Polysialylation of NCAM

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Introduction

The precise orchestration of cell adhesion and cell communication is indispensable for development, and particularly important for nervous system wiring and plasticity. Among the numerous cell adhesion molecules of the immunoglobulin superfamily dedicated to this task, the neural cell adhesion molecule NCAM stands out as a developmentally regulated switch in its pattern of glycosylation, which fundamentally alters its biophysical properties and, as a consequence, its binding abilities. The glycan responsible for the conversion of different NCAM isoforms from an interactive to an anti-adhesive state is a linear hompolymer of α2,8-linked *N*-acetylneuraminic acids called polysialic acid (polySia,[1] Fig. 1). The large negatively charged and highly hydrated structure can extend beyond the protein core and double the hydrodynamic radius of the extracellular part of NCAM, thereby increasing the intermembrane space and disrupting the adhesive properties of NCAM and other cell adhesion molecules [1–3]. As highlighted by several recent reviews, polySia is a prominent regulator of neural cell migration and differentiation during nervous system development, and tightly associated with neurogenesis and synaptic plasticity in the adult brain [4–8]. Here, we briefly review the patterns of polySia expression during ontogenesis and its potential role in tumor progression before discussing its regulation by the two polysialyltransferases ST8SiaII and ST8SiaIV, as well as the latter's the individual and combined impact of these enzymes on NCAM polysialylation during brain development.

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[1] The most commonly used abbreviation for polysialic acid in neuroscience is PSA, but in tumor biology, PSA stands for prostate specific antigen. To avoid confusion, we therefore prefer to use polySia to abbreviate polysialic acid.

Developmental Regulation of NCAM Polysialylation

As first described 25 years ago, the hallmark of NCAM polysialylation is its regulation during development [9, 10]. Although essentially confined to the nervous system development and plasticity, polySia is transiently expressed in mesodermal and endodermal derivatives during organogenesis [11, 12]. NCAM does not carry polySia during the time of its first appearance on embryonic day 8–8.5 in the mouse, but shortly thereafter, polysialyated NCAM becomes predominant, reaching its maximum in the perinatal phase [13–15]. As shown by Western blot analyses of whole brain lysates, polySia expression keeps pace with the rapid increase in brain weight until day 9 of postnatal development, and almost all of the NCAM is polysialylated [16]. Subsequently, polySia drops rapidly, by approximately 70% within 1 week, accompanied by the first occurrence of polySia-free NCAM-140 and NCAM-180, the two transmembrane isoforms (see Fig. 1) that are the major
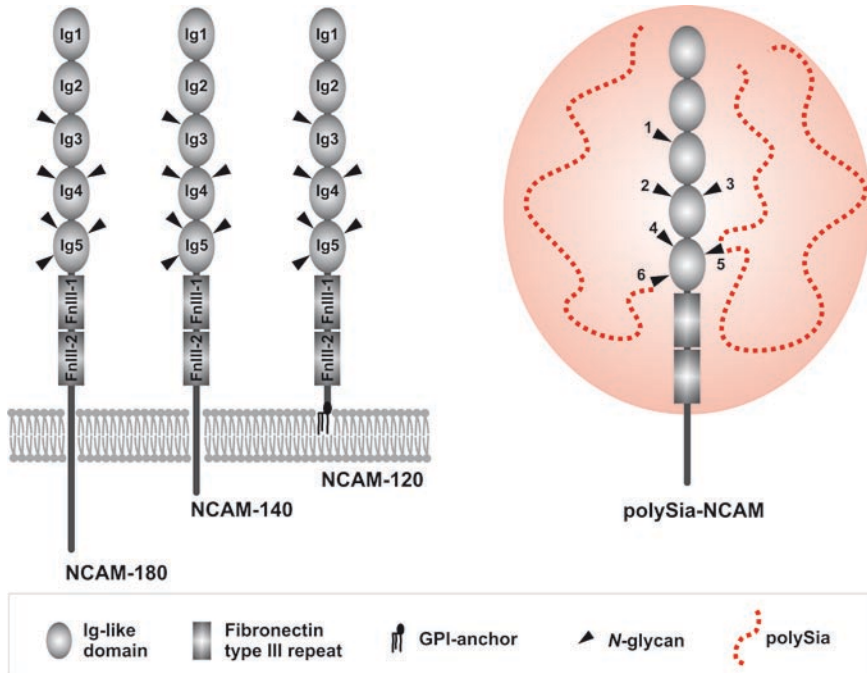

Fig. 1 Scheme of the three major NCAM isoforms (*left*) and the polysialylated form of NCAM (*right*). The extracellular part of NCAM is composed of five immunoglobulin(Ig)-like domains and two fibronectin type III (FnIII) repeats. NCAM-180 and NCAM-140 are transmembrane proteins which differ in the length of their intracellular part, whereas NCAM-120 is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. NCAM is a glycoprotein containing 6 *N*-glycosylation sites. In the polysialylated form of NCAM (polySia-NCAM), the *N*-glycans located at the 5th and 6th *N*-glycosylation site are modified by one or more polySia chains. The hydrodynamic radius of polySia is depicted as a shaded sphere

polySia carriers in mouse brain. By contrast, glycosylphosphatidylinisotol-anchored NCAM-120, the characteristic isoform of mature oligodendrocytes, is devoid of polySia during its massive upregulation in the early postnatal brain, which is associated with the onset of myelination [16]. Thus, the time-course of polySia downregulation and the dramatic increase of polySia-free NCAM coincide with the completion of major morphogenetic events within the first 3 weeks of postnatal brain development. However, as reviewed in great detail elsewhere, the expression of polysialylated NCAM persists into adulthood and is maintained at sites of ongoing neurogenesis or plasticity [5, 17]. In the absence of any known polySia-specific degrading enzyme to modulate polySia on the surface of vertebrate cells, the state of NCAM polysialylation depends predominantly on the biosynthetic pathway. This is underscored by the observation that the largely overlapping expression patterns of the polysialyltransferases ST8SiaII and ST8SiaIV are closely correlated with polySia immunoreactivity (see [18] for review). In addition to transcriptional control, polysialylation of NCAM is likely to be regulated by nontranscriptional mechanisms. As shown in the developing chick, polySia synthesis depends on calcium from intracellular compartments [19] and based on pharmacological and correlative studies, a protein kinase C-dependent regulation of polysialyltransferase activity has been suggested [20, 21]. Moreover, experiments with cultured neurons and insulin secreting β-cells indicate the possibility of a rapid mobilization of polysialylated NCAM to the cell surface by an activity- and calcium-dependent mechanism suggesting regulation of an exocytotic pathway [22]. Similarly, regulated exocytosis may contribute to the activity-dependent modulation of polySia required for hippocampal synaptic plasticity [23].

Re-expression of Polysia in Tumors

Although polySia is diminished in the majority of tissues during development, various tumors are known to re-express polySia [24–26]. Among the polySia-positive tumors are small cell and non-small cell lung carcinomas, multiple myeloma, Schwann cell tumors, pituitary tumors, Wilms' tumor, rhabdomyosarcoma and neuroblastoma [27–36]. A comparative study carried out with isogenic cell lines expressing NCAM with or without polySia identified polySia as a modulator of the malignant potential of small cell lung carcinoma [37]. The occurrence of polySia seems to facilitate the detachment of tumor cells from the primary tumor and presumably promotes the invasion and metastatic potential of these tumors [36–40]. As indicated by results obtained in vitro, polySia supports the undifferentiated state of tumor cells [41, 42]. In patients with neuroblastoma or rhabdomyosarcoma, high polySia serum levels have been correlated with poor prognosis and polySia itself as well as the transcript level of the polysialyltransferase ST8SiaII were suggested as molecular markers to monitor metastatic neuroblastoma [31, 32, 43, 44]. On the other hand, nonpolysialylated NCAM suppresses tumor progression in xenografted tumor cells and correlates inversely with malignancy [45–47]. Thus, polySia

represents an oncodevelopmental antigen, which significantly contributes to tumor growth and metastasis.

PolySia Biosynthesis

Biosynthesis of polySia is catalyzed by two Golgi resident enzymes, the polysialyl-transferases ST8SiaII and ST8SiaIV (formerly named STX and PST, respectively, Fig. 2) [48–51]. Both enzymes show 59% identity on the amino acid sequence level and share the typical features of eukaryotic sialyltransferases. They are type II transmembrane glycoproteins with a short *N*-terminal cytoplasmic tail, a transmembrane domain, a stem region, and a large C-terminal catalytic domain that resides in the lumen of the Golgi apparatus. The catalytic domain includes three consensus sequences called sialylmotifs L, S, and VS that are found in all mammalian sialyl-transferases and are involved in substrate binding [52, 53]. Although ST8SiaII and ST8SiaIV are typical members of the sialyltransferase family, they are unique with respect to their catalytic ability to synthesize polySia, i.e. α2,8-linked sialic acid
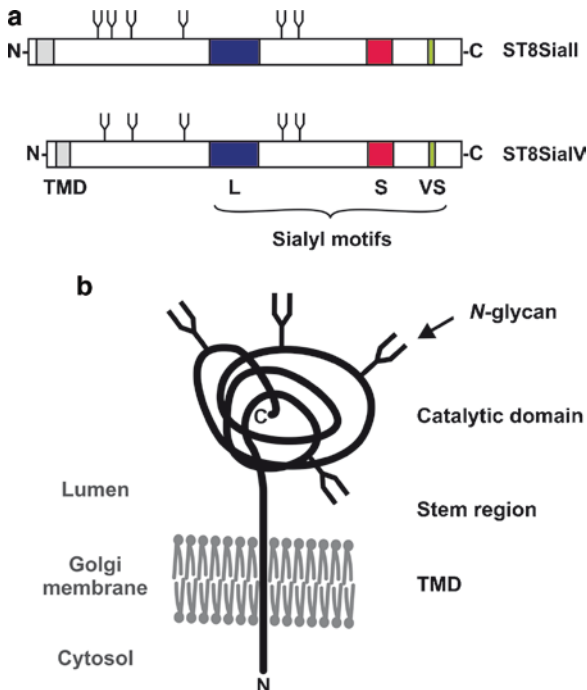

Fig. 2 (**a**) Schematic representation of the polysialyltransferases ST8SiaII and ST8SiaIV showing the transmembrane domain (TMD) and the sialylmotifs large (L), small (S), and very small (VS) of the catalytic domain. The relative positions of the *N*-glycans are indicated by Y-shaped symbols. (**b**) Type II transmembrane topology of polysialyltransferases

polymers which can exceed 50 residues [15, 54–56]. In accordance with this, only sialic acid oligomers with ≤7 residues were observed in ST8SiaII/ST8SiaIV double deficient mice [15]. Since no mammalian sialyltransferase has been crystallized so far, insight in the structural and/or mechanistic differences between mono-, oligo-, and polysialyltransferases is missing.

In contrast to most glycosyltransferases, which modify glycan structures irrespective of the carrier protein, the polysialyltransferases ST8SiaII and ST8SiaIV are highly selective for NCAM, which is by far the predominant polySia acceptor. Besides NCAM, a limited number of other polysialylated proteins have been described including the α-subunit of the voltage-gated sodium channel in rat brain [57], the scavenger receptor CD36 in human milk [58], neuropilin-2 on human dendritic cells [59], and the polysialyltransferases themselves, which can polysialylate their own *N*-glycans in a process termed autopolysialylation [60–62]. However, the complete loss of polySia in the brain of ST8SiaII/ST8SiaIV double deficient mice indicates that in the central nervous system polysialylation of potential alternative acceptor molecules also depends on ST8SiaII and ST8SiaIV activity [63]. Future studies are required to elucidate the enzyme responsible for polysialylation of these molecules. With regard to NCAM, both enzymes ST8SiaII and ST8SiaIV have been shown to catalyze the transfer of multiple α2,8-linked sialic acid residues to terminally α2,3- or α2,6-sialylated galactose residues that are bound in α1,4-linkage to *N*-acetyl glucosamine [64, 65]. Although NCAM carries six *N*-glycosylation sites, the addition of polySia is restricted to *N*-glycans at the 5th and 6th site which are located in the 5th Ig-like domain (Fig. 1) [66–68]. Structural analysis of polysialylated *N*-glycans of NCAM revealed complex structures with a high degree of heterogeneity. PolySia was found on di-, tri-, and tetraantennary glycans that were, in part, additionally modified by fucose, sulfate and uronic acid residues [67–71]. These findings indicate that the pronounced acceptor specificity of polysialyltransferases is not mediated by the recognition of a particular glycan structure, but by the specific interaction with the NCAM protein core. Crystallization of the first FNIII domain of NCAM revealed a unique acidic surface patch and a novel α-helix between β-strand 4 and 5, two structural motifs essential to allow polysialylation of the *N*-glycans in the adjacent 5th Ig-like domain [72]. In accordance with a specific enzyme-acceptor protein interaction, in vitro studies demonstrated that both ST8SiaII and ST8SiaIV polysialylate *N*-glycans attached to NCAM with a much higher efficiency than isolated *N*-glycans released from NCAM [73, 74]. The majority of polysialylated NCAM glycans in perinatal mouse brain was found to carry two polySia chains [56]. However, incomplete diantennary *N*-glycans with only one polySia polymer as well as a small proportion of glycans that appeared to carry three or even four chains were also observed [56]. This highlights that the number of polySia chains per NCAM molecule can vary. At this level, heterogeneity depends not only on the polysialyltransferases but also on the glycosylation machinery, which determines the number of polySia acceptor sites on NCAM, i.e. the number of terminally sialylated antennae provided by the *N*-glycans at site five and six (Fig. 3).
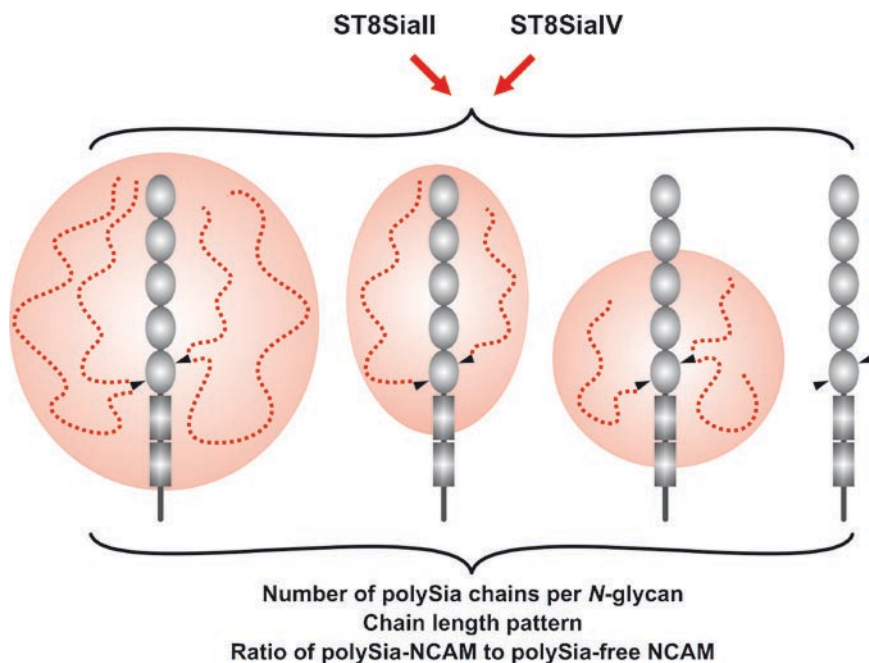
Fig. 3 Potential for variability in NCAM polysialylation. The polysialylation pattern of NCAM is defined by the interplay of ST8SiaII and ST8SiaIV and can vary with respect to the number of polySia chains per NCAM, the length of each individual polySia chain, and the ratio of polysialylated to polySia-free NCAM

Transfection and in vitro experiments unequivocally demonstrated that ST8SiaII and ST8SiaIV are individually able to synthesize polySia on NCAM [48–51, 64, 73], provoking the question why NCAM polysialylation is mediated by two enzymes. In vitro analyzes using soluble polysialyltransferases lacking their transmembrane domain revealed distinct differences between ST8SiaII and ST8SiaIV. Under the in vitro conditions used, ST8SiaII produced shorter polySia chains than ST8SiaIV and appeared to be less efficient in NCAM polysialylation [75, 76]. If both enzymes worked together, a synergistic effect was observed, yielding higher numbers of polySia chains and a higher degree of polymerization [65, 75]. Using N-glycosylation site mutants of NCAM, Angata et al. observed that ST8SiaIV strongly preferred the sixth over the fifth N-glycosylation site, whereas this preference was only moderate for ST8SiaII [65].

To understand the impact of ST8SiaII and ST8SiaIV in vivo, genetic mouse models lacking either ST8SiaII or ST8SiaIV were generated, which show only partial loss of polySia [77, 78]. The biochemical analysis of the polySia pattern in perinatal brain of polysialyltransferase-deficient mice revealed striking differences in the ability of the two enzymes to polysialate the complete NCAM pool and highlighted that, in contrast to the in vitro findings, ST8SiaII but not ST8SiaIV is

much more efficient in NCAM polysialylation. Whereas in wild-type and ST8SiaIV-null mice almost all NCAM is kept in the polysialylated state at postnatal day one, 45% of the brain NCAM was found polySia-free in ST8SiaII-deficient mice [15]. The quality of the polysialylated NCAM, however, was remarkably similar in all three genotypes [56]. Independent of the enzyme setting, *N*-glycosyation sites 5 and 6 were almost completely polysialylated and the same set of heterogeneous *N*-glycans served as polySia acceptors, excluding differential glycan acceptor specificities for ST8SiaII and ST8SiaIV. In vivo, ST8SiaII and ST8SiaIV are both able to synthesize polySia chains with up to 90 sialic acid residues [56]. However, at the fifth *N*-glycosylation site, loss of either enzyme resulted in slight alterations of the chain length pattern and the highest amount of long polySia chains was found in the presence of both enzymes. Thus, in line with the in vitro findings a synergistic action in the synthesis of polySia with a high degree of polymerization was observed, although in vivo, this effect is restricted to the fifth *N*-glycosylation site [56]. A comprehensive study of the NCAM polysialylation pattern in perinatal brain of mice with variant allelic combinations of ST8SiaII and ST8SiaIV demonstrated that alterations in the expression of the two polysialyltransferases affect the total amount of polySia, the chain length distribution, the ratio of polysialylated to polySia-free NCAM, and the amount of polySia per NCAM molecule [15]. Thus, the degree of NCAM polysialylation can be precisely adjusted by alterations in the ST8SiaII and ST8SiaIV level (Fig. 3).

Although the data are not consistent in all details, there is a close correlation between polySia immunoreactivity and the combined mRNA expression of polysialyltransferases [14, 33, 79–82]. Despite considerable overlap, there are marked differences in tissue- and time-specific mRNA expression patterns suggesting an independent regulation of ST8SiaII and ST8SiaIV at the transcriptional level. Most notably, ST8SiaII is predominant during embryonic development, while ST8SiaIV is the major polysialyltransferase of the adult brain [16, 79, 81, 82]. In contrast to earlier Northern blot analyzes indicating manifold higher ST8SiaII levels in the embryonic and perinatal phase [14, 81, 82], recent studies using real-time quantitative RT-PCR determined that the ST8SiaII transcript level in perinatal mouse brain is less than twofold higher than the level of ST8SiaIV [15, 16]. The factors responsible for the joint but sometimes distinct regulation of the two polysialyltranserases are largely unknown. Initial investigations of the proximal promoter regions of the polysialyltransferases provided first evidence for specific regulatory elements [83–85]. As shown in human tumor cells two drugs, retinoic acid and valproic acid, are able to differentially affect polysialyltransferase mRNA levels [86, 87] and elevated polySia levels due to overexpression of the developmentally regulated transcription factor Pax3 could be assigned to a specific increase of ST8SiaII mRNA [88]. However, the data available so far are not sufficient to explain how the spatial and temporal expression patterns of ST8SiaII and ST8SiaIV are regulated.

In contrast to the mouse system, expression analysis, gene-targeted knockdown experiments, and in vitro catalytic assays indicate that in the developing and adult zebrafish ST8SiaII is the major, if not the only enzyme capable of performing NCAM polysialylation [89]. The evolutionary divergence of ST8SiaIV in bony-fish

supports the assumed functional loss of ST8SiaIV [89]. Despite the very low expression levels of ST8SiaIV reported by Marx et al. [89], a recent study describes partially overlapping expression domains of ST8SiaII and ST8SiaIV throughout the mature zebrafish brain [90]. Evidently, the function of ST8SiaIV in zebrafish remains to be elucidated.

Phenotype of Polysia-deficient Mice

The finding that, at least in mammals, both polysialyltransferases can partially compensate for each other is reflected by the mild but distinct phenotypes of mice lacking only one polysialyltransferase [77, 78]. In perfect agreement with the pre-dominance of ST8SiaII during embryonic and early postnatal development, ST8SiaII-deficient mice display neurodevelopmental defects manifesting in the aberrant topology of hippocampal mossy fiber projections [78]. In contrast, and consistent with the prevalent expression of ST8SiaIV in the adult, the lack of ST8SiaIV gives rise to markedly impaired synaptic plasticity in the CA1 subregion of the hippocampus without detectable morphological defects [77]. In extension of this finding, a prominent role of polySia in regulating ionotropic receptor functions involved in long-term potentiation and memory formation was unraveled [91–94].

Since NCAM is the major carrier of polySia in mammalian brain development, mice with genetic ablation of NCAM are almost completely devoid of polySia [95]. While the overall brain architecture of these mice is surprisingly normal, two major morphological aberrations have been described and extensively studied. One is a dramatic reduction in the size of the olfactory bulbs caused by a migration deficit of newly born olfactory bulb interneurons derived from the subventricular zone [96–99]. The other is a defective lamination of mossy fibers projecting from the dentate gyrus to the CA3 subfield of Ammon's horn [96, 100, 101]. Both pheno-typic traits must be explained by the loss of polySia and not NCAM because they could be copied by enzymatic removal of the sugar polymer leaving the NCAM protein backbone unaltered [97, 101]. It was therefore not surprising that the mal-formations found in NCAM$^{-/-}$ animals also develop in the polySia-deficient ST8SiaII$^{-/-}$ ST8SiaIV$^{-/-}$ double knockout mice [63]. Since polySia impairs not only NCAM but also other cell surface interactions [1–3], the prevailing view is that the structural deficits shared by polysialyltransferase-deficient and NCAM-knockout mice are due to aberrant, NCAM-independent cell surface interactions induced by the absence of polySia [8]. In the particularly well-studied case of precursor migra-tion toward the olfactory bulb, the lack of polySia in NCAM$^{-/-}$ mice disturbs not only contacts within the chains of migrating cells but also the interactions with their stationary environment, which is dramatically altered due to a massive astrogliosis [99]. A recent report on polysialyltransferase double-knockout mice confirmed that defective chain migration in the absence of polySia is associated with altered mor-phology of astroglia [102]. Interestingly, this study describes more widespread defi-cits of precursor cell migration during cerebral cortex development together with a

general up-regulation of the astrocytic marker GFAP in the forebrain of ST8SiaII$^{-/-}$ ST8SiaIV$^{-/-}$ mice and reveals that the absence of polySia promotes PDGF-induced differentiation of astroglia in vitro.

Considering the prominent role assigned to polysialylation of NCAM during developmental plasticity (see [8] for a recent review), the overall mild phenotype of mice lacking polySia due to NCAM deficiency was puzzling. Only after poly-Sia could be ablated independent from NCAM the vital role of polysialylation became apparent. Beyond the recapitulation of major defects in brain morphology as described for NCAM deficient mice, the block of polysialic acid biosynthesis in ST8SiaII$^{-/-}$ ST8SiaIV$^{-/-}$ double knockout animals leads to additional, far more severe defects [63]. Although born overtly normal and at the expected Mendelian ratio, the polysialyltransferase-deficient mice suffer from drastic growth retardation in the early postnatal phase and more than 80% of these mice die during the first 4 weeks. With regard to brain development ST8SiaII$^{-/-}$ ST8SiaIV$^{-/-}$ animals are characterized by a high incidence of hydrocephalus and severe malformations of a specific set of brain fiber tracts. Remarkably, all of these defects found in the ST8SiaII$^{-/-}$ ST8SiaIV$^{-/-}$ but not in the NCAM$^{-/-}$ mice are rescued by the additional ablation of NCAM demonstrating that the untimely appearance of "naked," i.e. polySia-free NCAM causes the fatal developmental phenotype [7, 63]. These findings prove that the abundant expression of polySia during development is an essential control mechanism to specifically regulate NCAM interactions.

Future Directions

Future work will aim at dissecting the different molecular and cellular mechanisms underlying the changes induced by regulating polysialylation of NCAM in vivo and at making use of this knowledge in novel experimental and therapeutic approaches. Particularly interesting is the recent progress made in animal models of peripheral and central nervous system repair by applying polySia via engineered overexpression of polysialyltransferases as well as in steering the migratory capacity of Schwann cells and embryonic stem cell-derived glial precursors [103–110]. Complementary, first studies indicate that purified or synthetic polySia may prove useful as a biocompatible, and bioresorbable material for nerve tissue engineering [111–114]. Further challenges involve testing if enzymatic degradation of polySia by target-oriented application of phage-derived endosialidases [115–117], manipulation of endogenous polysialyltransferase activity and/or the use of NCAM or polySia peptide mimetics [118–120] have the potential to affect tumor development or to trigger endogenous brain repair processes.

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