated protein

Histone H1 is a basic protein and is present as a linker histone, usually localizing to the nucleus. In several types of cell, H1 has been observed extracellularly, near the cell surface (Watson et al., 1999). The binding of polySia toward H1 was first shown by the use of a polySia-mimicking scFv antibody and by ELISA (Mishra et al., 2010). Later, polySia was shown to be involved in neutrophil extracellular trap (NET)-mediated cytotoxicity, through extracellular histone present as a component of (NET) (Saffarzadeh et al., 2012). It is also interesting that polySia-NCAM has been found inside the nucleus, and was shown to be involved in the regulation of circadian rhythm (Westphal et al., 2016). Cytosolic and nuclear polySia will expand the world of attractive field through binding to the transcription-regulation molecules.

5.3. Regulatory role for receptors

PolySia was reported to regulate AMPA-R and GluN2B-containing NMDA-Rs, by evidence from *in-vitro* electrophysiological methods. A direct relationship was shown between polySia-NCAM expression and AMPA-R, in immature pyramidal neurons isolated from the CA1 region of the HP (Vaithianathan et al., 2004). 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 the single-channel conductance of AMPA-R (Vaithianathan et al., 2004). Later, it was reported that treatment with polySia, or polySia-NCAM, inhibited the activation of GluN2B-containing NMDA-receptors at low micromolar concentrations of glutamate (Hammond et al., 2006). It is strongly suggested that polySia regulates GluN2B-containing NMDA-Rs. Although no direct evidence has been provided yet, polySia might interact directly with these receptors or might indirectly regulate them via the formation of membrane domains. Ion channels are very important in relation to learning and memory.

Another type of receptor that might be regulated by polySia is the sialic acid receptor, Siglec, especially inhibitory receptor Siglec-11. Siglec-11 and -16 are present on microglia in the brain (Crocker et al., 2007). In mouse and human co-culture systems, polySia ($\text{DP}_{\text{av}} = 20$) was shown to bind to Siglec-11 and inhibit immune responses, such as the inflammatory neurotoxicity of phagocytosis (Wang and Neumann, 2010).

6. Bacteria and polySia

PolySia was firstly found in bacterial polysaccharides, as described above, in neuroinvasive and pathogenic bacteria. *E. coli* K1 and *Neisseria meningitidis* are two famous polySia-carrying neuroinvasive bacteria, and their polySia capsules are shown to be a determinant of neurovirulence, associated with meningitis (Troy, 1979). These bacteria are considered to be shielded with a polySia capsule, in order to avoid detection by the human immune system. It is considered that polySia is usually recognized as "self" by the human immune system because polySia-NCAM is expressed in the host and the tolerance of polySia is naturally established. Therefore, bacteria use this unique epitope to invade human host cells (Adlam et al., 1987; Vimir et al., 2004). Expression of the polySia-glycocalyx helps neuroinvasive bacteria to penetrate the blood brain barrier and to colonize the meninges of neonates (Stein et al., 2006; Zelmer et al., 2008), although the detailed molecular mechanism is still unclear. The O-acetylation of polySia residues on capsular polysaccharide was reported in *E. coli* K1 and was demonstrated to increase the immunogenicity and invasiveness of cells into host neurons (Higa and Varki, 1988; Orskov et al., 1979), probably due to the protection of polySia against degradation by sialidase or acidic conditions. So far, Neu5Gc has not been found in bacteria.

Among diseases caused by polySia-carrying bacteria, invasive meningococcal disease (IMD) is the major and most serious disease of humans. Meningitis can be caused by several bacteria, *E. coli*, *Streptococcus agalactiae*, and *Listeria monocytogenes* (new born and early stage infant), *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* b (infant at late stage, child, and young adult) (Astronomo and Burton, 2010). Vaccination is a powerful way to protect against bacterial meningitis, and many attempts have been made to produce vaccines toward these capsular polysaccharides. The vaccines for *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (PCV and Hib, respectively) were developed and are available worldwide, especially for infants. Meningococcal infection is also caused by *Neisseria meningitidis*, which usually resides harmlessly in the human pharynx. Under certain conditions, asymptomatic carriage can progress to IMD, resulting in meningitis, fulminant septicemia or both. *Neisseria meningitidis* has 13 serogroups, based on its capsular polysaccharides, and 5 of these (A, B, C, Y, and W) comprise the major causative meningococci serogroup. The vaccines for A, C, W-135, and Y were developed and are available now. Both meningococcal polysaccharide vaccine (MPSV4) and meningococcal conjugate vaccine (MCV4) are available, although the antibody persistence produced by MCV is longer than that of MPSV. The most challenging vaccine is that for *Neisseria meningitidis* group B because the antibodies against polysaccharides from *Neisseria meningitidis* group B were not easy to obtain, although antibodies against those from *Neisseria meningitidis* group C were more readily obtained (Frosch et al., 1985). The difference is in the linkage of polySia. *Neisseria meningitidis* group B has an $\alpha 2,8$ -linked polyNeu5Ac, while *Neisseria meningitidis* group C has an $\alpha 2,9$ -linked polyNeu5Ac. The point of difficulty is the tolerance of the host, due to the structural similarity between bacterial glycan antigen and host glycans. Attempts have been made to gain a good vaccine for *Neisseria meningitidis* group B, and the breakthrough was the change from $\alpha 2,8$ -linked polyNeu5Ac to $\alpha 2,8$ -linked polyNeu5NPropionyl. Introduction of *N*-propionyl instead of *N*-acetyl to polySia, conjugated to the tetanus toxoid, succeeded in increasing immunogenicity and to produce anti-polyNeu5NPr antibody. In the case of rodents, less auto-antibody to polyNeu5Ac was observed (Jennings et al., 1986). Poly-Neu5NPr was used to evaluate the vaccine as a phase I trial in 17 healthy male volunteers, aged 18–40 years, and the authors demonstrated that a polyNeu5NPr conjugated vaccine has a good short-term safety profile and is able to induce specific IgG and IgM against polyNeu5NPr and IgM for polyNeu5Ac. However, *in vitro* testing (serum bactericidal assay, passive protection and opsonophagocytic tests) found that the sensitivity is not enough to detect functional activity (Bruge et al., 2004). Other attempts were made to develop vaccines using other components

of *Neisseria meningitidis* group B, such as the outer membrane protein factor H binding protein (fHBP) (McNeil et al., 2013). The successful outcome of these tests has led to the development of the currently available vaccine, anti-MenB (Masignani et al., 2019).

7. Cancer and polySia

PolySia is associated with various types of cancer (Colley et al., 2014). NCAM is thought to be the main carrier protein of polySia in cancer cells, although some cells do not express NCAM (Martersteck et al., 1996). Because of the expression of polySia during embryogenesis and cancer, polySia is recognized as an oncodevelopmental antigen. Neuroblastomas (Livingston et al., 1987, 1988), Wilms' tumors (Roth et al., 1988), medulloblastomas (Figarella-Branger et al., 1990), pheochromocytomas (Lahr et al., 1993), medullary thyroid carcinomas (Komminoth et al., 1994), non-small cell lung (NSCL) carcinomas (Moolenaar et al., 1990; Tanaka et al., 2001), pituitary adenomas (Figarella-Branger et al., 1990), teratomas (Metzman et al., 1991), childhood rhabdomyosarcomas (Glüer et al., 1998), malignant lymphomas (Kern et al., 1993), pancreatic cancer (Kameda et al., 1999), and breast cancer (Martersteck et al., 1996) are all shown to have polySia on the cell surface. The relationship between the expression of polySia and cancer progression was first reported for SCLC, NCI-H69-derived cell lines, E2, and F3 cells. While E2 cells did not have a polySia structure, F3 cells have a large amount of polySia on the cell surface, which is consistent with the high expression of ST8SIA2 (Scheidegger et al., 1995). E2 cells can aggregate easily, but F3 cells tend to disperse. After removal of polySia from the F3 cell, they can also aggregate. F3 cells form many more colonies than E2 cells on soft agar or in the methylcellulose test. Even *in vivo*, using nude mice, F3 cells show high metastasis, compared with E2 cells (Scheidegger et al., 1994). As polysialylation has an anti-adhesive effect on cell-cell interactions, it is likely to be involved in the detachment and metastasis of cancer cells, although the molecular mechanism is still unclear. Because of the highly invasive and proliferative features of polySia-expressing cancers, polySia-NCAM is used as diagnostic marker, not only for lung carcinoma but also other cancers, such as neuroblastoma (Cheung et al., 2006) (Korja et al., 2009) (Seidenfaden et al., 2003), glioblastoma (Amoureaux et al., 2010) (Suzuki et al., 2005), and pituitary tumors (Trouillas et al., 2003).

The regulation of polySia-NCAM expression for anti-cancer therapy, and the targeting of drugs in cancers where polySia-NCAM is expressed, are important therapeutic approaches. Therefore, inhibitors and antibodies specific to polySia-NCAM have been used as powerful tools (Roth et al., 1996). The addition of Man2NPro to basophilic leukemia cells in rats converted cell surface native polyNeu5Ac to unnatural poly-Neu5NPro (Liu et al., 2000). Natural polySia is not immunogenic (Finne et al., 1983), but unnatural polyNeu5NPro is highly immunogenic for humans. Once polyNeu5Ac is converted to polyNeu5NPro, the anti-polyNeu5NPro antibody could be used as a targeted anti-cancer drug. Antibody-dependent cytotoxicity, using anti-polySia antibody, was observed *in vitro* and *in vivo* (Krug et al., 2004, 2012). Cancer vaccines and antibody-drug conjugated therapy are reviewed in several published sources (Krug et al., 2012) (Cox et al., 2019; Heimborg-Molinaro et al., 2011).

Another therapeutic approach for regulating the polySia structure on the cell surface is to use ammonia. Ammonia is shown to regulate polySia-expression by changing the nucleotide sugar pools (Zanghi et al., 1998b), and expressions of polySia using CHO and SCLC were inhibited by ammonia (Zanghi et al., 1998a). Valproic acid (2-propylpentanoic acid, VPA) is one of candidate drugs for anti-cancer activity, because VPA downregulates ST8SIA2 mRNA expression in neuroblastoma cell lines UKF-NB-3, UKF-NB-4, BE(2)-C, and MHH-NB-11, but upregulates mRNA expression for ST8SIA4 (Beecken et al., 2005). Another chemical that regulates polySTs is CMP. As CMP acts on ST8SIA2, via product inhibition, the addition of CMP to

neuroblastoma (SH-SY5Y) and ST8Sia2-overexpressed C6 glioma cells, inhibited migration (Al-Saraireh et al., 2013).

8. Mental disorders and polySia

8.1. Schizophrenia

Schizophrenia (SCZ) is a mental disorder with multiple factors contributing to its pathogenesis. Its diagnosis is based on the Diagnostic and Statistical Manual of Mental Disorder (DSM)-5 from the American Psychiatric Association (American Psychiatric Association, 2013) or/and the International Statistical Classification of Diseases and Related Health Problems (ICD)-10 from the World Health Organization (World Health Organization, 1993). SCZ is a worldwide disease, and the socio-economic problems associated with this disease are increasing in every country. At a molecular level, the cause of SCZ is highly complex and the causes include genetic and environmental factors, acting cumulatively. The symptoms of SCZ are classified into three types: positive, negative, and cognitive symptoms (Owen et al., 2016). Positive symptoms are subcategorized into thought disorder, hallucination, and delusion. Negative symptoms show a decrease in or loss of normal activity, such as flat emotions, poor conversation, loss of motivation and continuousness, anhedonia, and social withdrawal. Cognitive symptoms are lack of retention or attention, learning and memory disorders, impairment of abstract thinking, and problem-solving disability. People with injuries to the prefrontal cortex also show negative and cognitive symptoms, and these symptoms are highly related and are considered to be caused by abnormalities in the same region of the brain, especially the prefrontal cortex. These are considered as abnormal functions in the specific area of the brain, due to abnormal brain development or abnormal denaturation processes (Owen et al., 2016) (Weinberger, 1988). In contrast, the positive symptoms are considered to be due to hyperactivity of the dopamine-related neural circuit (Owen et al., 2016), indicating an abnormality of the dopaminergic neurons (or of regulatory neurons towards the dopaminergic neurons).

The first report on the relationship between mental disorder and polySia was that the number of polySia-NCAM-immunostained cells derived from the HP of SCZ brains had decreased, in comparison with normal brains (Barbeau et al., 1995). Later, decreased polySia-NCAM expression was reported at layers IV and V of the dorsolateral PFC of SCZ brains (Gilabert-Juan et al., 2012). Interestingly, no such decrease was observed in the AMG (Varea et al., 2012), indicating that region-specific polySia impairments are characteristic of schizophrenic brains.

It is well known that SCZ aggregates in families, and causative genes for SCZ have long been suggested and studied (Millar et al., 2000) (Tandon et al., 2008b). Recently, causative factors have been considered from the twin viewpoints of genetic risk factors and environmental risk factors. In the case of SCZ, around 50% of total causation is genetic and 50% environmental, based on adopted offspring and mono- and di-zygotic twin studies (Tsuang et al., 1991). Onset of the disease is the result of accumulating these causative risk factors. An early stage of the study was the hunt for causative genes, but no single gene has been found for this disorder. Thus far, from the results of genome-related studies, several risk genes have been reported. *DISC1* (Disruption of the schizophrenia 1) (Devon et al., 2001) was first identified in Scottish families. This gene is disrupted in a t(1; 11)(q42.1; q14.3) translocation which segregates with SCZ and has been related to mental disorders, such as bipolar disorder (BD) and major depressive disorder (MDD) in a large Scottish family. This gene encodes a scaffold protein and, based on interactome analyses, several signaling molecules and microtubules were identified: NDEL1 (Nuclear distribution element-like 1), 14-3-3, GSK3 β , Girdin, PDE4 (phosphodiesterase 4) (Lipina et al., 2012), CCDC88A, PCM1 (Pericentriolar material 1), BBS4 (Bardet-Biedl syndrome 4), KAL7, and TNIK (Bradshaw and Porteous, 2012). *DISC1* SNPs are considered to lead to various disorders due to the disorganization of

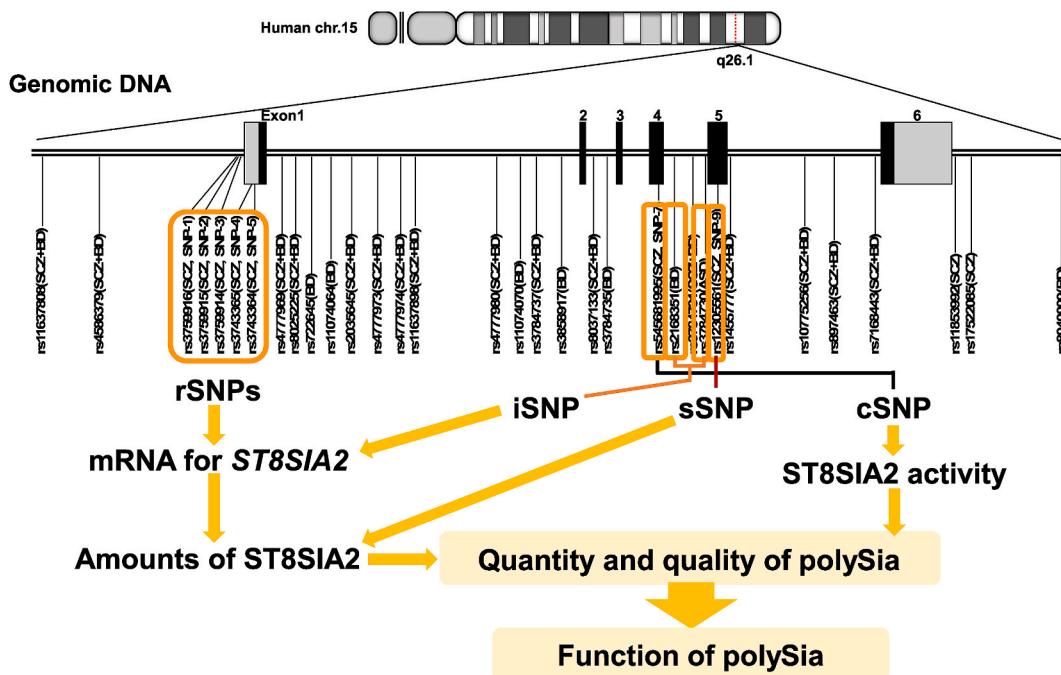


Fig. 5. SNPs of the ST8SIA2 gene and biochemical analysis of the 4 types of the SNPs. The ST8SIA2 gene is located on chromosome 15q26. All four types of SNPs (mis-sense mutations, cSNPs; silent SNPs, sSNPs; regulatory SNPs, rSNPs; and intronic SNPs, iSNPs) of the ST8SIA2 gene are found in SCZ and BD patients. Black boxes indicate the coding region and gray boxes indicate the untranslated region of each exon. The biochemically analyzed SNPs are shown with the results of the change of SNPs after comparing with the results derived from normal ST8SIA2.

cytosolic proteins, which leads to impairment of axon and neural migration. Proteins NDEL-1 (Kamiya et al., 2006) and 14-3-3 (Taya et al., 2007) are known to be important for axon regulation and neuronal migration. Gamma-amino butyric acid (GABA) is an important molecule for the excitatory-to-inhibitory (E/I) balance which regulates normal brain function. The imbalance of E/I is one of the phenotypes of SCZ. The disorders are thought to be caused by glutamic acid decarboxylase (GAD67) (Akbarian and Huang, 2006) encoded by *GAD1*, a key enzyme involved in the synthesis of GABA, and NRG1 (neuregulin 1) (Stefansson et al., 2002) which is involved in the regulation of GABA inhibitory neurons via binding to ErbB4. Other striking causative genetic factors are dopaminergic genes, such as *Akt-1* (Joo et al., 2009), *COMT* (for catechol-O-methyltransferase) (Nicodemus et al., 2007), and *DRD4* (for dopamine receptor) (Allen et al., 2008). Because of the importance of dopamine concentration and signaling in the PFC and the ventral tegmental area (VTA), dysregulation of DA is considered to lead to disorders. Recently, a deletion of chromosome 22q11 has focused attention on this region of the genome (Tandon et al., 2008a).

Regarding the relationship between one of the polysialyltransferase genes (*ST8SIA2*) and SCZ, it was first reported that chromosome 15q26, which is the genomic region where the gene encoding ST8SIA2 localizes, was related to SCZ and BD in the population of Eastern Quebec (Maziade et al., 2005). Later, from genome-wide studies among Japanese populations, a relationship was observed between SNPs in the promoter region of *ST8SIA2*, rs3759916 (SNP-1) and rs3759914 (SNP-3), and SCZ (Arai et al., 2006) (Fig. 5). Based on the results of promoter assays, using a human neural cell line, the risk haplotype (T-G-T-G-C/SNP1-2-3-4-5) was found to be associated with higher promoter activity than the protective haplotype (C-G-C-G-C/SNP1-2-3-4-5), indicating that overexpression of *ST8SIA2* may occur in brain. This result is contradictory to a previous report of polySia immunostaining cells in human SCZ brain tissue (Barbeau et al., 1995). One study reported, in rodents, that the overexpression of *ST8SIA2* induced apoptotic cell death of neurons in the HP (Brocco et al., 2003). Our recent promoter assay, including these risk haplotypes with several neuronal and non-neuronal cells, showed that the promoter activity of *ST8SIA2* is cell-type dependent. Interestingly,

the migration of embryonic neural cells from the SVZ was inhibited by the overexpression of *ST8SIA2*, indicating that phase formation of the PFC should be impaired (Hane et al., unpublished results). All of these results are consistent with the observations from human SCZ brains (Gilabert-Juan et al., 2012). Later, several SNPs from the promoter region of *ST8SIA2*, were revealed to be associated with SCZ in populations from China (Tao et al., 2007) and Spain (Gilabert-Juan et al., 2013b). Some of these SNPs were shown to be associated with BD-I. All these data indicate that the highly regulated expression of *ST8SIA2* is important for normal brain function. In Arai's report (Arai et al., 2006), other interesting SNPs, SNP-7 (rs545681995) and SNP-9 (rs2305561), were shown. In a schizophrenia patient whose parents and a brother were all diagnosed with SCZ, rs545681995 led to a mis-sense mutation (cSNP), and a single amino acid change (E141K). SNP-9 (rs2305561; c621g) is a silent SNP (sSNP), which leads to a change of codon for proline, from ccc (most abundant) to ccg (less abundant). rs2305561 was reported not to have a significant effect in SCZ patients, in a Japanese population. However, an association was found between rs2305561 and SCZ, in a Spanish population (sex-dependent), based on a dominant model of inheritance, although the association was not significant, according to Bonferroni correction for multiple testing. The statistical analyses are useful but more important is the evaluation by biochemical analyses of the gene.

The rs545681995 reported from an SCZ patient, was the first SNP of the gene *ST8SIA2* to be critically and fully evaluated by biochemical studies. SNP-7 (E141K) is located between PBR and PSTD, which are both involved in NCAM-polysialylation. *In vitro* and *in cell* enzymatic activities of *ST8SIA2* (SNP-7) were significantly reduced, to just 20% (Sato and Kitajima, 2019). Precise analyses of the quantity and quality of the soluble type of polySia-NCAM-Fc, synthesized by normal and SNP-7 enzymes, were evaluated. The polySia-NCAM synthesized by *ST8SIA2* (SNP-7) was significantly impaired in terms of quantity, DP and negative charge (Sato and Kitajima, 2019) (Isomura et al., 2011) (Fig. 5). In addition, both repulsive and attractive fields of polySia-NCAM-Fc synthesized by *ST8SIA2* (SNP-7) were compared with that from normal *ST8SIA2* and both functional fields were impaired, as evaluated by the

SPR and FAC methods. Especially, the attractive fields (molecule binding properties) of polySia for BDNF, FGF2, and DA are completely impaired in polySia-NCAM-Fc synthesized by the SCZ-type protein (Isomura et al., 2011) (Hane et al., 2012) (Hane et al., 2015) (Mori et al., 2017) (Fig. 5). It is noteworthy that not only the binding amounts of FGF2 and BDNF, but also the K_D and k_{on} , especially for BDNF-binding with polySia-NCAM synthesized by ST8SIA2 (SNP7) are changed. This is strong evidence that the functional polySia epitope is impaired and this leads to the impaired supply of BDNF to the surroundings, which is important for many functions and is potentially involved in the development of SCZ directly and indirectly. As mentioned, because polySia can regulate many molecules at the same time, the impairment of polySia leads to multiple effects on the functions regulated by these neurologically active molecules, such as BDNF, FGF2, and DA. All these biochemical lines of evidence strongly support the molecular basis of polySia-involved phenomena: (i) polySia enhances neural cell proliferation; (ii) polySia, but not HS, primarily regulates the FGF2-driven cell proliferation via its molecule binding function; (iii) polySia regulates the Akt signals through DA; and (iv) polySia-NCAM is involved in BDNF-dependent cell survival, differentiation, and the LTP of CA1. In addition, the decrease of polySia expression observed in the HP and PFC, in SCZ brains, is consistent with the results obtained by the biochemical approach using SNP-7. The evaluation of SNP-9 (rs2305561; c621g) gave further interesting results. As the amino acid was unchanged, it was predicted that the biochemical results would not be influenced. However, this SNP also impaired the quality and quantity of polySia and functions of polySia, indicating that the SNP-9 might be functional, although the change was milder than that of SNP-7 (Isomura et al., 2011). In addition, this SNP is not significant in Japanese populations but was later shown to be significant in Spanish SCZ males and in other BD patients, as described above, indicating that the slight change in ST8SIA2 might influence the product and its effects in a population-dependent manner. Biochemically, SNP-9 is a silent SNP, but the codon pool for Pro was changed from major to minor and the speed of ST8SIA2 translation might be changed, due to the codon usage ratio being altered from the highest to the lowest.

Anatomically, the volume of OB derived from schizophrenic brains is reduced (Turetsky et al., 2003), which is a similar phenotype to that of *Ncam*-KO mice (Cremer et al., 1994). This phenotype was observed in *Ncam*-KO mice during migration of neural precursor from the SVZ, through the RMS, to the OB, and is believed to result from a requirement for polySia function. The functional impairment and disturbed organization of the HP are also observed in the SCZ brain, although brains with other mental disorders show the same phenotypes (Harrison, 2004). In this regard, it is interesting that the loss of *ST8Sia2* or *Ncam* results in the misguidance of infrapyramidal mossy fibers and formation of ectopic synapses in the HP (Angata et al., 2004). These changes to LTP and the HP are closely implicated in impairments to learning and memory, and cognitive deficits are commonly observed in SCZ patients (Salavati et al., 2015). *Ncam*-, *ST8Sia2*-, *ST8Sia4*-KO mice showed impaired LTP, with different features. Interestingly, the impairments of spatial learning (Cremer et al.), circadian rhythms (Shen et al., 1997, 2001), and social interactions (Calandreau et al., 2010), observed in SCZ patients and other mental disorders, were frequently observed in polySia-impaired mice. Precise neuroanatomical analyses of *ST8Sia2* and *ST8Sia4*-KO mice and their littermates, toward SCZ endophenotypes, were performed by Krocher et al. (Krocher et al., 2015). They found a size reduction in the thalamus, accompanied by a smaller internal capsule. In addition, they found that the mice showed a highly disorganized pattern of fibers connecting the thalamus and cortex. The *ST8Sia2*- and *ST8Sia4*-KO mice showed impaired short- and long-term recognition memory. Interestingly, impaired working memory and deficits of prepulse inhibition were only observed in *ST8Sia2*-KO mice. In addition, anhedonic behavior and amphetamine-induced hyperlocomotion were also observed. *ST8Sia2*-KO mice show SCZ-like phenotypes (Krocher et al., 2015). *Ncam*-KO mice were also demonstrated to be useful for

studying specific endophenotypes related to SCZ, although these mice do not display typical SCZ-like phenotypes (Albrecht and Stork, 2012).

The contribution of environmental factors to the development of SCZ has been demonstrated. In the antenatal period, parental environment is important. Infection of the mother (influenza and toxoplasmosis) and nutritional deficiency of the mother during the first and early second trimesters of pregnancy were shown to be related to an increased risk of SCZ development (Tandon et al., 2008a). Maternal stress, higher paternal age, trauma, head injury, parental separation, migration, adverse child rearing, infection in childhood, cannabis abuse during adolescence, social adversity, and a stressful lifestyle have all been shown to increase the risk of developing SCZ. However, none of these environmental risk factors appears sufficient or necessary to cause SCZ. The relationship between environmental factors and polySia expression has been demonstrated. A double-hit model, by administration of MK-801 (NMDA receptor antagonists) and post-weaning social interaction, was used as an animal model for causation of SCZ (Gilabert-Juan et al., 2013a). The model rodents showed reduced mPFC and HP volumes, reduced polySia-NCAM expression in the mPFC and in the *stratum lucidum* of the HP. Acute stress is known to be an important environmental factor, causing several mental disorders, and study of the effect of acute stress on polySia expression has also been made in mice (Abe et al., 2019). Reductions in both the quantity and quality of polySia expression in the OB and PFC were observed after a 7 min exposure to acute stress, but expression was recovered within 3–24 h. This was the first observation of acute change of polySia expression in animals. The reduction was due to the action of sialidase. Microglia in the OB and astrocytes in the PFC are involved in the secretion of sialidase. All these data suggest that the quality and quantity of polySia is highly regulated and, therefore, abnormal expression of polySia or impairment of its recovery to normal can increase the risk of SCZ.

Regarding the therapeutic aspects, the effect of chlorpromazine (CPZ) on polySia expression has been reported (Abe et al., 2017). CPZ is a well-known first-generation medicine for schizophrenia that has been shown to be effective in reducing the positive symptoms, which are considered to be due to excess DA signals in patients. The pharmaceutical mechanism of CPZ involves reduction of DA signals, due to inhibition of D₂R (Miyamoto et al., 2012). Interestingly, reduction of polySia in the PFC is reported in SCZ patients (Gilabert-Juan et al., 2012). However, the effects of CPZ on polySia expression in human cells and animals remain unclear. One report showed that CPZ treatment in polySia-expressing human neural cells increased cell-surface expression (Abe et al., 2017). In mice, the administration of CPZ led to upregulated expression of polySia only in the PFC, and unaltered expression in other areas. This may lead to ideal conditions for the emergence of the positive symptoms of SCZ, which are closely related to DA neurons. To this point, upregulation of polySia expression in the PFC, by CPZ, may contribute to the treatment of the positive symptoms of SCZ. The measurement of fine-tuned polySia-NCAM might be important marker for this treatment.

8.2. Bipolar disorder (BD)

Bipolar disorder (BD) is an important mental disorder, affecting 1% of the world population, irrespective of nationality, ethnic origin, or socioeconomic status. BD represents one of the leading causes of disability among young people. BD is a lifelong episodic illness with a variable course, that often results in functional and cognitive impairment and a reduction in quality of life (Grande et al., 2016). BD is a severe, chronic mood disorder, characterized by episodes of mania, hypomania, and alternating or intertwining episodes of depression.

BD is one of the most heritable mental disorders. However, multiple risk factors, genetic risk factors and environmental risk factors, are involved. Many risk alleles have a small effect and sometimes overlap with those for SCZ and other mental disorders. For example, *DISC1*, *NRG1*, and *BDNF* (Gutiérrez-Fernández et al., 2014; Kerner, 2014) were shown to be related to BD. The involvement of *ST8SIA2* is also reported

to be a generalized susceptibility marker for psychotic and mood disorders on chromosome 15q25-26 (Park et al., 2004). *ST8SIA2*, SNP-2 (rs3759915) and SNP-1 (rs3759916) were shown to be associated with SCZ in a Chinese population and in a Spanish population, respectively. In addition, many SNPs were shown to be associated with Korean SCZ patients (Yang et al., 2015). The interesting point is that these SNPs were also shown to be associated with bipolar 1 disorder (BD-I). Sometimes, the phenotypes and genotypes overlap between SCZ and BD, and the same neural circuit might be involved. Expression analysis of *ST8SIA2*, from adult postmortems, showed lower expression of *ST8SIA2* in the dorsolateral PFC of BD and SCZ patients (Gilabert-Juan et al., 2012), indicating that this brain area might also have lower polySia-NCAM expression levels in these patients. In one report, an increased expression of polySia was observed in the AMG of BD patients (Varea et al., 2012). To this point, one interesting intronic SNP (rs2168351), related to BD patients, was biochemically evaluated (Hane et al., 2016) (Fig. 5). Using mouse and human neural cells, an iSNP (rs2168351) changed the level of pre-mRNA for *ST8SIA2*, *ST8SIA2*, and polySia-NCAM. This could explain the AMG case, although the underlying mechanism of action remains unknown. Therefore, further studies of rs2168351, the only nucleotide changed in the 6-kbp long intron 4, need to be carried out because this mutation clearly affects polySia expression.

8.3. Autism spectrum disorder (ASD)

Autism spectrum disorder (ASD) is considered to be a developmental disorder that affects communication and behavior, characterized by (early) onset in the first two years of life. ASD patients may have difficulties in social communication, displaying little interest in others, restricted interests, repetitive movements, and difficulties in communication through spoken language (Constantino and Charman, 2015). It is also noted that ASD patients are too sensitive or insensitive toward sensory stimuli, such as light and sound. Autism is known as a spectrum disorder based on the wide range of symptoms derived from the dysfunction of brain structure. ASD is considered to be influenced largely by genetic factors. The accumulation of SNPs or mutations in the causative genes and/or impairment of epigenetic regulation results in altered brain structures (Bourgeron, 2015). The causative genes, such as *SHANK3* (SH3 and multiple ankyrin repeat domain 3), *CHD8* (chromo-domain helicase DNA binding protein 8), and *TBR1* (T-box brain 1) are well documented in the literature (Liu and Takumi, 2014). In anatomical analyses, insufficient development of PFC and Purkinje cells in the cerebellum are reported (McKimm et al., 2014) and also impairments of the cortex, pons, and limbic area (Itahashi et al., 2015; Lai et al., 2015). The impairment of a wide range of components of the network might be the cause, but the underlying mechanism of the cause is still unclear.

Valproic acid (VPA) exposed rats, which are a model for ASD, showed decreased polySia staining in the mPFC and HP, especially CA3, where early structural and molecular changes, coincident in time with the behavioral alterations, were observed (Codagnone et al., 2015). Prenatal exposure to VPA, in mice, led to abnormal polySia expression in the HP (Natori et al., 2008).

In terms of polySia-related genes, a GWAS of 1558 ASD families (4712 subjects) from the database of the Autism Genome Project (AGP) consortium, which consists of more than 50 centers in North America and Europe, demonstrated, by exploratory analysis, that a SNP (rs3784730) of *ST8SIA2* was associated with ASD. However, the SNP was not found to be significant, after correction for multiple tests of diagnostic groups and sub-phenotypes (Anney et al., 2010). One child diagnosed with ASD and epilepsy was then found to have a heterozygous 520-kb deletion on chromosome 15q26.1 (chr15:90,517,962–91,039,825; NCBI36/hg18), where three genes, *ST8SIA2*, *C15orf32*, and *FAM174B*, are located (Kamien et al., 2014). The functions of *C15orf32* and *FAM174B* are still unknown, although *C15orf32* is predicted not to express in the brain. Later, the father of the child was also found to have the same deletion (Kamien et al., 2015), although, based on his

self-report, he was not diagnosed with ASD or epilepsy. Interestingly, he did have a history of attention deficit hyperactivity disorder (ADHD), which shares a common phenotypic spectrum with ASD. PolySia might be involved in the common phenotype between ASD and ADHD. There are likely to be several genetic risk factors other than a single causative gene, and also environmental factors, which might contribute to the development of ASD. The child in this study was in a chaotic home environment until the age of 18 months, which could have contributed to the development of ASD. The effect of rs3784730 on *ST8SIA2* has been critically evaluated (Hane et al., 2016) (Fig. 5). The mRNA and the protein *ST8SIA2* derived from rs3784730 SNP were both reduced in human cells, compared to that of wild-type, indicating that a change might occur in the brain. It is noteworthy that the *ST8Sia2-KO* mice show hyperactivity and aggressiveness (Angata et al., 2004; Calandreau et al., 2010).

9. Neurodegenerative disorders

9.1. Parkinson's disease (PD)

Parkinson's disease (PD) is a neurological degenerative disorder with evolving layers of complexity. It leads to shaking, stiffness, and difficulty with walking, balance, and coordination (Kalia and Lang, 2015). Symptoms of PD gradually progress until the patients are suffering not only from difficulty in walking and talking but also mental and behavioral changes, including sleep problems, depression, memory impairment and fatigue. One risk factor of PD is age. The development age of PD in most patients is around 60, although a small percentage show early onset, before age 50. It is not always inherited but some cases are considered to be linked with specific gene mutations, such as *SNCA* which encodes α -synuclein, *LRRK2* which encodes leucine rich repeat kinase 2, *parkin* which encodes Parkin, and *GBA* which encodes β -glucuronidase, the lysosomal enzyme deficient in Gaucher disease. The majority of PD cases have no common genetic cause, and there are many environmental risk factors, including exposure to pesticides, consumption of dairy products, history of melanoma, and traumatic brain injury (Ascherio and Schwarzchild, 2016). An important point is that there are also factors which convey a reduced risk, including smoking (nicotine), caffeine consumption, higher serum urate concentration, physical activity and the use of ibuprofen and other common medications (Ascherio and Schwarzchild, 2016). The crucial pathology of PD is the loss of dopaminergic neurons within the SN, which leads to a shortage in DA, an important chemical neurotransmitter for motor response. Moderate to severe dopaminergic neuronal loss, within the area which contains neurons projecting to the dorsal putamen of the striatum, is considered to cause motor features of advanced PD, such as bradykinesia and rigidity. An important hallmark of PD is Lewy pathology, the aggregation of abnormally folded proteins. A key protein, α -synuclein, is misfolded, becomes insoluble, and the aggregates form intracellular inclusions within the cell body, named Lewy bodies. Lewy pathology is not restricted to the brain but is also found in the peripheral nervous system.

The relationship between PD and polySia was first reported in 2005 (Yoshimi et al., 2005). The authors tried to stain polySia using SN section and anti-polySia antibodies (12E3) from human, monkey and rodent. PolySia was stained clearly in substantia nigra (SN) in humans and rodents. The polySia-positive cells were occasionally co-immunostained with TH (tyrosine hydroxylase), although this was rare. Although no difference was observed in the number of polySia-positive cells in the SNc of patients with different conditions, probably due to the dense polySia-positive fibers, the cell numbers in PD tended to be higher than those of the disease control group ($p < 0.06$, two-tailed t-test). Similar results were observed using monkeys which, six months after MPTP infusion into the left caudate, showed less TH staining and more staining of polySia. In addition, in rats deprived of using dopaminergic neurons by applying 1-methyl-4-phenylpyridinium salt to left sphere, TH

staining in the left was reduced and polySia was increased. Later, close analysis using human brains showed no significant difference in middle temporal gyrus, superior frontal gyrus, the caudate nucleus or HP sub regions (Murray et al., 2016).

The pathology of the PD is the loss of dopaminergic neurons within the SN, as described, so the treatment of the PD is focused on the dopaminergic neurons. PolySia-related treatment has been attempted to establish engraftable, authentic midbrain DA-neurons *in vitro* from human embryonic stem cells, as cell replacement therapy, in models of PD (Battista et al., 2014). Authors successfully established cells, enhancing the polySia by overexpressing *ST8SIA4*, and these (*ST8SIA4*-modified DA/Nurr1 neurons) showed three important improvements: more efficient amelioration of PD-associated behavior, better cell survival, and enhanced neurite outgrowth of TH-positive processes into host tissues.

9.2. Alzheimer's disease (AD)

Alzheimer's disease (AD) is another major neurodegenerative disease and one of the great healthcare challenges of the 21st century (Scheltens et al., 2016). Amyloid β and tau, the main components of plaques and tangles, respectively, are two main target molecules for AD therapy (Scheltens et al., 2016).

As for the relationship between AD-related brains and polySia, the first report was published in 1999 (Mikkonen et al., 1999) and the author found polySia-immunoreactivity to be increased in the HP formation of AD patients, particularly in the outer two thirds and the inner third of the molecular layer of the DG. The author also found that the subiculum and entorhinal cortex (EC) of AD patients showed disorganization of polySia-immunoreactive fibers. It was the first observation of polySia staining in EC. Then, increased expression of immature neuronal marker proteins, such as doublecortin (DCX), polySia-NCAM, TUC-4, and NeuroD (neurogenic differentiation factor) in the HP of AD brains was reported, with immuno-histochemical localization to hippocampal sites of neurogenesis at DG and of AD pathology at CA1 (Jin et al., 2004). Interestingly, polySia-NCAM expression increased with severity of disease. The increased expression of polySia at high Braak stages, especially at the SGL and the GCL, but not at the SVZ of the lateral ventricle, was also reported (Perry et al., 2012). In both areas, polySia was significantly higher in Braak stage VI compared to I-III. The polySia staining at layer II/III and V of the EC was decreased, especially at layer II, which is severely affected by the disease (Murray et al., 2016). The authors reported that a decrease in the number of polySia-expressing interneurons in the EC of AD was particularly observed in the subpopulation of parvalbumin-expressing NeuN positive interneurons (Murray et al., 2018). This result may be related to the dysregulation of the neuronal network, which contributes to cognitive decline observed in AD patients, even before the appearance of clinical symptoms.

Another region of neurogenesis is the OB system and the relationship between OB neurogenesis and AD has been investigated by a comparison of brain tissues between normal mice and Tg2576 mice (Guérin et al., 2009). Tg2576 mice, expressing the Swedish mutation of the human Amyloid Precursor Protein, were the animal models for AD (Hsiao et al., 1996). They usually develop an age-dependent elevation of $\text{A}\beta$, with onset at 8 months of age and with $\text{A}\beta$ -containing plaques occurring in the neocortex and HP by 10–16 months, and impairments of spatial and fear memory at 9–10 months. The author analyzed polySia-NCAM expression, especially in the OB (Guérin et al., 2009), because functional olfactory deficits associated with the earliest symptoms of AD were considered to be related to an alteration of neurogenesis, induced by a dysfunction of the LC/NA (*locus coeruleus/noradrenaline*) system (Hawkes, 2003; Mesholam et al., 1998; Murphy, 1999). Interestingly, Tg mice showed decreased polySia-expression in the OB.

For the clinical trial, several chemicals to improve cognition were reported and the expression of polySia was analyzed. The chronic administration of chemical reagents tacrine, nefiracetam, and deprenyl,

enhances cholinergic function and increases the basal frequency of dentate polysialylated neurons. This is similar to the improved neuroplasticity achieved through complex environment rearing in rats (Murphy et al., 2006). Memantine, classified as an NMDA receptor antagonist, is clinically used for the treatment of AD patients and promotes adult neurogenesis (Jin et al., 2006). When the effects of this reagent were tested (Maekawa et al., 2009), memantine treatment decreased polySia expression in the 50 mg/kg memantine-injected group at 7 days after BrdU-injection. The authors considered that the speed of maturation of newly generated neurons was different in the controls because, at day 28, the level was the same. The duration of the effect or dosage might also be important variables for study. T-817MA [1-{3-[2-(1-Benzothiophen-5-yl)ethoxy] propyl}-3-acetidinol maleate] (Kimura et al., 2009) is a chemical reagent for AD treatment, with neuroprotective effects against toxicity from the $\text{A}\beta$ peptide and activity in promoting neurite outgrowth *in vitro* (Hirata et al., 2005). The administration of T-817MA improved cognitive dysfunctions caused by neurodegeneration, in a rat model of AD. The reagent significantly increased hippocampal neurogenesis and improved spatial learning impairments. At the same time, the decreased expression of polySia, assessed by staining in AD model mice injected with $\text{A}\beta$, was recovered to the control level (Kimura et al., 2009). Recently, exosome-based treatment, using mesenchymal stem cell-derived exosomes, delivered enhanced neural plasticity and improved cognitive impairments. AD model mice were established by the administration of $\text{A}\beta$, and showed impaired memory and learning. The subsequent administration of mesenchymal stem cell-derived exosomes increased learning and memory function, with increased adult neurogenesis in SVZ, evaluated by polySia staining (Reza-Zaldivar et al., 2019).

9.3. Huntington's disease (HD)

Huntington's disease (HD) is a progressive brain disorder which causes uncontrolled movements, emotional problems and cognitive deficits that eventually lead to dementia. It is considered to be a fatal, hereditary disorder caused by the *HTT* gene (huntingtin). The patients usually have an expanded number of CAG repeats in the gene. The first sign of HD is the impairment of cognition and olfaction. Reduced neuronal plasticity in the striatum, HP and neocortex is a common feature of HD model mice.

The relationship between HD and polySia was first reported in 2007 using R6/1 and R6/2 HD model mice (Lazic et al., 2007; van der Borgh and Brundin, 2007). The R6 mouse lines express exon 1 of the human huntingtin gene. R6/1 mice and R6/2 mice, having 115 CAG repeats and 150 CAG repeats, respectively, develop many of the pathological features of HD, such as synaptic dysfunction, motor problems, and cognitive deterioration. With age, the number of polySia immunostained cells was decreased in the HP, in both WT and HD mice, and the R6 line showed a reduced number of polySia staining cells, all compared to their respective controls. The observation of a decline in polySia in HP happened at an early stage, before the granule cells showed any inclusions. In layer II of the primary olfactory (piriform) cortex, the age-related decline in polySia immunostaining was observed in both wild-type and HD mice but the decline was more profound in the case of HD mice, although the total cell number was the same between WT and R6/2 mice. In R6/1 mice and human HD patients, the transcripts of *ST8SIA3* that can synthesize oligoSia but not polySia were shown to be greatly reduced (Desplats et al., 2007). Later, close analysis of *ST8Sia3-KO* mice showed that adenosine A_{2A} receptor (A_{2A}R) and dopamine D₁ and D₂ receptors (D₁R and D₂R) were oligosialylated, and this is related to the localization of these receptors in lipid rafts and regulation of motor functions in the striatum (Lin et al., 2019).

9.4. Ischemia

Stroke is one of the most common causes of death and disability in

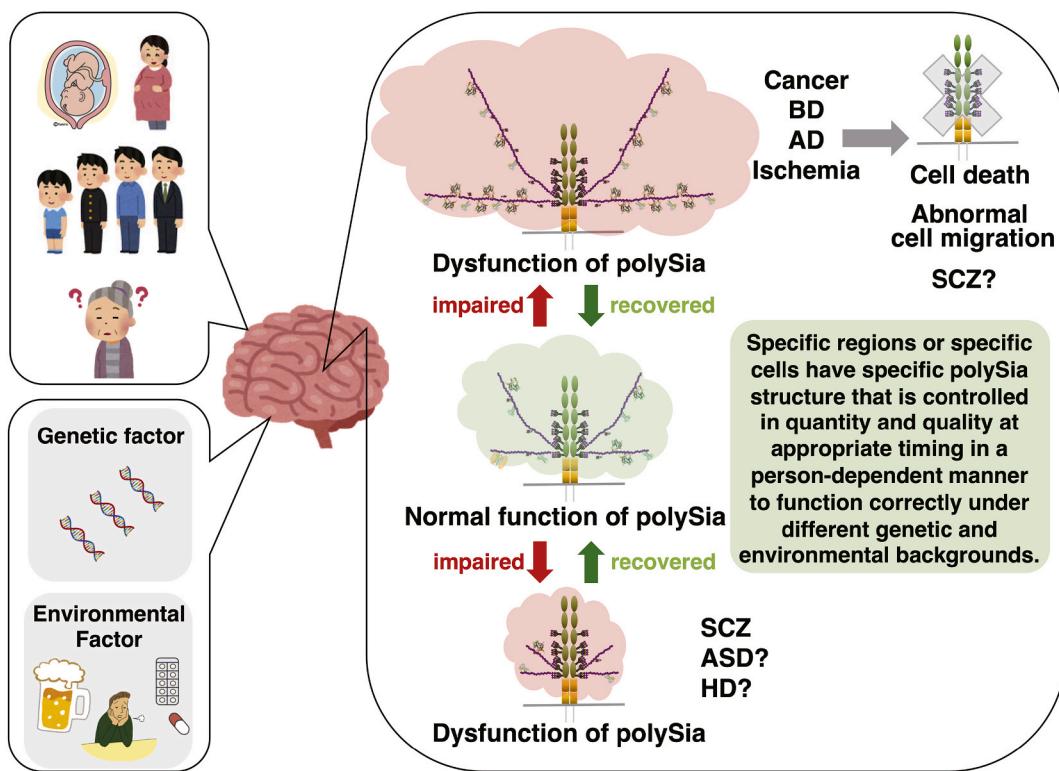


Fig. 6. Hypothetical relationships between polySia expression and diseases. PolySia expression is highly regulated by genetic factors (not only the ST8SIA2 gene but also other causative gene factors) and environmental factors. The effects of these causative factors are different between person to person, depending on the state of the person. If the polySia expression, which is usually regulated in quantity and quality at appropriate timing in specific cell types, is impaired (red arrows), then the functional fields (both repulsive and attractive fields) are impaired (red area). Therefore, dysfunction of polySia influences its own cells and the surrounding cells. The abnormal polySia state can sometimes recover to normal state (green area), probably due to several factors, including medicine, enriched environment, and food (green arrow); with respect to the recovery, the genetic background of persons might be relevant as well. Too much polySia leads to either cell death or abnormal cell migration, resulting in the mispositioning of polySia-expressing cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the world, and occurs when blood vessels are interrupted by a blood clot (thrombus) or when blood vessels rupture (hemorrhage) due to arteriovenous malformation or aneurysm.

The relationship between the polySia-expression and ischemic brain was reported using model animal mice, by carotid artery occlusion. The delayed cell death of pyramidal cells in CA1 was observed 5–7 days after occlusion. Interestingly, at 1–2 days post-occlusion, there was a significant increase of polySia-immunostained granule cells, then a subsequent decrease at day 5. In addition, the author observed a substantial increase in glial polySia staining in all HP regions at 1–7 days. Administration of a glutamate antagonist significantly blocked these phenomena (Fox et al., 2001). The extent of increased polySia staining differed, depending on the age and conditions (Sato et al., 2001). Not only mice but also primates, including humans, have shown an increased expression of polySia after ischemia (Macas et al., 2006).

For cell based therapy, the transplantation of human embryonic stem cells (Kim et al., 2014) and a bone marrow mesenchymal stem cell line (Shiota et al., 2018) have been used to treat ischemic brain damage in stroke. PolySia-positive transplanted cells promote neural tissue integrity and improve behavioral performance in a rat stroke model.

10. Application of polySia and polySia probes in medical fields

The first application of polySia or polySia-related molecules for a medical treatment was the production of vaccines for neuroinvasive bacteria, such as *Neisseria meningitidis* group B and C as described above. The vaccine for *Neisseria meningitidis* group C, in which polySia was used as its major component, was successfully developed. However, for the anti-*Neisseria meningitidis* group B vaccine, great efforts were made on

production, without complete success, and now the target has changed to the factor H protein. The underlying problem is the tolerance of α 2,8-linked polySia by human tissues. Owing to polySia, cancer cells having polySia on cell surface can also escape from the host immune system. Antibodies to the polySias can be used for cancer therapy or drug delivery. As the polySia is largely ignored by the host defense system, we noticed that polySia can be used as a safety material. Currently in the early stages, trials have been established for polySia-modification onto serum proteins, such as asparaginase (Fernandes and Gregoriadis, 1997) and EPO (Meng et al., 2018), to lengthen their useful duration in circulation. Recently, polySia with DP = 20 was shown to be a good material for the treatment of age-related macular degeneration (AMD), based on the observation that sialic acid polymers prevent ROS production of human mononuclear phagocytes, via the inhibitory SIGLEC11 receptor (Karlstetter et al., 2017). The author discussed whether polySia can be used as a reagent to prevent AMD-related inflammation and angiogenesis, after laser-induced damage in the retina. More recently, liposomes carrying polySia are being considered in cancer therapy (Liu et al., 2018). Another aspect to consider is cell/tissue-based polySia regulation. Several reagents, or environmental and genetic factors, as described above, can change polySia expression in a region-specific manner. Regulation of region-specific polySia expression is the key to successful therapy, but we first need to fully understand the basic molecular features of polySia and polySia expression. Enriching the environment is also an interesting factor to be considered for therapy. After exposure to an enriched environment, ST8Sia4-KO mice with a LTP deficit in CA1 recovered and increased the number of polySia-NCAM-expressing neurons in the DG (Zerwas et al., 2016).

11. Conclusion

The expression of polySia is restricted to certain bacteria and vertebrates. PolySia-containing bacteria are highly pathogenic and neuro-invasive. These bacteria cover their surface with polySia to protect themselves from vertebrate immune systems as well as to facilitate their invasion through the blood brain barrier. The use of polySia by the bacteria is pertinent to the fact that vertebrates cannot avoid using the epitope because, like sialic acid itself, polySia is an essential component. Interestingly, the diversity of polySia structure becomes less as we go from invertebrates to vertebrates; for example, human exclusively contains a polymer of Neu5Ac with α 2,8-linkage, except for the DP, while echinoderms contain a variety of polySia structures with diversities in the Sia component, DP and linkages. The DP of polySia synthesized by human polysialyltransferases is the longest of those synthesized by vertebrate enzymes, thus indicating that longer polySia might be of higher quality.

In addition to the merit of long DP, the importance of restricted DP should also be emphasized, because polySia specifically binds with BDNF and FGF2 in a DP-dependent manner. As for the quantity, the amount of polySia is highly regulated, specific to the brain region. Collectively, all these data indicate that the polySia structure is highly regulated in quantity and quality as well as in localization and timing. Since it is highly regulated by genetic and environmental factors, the state of polySia may change in various diseases. So, one can easily imagine why polySia is so frequently associated with diseases. Once polySia structures are impaired in certain brain regions, they try to recover from those abnormal states. However, if abnormal states do not recover, then the functional fields of polySia, both repulsive and attractive fields, remain impaired. No recovery of the state of polySia would eventually lead to the appearance of symptoms of disease (Fig. 6). Due to its complex features in structure and function, polySia is difficult to study and manipulate. However, once we will succeed in regulating polySia expression in quantity and quality through medicinal and pharmaceutical approaches, then some of the diseases discussed in this review might be treatable and curable. In this regard, it is the most important to understand the underlying mechanisms of interactions between polySia and polySia-recognition molecules, which leads to various polySia-related functions. Unraveling of those mechanisms should be the high priority now and in the future.

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