Topics in Current Chemistry 366

Rita Gerardy-Schahn
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SialoGlyco Chemistry and Biology I

Biosynthesis, structural diversity and sialoglycopathologies

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366
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SialoGlyco Chemistry and Biology I

Biosynthesis, structural diversity and sialoglycopathologies

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Preface

Nature's enormous potential for the shaping of structures is made possible by the use of sugars.[1] These molecular building blocks are unique in providing permutation capacity. From a chemical point of view, sugars are polyhydroxy-aldehydes and -ketones which, under physiological conditions, form ring structures (hemiacetals). The relative position of hydroxyl groups (OH– groups) to the plane of the cyclic scaffold determines their chemical and biological properties. For instance, sugars with distinct biological functions such as glucose and galactose differ chemically in no more than the relative positioning of a single OH– group. Moreover, sugars are multivalent and, because of the anomeric freedom of the reducing end, can generate α- or β-linkages to any one of several positions on a second monosaccharide. Thus, the theoretical number of distinct trisaccharides that can be built by the combination of 3 monosaccharides can reach 27,648 whereas 3 different nucleotides or amino acids can form only 6 trimers (for review see [1]).

Sialic acids are acidic nine-carbon sugars that meet all the above discussed aspects and are special because the addition of sialic acid to glycoconjugates occurs exclusively at the non-reducing end. Because of this 'outstanding' position, sialoglycoconjugates form the'communication front' of animal cells. Mandal and colleagues review this richness of the sialome with a focus on the O-acetylation of sialic acids. O-Acetylation represents a developmentally regulated modification and a marker of some cancer cells such as lymphoblasts in acute lymphoblastic leukemia.

In terms of evolution, sialic acids have an interesting history with an abundant occurrence in the deuterostome lineage and a scattered expression in bacterial pathogens. Importantly, viruses infecting these bacteria bear highly specific receptors recognizing the bacterial sialoglycans. In fact, the use of these unique viral tools in biochemistry laboratories has been of enormous value for the detection and characterization of sialoglycans, as is discussed by Jakobsson and colleagues

[1] The term is synonymously used with the term carbohydrates.

(see the chapter titled "Endosialidases: Versatile Tools for the Study of Polysialic Acid"). Moreover, as reviewed by Matrosovich and colleagues, many viral pathogens recognize and intrude on their hosts by exploiting cellular sialoglycans (see the chapter titled "Sialic Acid Receptors of Viruses").

An organ particularly rich in sialoglycans is the brain. As extensively reviewed by Hildebrandt and Dityatev, a large number of reports has demonstrated the essential nature of sialic acid for brain development and function. While the major form of sialic acid in humans is *N*-acetyl-neuraminic acid (Neu5Ac), most vertebrates, including the great apes, produce *N*-glycolyl-neuraminic acid (Neu5Gc) at similar or even higher concentrations. However, remarkably, in species where Neu5Gc represents the major sialic acid in peripheral tissues, Neu5Gc is rarely found in brain structures, thus raising the question as to whether Neu5Gc could be toxic in the brain (see the review by Davies and Varki). In the light of evolution, this tissue-selective expulsion of Neu5Gc is exciting because the gene encoding the hydroxylase needed for the conversion of CMP-Neu5Ac to CMP-Neu5Gc was lost before the emergence of the genus *Homo*. However, humans can acquire Neu5Gc from dietary sources and a number of studies have shown the presence of Neu5Gc in peripheral tissues. Whether the integration of Neu5Gc into human tissue causes major immune reactions is a matter of debate. Shilova et al. present a primary study in this volume, in which large cohorts of probands were screened for their patterns of natural antibodies. Surprisingly, only low concentrations of natural antibodies against sialoglycans were identified.

The cloning of the major components of the sialylation machinery in mammals generated new targets for the generation of knockout models which have been used to interrogate the role of sialic acids and sialoglycans in organ development and homeostasis. The lessons learned by the use of these mouse models to re-enact sialoglyco pathologies identified in humans are reviewed by Hinderlich and colleagues and Sellmeier and colleagues.

Control of sialoglycoconjugate expression in the mammalian system involves the activity of sialidases as well as anabolic pathways. The interest in this important group of enzymes was underestimated in the past but is currently escalating. This volume is directing major attention to these developments by providing a detailed review on structure-function and phylogenetic analyses prepared by Monti and Miyagi.

Considering that the outside of the animal cells is dominated by the presence of sialoglycans, it is not difficult to deduce that there must be numerous counter-receptors to decipher the information presented in the sialome. This fact requests both novel analytical techniques permitting the quantitative determination of individual glycotopes and techniques that allow a holistic monitoring of variations in the cellular sialome such as those occurring during cell differentiation or in cancerogenesis. This analytical area has generated a new research field which is reviewed in this volume by outstanding experts (Kitajima et al., see the chapter titled "Advanced Technologies in Sialic Acid and Sialoglycoconjugate Analysis" and Hirabayashi et al., see the chapter titled "Development and Applications of the Lectin Microarray"). The search for proteins (factors in general) that specifically

bind to sialoglycans is a success story that impressively demonstrates the added value of interdisciplinary collaborative research activities in the field of glycobiology. Innovative array technologies developed by synthetic organic chemists have paved the way for the search of binding molecules not only at the cellular level but also systematically. These topics are reviewed in three different chapters by Liang et al. (see the chapter titled “Sialoside Arrays: New Synthetic Strategies and Applications”), Tanaka and Fukase (see the chapter titled “Chemical Approach to a Whole Body Imaging of Sialo-N-Linked Glycans”), and Meinke and Thiem (see the chapter titled “Trypanosomal Trans-sialidases: Valuable Synthetic Tools and Targets for Medicinal Chemistry”). Last, but certainly not least, Schwadt et al. (see the chapter titled “SIGLEC-4 (MAG) Antagonists: From the Natural Carbohydrate Epitope to Glycomimetics”) focus on the specificity of sialylated carbohydrate structures and the development of sialic acid derivatives that mimic these structures. With myelin-associated glycoprotein (MAG) as an example, the development of mimetics that bind with low nanomolar affinity is described. The leads identified show great promise to be developed further to prevent the inhibitory activity of MAG on nerve regeneration.

This edition entitled SialoGlyco Chemistry and Biology I and SialoGlyco Chemistry and Biology II combines 15 chapters from distinguished authors that, together, form a unique reference book which should be of great interest to researchers and teachers.

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Functions and Biosynthesis of *O*-Acetylated Sialic Acids

Chitra Mandal, Reinhard Schwartz-Albiez, and Reinhard Vlasak

Abstract Sialic acids have a pivotal functional impact in many biological interactions such as virus attachment, cellular adhesion, regulation of proliferation, and apoptosis. A common modification of sialic acids is *O*-acetylation. *O*-Acetylated sialic acids occur in bacteria and parasites and are also receptor determinants for a number of viruses. Moreover, they have important functions in embryogenesis, development, and immunological processes. *O*-Acetylated sialic acids represent cancer markers, as shown for acute lymphoblastic leukemia, and they are known to play significant roles in the regulation of ganglioside-mediated apoptosis. Expression of *O*-acetylated sialoglycans is regulated by sialic acid-specific *O*-acetyltransferases and *O*-acetylesterases. Recent developments in the identification of the enigmatic sialic acid-specific *O*-acetyltransferase are discussed.

Keywords 9-*O*-Acetylated sialic acids · Anemia · Anti-9-*O*-acetylated sialoglycoprotein antibody · Apoptosis · Erythrocytes · Indian visceral leishmaniasis · Leukemia · *O*-Acetylated disialoganglioside · *O*-Acetylated sialoglycoproteins · Sialate *O*-acetylesterase · Sialate *O*-acetyltranferase · Sialic acids

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Abbreviations

9-*O*-AcGD3	9-*O*-Acetylated ganglioside GD3
ALL	Acute lymphoblastic leukemia
GC/MS	Gas chromatography/mass spectrometry
GP	Glycoprotein
HPLC	High performance liquid chromatography
MAb	Monoclonal antibody
$Neu4,5Ac_2$	5-*N*-Acetyl-4-*O*-acetyl neuraminic acid
$Neu5,7(8),9Ac_3$	5-*N*-Acetyl-7(8),9-*O*-acetyl neuraminic acid
$Neu5,9Ac_2$	5-*N*-Acetyl-9-*O*-acetyl neuraminic acid
$Neu5,9Ac_2$-GPs	Glycoproteins with 5-*N*-acetyl-9-*O*-acetyl neuraminic acid
Neu5Ac	5-*N*-Acetyl neuraminic acid
Neu5Gc	5-*N*-Glycolyl neuraminic acid
O-Ac	*O*-Acetyl
O-Ac-Sia	*O*-Acetylated sialic acid
SIAE	Sialate-*O*-acetylesterase, sialic acid *O*-acetylesterase
SOAT	Sialate-*O*-acetyltransferase, sialic acid *O*-acetyltransferase

1 Introduction

The generic term sialic acid defines a large family of 9-carbon monosaccharides which commonly occur as terminal residues of the oligosaccharide moiety of glycoconjugates. The diversity of sialic acids results from differential *N*- and *O*-substitutions of the two basic molecules, i.e., *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). The most common modification of sialic acids is *O*-acetylation preferentially at the hydroxyl groups of carbon C4, C7, C8, and C9, whereas that at C9 seems to be the most frequent one.

The complexity of sialic acids is further augmented by their different glycosidic linkage types (for example, α2,3, α2,6, α2,8, and α2,9) to the oligosaccharide chain. Both modification of the terminal sialic acid and the anomericity of linkage determine their functional impact in many biological interactions, such as, e.g., virus attachment, cellular adhesion, and regulation of proliferation and apoptosis.

2 Occurrence and Functions of *O*-Acetylated Sialic Acids

2.1 Analysis of O*-Acetylated Sialic Acids*

Detection and analysis of *O*-acetylated sialic acids (*O*-Ac-Sias) requires several precautions in sample preparation. Even by taking special care to avoid the loss of *O*-acetyl groups during the purification and analysis process of sialoglycans, results may lead to an underestimation of their *O*-acetylation. Exposure of *O*-Ac-Sias to moderate alkaline conditions results in migration of acetyl groups from carbon C7 to carbons C8 or C9 [1, 2]. Procedures for isolation of gangliosides often involve incubation with NaOH to remove phospholipids, which results in a complete loss of *O*-acetyl groups due to saponification.

Histochemical staining with mild periodic acid-Schiff reaction (mPAS) is a general method to detect sialic acids. *O*-Ac-Sias with acetyl groups at C-8 or C-9 do not react under these conditions, but will stain after saponification with NaOH [3]. Mild oxidation in combination with detection of liberated formaldehyde with acetyl-acetone and ammonium acetate, yielding a fluorogen, has been described as a sensitive method to determine sialic acid concentration. After removal of *O*-acetyl groups at C-8 and C-9, an increase in formaldehyde production can be observed, which allows an indirect quantitative determination of *O*-Ac-Sias [4]. Lectins from the crab *Cancer antennarius* [5] and Achatinin-H derived from the snail *Achatina fulica* [6, 7] were shown to bind to 9-*O*-Ac-Sias. Viral lectins have also been used to detect this sialic acid derivative. The influenza C virus surface hemagglutinin-esterase glycoprotein termed HEF specifically binds to 9-*O*-Ac-Sias [8–10]. Influenza C virus preparations were used to stain cells and tissues of different sources [11–18]. To avoid the use of infectious virus, the influenza C virus surface glycoprotein was expressed as a chimeric protein fused to an Fc immunoglobulin domain [19] or to enhanced green fluorescent protein [20]. The chimeric proteins are useful to remove specifically 9-*O*-acetyl groups from sialic acids [10, 19–27]. The chimeric protein was also useful as lectin: by inhibition of the esterase domain with diisopropyl fluorophosphate, a general serine hydrolase inhibitor, the lectin function of the CHE-Fc protein was employed to detect 9-*O*-Ac-Sias [19, 25, 28, 29].

In addition to lectins, monoclonal antibodies with specificity for *O*-acetylated gangliosides are available, which can be used for immunodetection and fluorescence activated cell sorting. Examples are MAb Jones [30], MAb UM4D4 [31],

MAb U5 [32], and MAb 7H2 [33]. These antibodies can also be used for immunodetection of *O*-Ac gangliosides on HPTLC plates using a method originally described by Saito et al. [34]. Most recently, a method to determine the distribution of *O*-Ac gangliosides in rat brain by matrix assisted laser desorption/ionization (MALDI) mass spectrometry imaging was published [35]. This procedure combines high resolution mass spectrometry with imaging software to map and image gangliosides with detailed structural information and histological accuracy.

A highly sensitive method to analyze *O*-Ac-Sias relies on fluorescence detection of sialic acids derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB), followed by fluorometric high-performance liquid chromatography (HPLC) [36]. For analysis of *O*-Ac-Sias the acidic hydrolysis of glycosidically bound sialic acids should be performed with propionic instead of acetic acid [37]. The presence of *O*-Ac-Sias can be determined by their respective Rf values [38]. In biological samples carboxyl groups of other compounds may result in additional peaks in chromatograms. Therefore, the presence of *O*-Ac-Sias in HPLC peaks should be confirmed by saponification, specific de-*O*-acetylation with influenza C virus esterase (for Neu5,9Ac_2) or rat coronavirus esterase for Neu4,5Ac_2, or by mass spectrometry. The position of side groups of sialic acids can be determined by coupled gas chromatography/mass spectrometry (GC/MS) [39–43]. Very recently another highly sensitive method to identify different forms of *O*-acetylated sialic acids by electrospray ionization travelling wave ion mobility mass spectrometry coupled with low-energy collision-induced dissociation was described, allowing unambiguous assignment of the position of *O*-acetylation [44].

2.2 O-Acetylated Sialic Acids in Bacteria

Sialic acids are constituents of different opportunistic human pathogens. Bacteria can obtain sialic acids by either de novo biosynthesis or by acquisition from the environment. Different Gram negative bacteria like *Escherichia coli K1*, *Neisseria meningitidis*, *and Campylobacter jejuni*, and Gram positive bacteria like Group B *Streptococcus* (GBS) can synthesize sialic acids [45]. Sialic acid uptake in other Gram negative bacteria like *Haemophilus Influenza, Pasteurella multocida*, *Haemophilus ducreyi*, and *Pseudomonas aeruginosa* from exogenous sources has recently been established [45, 46]. These bacteria use their sialic acids for a variety of different purposes that play important roles in their ability to colonize and persist in organs of their hosts. For instance, sialic acids were shown to subvert immune clearance mechanisms by restricting complement C3b deposition on its surface [47]. Once synthesized, sialic acid residues in the capsular polysaccharide or oligosaccharide of *E. coli* K1, GBS serogroup III, *N. meningitidis*, one or more of the hydroxyl groups in positions 4, 7, 8, and 9 are substituted by acetyl groups [48]. Sia *O*-acetylation and de-*O*-acetylation is regulated by the gene *neuD* and *neuA,* respectively, to reach a final level of the surface expressed *O*-Ac-Sia modification [47, 49, 50]. In addition to *neuD*, which *O*-acetylates monomeric sialic acid, *E. coli*

K1 strains harboring the prophage CUS-3 express *neuO*, a polysialic acid-specific *O*-acetyltransferase [51, 52]. Expression of *neuO* is regulated by phase variation [53]. In *E. coli* K1 strains *O*-acetylation increases immunogenicity of the K1 capsule and correlates with increased virulence in patients [54]. *O*-Acetylation of polySia increases desiccation resistance, which may favor survival in the environment [55]. In *N. meningitides* the genes for *O*-acetyltransferases are located immediately downstream of the capsule synthesis genes *siaA* – *siaD* [48] and termed *oatC* and *oatWY* [48, 56, 57].

Most bacterial *O*-acetyltransferases are characterized by a hexapeptide repeat sequence folding into a left-handed β-helix [48, 57, 58]. OatC, transferring acetyl groups exclusively onto polysialic acid joined by α2,9-linkages, apparently evolved separately and is characterized by an α/β hydrolase fold topology [56]. The presence of the sialic acids and Neu5,9Ac$_2$α2-6GalNAc sialoglycotope has been demonstrated on *P. aeruginosa* [46]. Molecular analysis of GBS serogroup III strain indicates that high levels of Sia *O*-acetylation disrupt interactions with human Siglec-9 present on neutrophils and block removal of capsular polysaccharide Sia by bacterial sialidase, but do not alter deposition of complement on its surface [47, 59]. Blocking the interaction of GBS with neutrophils increases their activation followed by increasing bacterial killing.

2.3 O-Acetylated Sialic Acids as Virus Receptors

Many viruses use sialic acids as receptors for binding to target cells, which is the critical first step of infection. Influenza C viruses bind to cells via their surface glycoprotein termed hemagglutinin-esterase-fusion (HEF) protein. These viruses bind to Neu5,9Ac$_2$ via the hemagglutinin function of the HEF protein [9, 10]. In addition, the HEF protein has a sialate-9-*O*-acetylesterase activity [8, 10]. Infectious salmon anemia viruses, another genus of the *Orthomyxoviridae,* encode a hemagglutinin-esterase (HE) surface glycoprotein which binds to Neu4,5Ac$_2$ [60]. The esterase of these viruses is also specific for Neu4,5Ac$_2$ [24]. Several coronaviruses and toroviruses also express HE proteins which interact with *O*-Ac-Sias (Table 1). A more comprehensive review of "Sialovirology" will be published in another chapter of this issue.

2.4 Functions and Biosynthesis of O-Acetylated Sialic Acids in Parasites

Extensive research for the past decade has associated 9-*O*-acetylated sialic acids with promastigotes and amastigotes kinetoplastid parasites *Leishmania sp.* [77–83]. During the disease manifestation of visceral, cutaneous, and mucocutaneous

Table 1 Viruses recognizing *O*-acetylated sialic acids

Virus	Viral sialic acid binding protein	Sialic acid recognized	Reference
Influenza C virus	HEF	Neu5,9Ac$_2$	[8–10, 61]
Infectious salmon anemia virus	HE	Neu4,5Ac$_2$	[24, 60]
Human coronavirus OC43	HE, S	Neu5,9Ac$_2$	[62–64]
Bovine coronavirus	HE, S	Neu5,9Ac$_2$	[64–69]
Hemagglutinating encephalomyelitis virus	HE	Neu5,9Ac$_2$	[70, 71]
Murine coronavirus	HE	Neu4,5Ac$_2$	[72, 73]
Murine coronavirus strain DVIM	HE	Neu5,9Ac$_2$	[74]
Puffin coronavirus	HE	Neu4,5Ac$_2$	[73, 75]
Sialodacryo-adenitis virus	HE	Neu4,5Ac$_2$	[20, 73]
Porcine torovirus	HE	Neu5,9Ac$_2$	[67, 76]
Bovine torovirus	HE	Neu5,7(8),9Ac$_3$	[67]

leishmaniasis, increased presence of 9-*O*-acetylated sialic acids is observed in virulent strains of *Leishmania sp*., indicating their probable relevance in pathogenesis. In contrast minimal or undetectable presence of 9-*O*-acetylated sialic acids on a virulent strain of UR6 also signifies the role of 9-*O*-acetylated sialoglycotope as markers of virulence [78, 81]. The function of parasite-associated 9-*O*-acetylated sialic acids for entry of promastigotes into macrophages has been demonstrated in comparison to the minimal internalization of de-*O*-acetylated promastigotes. Analysis of the sialylation during the differentiation of internalized virulent promastigotes into amastigotes indicates that 9-*O*-acetylated sialic acids not only facilitate promastigote-entry but also play a probable role in differentiation and persistence of infection [81]. Additionally, increased presence of 9-*O*-acetylated sialic acids during metacyclic stages of virulent promastigotes has pointed to the direct correlation between the association of 9-*O*-acetylated sialic acids and virulence. Apart from being a marker of virulence, 9-*O*-acetylated sialic acids also play an important role in conferring nitric oxide resistance in virulent *Leishmania sp*. having enhanced distribution of the sialoglycotope. Furthermore they also influence the intracellular survival of the parasite within macrophages and modulate the host responses in their favor as evidenced by decreased level of IL-12 and IFN-γ, the signature TH1 cytokines [81]. In contrast, macrophages show increased levels of these cytokines, when they are infected with de-*O*-acetylated promastigotes. This observation suggests that the parasite is capable of modulating the host responses via 9-*O*-acetylated sialic acids for the successful infection. Apparently they act as effective ligands whose expression supports parasite internalization, intracellular differentiation.

De novo synthesis of sialic acids usually occurs as a result of the fine-tuning of four enzymes, namely sialidase, trans-sialidase, esterase, and *O*-acetyltransferase. Extensive work from the author's group (CM) has convincingly demonstrated that these parasites lack an active machinery for the biosynthesis of this unique sialoglycotope as corroborated by the absence of activity of UDP-GlcNAc

2-epimerase which catalyzes the first step of sialic acid synthesis [77, 79]. The presence of *N*-acetyltransferase in *L. amazonensis* has indicated the presence of enzymes for acetylation [84]. However, any such claim for the presence of *O*-acetyltransferase requires the identification of the respective genes that, at present, is lacking. Direct transfer of sialoglycoproteins from the serum demands extensive study of proteomic characterization of surface proteins on promastigotes and is a subject of future research.

2.5 O-*Acetylated Sialic Acids During Development and Differentiation*

As there are multiple changes known to occur during the development of organs and tissues, glycosylation patterns are also subject to change [85]. As an example, aberrant expression of sialyltransferases can result in displacement of cells, as shown for sialyltransferase ST6GalNAc5, which is normally expressed only in brain. Expression of this sialyltransferase in breast cancer cells alleviates their migration into the brain by mediating cell passage through the blood–brain barrier [86].

In order to identify the functional impact of sialic acids, knockout mice with deleted sialyltransferase genes were created which exhibited a number of developmental changes. Knockout of sialyltransferase ST8Sia1, also known as GD3 synthase, resulted in a complete absence of b and c series gangliosides. These mice appeared to undergo normal development and had a normal life span. Double knockout mice with an additional disruption of the *GalNAcT* gene encoding β1,4-*N*-acetyl-galactosaminyltransferase, thereby expressing GM3 as the sole ganglioside, were extremely sensitive to sound stimuli even leading to sudden death [87]. Mice with a knockout of the ST6Gal1 gene exhibited tissue specific alterations in sialylation, concomitant with highly selective losses of 9-*O*-acetylation of sialic acid residues [88]. Knockout of polysialyltransferase ST8Sia4 allowed for the first time a discrimination of the roles of neural cell adhesion molecule protein and polysialic acid in neural development and synaptic plasticity [89]. Addition of polysialic acid is an important modification of the neural cell adhesion molecule NCAM, directing migration and differentiation of neuronal cells within the central nervous system [90]. Recent investigations point to a role of polysialic acids in the development of social interactions and aggression in mice [91].

Due to the fact that the understanding of the molecular functions of sialic acids in development and differentiation are just emerging, it is not surprising that the functions of *O*-acetylation of sialic acids are even less well understood. However, some examples pointing to specific roles of *O*-AcSias are available.

By expression of the influenza C virus sialate-9-*O*-acetylesterase in transgenic mice, it was shown that *O*-acetylation is a prerequisite for normal development. Mouse embryos constitutively expressing the esterase were arrested as early as in

the two cell stage [92]. In the rat nervous system the 9-*O*-acetylated ganglioside GD3 (9-*O*-AcGD3) was shown to exhibit discrete patterns during neuronal development [30]. In the fetal mouse cortex 9-*O*-acetylated ganglioside GT3 is strongly expressed and decreases to undetectable levels after birth [93]. In another study glycolipid-bound Neu5,9Ac_2 was highest in embryonic mouse brain E13, and gradually decreased until birth. Significant amounts of Neu5,9Ac_2 were found in adult mouse brain in the glycolipid fractions of the olfactory bulb, hippocampus, and telencephalon [40]. In pig brain, an increase of Neu5,9Ac_2 was observed during the maturation of the cortex and cerebellum [94]. In patients with Guillain-Barré and Fisher´s syndromes, which are manifested as acute inflammatory demyelinating polyneuropathies, antibodies against *O*-acetylated gangliosides were found [95]. For 9-*O*-Ac-GD3, roles in neuronal motility were suggested [96, 97]. Antibodies to 9-*O*-Ac-GD3 induce microtubule depolymerization in growing neurits [98], and 9-*O*-Ac-GD3 was found in point contacts of neuronal growth cones [99]. Cerebellar granule neuron migration was blocked by the 9-*O*-Ac-GD3 mAB Jones in live animals [100]. This block was also observed in GD3 synthase knockout mice [101]. Results from the latter study indicated that the inhibitory effect of mAB Jones may be caused by binding to β1-integrin.

In chicken erythrocytes *O*-Ac-Sias represent a differentiation marker, which appears in 6-day-old birds and is fully developed in 20-day-old chickens [102]. During the early stages of human development, 9-*O*-AcGD3 is present in different tissues. In contrast, during the erythropoiesis, 9-*O*-AcGD3 level is decreased during maturation in the erythroid progenitor cells in bone marrow. Mature erythrocytes show lower 9-*O*-AcGD3 levels than immature cells.

Alterations of membrane characteristics and morphology occur in mature erythrocytes via 9-*O*-AcGD3 mediated signaling [103]. Such signaling via 9-*O*-AcGD3 also induces membrane alterations, vesicularization, phosphatidyl serine exposure, and activation of cysteine proteases like caspase 3, suggesting a programmed cell death like pathway in mature erythrocytes. In contrast, enhanced level of 9-*O*-AcGD3 is observed in lymphoblasts whereas GD3 expression is insignificant compared to normal lymphocytes. The anti-apoptotic role of 9-*O*-AcGD3 in lymphoblasts in contrast to mature erythrocytes suggests a cell specific role of 9-*O*-AcGD3 [104].

2.6 Role of O*-Acetylated Sialic Acids in Immunological Processes*

The major task of the immune system is to fight against invading microorganisms, to differentiate between self and non-self, i.e., to control autoimmune reactivity, and to eliminate defective or mutated cells such as tumor cells. The immune detection of tumor cells is often hampered by the fact that they are able, by many mechanisms, to disguise themselves as being normal. In a wider sense repair mechanisms such as wound healing and angiogenesis as part of the inflammatory process should also be included within the range of immune reactions. In general,

immune reactions can be divided into the branches of innate immunity as first line of defense and adaptive immunity for highly specific reactions including immunological memory. However, there are many molecular structures which bridge these two systems.

During recent years it has become increasingly apparent that carbohydrate–lectin interactions play a vital role in many of these immune reactions regulated by the innate and the adaptive branch of the immune system [105]. This includes both the recognition of microbial structures by immune cells and the intricate crosstalk between immune cells. It was hypothesized that attachment to and invasion of microorganisms into host cells taking advantage of carbohydrate–lectin interactions had induced a selective pressure to modulate their carbohydrate structures and the respective lectin receptors in order to prevent infection or on the side of the microorganisms to counteract these alterations on the host's side [106]. By means of this putative evolutionary process the complexity of carbohydrate structures expressed on the cell surface might have developed. Interestingly, a majority of these protein-carbohydrate interactions depends on the presence of terminal sialic acids as decisive recognition elements of the oligosaccharide ligand. Accordingly there are at least two lectin families known, the selectins and the Siglecs, which are specialized in the recognition of sialoglycans in various anomeric linkage patterns [107, 108]. Within the evolutionary development of carbohydrate complexity, *O*-acetylation of sialic acids seems to play a major role in infections and possibly also in defense mechanisms of immune cells. For example, coronaviruses have adapted specialized hemagglutinins to attach to cell surface expressed *O*-acetylated sialoglycans and to destroy further the linkage using sialic acid specific *O*-acetylesterases by removing the respective *O*-acetyl group [64, 67–69, 109].

When analyzing *O*-acetylation of lymphocytes it became evident that these immune cells preferentially synthesize 7-*O*-acetyl- and 9-*O*-acetyl sialoglycans [22, 32, 110–112].

O-Acetylated sialic acids resulted in increased susceptibility to alternate complement pathway-mediated lysis of murine erythrocytes. Progressive loss of Neu5,9Ac_2-GPs with differentiation is concomitantly associated with an increasing resistance to alternate complement pathway activation. Increased presence of Neu5,9Ac_2-GPs on erythrocytes of patients with visceral leishmaniasis may be responsible for ~ two- to threefold greater susceptibility to alternate complement-mediated hemolysis as compared to healthy individuals [80, 113].

2.6.1 *O*-Acetylation of Ganglioside GD3

Although theoretically *O*-acetyl sialoglycans can occur on glycoproteins and glycosphingolipids, the majority of studies focused on the disialo-ganglioside GD3 as the major carrier of terminal *O*-acetylated sialic acid. GD3 itself has been described to be involved in several immune reactions. These results were obtained to a large extent by application of specific monoclonal antibodies against GD3 and

its 9-*O*- and 7-*O*-acetylated variants (designated as CD60a (GD3), CD60b (9-*O*-acetyl GD3), and CD60c (7-*O*-acetyl-GD3) [114]. By means of these CD60 antibodies the expression of these gangliosides can be observed in the sterical context of the cell surface of live cells. In addition these anti-ganglioside antibodies are useful tools to study the content of GD3 and its *O*-acetylated variants in distinct cellular compartments.

In earlier in vitro studies it was shown that gangliosides such as GD3 shed by tumors can inhibit the activity of natural killer (NK) cells [115, 116]. It was proposed that anti-tumor cytotoxicity of NK cells was blocked, thereby alleviating undisturbed tumor growth. While at that time the mechanism of this possible inhibition was not clear, consecutive work may explain the mechanism. Nicoll et al. described that GD3 expressed on target cells is able to modulate NK cell cytotoxicity by interaction with Siglec-7 expressed on NK cells. Siglec-7 has a preference for binding of α2,8 linked disialo glycans and seems to be one of the inhibitory NK cell receptors [117]. In the case of melanoma cells the inhibitory function of GD3 could not be verified since GD3 expressed on melanoma cells can induce another class of cytotoxic cells, the NKT cells [118]. It was further elucidated that the fine specificity of GD3 reactive NKT cells is mediated by binding to CD1d, a surface molecule structurally related to the major histocompatibility antigen (MHC) which is expressed on T cells. CD1d has a preference for carbohydrate ligands. It is not clear at the moment whether in the in vivo situation there may be a balance between anti-melanoma NK and NKT cell-mediated immunity which affects the outcome of an effective immune surveillance in melanoma patients. Another mechanism to explain immune escape of melanoma cells may be that GD3 seems to be able to impair dendritic cell differentiation from monocytes and may induce their apoptosis [119]. On the other hand it is known that melanoma cells not only express GD3 but also its *O*-acetylated variants in various degrees [16]. One may speculate that *O*-acetylation of GD3 can provide another protective mechanism of melanoma cells against cytotoxic attack of immune cells.

2.6.2 Role of *O*-Acetylated Sialic Acids on Glycoproteins

O-Acetylated sialoglycans may also be part of glycoproteins as shown for the mucin family of *O*-glycosylated glycoproteins. In the colon, sialyl Lewis^{x} (CD15s) moieties of MUC1 and MUC2 were found to be differentially *O*-acetylated [120, 121]. It may be that *O*-acetylated CD15s is a negative regulator of the metastasis process because it may block the recognition of CD15s by E-selectin (CD62E) expressed on vascular endothelial cells and thereby inhibit the attachment of metastasizing cells to the vessel wall as a prerequisite to invade the host tissue. Krishna and Varki described the presence of 9-*O*-acetylated sialomucins on murine CD4 T lymphocytes [28].

Whether sialic acid *O*-acetylation has an effect on the binding capacity of Siglec proteins is still not unequivocally resolved. In an earlier report Sjoberg et al. stated that semisynthetic *N*-linked oligosaccharides with terminal *O*-acetylation have

reduced binding to CD22 (Siglec2) and, further, treatment of murine lymphocytes with a chimeric influenza C esterase (CHE-Fc) clipping off *O*-acetyl residues increased binding of CD22 to various murine lymphocyte subsets [27]. CD22 is a B lymphocyte specific lectin which recognizes preferentially terminally α2,6 sialylated lactosaminyl oligosaccharides. It does not react with α2,3 sialylated structures. In the murine system CD22 has a preference for Neu5Gc whereas in humans CD22 solely recognizes Neu5Ac. Whether Neu5Gc is synthesized and expressed in the human system is most unlikely although some reports described its presence though in small quantities [122].

The conceptional problem with the above-mentioned results of Sjoberg et al. is that natural *O*-acetylated α2,6 sialylated lactosaminyl ligands have not yet been identified. Effects of the influenza esterase on CD22 binding as described may be a result of steric alterations in the composition of surface oligosaccharides.

To clarify the influence of 9-*O*-acetylated sialoglycans on the intracellular functions of CD22 as a negative regulator of B cell activation, Cariappa et al. used murine mutants with a defect in the cellular sialate *O*-acetylesterase and investigated the function of CD22 in B cell signaling [123]. Indeed, they found increased 9-*O*-acetylation in B cells of these mouse mutants and subsequently also enhanced B cell receptor signaling, pointing to a possible suspension of CD22 control. Although these results prove a regulatory role of the sialate *O*-esterase towards B cell activation, the direct effect of 9-*O*-acetylation of CD22 ligands is still to be shown. Interestingly, mutations in the sialate *O*-esterase gene can be linked to certain human autoimmune disorders [124].

The overall expression of *O*-acetylated sialoglycoconjugates at a given stage of lymphocyte differentiation depends on the intricate balance of enzymes involved both in synthesis and degradation of these oligosaccharides. Wipfler et al. recently measured the transcription of GD3 synthase *ST8SIA1*, the putative sialic acid-specific *O*-acetyltransferase *CASD1*, the human sialidases *NEU1* and *NEU3*, and the sialic acid *O*-esterase *SIAE* in various human lymphocyte subsets representing various differentiation and activation stages in comparison to the expression of GD3 and its 7-*O*-acetylated and 9-*O*-acetylated variants (CD60a,b,c) [112]. It became apparent that the transcription of anabolic and catabolic enzymes was different in lymphocytes of various stages which had an impact on the intracellular and surface expression of CD60 structures.

Reduced *O*-acetylation may help tumor cells to escape from complement-mediated lysis because recognition of carbohydrates by elements of the alternative complement pathway may be inhibited by *O*-acetylation [26].

2.7 O-*Acetylated Sialic Acids in Cancer*

In malignant cells glycosylation is often altered in different ways. Examples are changes in glycosaminoglycans, altered branching on *N*-glycans, changes in mucin *O*-glycans, and in many instances elevated expression of sialic acids [125]. Changes in *O*-acetylation are also observed regularly in cancer cells.

2.7.1 9-*O*-Acetyl GD3 and Cancer

The identification of the disialo ganglioside 9-*O*-acetyl GD3, considered as an oncofetal marker, has been achieved using a lectin derived from the crab *Cancer antennarius* that recognizes sialic acids which are *O*-acetylated at both C4 and C9 positions and have been shown to be a biomarker in human melanoma [5]. Enhanced presence of *O*-acetylated GD3 has been reported in breast cancer, basaliomas, tumors of neuroectodermal origin [17, 126], childhood lymphoblastic leukemia (ALL) [104], and glioblastoma [127].

Ravindranath et al. [128] found that in one melanoma patient only metastatic lesions expressed the *O*-acetylated forms of GD2 and GD3 whereas the primary tumor expressed exclusively the non-*O*-acetylated gangliosides. The same was observed in basalioma. In basalioma the expression of this antigen was generally up to 60-fold higher than in surrounding normal skin [17]. However, in breast carcinomas the situation seems to be more complex. In normal ducts and in benign lesions 9-*O*-acetylated sialoglycans were present in Golgi regions and at the plasma membrane as detected by reaction with a CD60b antibody. This is in contrast to carcinomas of the breast where 9-*O*-acetylated sialoglycans were distributed in the cytoplasm in a disorderly fashion [129]. Cell surface expression of CD60b structures was only observed in well-differentiated carcinomas and overall expression decreased with progression of malignancy. Similar changes in distribution of sialoglycans and the sialyltransferase ST6Gal1 responsible for α2,6 sialylation have been found in hepatocellular carcinomas in which the disorder of sialoglycan distribution was correlated to the grade of malignancy [130]. It is still unclear whether loss of *O*-acetylation in malignant colon carcinomas is an advantage for tumor progression.

Surface-expressed 9-*O*- and 7-*O*-acetylated GD3 are abundantly expressed on human T lymphocytes [110]. Expression of both CD60b and CD60c (7-*O*-acetylated GD3) has also been detected on small resting lymphocytes of peripheral blood and on mature, activated T lymphocytes in lymph nodes [22, 112]. Tonsillar B lymphocytes, though to a smaller extent than T lymphocytes, express CD60b and c [22, 111]. Additionally the occurrence of CD60c was described on CD16+ NK cells, monocytes, and granulocytes to various extents [32] and on human CD34 hematopoietic progenitor cells derived from bone marrow (Schwartz-Albiez, unpublished). Cell surface-expressed CD60b and c on T and B lymphocytes may have a functional role in the lymphocytic activation process because anti-CD60b and c monoclonal antibodies can influence lymphocytic proliferation [22, 32, 131]. An interesting observation was that T and B lymphocytes react towards stimulation with CD60b and c antibodies in a different way. While in T lymphocytes CD60c antibodies alone, like a mitogen, can stimulate proliferation in B cells, additional signals such as addition of the cytokine Il-4 and triggering of the B cell receptor (surface expressed immunoglobulin) are required. For stimulation with antibodies against CD60b in T and B lymphocytes, additional signals are required [22]. This differential behavior may have a basis in a different surface distribution of the

antigens. While CD60b structures are found in dot-like formations, possibly as components of rafts, both in T and B cells, CD60c on T cells showed a more homogenous distribution on the cell surface [22]. It is most likely that CD60b is a cno-stimulatory signal for raft-concentrated receptors while CD60c in T cells acts as a mitogen. It is rather unlikely that gangliosides themselves confer transmembrane signaling because they do have neither a transmembrane nor an intracellular domain. An explanation may be that distinct glycosphingolipids cross-linked by antibodies can contribute to raft formation by pulling together a certain array of receptors.

2.7.2 Anti-apoptotic Role of *O*-AcGD3

The disialoganglioside GD3 is a well-known inducer of the apoptotic-program and its proapoptotic-effects can be counteracted by *O*-acetylation. Exogenous addition of GD3 to lymphoblasts promotes the apoptotic program whereas 9-*O*-acetyl-GD3 has anti-apoptotic effects. Unlike GD3, 9-*O*-acetyl-GD3 fails to depolarize mitochondrial membranes followed by the release of cytochrome c and caspase 9 and 3. The removal of *O*-acetyl groups by sodium salicylate in lymphoblasts re-establishes the GD3-responsiveness to apoptotic signals. Thus, the balance of de novo synthesized GD3 and 9-*O*-acetyl-GD3 plays important roles in the survival of lymphoblasts in leukemia [104].

Recently, differential expression and possible function of 9-*O*- and 7-*O*-acetylated GD3 during apoptosis of human tonsillar B and T lymphocytes has also been reported [22]. Malisan et al. [132] also demonstrated that acetylation suppresses the proapoptotic activity of ganglioside GD3.

Interestingly, this ganglioside and its *O*-acetylated variants also have a function inside the cell, breaking the dogma that the final destiny for gangliosides is the cell surface. Intracellular GD3 is involved in CD95- and ceramide-mediated apoptosis [133]. Upon receptor-triggering, de novo synthesized GD3 accumulates intracellularly which can be demonstrated by increased activity of GD3 synthase. GD3 as a rule is restricted to be present in the Golgi network and at the plasma membrane it is transferred to mitochondria via endosomal transport [134] to raft-like mitochondrial membrane domains [135]. The mechanisms of GD3-induced apoptosis can be traced back to a GD3-mediated change in the mitochondrial membrane potential and consequently increased production of reactive oxygen species [136, 137]. Oxidation of GD3 to GD3-7-aldehyde was shown to increase the apoptotic effect by targeting adenine nucleotide translocase [138]. *O*-Acetylation of GD3 suppresses this pro-apoptotic function of non-acetylated GD3 and does not have the deleterious effects of GD3 on mitochondria membranes [132]. Cells which are resistant to over-expression of GD3 convert existing GD3 more readily to 9-*O*-acetylated GD3 [132]. In further confirmation of the anti-apoptotic effects of *O*-acetylated GD3, Kniep et al. found that exogenous *O*-acetylated GD3 given to cells in vitro is internalized and can prevent apoptosis [139]. It was also observed that a T leukemia cell line resistant to apoptosis induced by *N*-acetyl-sphingosine or daunorubicin

transferred GD3 more readily into 9-*O*-acetyl GD3 [139]. Targetting 9-*O*-acetylated GD3 with sialate-9-*O*-acetylesterase results in apoptosis of biopsy-derived human glioblastoma cells. Compared to treatment of cells with exogenous *O*-acetylesterase, the effect of de-*O*-acetylation is more pronounced when the esterase is expressed within the cells from a recombinant baculovirus vector [127]. Thus, these data strongly point to an anti-apoptototic function of intracellular 9-*O*-acetyl GD3 that may protect tumor cells from apoptosis. While data have been gathered on the anti-apoptotic effect of 9-*O*-acetyl GD3, no data are available on possible effects of 7-*O*-acetyl GD3. We have observed that in T and B cells 7-*O*-acetyl GD3 followed in its expression intensity by 9-*O*-acetyl GD3 is present in an intracellular pool [112]. Given that intracellular 9-*O*-acetyl GD3 confers anti-apoptotic capacity, can this protective effect possibly be accelerated by conversion of 7-*O*-acetyl- into 9-*O*-acetyl GD3?

We also have no knowledge of what regulates the apparently differential transport of both acetylated forms of GD3 to the cell surface and what mechanisms with regard to the balance between the intracellular pool and cell surface expression of CD60b and c are decisive for regulation of either anti-apoptotic or proliferative effects. It was shown that human tonsillar B lymphocytes undergoing in vitro either spontaneous or staurosporine-induced apoptosis are characterized by surface expression of CD60b but not CD60c [22]. It may be that *O*-acetylated gangliosides fulfil different tasks at the cell surface and in intracellular compartments.

2.7.3 Neu5,9Ac$_2$-GPs and Cancer

In the gastrointestinal tract, the concentration of *O*-acetylated sialic acids of colonic mucin decreases in colorectal carcinomas, colonic adenomas, ulcerative colitis, and Hirschsprung's disease, suggesting its reversal to the embryonic form. However, human skin contains very little *O*-acetylated sialic acids. This decrease in *O*-acetylated sialic acids is associated with a concomitant increase in expression of the sialylated antigens, sialyl Tn, sialyl Lewis (a), and sialyl Lewis (x), which are considered to be adverse prognostic indicators.

An increased amount of *O*-acetylated sialic acids (Neu5,9Ac$_2$-GPs$_{ALL}$) on erythrocytes [140] and peripheral blood mononuclear cells (PBMC) of patients suffering from childhood acute lymphoblastic leukemia (ALL) [141–145] has been demonstrated using the preferential specificity of a lectin, Achatinin-H, towards Neu5,9Ac$_2$-α2,6-GalNAc [6, 7]. The absence of Neu5,9Ac$_2$-GPs and corresponding anti-Neu5,9Ac$_2$-GPs antibodies in corresponding cells of healthy children or in patients with other cross-reactive hematological disorders such as acute myelogenous leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, non-Hodgkin's lymphoma, thalassemia, and aplastic anemia confirmed the specificity of these biomarkers [141–145]. The binding of Neu5,9Ac$_2$-GPs$_{ALL}$ with Achatinin-H in the presence of several synthetic sialic acid analogs further confirmed the presence of this sialoglycotope on lymphoblasts in leukemia [144].

2.7.4 Function of Neu5,9Ac$_2$ for Detection of Minimal Residual Disease

In spite of successful treatment, patients may retain small numbers of malignant cells which is referred to as minimal residual disease (MRD) responsible for relapse. MRD is the main cause of a relapse of the disease. Neu5,9Ac$_2$-GPs are strongly expressed in childhood ALL at the onset of disease, then they decrease with chemotherapy and reappear with relapse. This observation makes Neu5,9Ac$_2$-GPs a potential biomarker for diagnosis and monitoring the disease status in childhood ALL (Fig. 1) [146, 151–155].

A 6-year longitudinal follow-up study reveals that the expression of three newly induced leukemia-associated Neu5,9Ac$_2$-GPs$_{ALL}$ (90, 120, and 135 kDa) disappears after treatment in patients who have disease free survival [143, 144]. The 90-kDa band persists in a few patients who subsequently relapse with the re-expression of the 120-kDa band. Early clearance of Neu5,9Ac$_2$-GPs$_{ALL}$$^+$ cells, during 4–8 weeks of treatment, shows a good correlation with low risk of relapse [143]. Therefore, close monitoring of 90- and 120-kDa 9-*O*-AcSGs may serve as a reliable index for long-term management of these children and merits therapeutic consideration.

Subsequently, a suitable template has been established by using the differential expression of Neu5,9Ac$_2$-GPs$_{ALL}$ along with other known CD antigens to monitor

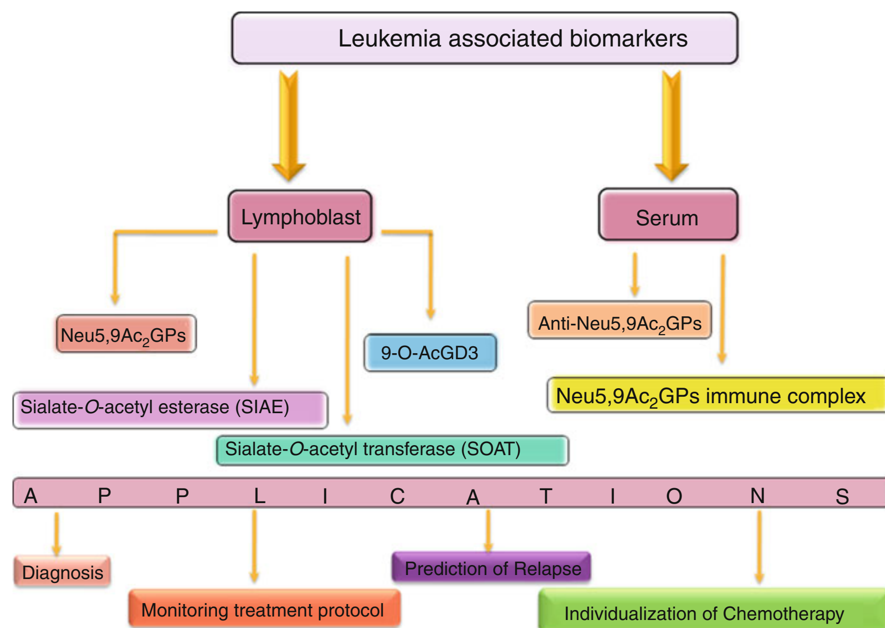

Fig. 1 Schematic overview of enhanced sialylation, Neu5,9Ac$_2$GPs [141–145], 9-OAcGD3 [104] on lymphoblasts, SOAT in microsomes [146], and anti-Neu5,9Ac$_2$GPs antibodies [147–150] in serum as signature molecules useful for diagnosis and monitoring childhood ALL

MRD [151]. A 2-year longitudinal follow-up study of 89 patients [B- ($n = 75$) or T- ($n = 14$) ALL], from the onset of the disease until the end of chemotherapy, reveals the sensitivity of MRD detection reaching 0.01% for a patient in clinical remission using flow cytometry. Presence of enhanced MRD due to failure in early clearance of lymphoblasts is implicated in an elevated risk of relapse. Elevated MRD during the chemotherapeutic regime predicts clinical relapse, at least 2 weeks before clinical manifestation. Therefore, these templates can function for MRD detection, during and post-chemotherapy for proper patient management strategies, thereby helping in designing tailor-made chemotherapy.

2.7.5 Function of Neu5,9Ac_2 for the Survival of Lymphoblasts

The role of *O*-acetylated Sias for the survival of lymphoblasts has been reported (Fig. 2) [23, 104, 156–158, 160].

2.7.6 Anti-Neu5,9Ac_2-GPs_{ALL} Antibodies

Enhanced levels of antibodies against Neu5,9Ac_2-GPs in leukemia patients as compared to normal individuals have also been used for monitoring disease status [147–150, 152, 160].

An enhanced amount of Neu5,9Ac_2-GPs specific IgG2 in leukemia was unable to trigger the complement cascade, activation of FcγR and cell-mediated cytotoxicity, although its sialoglycotope binding ability remains unaffected [160]. Interestingly, only Neu5,9Ac_2-GPs specific $IgG1_N$ purified from normal human serum emerged as the potent mediator of cell-mediated cytotoxicity, complement fixation, and activator of effector cells through FcγR. Therefore, the generation of customized *O*-acetylated sialic acid specific chimeric anti-9-Neu5,9Ac_2-GPs-IgG1 antibody constructs bearing functional normal Fc domain, having a homogenous glycoform and a pre-determined profile of functional potential would be ideal for therapeutic applications. Such customized antibodies might lead to their proper functioning and therefore possibly being useful along with cytokine therapy to activate in vivo anti-cancer pathways for proper immune-surveillance in leukemia.

Subclass switching of anti-Neu5,9Ac_2-GPs specific to IgG_2, alteration in their Fc-glycosylation profile, along with impairment of a few Fc-glycosylation-sensitive effector functions hint towards an unbalanced homeostasis helpful for evading the host's immune defense, suggesting a possible mechanism for functional unresponsiveness of tumor antibodies in general [160].

An interesting phenomenon is that patients suffering from certain tumors, for instance medullablastomas, a neural tumor disease, carry antibodies of the IgM subtype against *O*-acetylated GD3 in their serum [161].

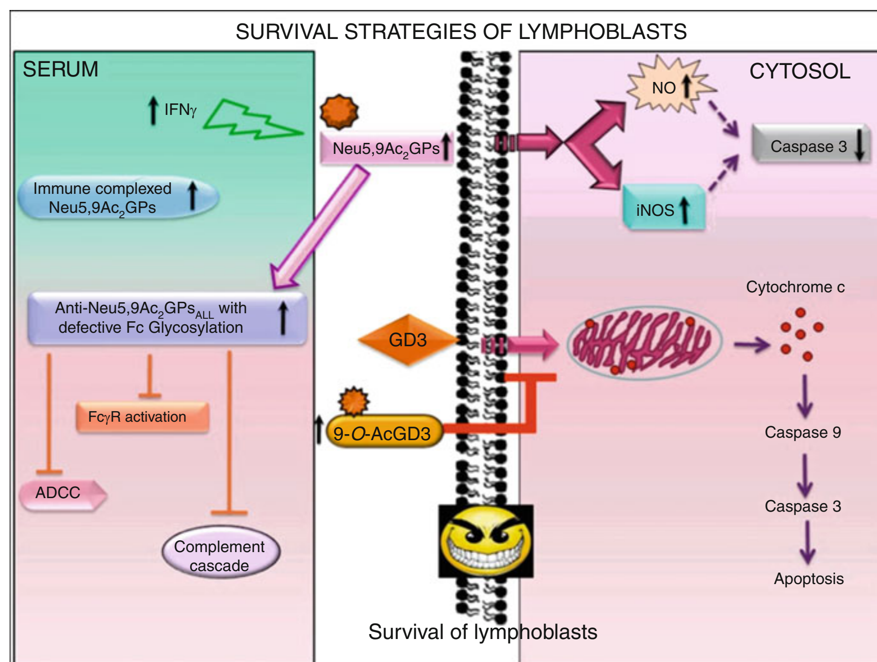

Fig. 2 Immune escape of lymphoblasts possibly due to enhanced sialylation, *O*-acetylation of glycoproteins or disialo gangliosides GD3 [156–158], SOAT [146], and reduced membrane bound sialidase (Neu 3) [159] in ALL. Subclass switching of anti-Neu5,9Ac$_2$GPs antibodies from IgG1 to IgG2, modulation of Fc-glycosylation, and weakening a few Fc-glycosylation-sensitive effector functions seem to be responsible for evading the host's immune response [160]

3 Biosynthesis and Degradation of *O*-Acetylated Sialic Acids

Appearance of *O*-acetylated sialoglycoproteins or glycosphingolipids is cell type specific and developmentally regulated. Their synthesis and turnover is a finely tuned phenomenon. Following the translocation of cytidine monophosphate (CMP)-sialic acid residues into the Golgi apparatus, sialyltransferases catalyze the transfer of sialic acid onto an acceptor like galactose or *N*-acetylgalactosamine or less commonly *N*-acetylglucosamine or 5-*N*-acetyl neuraminic acid of an appropriate oligosaccharide chain as part of a nascent glycoconjugate in α2,3, α2,6, α2,8, or α2,9 linkages. Subsequently, sialate *O*-acetyltransferases (SOAT) transfer the acetyl group from acetyl-CoA onto sialoglycoconjugates at the C-7/8/9 positions, generating *O*-acetylated sialoglycoconjugates. The primary insertion site for the *O*-acetyl group may well be the C7–OH group, from where it can non-enzymatically migrate to the C-9 position, presumably via the C8–OH group, leaving the C7–OH group available for a new transfer [1, 162].

These *O*-acetyl esters are removed by a family of other important enzymes in sialic acid metabolism, the sialate-*O*-acetylesterases (SIAE). Both SOAT and SIAE

are the two main enzymes responsible for the quantity of the *O*-acetyl ester groups on sialic acids. Therefore the activities of sialyltransferases and SOAT at one end of the spectrum, and the SIAE and a group of another key catabolic enzyme (sialidases), responsible for cleaving sialic acid residues from glycoproteins and glycolipids, at the other end of the spectrum, regulate the expression of *O*-acetylated sialoglycoconjugates.

*3.1 Sialate-*O*-Acetyltransferase*

Cancer cells frequently alter the regulation of sialylation processes leading to the appearance of characteristic sialoglycoproteins and sialoglycosphingolipids. A reduced SOAT enzyme activity in human colon and colorectal carcinoma is corroborated with decreased *O*-acetylation in the course of tumor development [120, 163, 164]. In contrast, enhanced SOAT in microsomes of lymphoblasts from bone marrow of children with leukemia, irrespective of their lineage, is corroborated with increased Neu5,9Ac_2-GPs [146]. The *O*-acetylation of exogenously added GD3 by ALL-microsomes extends the specificity of this SOAT towards gangliosides. Enhanced activity of SOAT with higher V_{max} in leukemia is one of the few descriptions of an enzyme of this type in human. However, a higher acetylation rate may also be partly due to differences in transporters and natural acceptors.

Besides endogenous acceptors, exogenous substrates like different sialoglycoproteins, CMP-Neu5Ac and GD3, are substrates for the enzyme. However, it is difficult to discover the selectivity of the SOAT in vivo. The reaction products are mainly Neu5,7Ac_2 and Neu5,8Ac_2, suggesting the primary insertion site of the *O*-acetyl group to be at C-7, followed by C-8 of Neu5Ac. The acetyl group possibly migrates from the seven position to the primary alcohol group of sialic acid at C-9 presumably via C-8. This is corroborated by enhanced Neu5,9Ac_2 exclusively in isolated microsomes of these lymphoblasts. Accordingly, the leukemia SOAT was denoted as sialate-7(9)-*O*-acetyltransferase [146].

The possibility that a number of distinct SOATs are controlling *O*-acetylation of sialic acids attached to glycans via different linkages cannot be ruled out, suggesting another level at which *O*-acetylation is possibly controlled in cancer and normal tissue. This has been supported by the observations wherein SOAT activity with high specificity for terminal α2,8-linked sialic acid residues and no detectable activity for α2,3-linked sialic acids is reported [165].

Interestingly, expression of 9-*O*-AcGD3 is higher in leukemic cells, which gives the plausible answer that increased amounts of GD3 might be converted to 9-*O*-AcGD3 by means of enhanced SOAT and reduced membrane-bound sialidase (Neu3) [159], thereby reducing the GD3 content. Complex regulations of the overall metabolism of sphingolipids through different activation of other enzymes are involved in association with sialyltransferases in leukemia.

Augmented lactosylceramide might contribute to the increased resistance of malignant lymphocytes towards apoptosis.

The SOAT activities increase rapidly with the onset of disease, decrease with clinical remission, and increase sharply again with clinical relapse and correlate well with high levels of cell surface Neu5,9Ac$_2$-GPs and 9-*O*-AcGD3 on lymphoblasts. Thus understanding the mechanisms of *O*-acetylation of sialic acids will enhance our knowledge of the functions of sialic acids in animals and humans in general and not only in cancer. Analysis of SOAT may provide insight into the pathogenesis of disease and its progression, and may even provide clues for designing new drugs. Clearly, further studies are needed to unravel the sialic acid linkage specificity of SOAT in cancer.

As already indicated, *O*-acetylation depends on multiple factors, including the origin of tissues or the type of cell lines used for SOAT assays. Attempts to isolate the enzyme by biochemical procedures [2, 166–169] led to the identification of at least two different SOAT activities: partially purified SOAT from bovine submandibular glands transfers acetyl groups to C7 of Sias. It was proposed that migration of acetyl groups from C7 to C9 might be enzymatically catalyzed [2]. In a later publication the existence of a "migrase" could not be substantiated [167]. A second type of SOAT was found in Golgi-enriched fractions of guinea pig liver, which transfers acetyl groups to C4 [170]. SOAT activity isolated from guinea pig liver preferred gangliosides as substrate. In addition, a heat-stable low molecular weight cofactor, which could be separated from SOAT activity by ultrafiltration, was proposed to enhance 4-*O*-SOAT activity [166]. In vitro several substrates, including free sialic acid, CMP-Sia, gangliosides, and glycoprotein-bound Sias could be acetylated by SOAT derived from different sources. In vivo free Sias most likely do not represent natural substrates for SOAT, because Sia is transferred into the Golgi as CMP-Sia by a nucleotide-sugar transporter [171, 172].

Higa and Paulson isolated CMP-Sia synthase and used this enzyme to prepare 9-*O*-Ac-CMP-Sia [173]. Interestingly, 4-*O*-Ac-CMP-Sia could not be synthesized with this enzyme. Sialyltransferases were able to transfer Neu5,9Ac$_2$ from the donor 9-*O*-Ac-CMP-Sia to glycoproteins, but at lower rates than Neu5Ac or Neu5Gc. It was concluded that a direct transfer of *O*-Ac-Sias to glycoproteins like bovine mucin from 9-*O*-Ac-CMP-Sia could account only for a fraction of the total *O*-Ac-Sias found. Moreover, it was proposed that 4-*O*-acetylation would result from the action of an *O*-acetyltransferase on the glycosidically-bound Sia [173]. Later it was found that both the CMP-Sia and acetyl-CoA transporters are critical components for the *O*-acetylation of CMP-Sia in the Golgi lumen. In addition, it was also suggested that a sialyltransferase exists that preferentially utilizes CMP-Neu5,9Ac$_2$ as the donor substrate to sialylate Galβ1,3(4)R- residues [164]. This finding was in contrast to earlier observations that acetyl-CoA does not enter isolated rat liver Golgi vesicles which are able to perform the acetylation of α2,6 linked sialic acids on *N*-glycans [174]. Additional data had led to the proposal that *O*-acetylation is the product of a trans-membrane reaction, involving a membrane protein with essential histidine and lysine residues [175]. In summary, it has

not yet been possible to obtain a purified eukaryotic SOAT preparation suitable to determine its amino acid sequence and the gene(s) encoding SOAT.

Other laboratories have tried to identify SOAT by expression cloning. Expression of different cDNAs was found to stimulate *O*-acetylation of sialic acids. With such experiments, Ogura and coworkers reported the cloning of an *O*-acetyl ganglioside synthase with a significant homology to milk fat globule membrane protein [176]. Kanamori et al. isolated a trans-membrane protein that most likely represents an acetyl-CoA transporter. Interestingly, they found that expression of this transporter induced the formation of *O*-Ac-GD3 [177]. During the search for the SOAT gene Shi et al. also isolated cDNAs which most likely are not directly involved in transfer of *O*-acetyl groups: a cDNA clone encoding a chimeric protein composed of a bacterial tetracycline resistance gene repressor and a plasmid sequence was found to enhance *O*-acetylation. Also, a clone encoding a truncated form of vitamin D binding protein was isolated. In both cases, expression of the recombinant proteins was required to observe increased *O*-acetylation [178]. This finding may indicate that the expressed mRNAs or proteins are recognized by pattern recognition receptors that then induce an "alarm" pathway, finally resulting in *O*-acetylation of sialic acids. Binding to the bacterial tetracycline resistance gene repressor may have triggered the initiation of cells to become pre-apoptotic by inducing the expression of 9-*O*-Ac-GD3. To speculate further, induction of apoptosis would then just require activation of the cellular SIAE to generate the pro-apoptotic ganglioside GD3. Another molecule possibly involved in *O*-acetylation of GD3 was identified as Tis21, a cell cycle regulator and cell death molecule [179]. It is possible that Tis21 may be a mediator of *O*-acetylation, but it appears unlikely that Tis21 represents the elusive SOAT. In GM2/GD2 knockout mice high amounts of 9-*O*-Ac-GD3 were found to accumulate in nerve tissue [180]. In this publication expression levels of the previously reported inducers of *O*-acetylation were also examined. No up-regulation was found for vitamin D binding protein, acetyl-CoA transporter, or the putative *O*-acetyl ganglioside synthase, while Tis21 was partially down regulated.

Recently, a new player in the field was identified. A screening of the human genome database revealed the *CASD1* gene as a candidate to encode a key enzyme involved in sialic acid *O*-acetylation [181]. When the Cas1 protein was expressed in COS cells together with ST8Sia1, a substantial increase of synthesis of 7-*O*-Ac-GD3 was observed. Human Cas1p was shown to co-localize with the Golgi marker ST6Gal1. At the mRNA level, elevated *CASD1* expression was concomitant with increased levels of *O*-Ac-GD3 in primary human cells and in cell lines derived from human melanoma and liver cancers. Transfection of *CASD1* specific siRNA resulted in a reduction of *O*-Ac-GD3 expression. On the other hand, expression of Cas1p in COS cells is not sufficient to direct *O*-acetylation of sialic acids on *N*- or *O*-linked glycans on the model glycoprotein erythropoietin (manuscript in preparation).

Cas1p is encoded by the *CASD1* gene located on chromosome 7q21.3. The gene is homologous to the *CAS1* gene of *Cryptococcus neoformans*. The designation is derived from the function of its encoded protein in the formation of the fungal

Capsule Structure. Genetic evidence strongly indicates that the fungal Cas1 protein is involved in the *O*-acetylation of glucorono-xylomannans [182]. Deletion of the *CAS1* gene resulted in a loss of *O*-acetylation on C-6 of either Man or Man^{GlcA} residues. *O*-Acetylation could be restored by expression of *CAS1* from a plasmid.

The human and fungal Cas1 proteins are composed of an N-terminal serine–-glycine–asparagine–histidine (SGNH) domain and a C-terminal trans-membrane domain with 8–12 trans-membrane regions (Fig. 1). Homologs of *CAS1* are also present in plants. They were shown to be directly involved in *O*-acetylation of plant cell walls. This modification is present in high amounts in different plant polysaccharides. *O*-Acetylation is a hurdle in the processing of plant material into biofuels, because glycosidases used for degradation of plant polysaccharides are negatively affected by *O*-acetylation [183]. On top of that, acetate and its conversion products are inhibitory to microorganisms used for fermentation [184].

During the screening of *Arabidopsis* mutants, plants were identified with insertional mutations in the plant *CAS1* genes, which were termed *RWA* (reduced wall acetylation) [185]. In Arabidopsis four *RWA* genes are present, and their expression in different plant tissues partially overlaps. The *rwa2* mutant exhibited a 15–30% reduction in cell wall acetylation. Different polysaccharides were affected at similar rates. Therefore, it was speculated that rwa proteins act immediately upstream of the transfer of acetyl groups to different acceptors. Interestingly, the plant Cas1 proteins lack the N-terminal SGNH domain. Instead, a large number of proteins with similarity to the N-terminal domain of Cas1p was identified in a bioinformatics approach [186]. In plants, the DUF231 (domain of unknown function) family of proteins may be involved in the transfer of *O*-acetyl groups to specific acceptors, possibly by a trans-esterase mechanism [185]. In mammals, other proteins, including members of the FAM55 and FAM113 families, and C7orf58, exhibit similarities to the SGNH domain of Cas1p [186]. Thus, it may be speculated that these proteins are candidates to direct *O*-acetyl groups to sialic acids in different glycosidic linkages and/or to different carbons of sialic acids (Fig. 3).

In addition, other yet unidentified components of the cellular SOAT activities may be required for the fine specificity of *O*-acetylation. Moreover, it is also unclear how SOAT and SIAE work in concert to regulate acetylation. This regulation may be at transcriptional level, but interactions at the protein level may also play a role.

3.2 *Sialate-*O*-Acetylesterase*

SIAE hydrolyses either C4- or C9-*O*-acetyl groups of glycosidically-linked or free sialic acid released from glycoconjugates by sialidases [162, 187–190]. At least two forms of cellular 9-*O*-acetyl-SIAE, one in the cytoplasm and the other in the lysosomal compartment (i.e., membrane-bound), exist in mammals. The secreted form, originally termed luminal sialic acid esterase [191, 192], was later termed lysosomal sialic acid *O*-acetylesterase (Lse) [193], because it was shown by immuno-electron microscopy to co-localize with acid hydrolases and lysosomal

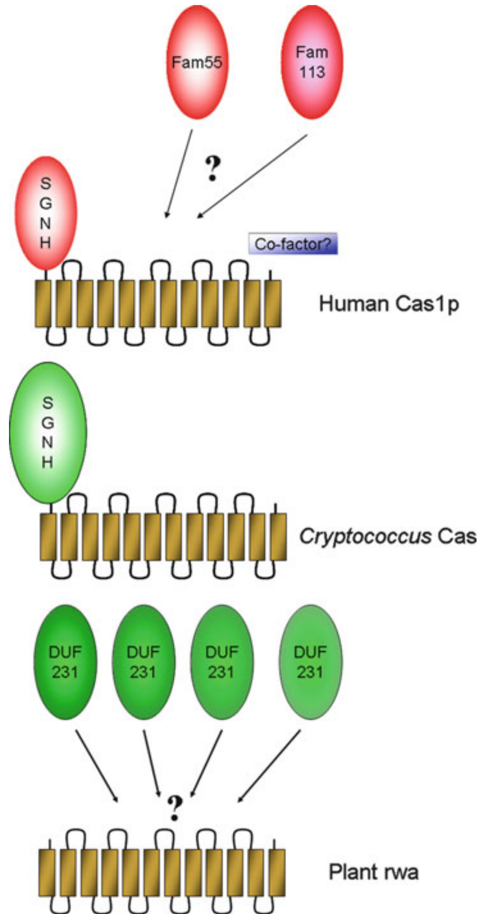

Fig. 3 Proposed schematic structures of Cas1p and rwa proteins and possible recruitment of FAM55, FAM113, or DUF231 proteins for catalytic activity of *O*-acetyltransferases. The human and cryptococcal Cas1 proteins consist of N-terminal serine–glycine–asparagine–histidine (SGNH) and C-terminal transmembrane domains. Plants express similar proteins termed reduced wall acetylation (rwa) lacking the N-terminal SGNH domain. Rwa may recruit DUF231 proteins to deliver acetyl groups from acetyl-CoA to different polysaccharides of the plant cell wall. In mammalian systems members of the FAM55 or FAM113 proteins may interact with Cas1p and a cofactor to transfer acetyl groups to sialic acids in different linkages

membrane glycoproteins [191]. This SIAE contains a cleavable N-terminal signal sequence and is secreted from COS cells as a glycoprotein with an apparent molecular mass of 62 kDa [193], whereas in rat liver it is found predominantly as a heterodimer composed of a small (28-kDa) and large (38-kDa) subunit connected by disulfide bridges. The small subunit could be labeled with the serine hydrolase inhibitor diisopropyl fluorophosphate, indicating that SIAE is a serine esterase. No sequence similarities to other known serine esterases were found [192]. Amino acid

residues, important for catalytic activity and secretion, were determined by expression of mutated cDNA. The mutations tested were found in the *SIAE* gene of patients with autoimmune disease [124]. Both subunits are encoded by the same mRNA in the order signal sequence – small subunit – large subunit. The precursor is presumably cleaved in lysosomal compartments [192, 194]. SIAE was identified independently by another research team as cDNA derived from a gene that is upregulated during B cell maturation. Several cDNAs were isolated which apparently are derived from differentially spliced SIAE mRNA [195]. One of the spliced mRNAs is lacking the exon for the signal sequence and encodes the cytosolic SIAE. While the mRNA for the secreted SIAE is widely expressed in different adult tissues, the expression of the cytosolic SIAE is restricted. Elevated expression of the latter was found mainly in liver, ovary, and brain [196]. SIAE regulates B cell antigen receptor signal strength and peripheral B cell development in mice [123], presumably by regulating recognition of α2,6-linked sialic acids by CD22 [27], a negative regulator of the B cell receptor [197–200] and Toll-like receptors [201]. Mutations in the *SIAE* gene resulting in a loss of function are strongly linked to autoimmune disease [124], while overexpression of SIAE is linked to pre-eclampsia [202], a condition of pregnant women who exhibit high blood pressure and proteinurea.

Concerning the function of SIAE, many open questions remain. While it can be envisaged that cytosolic SIAE is involved in de-*O*-acetylation of *O*-Ac-Sias delivered from lysosomes, the function of the "lysosomal" SIAE remains controversial due to the unfavorable pH in lysosomes. The pH optimum of SIAE is in the neutral to alkaline range. Data indicate that intracellular vesicles exist which contain mannose-6-phosphate positive glycoproteins [191]. Such entities possibly represent transport vesicles on the way from the Golgi to lysosomes. In any case, precise models of how SIAE is involved in tuning the balance of *O*-acetylation of sialic acids are currently not available. Consequently, a large field is open for future investigations. Another question concerns the enzymatic activity of secreted SIAE, which was shown to be increased upon proteolytic cleavage [193]. The protease required for cleavage activation was proposed to be a lysosomal enzyme, but the nature of this protease remains to be determined. Moreover, it is apparently not a common protease. COS cells were shown to express and secrete the uncleaved SIAE [193].

Another puzzle remains concerning the distribution of the secreted SIAE. Available data indicate that it is intracellularly concentrated in lysosomal compartments [191]. On the other hand it is efficiently secreted into the culture supernatant of cells over-expressing SIAE, and therefore it has access to *O*-Ac-Sias at the cell surface [193, 195]. Furthermore, the substrate specificity was shown for *O*-acetyl groups on free sialic acids [193]. In *Siae* knockout mice, an increase in α2,6-linked *O*-Ac-Sias was observed by using the influenza C virus lectin [123]. Strictly speaking, the influenza C virus lectin binds to any accessible *O*-Ac-Sia regardless of the underlying linkage. Thus, it remains an open question whether α2,3- or α2,8- linked sialic acids are also de-*O*-acetylated by SIAE. Furthermore, no SIAE hydrolyzing Neu4,5Ac_2 has been purified to homogeneity allowing the

determination of its relationship to SIAE specific for Neu5,9Ac_2. In summary, the expression of 9-*O*-acetylated sialoglycoproteins seems to be controlled by the relative activities of SOAT and SIAE. Many details on *O*-acetylation and de-*O*-acetylation still require clarification.

4 Future Perspectives

The existence of diverse *O*-acetylated Sias together with the enormous variations in sialoglycotopes having *O*-acetylated Sias in different linkages, with different sub-terminal sugars and their regulative functions in proliferation, and their controlled expression may be explored in view of possible applications in cancer therapy. Despite huge efforts to elucidate the factors mediating the escape of cancer cells from immunological surveillance, our knowledge in this regard is still rather limited. Exploring the function of *O*-acetylated sialic acids on the immune cells may contribute to our understanding of this problem.

In this direction, much emphasis of current research is invested in development of target-oriented anti-cancer drugs. Future investigation using both enzymological and molecular biology approaches of some key enzymes like SOAT, SIAE, sialyltranferases, and sialidases as drug targets may also lead into the direction of therapeutic interventions. For instance, deeper understanding of their functioning could be explored in a more practical direction for pharmacological manipulation of the apoptotic pathways.

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Why Is *N*-Glycolylneuraminic Acid Rare in the Vertebrate Brain?

Leela R.L. Davies and Ajit Varki

Abstract The sialic acids *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) differ by a single oxygen atom and are widely found at the terminal position of glycans on vertebrate cell surfaces. In animals capable of synthesizing Neu5Gc, most tissues and cell types express both sialic acids, in proportions that vary between species. However, it has long been noted that Neu5Gc is consistently expressed at trace to absent levels in the brains of all vertebrates studied to date. Although several reports have claimed to find low levels of Neu5Gc-containing glycans in neural tissue, no study definitively excludes the possibility of contamination with glycans from non-neural cell types. This distribution of a molecule – prominently but variably expressed in extraneural tissues but very low or absent in the brain – is, to our knowledge, unique. The evolutionarily conserved brain-specific suppression of Neu5Gc may indicate that its presence is toxic to this organ; however, no studies to date have directly addressed this very interesting question. Here we provide a historical background to this issue and discuss potential mechanisms causing the suppression of Neu5Gc expression in brain tissue, as well as mechanisms by which Neu5Gc may exert the presumed toxicity. Finally, we discuss future approaches towards understanding the mechanisms and implications of this unusual finding.

Keywords Brain · Central nervous system · *N*-Glycolylneuraminic acid · Sialic acid · Vertebrate

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1 The Evolutionary Origins of Sialic Acids

Sialic acids (Sias) commonly occupy the terminal position on the glycoconjugates that cover all vertebrate cell surfaces, and as such are major determinants of the molecular cell surface phenotype [1]. The term Sia refers to derivatives of *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and ketodeoxynonulosonic acid (Kdn) (Fig. 1). In 1963, Leonard Warren used the newly developed thiobarbituric acid test to screen for Sias in a wide variety of animals, algae, plants, and fungi [2], detecting them almost throughout the deuterostome lineage of animals, a group that includes vertebrates and so-called “higher” invertebrates. Sias were found to be present in vertebrates, echinoderms, hemichordates, and cephalochordates, although they were not found in urochordates. Outside the deuterostomes, Warren also noted small amounts of Sias within two species of flatworm and the digestive glands of squid and lobster,

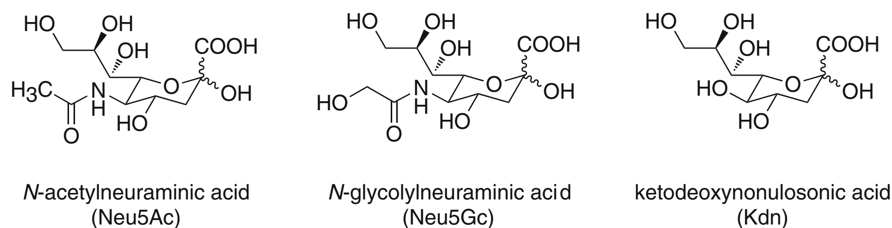

Fig. 1 Structures of the three common sialic acids in vertebrates

but considered these likely to be of dietary origin rather than endogenously synthesized. Based on these and later data, Sias were once considered both unique and universal to the deuterostome lineage [3].

In keeping with this concept, neither Sias nor the genes encoding their biosynthesis can be found in the model protostome *Caenorhabditis elegans* [4]. However, although early studies failed to identify any Sia-containing structures in invertebrates [5], more recent analyses identified small amounts of Neu5Ac in certain protostomes, including in gangliosides of squid and octopus [6], as well as in the slug *Arion lusitanicus* [7]. Furthermore the Drosophila genome encodes a sialyltransferase that functions in one phase of neural development [8]. Thus it seems likely that Sias originated before the deuterostome:protostome split, became prominent only in deuterostomes, and were mostly discarded in protostomes [9].

Outside the animal kingdom there are multiple instances of Neu5Ac production in various species of bacteria [10, 11]. However, the synthetic pathways for Neu5Ac in these species have evolved independently from those of animals, likely as a means of host immune evasion. These bacterial pathways appear to have arisen by convergent evolution, taking advantage of the more ancient pathways for biosynthesis of nonulosonic acids (NulOs), which are likely also the evolutionary precursors of the animal sialic acids [11].

2 Neu5Gc Is a Major Sialic Acid in Many Mammals

Although Kdn can be prominent in some fish, the two major Sias of most mammalian species are Neu5Ac and Neu5Gc. The CMP-Neu5Ac nucleotide sugar donor molecule originates from UDP-GlcNAc, via ManNAc and free Neu5Ac intermediates [3]. CMP-Neu5Ac can then be converted to CMP-Neu5Gc by cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH). This activity was first identified by Schauer more than 40 years ago [12], then shown to work specifically on CMP-Neu5Ac [13–15], and the enzyme was later cloned and further characterized [16, 17]. Gene knockout studies in mice [18, 19] have indicated that CMAH is likely to be solely responsible for Neu5Gc synthesis. The polypeptide sequence of CMAH is highly conserved across deuterostomes, but appears to be essentially confined to this lineage (Fig. 2).

```
HUMAN        --MGSIEQTTEILLCLSPVEVASLKEGINFFRNKSTGKDYVLYKNKSRLRACKNMCKHQGGLFIKDIEDLAGSC--------------------------   100
CHIMPANZEE   --MGSIEQTTEILLCLSPVEVASLKEGINFFRNKSTGKDYILYKNKSRLRACKNMCKHQGGLFIKDIEDLAGRSVRCTKHNWKLDVSTMKYINPPESFCQ   100
MACAQUE      --MGSTEQTTEILLCLSPVEVANLKEGINFFRNKSTGKDYILYKSKSRLRACKNVCKHQGGLFIKDIEDLAGRSVRCTKHNWKLDVSTMKYINPPESFCQ   100
MOUSE        --MMDRKQTAETLLTLSPAEVANLKEGINFFRNKTTGKEYILYKEKDHLKACKNLCKHQGGLFMKDIEDLDGRSVKCTKHNWKLDVSTMKYINPPGSFCQ   100
RAT          --MMDRKQTAETLLSLSPADVANLKEGVNFCRNKTTGKEYILYKEKDHLKACKNLCKHQGGLFMRDIEDLDGRSVKCTKHNWKLDVSTMKYTNPPGSFCQ   100
PIG          --MSSIEQTTEILLCLSPAEAANLKEGINFVRNKSTGKDYILFKNKSRLKACKNMCKHQGGLFIKDIEDLNGRSVKCTKHNWKLDVSSMKYINPPGSFCQ   100
COW          --MGSIEQTAELLLCLSPAEVANLKEGINFVRNKSTGKDYILYKSKSLLRACKNMCKHQGGLFIKDIEDLDGRSVRCTKHNWKLDVSTMKYINPPGSFCQ   100
XENOPUS      MEQSNDGQTAHTLLHLASAEVESLKEGITFLRNKESGKNFIIYKNGEELRACKNLCKHQGGTFIKDIEDLGNRTVRCTKHNWKLDVSSMKYVNPPDSFCQ   100
ZEBRAFISH    ----MAAQVSHTVLRLEAEDVRNLKDGINFQKNNKDGKCYIIYKANGELRACRNQCKHQGGLFIKDIEDMDGRTVRCTKHYWKLNVATMQYVNPPDSFMQ   100
SEA URCHIN   --------------------------------------MYFVSRP-LHVPAAGQTC-----------------VIKCTKHGWKLDAKTMRYTNPPDSFRQ   100

HUMAN        ----------------------------------------------------------------------------------------------------   200
CHIMPANZEE   DELVVEMDENNR-LLLLELNPPNPWDLQPRSPEELAFGEVQITYLTHACMDLKLGDKRMVFDPWLIGPAFARGWWLLHEPPSDWLERLCQADLIYISHLH   200
MACAQUE      DELVVEMDENNG-LLLLELNPPNPWDSEPRSPEELDFGEVQITYLTHACMDLKLGDKRMVFDPWLIGPAFARGWWLLHEPPSDWLERLCQADLIYISHLH   200
MOUSE        DELVIEMDENNG-LSLVELNPPNPWDSDPRSPEELAFGEVQITYLTHACMDLKLGDKRMVFDPWLIGPAFARGWWLLHEPPSDWLERLCKADLIYISHMH   200
RAT          DELVVEMDENNG-LCLVELNPPNPWDSDPRSPEELAFGEVQITYLTHACMDLKLGDKRMAFDPWLIGPAFARGWWLLHEPPSDWLERLCKADLIYISHMH   200
PIG          DELVVEKDEENG-VLLLELNPPNPWDSEPRSPEDLAFGEVQITYLTHACMDLKLGDKRMVFDPWLIGPAFARGWWLLHEPPSDWLERLSLADLIYISHMH   200
COW          DELVVEESKENE-LLLLELNPPNPWDSEPRSPEDLAFGEVQITYLTHACMDLKLGDKRMVFDPWLTGPAFARGWWLLHEPPSDWLERLCQADLIYISHMH   200
XENOPUS      DELVIENDDENG-VSLVELSPPNPWDSDPRMAVCLEGGEVQVTYLTHACMDLKLGNKHMVFDPWLIGPAFARGWWLLHEPPCDWLERLCRADLIYISHMH   200
ZEBRAFISH    DELEAVLSETDGSLELLELNPPDPWTAEPREAQDLQAGEITLTYITHACMELKAGERRMMFDPWLTGPAFARGWWLLHEPPKDAMDRLMEADLVYISHMH   200
SEA URCHIN   EQLVSEVDDEGS-MSLIELKPPQPWETDAREKVPLEVGEVKITYLTHACMELNLGGTVMFTDPWLTGPAFARGWWLMHEPPADWLDRLAKADFIYISHVH   200

HUMAN        ----------------------------------------------------------------------------------------------------   300
CHIMPANZEE   SDHLSYPTLKKLAGRRPDIPIYVGNTERPVFWNLNQSGVQLTNINVVPFGIWQQVDKNLRFMILMDGVHPEMDTCIIVEYKGHKILNTVDCTRPNGGRLP   300
MACAQUE      SDHLSYPTLKKLAGRRPDIPIYVGNTERPVFWNLNQSGVQLTNINVVPFGIWQQVDKNLRFMILMDGVHPEMDTCIIVEYKGHKILNTVDCTRPNGGRLP   300
MOUSE        SDHLSYPTLKQLSQRRPDIPIYVGDTERPVFWNLDQSGVGLTNINVVPFGIWQQVDKSLRFMILMDGVHPEMDTCIIVEYKGHKILNTVDCTRPNGGRLP   300
RAT          SDHLSYPTLKQLSQRRPDIPIYVGDTERPVFWNLNHSGVQLTNINVVPFGIWQQVDKNLRFMILMDGVHPEMDTCIIVEYKGHKILNTVDCTRPNGGRLP   300
PIG          SDHLSYPTLKKLAERRPDVPIYVGNTERPVFWNLNQSGVQLTNINVVPFGIWQQVDKNLRFMILMDGVHPEMDTCIIVEYKGHKILHTVDCTRPNGGRLP   300
COW          SDHLSYPTLKKLAGRRPDIPIYVGKTERPVFWNLNQSGVQLTNINVVPFGIWQQVDKNLRFMILMDGIHPEMDTCIIVEYKGHKILNTVDCTRPNGGRLP   300
XENOPUS      SDHLSYPTLKKLSEKRPDIPIYVGKTERPVFWYLDKSGVKLTNINVVPFGIWQEVDENLRFMILMDGVHPEMDTCIIVEYKGNKILNTVDCTRPNGGKLP   300
ZEBRAFISH    SDHLSYPTLQHLSKKRPDIPIYVGNTSRPVFWYLEKSGVNLTNINVVPFGVWQNVDDHLRFMILMDGVHPEMDTCLIVEYKGHMILNTVDCTRPNNGRLP   300
SEA URCHIN   SDHLSYPTLELLSARNPNIPIYVGDTSMPVFCKLKQSGVKLNNINVLKFGKWHEINKDTRFMIMMDGVHPDMDTCILIDYKGHLILNTVDCTNPNGGRLP   300

HUMAN        ----------------------------------------------------------------------------------------------------   400
CHIMPANZEE   MKVALMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKTERKKLLNYKARLVKNLQPRIYCPFAGYFVESHPSDKYIKETNTKNDPNELNNLIKKNS-DVIT   400
MACAQUE      TKVALMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKTERKKLLNYKAQLVKNLQPRIYCPFAGYFVESHPSDKYIKETNTKNDPNELNNLIKKNS-DVIT   400
MOUSE        EKVALMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKAERRKLLNYKAQLVKDLQPRIYCPFAGYFVESHPSDKYIKETNTKNDPNQLNNLIRKNS-DVVT   400
RAT          EKVALMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKAERRKLLNYKAQLVKDLQPRIYCPFAGYFVEAHPSDKYIKETNTKNDPNQLNNLIKKNS-NVVT   400
PIG          MKVALMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKTERKKLLNYKARLVKDLQPRIYCPFAGYFVESHPADKYIKETNIKNDPNELNNLIKKNS-EVVT   400
COW          MKVDLMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKTERKKLLNYKARLVKDLQPRIYCPFAGYFVESHPSDKYIKETNIKNDPDELNNLIKKNS-DVLT   400
XENOPUS      TNVALMMSDFAGGASGFPMTFSGGKFTEEWKSQFIKTERKKLLNYKAQLVKDLNPRIYCPFAGYFVEEHPSDKYIKETNLKNDAAELNMLIRNTS-DVVT   400
ZEBRAFISH    HGVDVMMSDFAGGASGFPMTFHGGKYTESWKANFIKNERKKLLNYKAQLVKSLQPKIYCPFAGYFTEAHPSDRYIKETNTKNSPAELNELIRKSCLNTLT   400
SEA URCHIN   IDVDIMLSDFAGGASGFPMNFFGGKYTEEWKGQFIKRERNKLLYYKTQVVRDVNPTVFCPFAGYFVEAHPSDGYIRETNTKNDAASLNALINKYSPEIKT   400

HUMAN        ----------------------------------------------------------------------------------------------------   500
CHIMPANZEE   WTPRPGATLDLGRMLKDPTDSKGIIEPPEGTKIYKDSWDFEPYLEILNAAVGDEIFLHSSWIKEYFTWAGFKDYNLVVRMIETDEDFNPFPGGYDYLVDF   500
MACAQUE      WTPRPGATLDLGRMLKDPTDSKGIIEPPEGTKIYKDSWDFEPYLEILNAAVGDEIFLHSSWIKEYFTWAGFKDYNLVVRMIETDEDFNPFPGGYDYLVDF   500
MOUSE        WTPRPGAVLDLGRMLKDPTDSKGIVEPPEGTKIYKDSWDFGPYLEILNSAVRDEIFCHSSWIKEYFTWAGFKNYNLVVRMIETDEDFSPFPGGYDYLVDF   500
RAT          WTPRPGATLDLGRMLKDPTDSKGIVEPPEGTKIYKDSWDFEPYLKTLNSSVRDSIFLHSSWIKEYFTWAGFKNYNLVVRMIETDEDFSPVPGGYDYLVDF   500
PIG          WTPRPGATLDLGRMLKDPTDSKGIVEPPEGTKIYKDSWDFGPYLNILNAAIGDEIFRHSSWIKEYFTWAGFKDYNLVVRMIETDEDFSPLPGGYDYLVDF   500
COW          WTPRPGATLDLGRMLKDPTDSKGIIEPPEGTKIYKDSWDFGPYLKILNAAVGDEIFRHSSWIKEYFTWAGFKDYNLVVRMIETDEDFSPFPGGYDYLVDF   500
XENOPUS      WTPKPGAILDLGRLLIDPTDKNGIIDPPPGTKIFKDSWDYDTYLSIHSFSFDDEIFHYPSWIKEYFTWAGFKGYNLVLRMIETDEHFVPLPKGYNYLVDF   500
ZEBRAFISH    WTPLPGSVLDLAVALNNRSNETAITDPPHGTKIYKDNWEFDLYLNQLNASISAEIFKHKHWIQYYYNWAGFRNYNLVIRVIETDDDFQPLNGGFDYLVDF   500
SEA URCHIN   WTPRPGATLDLQKAIQG--DSDYIQESPPGTKVFKDSWDFEKYLNGVNESIEHEIFAYPEWIKVYYKWAKFHSYNLVVRMIETDDDFKDIDGGYNFLIDF   500

HUMAN        ----------------------------------------------------------------------------------------------------   600
CHIMPANZEE   --LDLSFPKERPQREHPYEEIHSRVDVIRHVVKNGLLWDELYIGFQTRLQRDPDIYHHLFWNHFQIKLPLTPPNWKSFLMCCEQNGPGILQECKTT----   600
MACAQUE      --LDLSFPKERPQREHPYEEIRSRVDVIRHVVKNGLLWDELYIGFQTRLQRDPDIYHHLFWNHFQIKLPLTPPNWRSFLTCCEQNGPGISQECKTT----   600
MOUSE        --LDLSFPKERPSREHPYEEIHSRVDVIRYVVKNGLLWDDLYIGFQTRLLRDPDIYHHLFWNHFQIKLPLTPPNWKSFLMHCD-----------------   600
RAT          --LDLSFPKERPSREHPYEEIRSRVDVIRHVVKNGLLWDDLYIGFQTRLQRDPDIYHHLFWNHFQIKLPLTPPNWKLFLMRCG-----------------   600
PIG          --LDLSFPKERPSREHPYEEIRSRVDVIRHVVKNGLLWDDLYIGFQTRLQRDPDIYHHLFWNHFQIKLPLTPPDWKSFLMCSG-----------------   600
COW          --LDLSFPKERPSREHPFEEIRSRVDVIRHVVKNGLLWDDLYIGFQTRLQRDPDIYHHLFWNHFQIKLPLTPPDWKSFLMYCG-----------------   600
XENOPUS      --LDLSFPTERPERDHPYEEISSRATVIRHVVKHGLLWDDLYIGFQTRIQRNPDIYHHQFWNHFQIKLPLTPPDWKVFLDREKENNATLQNCSIM-----   600
ZEBRAFISH    --LDLSFPTERPEREHAYEEIKNRVNVMRHVVVNGLLWDDLYIGFNNRMSRDPDVYHHKFWNHFQTELPLSAPDWQHFLQICSQTQENGSSNGCSVS---   600
SEA URCHIN   SGLEATFPTERPSREHNYLEIKNRIGVHRQTVLKGLFWDDLYIGFNNQISRTPDTFHYLFWNHMQILLPFEPPNWDEFLDEMKAKNSPKKDVWKPSHAQI   600

HUMAN        -------------------------------------------------------
CHIMPANZEE   -------------------------------------------------------
MACAQUE      -------------------------------------------------------
MOUSE        -------------------------------------------------------
RAT          -------------------------------------------------------
PIG          -------------------------------------------------------
COW          -------------------------------------------------------
XENOPUS      -------------------------------------------------------
ZEBRAFISH    -------------------------------------------------------
SEA URCHIN   TNGKHTVYAGGDNGLKNGGPGAAKPKGGNKGDLMKWLVPIGIAALAAGIMIWRMK
```

Fig. 2 Alignment of vertebrate *CMAH* sequences. Alignment was performed using CLUSTALW [119]

Despite high sequence conservation of vertebrate CMAH, there are some instances of its loss. The human genome is unique among the old world primates in containing a universal deletion spanning exon 6 in the CMAH gene [20, 21]. While one group suggested that this causes an N-terminal truncation of the polypeptide [20], the other showed that an *Alu*-mediated mutation introduces a premature stop codon and highly truncated polypeptide (see Fig. 2), consequently preventing endogenous production of Neu5Gc [21, 22]. However, very low levels of Neu5Gc can be found in human tissues, likely due to metabolic incorporation from the diet [23]. Meanwhile, a recent study has confirmed the previously suspected absence of Neu5Gc in sauropsids – i.e., birds and reptiles – as well as likely in monotremes [24]. In keeping with this, no sequence strongly homologous to vertebrate *CMAH* can be found in any of the sauropsid genomes so far sequenced. Although some Neu5Gc was noted in one lizard and one bird – the green basilisk and the budgerigar – this was also suspected to originate from food [24]. Some strains of dogs and cats are deficient in erythrocyte Neu5Gc [25, 26]; it is as yet unclear whether this is caused by genomic *CMAH* mutations. Changes in the *CMAH* promoter, however, have been identified in some cats that appear to define the feline type B blood group that expresses solely Neu5Ac on erythrocytes [27].

There are many consequences to the loss of Neu5Gc production in a mammal. A mouse model of the human *CMAH* mutation, for example, exhibits numerous phenotypes, including delayed wound healing and age-dependent hearing loss [19], heightened B cell responses [18, 28], and a tendency for decreased insulin production [29]. Like this mouse model, vertebrate lineages that have lost functional *CMAH* are viable and fertile. However, Neu5Gc loss can cause relative infertility with wild-type animals, because of female anti-Neu5Gc antibodies that attack Neu5Gc-positive sperm or embryos [30].

3 Rarity of Neu5Gc in the Vertebrate Brain

Despite a universally high concentration of Neu5Ac in the brain, Neu5Gc has long been noted to be rare in the central nervous system (CNS) [3, 31]. Although it is well known in the field that Neu5Gc is rare in the vertebrate brain, we were unable to ascertain when and where this observation was first recorded in writing.

Unlike the highly variable levels of Neu5Gc found in all other tissues, the suppression of brain Neu5Gc expression is remarkably conserved across all vertebrates that have been studied to date (see Table 1, reproduced from [32]). The highest published fraction of Neu5Gc in brain tissue is 10% in a sample of adult bovine neocortex and 5% in calf [33]. However, other published studies of bovine brain report much lower fractions of under 2% [31, 34, 35]. Notably, even species that otherwise have a substantial fraction of their total Sias as Neu5Gc in many non-neural tissues maintain this suppression of Neu5Gc expression within the brain.

Table 1 Distribution of Neu5Gc in vertebrate tissues

Species	Serum	RBC	Submaxillary gland	Liver	Kidney	Milk	Brain
Human	–	–	nr	nr	–	–	–
Chimpanzee	nr	++	nr	+	+	+[a]	trace[b]
Macaque	+	+	nr	nr	nr	+	–
Mouse	+	+[b]	–	++	nr	nr	trace[b]
Rat	+	+[b]	+	+	+	nr	trace[b]
Rabbit	trace	+	nr	–	+	nr	–
Pig	nr	++	++	+	+	nr	trace[b]
Cow	++	++	+	nr	++	trace	trace[b]
Sheep	+	++	trace	+	+	++	trace
Elephant Afr	nr	nr	nr	++[a]	nr	+	nr
Elephant Asian	nr	nr	nr	++[a]	nr	–	nr
Dolphin	nr	nr	nr	++[a]	++	+[a]	trace[a]
Horse	+	++	trace	–	+	nr	trace
Chicken	–	–	–	–	–	–	–
Xenopus	nr	nr	nr	nr	nr	nr	–

The Neu5Gc fraction of total Sias in tissues was compared across vertebrates. This table combines data from the literature with that obtained from samples studied in our laboratory. Neu5Ac and Neu5Gc fractions of samples in our lab were determined by total acid hydrolysis of tissue lysate followed by DMB-HPLC. Conserved suppression of Neu5Gc in the brain is unusual among vertebrate tissues

++: major fraction; +: minor fraction; –: absent; trace: present at 0.8–3%; nr: not reported

[a]Data from our laboratory

[b]Published data confirmed in our laboratory

This research was originally published in [32]. © The American Society for Biochemistry and Molecular Biology

To our knowledge, no other molecule exhibits such an unusual tissue distribution. The evolutionary conservation of strong CNS suppression seems to indicate that maintaining very low levels of Neu5Gc in the CNS is very important.

4 Widely Variable Expression of Neu5Gc in Non-neural Tissues of CMAH-Positive Mammals

The conserved regulation of Neu5Gc expression in a given tissue across species is found only in the brain. As shown in Table 1, the Neu5Gc fraction of total sialic acid otherwise varies widely both across tissues and across species. To take the horse as an example, the fraction of total sialic acid that is Neu5Gc is as low as 1–2% in submaxillary mucin [31] but almost 100% on erythrocytes [36, 37]. This remarkable variability can also be found in interspecies comparisons within a single tissue. For example, in pigs, 90% of sialic acid in submaxillary mucin is Neu5Gc [38], while in cows, it is only 15% [31]; in sheep and horses it is lower still at only 1–3% [31]. Meanwhile, the fraction of Neu5Gc on erythrocytes from these species

shows no correlation to that found on the mucins. Thus Neu5Gc expression appears to be extremely dynamic in its regulation and evolving rapidly in all tissues other than the brain.

Our understanding of the regulatory mechanisms establishing the fraction of Neu5Gc in a given cell type is incomplete. One study of the developmental regulation of Neu5Gc in rat tissues found that CMAH enzymatic activity is an imperfect correlate to the level of Neu5Gc, suggesting that other mechanisms may influence the presence of Neu5Gc in sialylated glycans [39]. A more recent study of porcine tissues found that CMAH enzyme activity correlated reasonably well with Neu5Gc levels; however, in lung and heart tissues a much larger amount of immunoreactive protein was present, suggesting that modification of CMAH may influence its activity [40]. Nevertheless, although other factors may be at play, CMAH expression appears to be the major determinant of Neu5Gc levels.

The factors that determine the optimal ratio of Neu5Gc to Neu5Ac for a given tissue in a given animal are not entirely clear; however, it is easy to imagine the types of evolutionary selection pressures that may have had an influence. Sialic acid-binding lectins, whether pathogenic or endogenous, typically prefer either Neu5Ac or Neu5Gc, and these may affect the balance of Neu5Ac and Neu5Gc presence. Indeed, the Neu5Ac binding preference of ancestral *Plasmodium* species is postulated to be the impetus for fixation of the *CMAH* deletion in humans [41–43]. Siglec-2 (CD22), an endogenous immune modulator found on B cells, preferentially binds Neu5Gc in mice [44], but will bind either Neu5Ac or Neu5Gc in humans and great apes [45]. Recent work on a mouse model of muscular dystrophy shows that the absence of Neu5Gc is required to produce a severe, human-like phenotype, indicating that the presence of Neu5Gc may be important in normal muscle physiology [46]. Thus both pathogenic and endogenous selective pressures may influence the balance of Neu5Gc and Neu5Ac that is ultimately expressed within a given tissue. The data make it clear, however, that most tissues do not have an intrinsic requirement for a specific proportion of Neu5Gc in total sialic acids, let alone an exclusive preference for Neu5Ac or Neu5Gc.

5 Sialic Acids in the Vertebrate Brain

In seeking to understand why the vertebrate CNS suppresses the presence of Neu5Gc so consistently, it is important to consider the existing knowledge of CNS sialic acid biology. The brain contains more Sias than any other tissue [3, 31, 47]. In fact it was from brain tissue that Ernst Klenk extracted what he then called “neuraminic acid” in 1939, only a few years after the first isolation of sialic acids from salivary gland [31]. Interestingly, Neu5Ac content has been found to be approximately 30% higher in the left hemisphere than in the right hemisphere of a chimpanzee, suggesting that brain Sia concentration may correlate with neurological function or hemispheric dominance [48]. It has also been postulated that sialic acid from dietary sources can increase the Sia concentration in the developing

brain, playing an important role in learning and memory [49, 50]. Conversely, however, other studies of the incorporation of maternal dietary Neu5Gc into pups in utero have not found uptake of Neu5Gc into brain tissue [51], suggesting that dietary Neu5Ac and Neu5Gc may differ in their ability to be incorporated into brain glycans. It is clear, however, that Sias are extremely important within the brain.

As is the case elsewhere in the body, Neu5Ac in the brain can be found on N- and O-linked glycoproteins and on glycolipids; however, the distribution is somewhat different. It has been estimated that about 65% of brain sialic acid is on gangliosides, 32% on glycoproteins, and only 3% remains unbound [52]. This preponderance of ganglioside-bound, rather than glycoprotein-bound, sialic acid is unique among all tissues [49]. The brain also contains several characteristic sialoglycoconjugates, which we will briefly consider here.

5.1 Polysialic Acid

Polysialic acid (polySia) is an unusual posttranslational modification found on a few mammalian proteins as well as in the jelly coat of certain fish eggs, the voltage-gated sodium channel of eel, and the capsules of certain pathogenic bacteria. The most well studied carrier of polySia in the brain is the neural cell adhesion molecule (NCAM). Brain polySia is composed of long α2-8-linked polymers of Neu5Ac synthesized by two polysialyltransferases, ST8SiaII and ST8SiaIV, within the Golgi apparatus [53–55].

There is significant developmental regulation of the expression of polysialylated NCAM (also called embryonic NCAM or PSA-NCAM); in mice, very high levels occur in early postnatal life, mediated primarily by ST8SiaII, that drop to low adult levels by about 3 weeks of age [56]. Polysialylated NCAM plays a wide range of crucial roles throughout development affecting migration of neural progenitors, neurite outgrowth, and formation of appropriate synapses [57]. These roles are mediated through two mechanisms. Through the formation of a highly hydrated anionic cloud, polySia attenuates the adhesive properties of NCAM [58] and other adhesion molecules. PolySia also mediates other effects, specifically sensitizing cells to brain derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF-2) through direct interaction [59–62]. Later in development, downregulation of polySia has been found to be necessary for myelination of axons [63, 64]. In adult mammals, polySia is expressed at much lower levels throughout the brain, but can still be found in areas such as the hippocampus that are associated with adult plasticity [65].

Although polySia-NCAM is the most well characterized polysialylated glycoprotein, polySia has been identified on other vertebrate carrier proteins. Within the brain, it has been found on the synaptic cell adhesion molecule (SynCAM) on a specialized subset of glial cells, where it is thought to modulate synaptic formation [66], and has been identified in rat brain on the voltage-gated sodium

channel [67]. Although one study claimed to find polySia on neural podocalyxin, this was actually an erroneous use of the term to describe a highly sialylated protein [68].

5.2 Oligosialic Acid

A related but less well understood structure is oligosialic acid, i.e., two to four residues of α2-8-linked Neu5Ac. Oligosialic acids are enriched in the brains of both embryonic and adult pigs [69]. A specific trisialic acid epitope found on mouse brain glycoproteins appears, like polySia, to be developmentally regulated [70]. However, the functions of oligosialic acids remain relatively unknown. Notably, the oligosialic acid epitope is recognized by Siglec-11, a protein altered by gene conversion in humans, that is found on tissue macrophages and shows human-specific expression exclusively in microglia in the brain [71–73]. Although its function is not well understood, Siglec-11 was recently found to decrease the transcription of inflammatory mediators and thus reduce neurotoxicity induced by bacterial lipopolysaccharide (LPS) [74].

5.3 Gangliosides

Gangliosides are sialic acid-containing glycosphingolipids expressed throughout the body but most highly in brain tissue. In mammals, the simple gangliosides GD3 and GM3 predominate early in development throughout formation of the neural tube and neural stem cell proliferation, but this profile changes by the major period of neurogenesis [75]. In adult mammals, GM1, GD1a, GD1b, and GT1b together comprise over 85% of total brain gangliosides in pigs and 95% in humans [76]. These were found to be most prevalent in the neuropil surrounding neurons, suggesting an enrichment in neurons, particularly in synaptic membranes, rather than in glial cells [77].

Brain gangliosides have been proposed to be important in neurogenesis, neurite outgrowth, synaptogenesis, and synaptic function; however, the mechanisms underlying these roles have not been completely elucidated. Abolishing all ganglioside production by knockout of glucosylceramide synthase causes abrupt embryonic lethality after division of the primitive germ layers [78]. Neural-specific disruption of glucosylceramide synthase in mice produces live births, but the animals have severe neural abnormalities and die at 2–3 weeks of age, suggesting a critical impairment of appropriate brain maturation [79]. However, mice lacking only complex gangliosides were reported not to have any major abnormalities in histology or behavior, only a decrease in neural conduction velocity at 10 weeks of age [80]. Thus it has been hypothesized that gangliosides play a role in neural membrane function, such as signaling, conduction, or stability [81]. However, another group

found that mice lacking complex gangliosides were found by 16 weeks of age to exhibit more severe phenotypes, including progressive axonal degeneration of optic and sciatic nerves, as well as decreased central myelination [82].

The phenotype found in this study was noted to be similar to that caused by a deficiency of another important sialic acid-binding lectin, myelin-associated glycoprotein (MAG, Siglec-4), suggesting a common pathway for these molecules [82]. MAG is found periaxonally on Schwann cells and oligodendrocytes in both peripheral and central nervous systems. Notably, it preferentially binds the epitope Neu5Acα2-3Galβ1-3GalNAc [83], found on the complex neuronal gangliosides GD1a and GT1. Deficiency of MAG is associated with progressive signs of peripheral demyelination and axonal degeneration, similar to demyelinating peripheral neuropathies found in human patients [84, 85]. Additionally, the binding of MAG to gangliosides has been implicated in inhibition of neuronal regeneration after injury [86].

6 Claims for Presence of Neu5Gc in Neural Tissues

A number of studies have claimed to find small amounts of Neu5Gc-containing glycans in neural cells and tissues. It is worth considering these studies in some detail.

6.1 Tumor Gangliosides

Increased Neu5Gc expression is a feature of human cancers [87], and it might be expected that neural tumors would express Neu5Gc. An extensive characterization of mouse brain tumor gangliosides by Seyfried and colleagues has indeed repeatedly found Neu5Gc present in these gangliosides. However, Neu5Gc is only found when the tumor cells are injected subcutaneously into the flanks of mice, and not when the tumor cells are cultured independently, suggesting that the observed Neu5Gc is exogenous [88]. Subcutaneous injection of an experimental ependymoma incapable of synthesizing endogenous GM2(Neu5Gc) results in a tumor that contains GM2 (Neu5Gc) as well other gangliosides suggestive of the presence of Neu5Gc-expressing macrophages [89]. These Neu5Gc-containing gangliosides are still found when the cells are injected into mice with severe combined immune deficiency (SCID), which have no B or T lymphocytes, further suggesting that the gangliosides present do originate from tissue macrophages and/or from metabolic uptake by the tumor cells [90]. To date, no characterization of naturally occurring neural tumors has demonstrated endogenous Neu5Gc synthesis.

6.2 Normal CNS Gangliosides

There have, however, been a number of claims that Neu5Gc exists in normal brain gangliosides of some mammals. In 1970, Yu and Ledeen used gas–liquid chromatography to analyze sialic acids in the brain gangliosides of several species. Although the specific gangliosides were not defined, this study found Neu5Gc at 1–2% of total ganglioside sialic acids in ox, bull, and calf, 0.1–0.2% in pig and sheep, 0.4% in goldfish, but undetectable in rat, rabbit, frog, and chicken [35]. More recently, a study of cetacean brain gangliosides found low percentages (<2%) of Neu5Gc in the total ganglioside content of cerebrum and cerebellum of three toothed whales (killer whale, Dall's porpoise, and sperm whale). No Neu5Gc was found to be present, however, in the brains of other members of the dolphin family or baleen whale species (minke whale and Bryde's whale) [91].

Other studies have been able to identify specific gangliosides containing Neu5Gc in the brains of certain species. The first and most common such ganglioside to be identified was GD1a containing both Neu5Ac and Neu5Gc, initially estimated to account for 1% of total ganglioside-bound sialic acids in bovine brain [92]. However, a later estimate put the fraction of GD1a(Neu5Ac/Neu5Gc) much lower, at only 0.1% of total bovine brain ganglioside [93]. Using a two-step DEAE-Sepharose and TLC approach to increase resolution, Iwamori and Nagai also identified Neu5Gc-containing GD1a, as well as GM1, in the brain of cow; these were not found in brains of human, chicken, cat, rabbit, rat, or dog [94]. Another early study of calf and pig brain tentatively identified two unknown gangliosides as Neu5Gc-containing GD1a as well as GM3 [95]. GM3(Neu5Gc) has also been identified in equine brain, where Neu5Gc was found to comprise 18% of total GM3 Sias [96]. Finally, Neu5Gc-containing GT1b has been identified in extracts from bovine brain [97]. There are, therefore, quite a variety of gangliosides identified in these reports.

A major challenge in interpreting all such studies is that they did not separate neural cells, i.e., neurons and glia, from the endothelial cells and blood contents that run throughout the brain. Are the Neu5Gc-containing gangliosides that are being isolated in these experiments truly neural, or do they instead arise from the non-neural vasculature? The only study to address this question directly is an examination of horse brain [96]. The authors allow that the presence of endothelial cells may affect their results; however, they did determine that the lipid composition associated with this ganglioside was 60% 18:0, a feature characteristic of brain gangliosides as compared to aortic endothelial cells, which express a wide range of fatty acids [96].

A comparison of the types of gangliosides found in neural and endothelial tissues may also help to clarify the results of these studies. It can be expected that brain microvasculature, comprising the blood–brain barrier, may have different characteristics than endothelium from other tissues; however, unfortunately, few characterizations of the gangliosides of these cells have been done. Immortalized and cultured human cerebromicrovascular endothelial cells have been shown to

express GM3 (62%), GM2 (18%), GM1 (3%), and GD1a (15%) as the major gangliosides [98]. A similar cell line has GM3 and LM1, with small amounts of GM1, GD1a, GD1b, and GT1b [99]. Cultured microvascular endothelial cells from bovine brain also express GM3 as the major ganglioside component, with approximately 58% of GM3 containing Neu5Gc [100], although it is important to note that Neu5Gc in these cultured cells could have originated from components of the growth medium. As noted above, the major brain gangliosides are GM1, GD1a, GD1b, and GT1b. Thus, the above identification in horse brain of GM3(Neu5Gc) as a neural ganglioside is puzzling, as GM3 is a very minor component of brain gangliosides but a major fraction of cerebromicrovascular cells. Unfortunately, the significant overlap in expression of the remaining gangliosides in the two tissues makes it impossible to clarify the published identification of Neu5Gc in GM1, GD1a, and GT1b any further.

The fact that all published studies purporting to find Neu5Gc in brain gangliosides involve a group of closely related species – the even- and odd-toed ungulates and the cetaceans – may support the validity of their findings. Perhaps there is an evolutionary adaptation to allow low percentages of Neu5Gc within the brains of these animals. However, these species also happen to be mammals with a large enough amount of brain tissue to detect very minor ganglioside fractions, and the observation may thus result from a sampling bias.

Overall, the data on Neu5Gc in CNS gangliosides are quite challenging to interpret. While it is impossible to rule out completely endothelial and/or blood contamination in any case, it is also impossible to rule out a low level of Neu5Gc presence in neural cells. Further work will be necessary to clarify this issue fully.

6.3 Normal CNS Glycoproteins

Although much work has been done to characterize nervous system gangliosides, to our knowledge no study has so far identified Neu5Gc on a brain glycoprotein. It is interesting to note here that polysialyltransferases are able to incorporate a number of unnatural sialic acids into polySia [101, 102]. Recently, our laboratory has demonstrated that cells from a murine neuroblastoma line are similarly able to incorporate Neu5Gc into endogenous polySia [32]. Although polymers of Neu5Gc are found in the glycoproteins of the eggs of salmonid fish [103], Neu5Gc has never been reported in mammalian polysialic acid, neural or otherwise.

6.4 PNS Glycoconjugates

It is possible that the central nervous system suppression of CMAH and Neu5Gc does not extend to the peripheral nervous system (PNS). Very little work has examined this question. A study of bovine spinal motor neuron gangliosides that

reacted to serum antibodies from patients with Guillain–Barré syndrome identified two unknown gangliosides that the authors suggested, although did not show definitively, were GD1a containing one or two Neu5Gc residues [104]. Additionally, a membrane mixture of the noradrenergic vesicles from bovine sympathetic nerve endings was found to contain close to 50% Neu5Gc [105]. It is therefore quite possible that Neu5Gc is expressed without consequence in peripheral nerves.

It remains to be seen whether Neu5Gc retains a small presence in – or is completely absent from – the vertebrate CNS. The repeated finding of Neu5Gc in characterizations of neural gangliosides may indicate a true neural presence. Regardless, the very difficulty of detection and interpretation of these studies makes it clear that any Neu5Gc present is maintained at an extremely low level within the vertebrate brain. Further studies are needed, including in situ staining and/or cell-sorted analyses.

7 Possible Mechanisms for the Rarity of Neu5Gc in the Brain

The published record makes it clear that, for as yet unknown reasons, vertebrate brains have very low levels of Neu5Gc, regardless of the levels in other cell types in the same organism. There are a number of possible mechanisms responsible for this suppression, which has apparently persisted for hundreds of millions of years of vertebrate evolution. The most likely explanation is simply transcriptional repression of *CMAH*. Indeed, northern blot analysis of *Cmah* in mouse tissues detected no message in brain [16]. Further, the Allen Brain Atlas, which stores images of in situ hybridization to mouse brain slices, shows no *Cmah* signal throughout the brain (Allen Mouse Brain Atlas [Internet]. Seattle (WA): Allen Institute for Brain Science. ©2009. Available from: http://mouse.brain-map.org). Microarray analysis of *Cmah* cDNA in mouse brain gives a low but detectable level [106], although again endothelial contamination cannot be ruled out in whole brain extracts. Absence of *CMAH* transcript from brain has also been found by RT-PCR analysis of pig tissues [107]. Interestingly, human tissue mRNA microarrays give similar results [108], indicating that *CMAH* continues to be transcriptionally regulated long after its pseudogenization in the human lineage.

However, the pathway by which *CMAH* might be transcriptionally repressed is completely unknown. It may be induced by an extracellular factor, either soluble or membrane-bound. Conversely, it could be cell-intrinsic, a feature of neuronal/glial differentiation. The latter explanation is supported by the studies of gangliosides of murine neural tumor cells, which remain unable to synthesize Neu5Gc even when grown in culture away from their normal extracellular environment [88, 89]. Further, this same group found Neu5Gc-containing gangliosides in murine solid tumors of neural origin grown intracerebrally and in brain metastases of subcutaneously grown tumors, demonstrating that although neural cells do not express

Neu5Gc, inflammatory cells can maintain Neu5Gc expression even within the cerebral compartment [109]. It is therefore probable that *CMAH* downregulation is a characteristic of the neural lineage.

Of course, other mechanisms may influence Neu5Gc levels in neural cell types. CMAH may be further regulated at the level of mRNA stability, translational rate, and/or by posttranslational modification. Additionally, it cannot be ruled out that there may be enhanced mechanisms for eliminating Neu5Gc after it is synthesized, either within the neural cell or on the cell surface. The apparent evidence that dietary Neu5Ac, but not Neu5Gc, is incorporated into the developing brain may indicate that such an elimination is in fact taking place [50, 51]. Regardless, we can think of no other molecule that has such an unusual distribution amongst vertebrates: expressed at widely variable levels throughout extra-neural tissues, and yet always at very low levels in the brain. Whatever the mechanism, the neural regulation of Neu5Gc is apparently very tightly controlled.

8 Is Neu5Gc "Toxic" to the Vertebrate Brain?

The unusual picture of Neu5Gc distribution is suspicious for harmful effects of Neu5Gc on the brain. The degree of suppression is remarkable, with the Neu5Gc fraction being no more than about 2% (see Table 1). This finding implies that Neu5Gc is quite severely detrimental to the brain. To date, no studies have examined whether this presumed toxicity of Neu5Gc does in fact occur.

It is interesting to consider whether toxicity might be cell-intrinsic or extrinsic – that is, whether the toxicity targets a unique property of individual neural cells or only exerts its effects on the overall organ of the CNS. There is significant evidence to suggest that the latter is the case. Standard cell culture methods for all cell lines, including neurons, frequently involve fetal calf serum, a source of Neu5Gc. It has been shown that cells grown in such conditions will take up free Neu5Gc by macropinocytosis and incorporate it into glycans [110]. This has been shown to be true of human embryonic stem cells as well [111]. Neurons are routinely grown and differentiated in culture within such Neu5Gc-rich conditions. It is thus likely that Neu5Gc is toxic only on an intercellular level within the whole brain.

Despite these indications of a detrimental effect of Neu5Gc to the brain, it would be an unexpected finding. Neu5Gc is expressed to no ill effect throughout a wide range of other tissues, so such an effect would have to target neural tissue specifically. Even more puzzlingly, Neu5Ac, which differs only by a single oxygen atom, is a highly prevalent and critically important molecule in brain glycoconjugates. It will be fascinating to explore this question in future work. A number of mechanisms by which Neu5Gc might exert toxicity in the CNS are considered in the next section.

9 Possible Mechanisms for the Presumed Toxicity of Neu5Gc in the Brain

9.1 Biophysical Properties

Neu5Ac and Neu5Gc have some differences in chemical properties. Notably, the presence of the additional hydroxyl group on Neu5Gc may alter its pK_a from that of Neu5Ac [112]. This structural change also increases the hydrophilicity of Neu5Gc. These effects are minor when considered on the level of individual molecules. However, a mammalian erythrocyte, for example, contains many millions of surface sialic acid residues [113] and neural cells likely have even more. The overall effect of replacing Neu5Ac with Neu5Gc may thus have major effects on the surface charge and/or hydropathicity of a neural cell as a whole.

9.2 Differentiation

It is possible that the toxic effect is one to which neural progenitors or differentiating cells are primarily vulnerable. However, there is no evidence that CMAH and Neu5Gc are expressed in these immature progenitors, nor that their presence is toxic to neural differentiation. In fact, cultured neurons can be differentiated in the laboratory with Neu5Gc-rich fetal calf serum with no apparent detriment. Further, this explanation would also not account for the continued CNS suppression of *CMAH* into adulthood. It therefore seems somewhat unlikely that the presumed toxicity of Neu5Gc is mediated at the level of differentiation.

9.3 Sialoglycoconjugates

The presence of Neu5Gc in one of the sialic acid-containing molecules previously discussed may cause a detrimental effect. Although most sialyltransferases exhibit a preference for CMP-Neu5Ac or CMP-Neu5Gc, most will utilize either substrate if available [114]. Does Neu5Gc incorporation into a characteristic brain sialoglycoconjugate cause toxic aberrant function? Possibilities include polysialic acid; one study found polymers of Neu5Gc to be less effective at binding BDNF, although the authors acknowledged these were also shorter than the control Neu5Ac polymers [60]. The conversion of Neu5Ac to Neu5Gc in neuroblastoma–glioma hybrid cells also abrogates MAG binding [115].

Alternatively, perhaps Neu5Gc inhibits synthetic or degradative enzymes of brain sialoglycoconjugates, mediating toxicity not by affecting the function but by blocking the normal turnover of Neu5Ac-containing glycans. Indeed, a recent

paper from our own laboratory demonstrated that Neu5Gc is relatively resistant to degradation by sialidase when present in the α2-8 linkage common in brain glycans, including polySia [32]. This phenomenon may allow for a very small fraction of Neu5Gc to be highly detrimental to the brain, as an entire chain of polySia can be rendered resistant to breakdown by the presence of a terminal Neu5Gc. To date, this is the only hypothesis explaining a potential mechanism for Neu5Gc toxicity.

9.4 Alternative Role of CMAH

Lastly, although speculative, it is conceivable that CMAH has an additional role aside from converting Neu5Ac into Neu5Gc, and that it is this alternative function that requires suppression to avoid toxicity. In fact, a recent study of human stem cells suggested that human CMAH, although inactive as a hydroxylase, increases cellular uptake of exogenous Neu5Gc and decreases Wnt/β-catenin signaling [116]. However, this work depended heavily on an incorrectly reported N-truncated cDNA sequence [20]; the full-length human cDNA actually contains a stop codon upstream of the incorrect start site used [21]. Moreover, the raw data presented do not seem to support the claimed correlations. With the exception of this study, there is no work examining the possibility of an alternative role for CMAH. However, the continued tissue-dependent regulation of *CMAH* mRNA in humans may indicate that one exists. Perhaps this alternative role is the true mediator of neural toxicity.

The fact that makes few of these explanations entirely satisfactory is that Neu5Gc is not merely low in the brain, but that it is almost – perhaps completely – nonexistent. Any model of Neu5Gc toxicity in the vertebrate CNS will have to explain an evolutionarily selective effect requiring that Neu5Gc levels remain below 1–2% of total brain Sias. We believe this feature makes an inhibition of degradation hypothesis the only viable possibility at present. The fact that α2-8 linked Sias are uncommon outside the nervous system and widely distributed in brain glycoconjugates supports this notion [69].

10 Evolutionary Implications

If Neu5Gc expression is truly toxic to the vertebrate CNS, the expression or lack thereof of CMAH in a given animal may have implications for its neural evolution. The ancestral origins of CMAH are unfortunately not well understood, yet they may be important to our understanding of Neu5Gc in modern vertebrates. *N*-Acetylmuramic acid hydroxylase (namH) has been identified in certain species of *Mycobacteria*, where it converts UDP-*N*-acetylmuramic acid to UDP-*N*-glycolylmuramic acid [117]. Although the homology between namH and murine CMAH is only 12% at the peptide level, namH remains the most likely explanation as to the ancestral source of vertebrate CMAH. Unlike vertebrates, certain early

sialic acid-expressing invertebrates, such as the echinoderms, actually express a predominance of Neu5Gc [2], perhaps indicating that the development of CMAH and Neu5Gc was of importance in early lineages.

Within vertebrates, however, species that have since lost Neu5Gc may have gained some neural advantage in the bargain. Admittedly, it is difficult to see what benefit the brains of birds and reptiles have gained from losing Neu5Gc. However, the concentration of sialic acid in brain tissue in humans is reported to be extremely high – two- to fourfold that of most other mammalian species, and slightly increased over that of chimpanzees [48, 118]. Particularly considering the critical role that polysialic acid plays in neural plasticity, outgrowth, and myelination, it is tempting to speculate that the outright loss of neural Neu5Gc in an already sialic acid-rich brain may have eliminated a residual structural constraint and enabled the evolution of a larger, more complex, and more plastic brain in humans.

11 Conclusions and Future Prospects

The rarity of Neu5Gc in the vertebrate brain is certainly a fascinating observation. The literature to date indicates that this absence is highly conserved, with no animal expressing more than 1–2% Neu5Gc on neural gangliosides. Given the prominent sialylation of important neural glycoconjugates, such as gangliosides and NCAM, the striking absence of CMAH and Neu5Gc from neural cells is highly unusual. Although these findings are suspicious for a detrimental effect of Neu5Gc on the CNS, there is a dearth of studies examining this question. A number of questions will therefore need to be addressed in future work.

11.1 How Conserved Is Neu5Gc Suppression?

It is not yet clear whether some species are able to maintain a low percentage of Neu5Gc expression within neural tissue. It will be interesting to see whether some Neu5Gc does persist in the neural tissue of certain species, and to consider what implications this may have for those species. Additionally, no studies to date have observed whether there is similar suppression of Neu5Gc expression in the nervous systems of invertebrate deuterostomes, such as starfish and sea urchin.

11.2 What Is the Precise Localization of Residual Neu5Gc Within the CNS?

From existing studies it cannot be established whether trace Neu5Gc presence is confined to endothelial and blood cells or extends across the blood brain barrier. If it

exists within the CNS itself, cell-specific analyses will be necessary to determine whether all or only some cell types are able to express it. We are currently studying this issue.

11.3 What Is the Developmental Regulation of CMAH and Neu5Gc?

No study to date has examined whether Neu5Gc is endogenously expressed in neural progenitor cells or embryonic stem cells. This knowledge may help to clarify the mechanisms and the significance of Neu5Gc absence in mature brain cells.

11.4 What Mechanisms Cause the Suppression of CMAH Expression and Neu5Gc Production in the CNS?

This question will be particularly interesting given the unique distribution of CMAH, which exhibits dynamic regulation in non-neural tissues but strict neural suppression. Rigorous examination of the transcriptional regulation of *CMAH* is a necessary initial step.

11.5 Does the Presence of CMAH and/or Neu5Gc Truly Have Detrimental Effects on the Vertebrate Brain?

Animal models are needed to address this question since, as previously discussed, Neu5Gc is not toxic in cell culture. It will further be necessary to study whole brain tissue as well as individual cells. It is worth examining overexpression of CMAH and Neu5Gc separately in this work, as an additional role of CMAH mRNA or protein has not been ruled out.

These questions are critical in determining whether the repeatedly observed "smoke" of CNS rarity of Neu5Gc truly represents clues to a real "fire." In our ongoing and future work we are beginning to explore this decades-old unexplained observation, using a variety of methods. Our preliminary studies are promising in this regard. The evidence to date is certainly intriguing, and further investigation may help our understanding of Sias and the CNS.

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Polysialic Acid in Brain Development and Synaptic Plasticity

Herbert Hildebrandt and Alexander Dityatev

Abstract Polymers of sialic acid can be produced by pro- and eukaryotic cells. In vertebrates polysialic acid consists of α2,8-linked *N*-acetylneuraminic acid and is most prominent during nervous system development. Polysialic acid is produced by two complementary sialyltransferases, ST8SiaII and ST8SiaIV. The major, but not the only, carrier of polysialic acid is the neural cell adhesion molecule (NCAM). In this review we highlight how polySia dictates the interactions of various cell types during development and plasticity of the vertebrate central nervous system on different molecular levels. Recent progress in generating mouse models with differential ablation of the polysialyltransferases or NCAM revealed the dramatic impact of polysialic acid-negative NCAM on brain development and elaborate electrophysiological studies allowed for new insights into the role of polysialic acid in regulating synaptic plasticity and learning. The implications of dysregulated polysialylation for brain disease and neuropsychiatric disorders are discussed.

Keywords Axon development · Brain disease · Cell surface glycosylation · Neural cell adhesion molecule (NCAM) · Synaptogenesis

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1 Introduction

Polysialic acid (polySia or PSA[1]) occurs as a capsular polysaccharide of neuroinvasive bacterial pathogens (see Jakobsson et al. [1]) and as a unique glycan structure of a small set of eukaryotic cell surface proteins [2, 3]. In mammals, polySia consists of linear chains of α2,8-glycosidically linked *N*-acetylneuraminic acid residues (Fig. 1a) with a variable degree of polymerization ranging from 8 up to approximately 90 sugar units and comprises approximately 10% of the total protein-bound neuraminic acid in the developing brain [4, 5]. Early physicochemical investigations predict that at least parts of the polySia chain exhibit an extended helical conformation with a basal unit of approximately nine sialic acids [6–8]. Due to the negative charge of the nine-carbon monosaccharide, polySia forms a hydration shell, which increases the hydrodynamic radius of the polySia carrier and enlarges the space between adjacent cells (Fig. 1b) [9–12].

The most prominent protein modified by polySia is the neural cell adhesion molecule (NCAM), the prototypic member of the immunoglobulin family of adhesion molecules. Discovered as a synaptic glycoprotein more than 35 years

[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 prefer to use polySia to abbreviate polysialic acid.

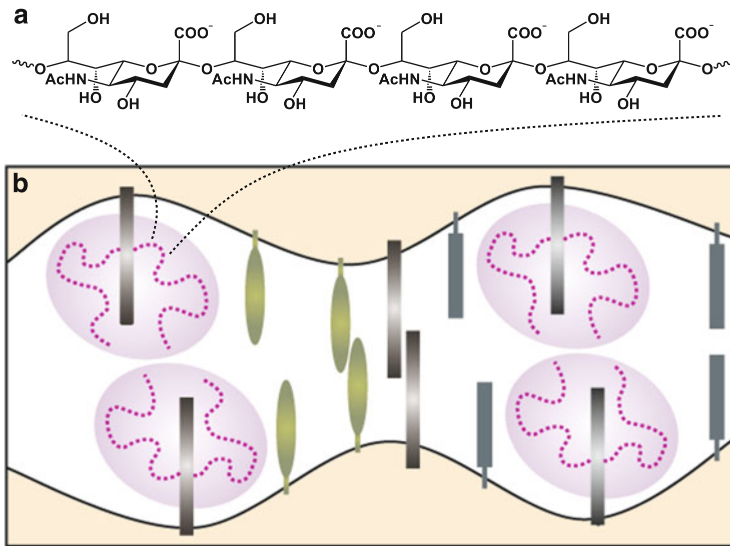

Fig. 1 Eukaryotic polySia structure. (**a**) α2,8-glycosidically linked *N*-acetylneuraminic acid residues. (**b**) PolySia increases the hydrodynamic radius, shields of interactions of its carrier protein and increases intermembrane space affecting interactions of various other cell surface proteins

ago [13], some of the first analyses already indicated striking differences in sialic acid content and biochemical properties of NCAM isolated from either embryonal or adult nervous tissue [14, 15]. At about the same time polySia was identified as a major source of sialic acid in the glycoprotein fraction of embryonic rat brain and hence could be assigned to NCAM [4, 16]. Since then, numerous studies in vitro and in cell-based approaches have shown that polysialylation decreases NCAM-mediated homophilic adhesion [11, 12, 17–20] as well as NCAM signaling functions induced by homophilic or heterophilic NCAM interactions [21–23].

However, NCAM is not the only carrier of polySia. A limited number of other polysialylated proteins have been described including the scavenger receptor CD36 in human milk [24], neuropilin-2 on human dendritic cells [25–27], and the polysialic acid synthesizing enzymes themselves, which can polysialylate their own *N*-glycans in a process termed autopolysialylation [28–31]. In the nervous system, occurrence of polySia on sodium channel alpha subunits of adult rat brain synaptosomal fractions has been reported [32] and most recently a subfraction of the synaptic cell adhesion molecule SynCAM 1 has been identified as a target for polysialylation in the early postnatal mouse brain [33]. The latter study also established that polysialylation attenuates homophilic adhesion of SynCAM 1 in a bead aggregation assay, implying that polySia serves as a potent regulator of Syn-CAM 1 interactions in vivo, as is known for NCAM (Fig. 1b).

2 Polysialic Acid Biosynthesis

In 1995 two different polysialyltransferase genes were independently characterized in four groups [34–37]. In these studies, each of the two enzymes was shown to be capable of producing polySia in vitro. Initially named STX [38] and PST-1 [34], the enzymes were designated ST8SiaII and ST8SiaIV according to a systematic nomenclature of sialyltransferases introduced in 1996 by Tsuji, Datta, and Paulson [39]. ST8SiaII and ST8SiaIV show a high sequence homology and are typical members of the mammalian sialyltransferase family with a type II *trans*-membrane topology, a short *N*-terminal cytoplasmic tail, a stem region, and a large catalytic domain facing the Golgi lumen (Fig. 2a) [40, 41]. The catalytic domain includes the sialylmotifs L, S, and VS, three conserved sequences that are found in all mammalian sialyltransferases and are involved in substrate binding [41–43]. The polysialyltransferases contain two additional structurally unique polybasic motifs, termed polysialyltransferase domain [44] and polybasic region [45, 46], respectively (PD and PBR; Fig. 2a). While the polysialyltransferase domain is part of the catalytic domain, the polybasic region is located in the stem region and seems to be involved in acceptor substrate recognition. Replacement of basic amino acids identified arginine residues within both motifs that are essential for polysialylation [45, 46]. In addition, interference with *N*-glycosylation of the polysialyltransferases and in particular the prevention of autopolysialylation leads to the formation of inactive enzymes [28, 47, 48].

Using cytidine 5′-monophosphate (CMP) – activated sialic acid as donor (see [49]), ST8SiaII and ST8SiaIV catalyze the transfer of multiple α2,8-linked sialic acid residues to, in the case of NCAM, a highly variable, di-, tri-, or tetraantennary *N*-linked core glycan [50–54] (Fig. 2b). As determined in vitro, terminally α2,3- or α2,6-sialylated galactose residues bound in α1,4-linkage to *N*-acetyl glucosamine can be used as acceptor sites for polysialylation [55, 56]. Although NCAM carries six *N*-glycosylation sites, the addition of polySia in vivo is restricted to sites 5 and 6, located in the fifth Ig-like domain (Ig5; Fig 2b) [52, 53, 57]. Mutational analyses identified an acidic patch in the first fibronectin type III repeat (FN1) that is critical for polysialylation [58] and hence might interact with the polybasic region of the polysialyltransferases [45]. Further deletion and replacement studies revealed the role of an alpha helix in Fn1 and the region linking FN1 and Ig5 in positioning of the Ig5 *N*-glycans for polysialylation with some but limited flexibility [58–60]. The latter studies suggest that not only protein–protein interaction but also proper spacing between the membrane and a particular *N*-glycosylation site are key determinants for site-specific polysialylation of only selected protein acceptors, such as NCAM and SynCAM 1 (Fig. 2b) [33, 58].

In the absence of specific enzymes that could degrade polySia at the cell surface, polySia expression in vertebrates seems to be regulated mainly by the balance between the synthesis of polysialylated structures and their internalization from the cell surface, which in the case of NCAM leads to either lysosomal degradation or recycling [61–63]. During mouse brain development, the expression of the two

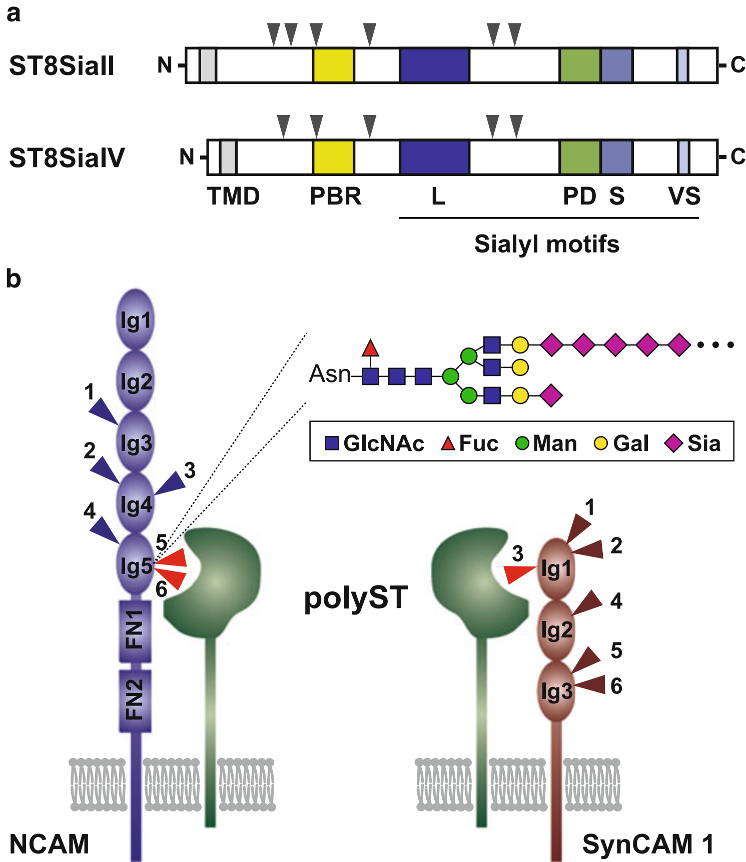

Fig. 2 Biosynthesis of polySia on NCAM and SynCAM 1. (**a**) Domain structure of the polysialyltransferases ST8SiaII and ST8SiaIV with the polybasic region (PBR), the polysialyltransferase domain (PD), and the sialylmotifs large (L), small (S), and very small (VS) of the catalytic domain. The relative positions of the *N*-glycans are indicated by *arrowheads*. *TMD* transmembrane domain. (**b**) Structure of the type I transmembrane proteins NCAM and SynCAM 1, example for a complex, here triantennary, core glycan with terminal sialic acid(s), and model of NCAM and SynCAM 1 in complex with a Golgi-resident polysialyltransferase (polyST) for site-specific polysialylation. *Ig* Ig domains, *Fn* fibronectin type III repeats, *triangles* *N*-glycosylation sites. Parts of panel **b** are modified from [33]

polysialyltransferases and the level of NCAM peaks during the third trimester [64] and in the perinatal phase, the entire pool of NCAM is polysialylated [65, 66]. Analyses of polysialyltransferase-deficient mice in this time window reveal that a loss of ST8SiaIV is completely compensated by the remaining activity of ST8SiaII. Conversely, in the absence of ST8SiaII more than 50% of the available NCAM are still fully polysialylated [65, 66]. Thus, biosynthesis of polySia under these conditions is limited by the availability of NCAM as the major acceptor. As detailed below (Sect. 4.2), an untimely appearance of polySia-negative NCAM causes severe defects

in brain development. The overly high enzyme levels in the embryonic and early postnatal period, therefore, might be crucial to guarantee that all NCAM is fully polysialylated during critical developmental periods. Possibly this overcapacity explains that a small fraction of SynCAM 1 is used as an alternative acceptor for polysialylation just during this phase [33].

3 Patterns of Polysialic Acid Expression

3.1 Developmental PolySia Patterns

PolySia is most prevalent during nervous system development. In mice the expression of both polysialyltransferases starts with neural tube closure at embryonic day (E) 8.5 and polySia is detected from E9 onwards [67, 68]. In general, polySia is widespread during embryonic and early postnatal brain development and most if not all neurons seem to be positive for polySia at some stage of their differentiation [69]. Staining has been detected on radial glia of the developing cortex and mesencephalon [64, 70] as well as on Bergmann and Müller glia, i.e., the radial glia of cerebellum and retina [71–73]. The most prominent polySia expression is found on interneuron precursors that migrate tangentially from the subventricular zone of the lateral ventricle to the olfactory bulb [74, 75]. This neurogenic niche is derived from the embryonic lateral ganglionic eminence and persists into adulthood. In the course of brain development polySia is also found on migrating precursors of cortical interneurons [76, 77] and cerebellar granule cells [71]. As shown for, e.g., Cajal–Retzius cells, the first neuron population growing out axons in the cortical primordium, or for fiber tracts like the optic nerve, the corticospinal tract, and thalamocortical fibers, developing axons of the rodent brain display strong polySia-immunoreactivity as well [72, 76, 78–80]. Finally, polySia is present during synapse formation of hippocampal neurons [81, 82] and plays a decisive role in the functional maturation of GABAergic inhibition which determines the time window of the so-called critical period of plasticity in the visual cortex [83] (see Sect. 5.2). Together, these findings point towards multiple functions of poly-Sia at all stages of neurogenesis. Correspondingly, the polysialyltransferases have broad and overlapping expression patterns in these developmental stages [68, 84].

As studied on the whole brain level and during development of the mesencephalic dopaminergic system in mice, mRNA expression of both polysialyltransferases increases dramatically after E10.5 and reaches plateau levels between E13.5 and E14.5, which are maintained until birth [64]. The time-course of ST8SiaII and ST8SiaIV upregulation is almost identical and precisely parallels a steep increase in the NCAM transcript level. In the course of postnatal brain development both polysialyltransferases are downregulated. In contrast to the moderate reduction of ST8SiaIV, a sharp drop of ST8SiaII mRNA occurs in a rather narrow time window between postnatal day (P) 5 and P11 followed by declining polySia [66]. At these

lower transcript levels a close correlation of polysialyltransferase expression and polySia formation becomes evident. Because the amount of NCAM remains almost constant, reduced polysialylation causes a gradual appearance of polySia-negative NCAM [66]. Highly consistent with these studies on the whole brain level, a decline of polySia has been observed in the prefrontal and visual cortex during the second and third week of postnatal development, which in the visual cortex is preceded by declining mRNA levels of ST8SiaIV and a particularly pronounced drop of ST8SiaII between P9 and P12 [83, 85, 86]. Moreover, postnatal downregulation of polySia but constant levels of NCAM were detected in the human prefrontal cortex [87].

Among the three major splice variants of NCAM only the two transmembrane isoforms NCAM-140 and NCAM-180 occur in their polysialylated form in brain lysates of embryonic and postnatal mouse brain [66, 67, 76]. In contrast, the glycolipid anchored isoform NCAM-120 is barely detectable at birth but massively upregulated during postnatal development without being polysialylated [66]. These findings contrast with in vitro data showing that all three NCAM isoforms can serve as polySia acceptors due to their identical extracellular domains [88]. Consistent with NCAM-120 being the characteristic isoform of mature oligodendrocytes and myelin sheaths, the increasing levels of NCAM-120 parallel the course of myelination during postnatal brain development [89, 90]. The lack of polysialylated NCAM-120 in postnatal mouse brain, therefore, may be explained by differential expression patterns of polysialyltransferases and NCAM-120. In contrast, oligodendrocyte precursors are positive for polySia in development and also during lesion-induced recruitment in the adult brain [91–96]. However, for both neuronal and oligodendrocyte precursors the NCAM isoform patterns remain to be determined.

3.2 *PolySia Patterns of the Mature Brain*

Under healthy conditions, polySia vanishes almost completely within the first 3 weeks of postnatal development, coinciding with the completion of major morpho-genetic events. There are, however, various hotspots of polySia expression in the mature brain. Most prominent, migrating neuroblasts arising from the neurogenic niches of the anterior subventricular zone [74, 75, 97, 98] and early postmitotic granular cell precursors in the subgranular layer of the hippocampal dentate gyrus [99–104] are characterized by their high polySia content and have been observed in all mammals including man [105–107]. Other major sources of polySia in the adult brain comprise widely spread subsets of interneurons and a population of immature neurons in layer II of the paleocortex. PolySia-positive interneurons were observed in different cortical areas, including prefrontal cortex [108, 109], piriform cortex [110], and hippocampus [111], as well as in the amygdala [112, 113]. Although polySia is best known by its intense expression in immature precursor stages, the polySia-positive interneurons of the cortex are mature neurons as evidenced by the presence of NeuN as an indicator of differentiated neurons, together with interneuron markers, mainly GAD67 and either calbindin, somatostatin, or parvalbumin

depending on the cortical area under consideration [114]. Compared to polySia-negative interneurons, these cells receive less synaptic input and have reduced dendritic arborization and spine numbers, suggesting that polySia is a negative regulator of interneuron connectivity and possibly allows for plasticity of inhibitory cortical networks [114]. As discussed in detail elsewhere [69, 115], the identity of the population of immature neurons in the paleocortex is enigmatic. Besides being polySia-positive, the immature cells display further features of neuronal precursors, like expression of doublecortin and the lack of NeuN [110, 116]. Despite a conflicting report [117], studies in rodents and cats provide substantial evidence that these cells are generated prenatally and maintain their immature phenotype into adulthood [118, 119]. Together with a comparative analysis of various mammalian and non-mammalian species [120], the data indicate that some immature polySia- and doublecortin-positive cells are also present in layers II and III of the mammalian neocortex. Most recently, the first evidence has been obtained that at least some of these immature neurons have the potential for maturation. After massive interference with olfactory processing by bulbectomy, the numbers of polySia- and doublecortin-positive cells in the piriform cortex layer II of adult rats were reduced in favor of increased numbers of differentiated, NeuN-positive neurons [121].

In addition to these examples of polySia immunoreactivity comprising the surface of neuronal cell somata and processes, some differentiated neurons of the mature brain are characterized by a polySia-negative soma while displaying polySia on their neurites. Most notably, most, if not all, hippocampal mossy fibers show intense polySia staining, although their somata in the granule cell layer are polySia-negative [77]. A similar situation was observed for pyramidal cells of the hippocampal CA1 region. Although the cell layer itself is polySia-negative [122], polySia immunoreactivity is detected on axons and dendrites of the CA1 pyramidal cells [123, 124]. Furthermore, as reviewed in great detail elsewhere [69, 125], wide areas of the adult brain retain a diffuse pattern of polySia staining. In thalamic and striatal regions this staining cannot yet be assigned to defined cell populations. In contrast, the more prominent diffuse polySia immunoreactivity of the adult hypothalamo-neurohypophysial system has been studied comprehensively [126–131]. Pronounced changes of polySia patterns occur during the glial and synaptic remodeling that accompany the physiological regulation of neuro-hormone release. While some of this polySia could be assigned to neurons of hypothalamic magnocellular nuclei, astrocytes and in particular their fine perineuronal processes are a major source of polySia in the hypothalamus. Strikingly, enzymatic removal of polySia by endosialidase injection prevents the rearrangement of synapses and astrocytic processes, indicating that polySia is a prerequisite for these changes [128, 130, 131]. Besides these hypothalamic astrocytes, polySia is found on other astrocytic cells of the adult brain, like the pituicytes of the neurohypophysis [132] and radial glia-like tanycytes in the ependymal layer of the third ventricular wall sending processes into the mediobasal hypothalamus [133]. Furthermore, polySia is also formed by reactive astrocytes, activated in response to various insults [92, 134–136].

3.3 Polysialyltransferase Activity in the Mature Brain

In general, polySia immunoreactivity and the combined mRNA expression of polysialyltransferases are well correlated [68, 84, 137–140]. 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 [66, 68, 84, 137]. Accordingly, polySia is drastically reduced in the brain of adult ST8SiaIV-negative mice as detected by Western blot analysis or immunohistochemistry [66, 141, 142]. However, polySia expression is retained on newborn neurons in the neurogenic niches of the subgranular zone of the hippocampal dentate gyrus and the subventricular zone of the lateral ventricle [141]. Loss of ST8SiaII has less effect on the polySia level but Western blot analysis of different brain regions indicates clear reductions in some parts of the brain [143]. First immunohistochemical data demonstrated a loss of polySia in the subgranular zone of the dentate gyrus, but normal levels of immunoreactivity were detected in the subventricular zone of the lateral ventricle and the descending stream of rostrally migrating-neuroblasts destined to become olfactory bulb interneurons [143]. Thus, both polysialyltransferases jointly produce polySia during subventricular zone neurogenesis and the loss of one enzyme can be largely compensated by the other. In contrast, ST8SiaII seems to be solely responsible for polySia synthesis in newborn granule cells of the adult dentate gyrus. However, the prominent polySia staining on the mossy fibers of the mature dentate granule cells is retained in the absence of ST8SiaII but completely abolished by the loss of ST8SiaIV [141, 143]. These findings match perfectly the ST8SiaII and ST8SiaIV mRNA expression patterns [84]. During the early stage of their life the newborn granule cell precursors in the subgranular layer express high levels of ST8SiaII, whereas only ST8SiaIV has been detected over the entire depth of the granular cell layer and consequently is associated with mature granule cells [84]. Interestingly, therefore, the expression patterns of the two polysialyltransferases during neurogenesis of dentate granule cells recapitulate the developmental profiles on the cellular level.

A direct comparison of polySia immunoreactivity in the cortex of young adult ST8SiaII- and ST8SiaIV-deficient mice corroborated the differential contribution of the two enzymes in the hippocampal dentate gyrus but also indicated a small overlap [142]. Minor populations of immature polySia-positive neurons remain in the ST8SiaIV-negative subgranular zone and some isolated polySia-positive fibers are still present throughout the granular cell layer of ST8SiaII-deficient mice. Moreover, this study clearly demonstrates that ST8SiaIV is solely responsible for polySia expression in mature cortical interneurons, whereas ST8SiaII is the major polysialyltransferase of the immature neurons in the paleocortex [142]. Remarkably, ST8SiaIV activity may drive maturation of these immature neurons, because ST8SiaIV deficiency leads to increased numbers of polySia- and doublecortin-positive immature neurons in the paleocortex layer II. In contrast, many of the

immature granule neurons displayed aberrant locations and morphology in ST8SiaII-deficient animals, suggesting a role for ST8SiaII in their terminal differentiation [142].

4 Role of Polysialic Acid in Brain Development

4.1 NCAM and PolySia are Implicated in Neural Tube Closure

A function of the earliest expression of polySia during neural tube closure is inferred from the premature polysialylation of NCAM observed in the splotch mutant mouse, a model of Waardenburg syndrome type I caused by pax3 mutations [144]. Indeed, pax3 mutations may affect the balanced expression of polysialylated NCAM, since NCAM and ST8SiaII are downstream targets of this transcription factor [145, 146]. The vital importance of tightly controlled NCAM interactions during these early stages of development was unequivocally demonstrated by the dominant embryonic lethality of mice in which all membrane-associated forms of NCAM were replaced by a soluble, secreted form of its extracellular domain [147]. Analysis of chimeric embryos revealed severe defects by E8.5–E9.5. The embryos were truncated with reduced numbers of poorly formed somites and neural tube defects. Embryos derived almost entirely from homozygous mutant ES cells exhibited the same lethal phenotype, indicating that the secreted NCAM is producing this phenotype through heterophilic rather than homophilic interactions [147].

Although not addressed in this study, the drastic effects of uncontrolled, overshooting NCAM interactions imply that the onset of polysialylation at E9 is used to limit NCAM interactions during neural tube closure. Noteworthy in this context are the pronounced effects of valproic acid and retinoic acid on the polySia-NCAM system. Both are potent teratogens in humans and cause defects of neural tube closure with different periods of sensitivity in mice [148]. Valproic acid increases the polySia to NCAM ratio, while retinoic acid accelerates polysialylation of NCAM, at least in cell culture experiments, by augmenting ST8SiaIV but decreasing ST8SiaII mRNA levels [149–151].

4.2 PolySia-Deficient Mouse Models Reveal Distinct Modes of PolySia Engagement in Neuronal Migration and Axon Tract Development

Analyzing mice with partial or complete ablation of polySia disclosed the crucial role of NCAM polysialylation for mammalian brain development [141, 143, 152–155]. The first models with an extensive loss of polySia were mice with

genetic ablation of all NCAM isoforms [151] or with a deletion of an exon specific for the 180 kD isoform of NCAM [152]. Surprisingly, these NCAM-deficient animals turned out to be viable and fertile and showed a grossly normal brain development. Both NCAM mutant mice, however, display two prominent neuroanatomical defects (for comprehensive review, see [156, 157]). First was a size reduction of the olfactory bulbs caused by a migration deficit of subventricular zone-derived olfactory interneuron precursors, the major polySia-positive cell type in the wild-type brain (see Sect. 3.2) [152, 158–160]. Second was a defective lamination of mossy fibers projecting from the dentate gyrus to the CA3 subfield of Ammon's horn [161, 162]. Both phenotypic 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 [158, 162].

Consistent with the potential of the polysialyltransferase ST8SiaII to compensate almost entirely for a loss of ST8SiaIV during the developmental phase (see Sect. 2), no defects of brain morphology were detected in the ST8SiaIV-negative mice [141]. Conversely, the partial reduction of polySia levels in the developing brain explains the malformation of the hippocampal mossy fiber tract observed in ST8SiaII-deficient mice, which is reminiscent of the respective phenotype of the *Ncam*-knockouts [143]. Since mice with genetic ablation of NCAM are almost completely devoid of polySia it is also not surprising that the major neurodevelopmental defects of $Ncam^{-/-}$ animals are recapitulated in *St8siaII*, *St8siaIV* double-knockout mice ($II^{-/-}IV^{-/-}$), which are polySia-negative but retain normal levels of NCAM expression [154, 155]. In marked contrast to the *Ncam*-knockout, however, the simultaneous deletion of both polysialyltransferases generates a postnatally lethal phenotype. Although born at Mendelian ratio and without overt morphological defects, $II^{-/-}IV^{-/-}$ mice fail to thrive and more than 80% die within the first 4 weeks of age [154].

The comparative analysis of $II^{-/-}IV^{-/-}$ and $Ncam^{-/-}$ brains then demonstrated that loss of both polysialyltransferases confers a phenotype that combines two types of defects: (1) defects that develop in polySia-negative mice irrespective of the presence or absence of NCAM and (2) defects that manifest exclusively in $II^{-/-}IV^{-/-}$ mice and therefore may be caused by the appearance of polySia-free NCAM [154]. The first category comprises defective rostral migration of subventricular zone precursors and smaller olfactory bulbs as well as delamination of mossy fibers. Besides postnatal growth retardation and precocious death, the second category includes a high incidence of progressive hydrocephalus and severe anomalies of a diverse set of brain fiber tracts, which occur regardless of ventricular dilatation. Affected are commissural and non-commissural axon tracts. Most conspicuous is the complete agenesis of the anterior commissure. As shown by anterograde tracing of the anterior limb, axons of the anterior commissure are present but lack normal fasciculation, deviate early from their normal trajectory, and therefore never cross the midline [154]. Morphometric analyses also revealed hypoplasia of the internal capsule, the major gateway of fibers to and from the cerebral cortex, and of the mammillothalamic tract. This tract projects from the mammillary bodies to thalamic nuclei as part of a circuit involving thalamus,

cortex, hippocampus, and mammillary body (Papez' circuit) and is essential for spatial working memory in the mouse [163]. Furthermore, size reduction but correct midline crossing of the corticospinal tract was detected. Although resembling the hypoplasia of the corticospinal tract in $Ncam^{-/-}$ [164] the defect was significantly more severe in $II^{-/-}IV^{-/-}$ mice [154]. In $II^{-/-}IV^{-/-}$ mice escaping from hydrocephalus, the corpus callosum reached its normal thickness in central sections, but, as demonstrated in a later study, is significantly shorter due to a marked hypoplasia of the splenium, the posterior end of the corpus callosum [165]. In contrast, other tracts, like lateral olfactory tract, optic tract, fasciculus retroflexus, or posterior commissure appeared to be normally developed.

Remarkably, all the fatal developmental defects specifically found in $II^{-/-}IV^{-/-}$ but not in $Ncam^{-/-}$ mice could be rescued by the additional deletion of NCAM in polysialyltransferase- and NCAM-negative triple-knockouts ($II^{-/-}IV^{-/-}N^{-/-}$). It therefore was hypothesized that the major function of polySia is to mask NCAM and to guarantee that NCAM mediated contacts take place in a highly organized, time- and site-specific manner [154]. To substantiate the assumption that untimely expressed polySia-negative NCAM causes malformation of brain axon tracts, the available mouse models with defects in NCAM, ST8SiaII, and ST8SiaIV were used to breed mice with different levels of polySia-negative, "naked" NCAM during brain development [165]. In addition to the entirely polySia-negative, NCAM-positive $II^{-/-}IV^{-/-}$ and the polySia- and NCAM-negative $II^{-/-}IV^{-/-}N^{-/-}$ animals, mice with different combinations of functional and mutant polysialyltransferase and NCAM alleles were screened. Out of the 27 possible allelic combinations, mice of nine genotypes with different levels of polySia, NCAM, and polySia-free NCAM at postnatal day 1 were selected for morphometric evaluation at the age of 4 weeks. Axon tracts like anterior commissure, internal capsule, and corpus callosum, for which morphological deficits have been identified in the brain of $II^{-/-}IV^{-/-}$ mice, were analyzed. As shown in Fig. 3 by the example of the corpus callosum, the degree of the axon tract defects correlated precisely with the amounts of untimely expressed polySia-free NCAM and not with the overall polySia or NCAM level at postnatal day 1 [165]. The premature occurrence of "naked" NCAM due to a loss of the shielding functions of polySia, therefore, causes inappropriate development of major axon connections, and addition of polySia to NCAM is needed for correct brain wiring. This strengthens the view that concealing NCAM is the key regulatory mechanism that makes polySia essential for brain development. In a broader perspective, these findings indicate that cell surface glycosylation can be used as a surveillance system to control interactions of the corresponding carrier protein.

In search for the cause of the internal capsule hypoplasia the development of thalamocortical and corticothalamic fibers was analyzed [80]. During normal embryogenesis the two fiber systems grow towards each other and intermingle to form the reciprocal connections between cortex and thalamus, which account for a major part of the internal capsule. Similar to the situation for the anterior commissure, labeling of thalamocortical axons revealed that the fibers are present but misrouted in the polysialylation-deficient $II^{-/-}IV^{-/-}$ but not in the NCAM-negative $II^{-/-}IV^{-/-}N^{-/-}$ mice. After correctly crossing the primordium

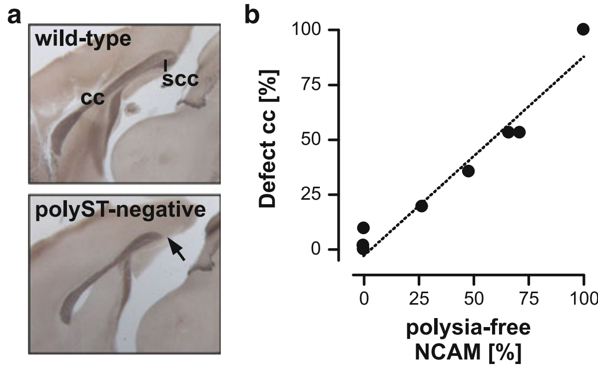

Fig. 3 Polysialylation of NCAM is essential for brain development. (**a**) In mice without functional polysialyltransferases (PolyST-negative) the splenium of the corpus callosum (scc) is markedly reduced (*arrow*), leading to an overall shorter corpus callosum (cc) as shown here in midsagital sections. (**b**) The severity of the corpus callosum defect in 4- to 6-week-old mice correlates linearly with the amounts of polySia-deficient NCAM at postnatal day 1. The strongest defect, i.e., the shortest cc, and the most polySia-free NCAM were detected in the polyST-negative *St8siaII*, *St8siaIV* double-knockout mice and set to 100%. Each of the data points stands for one of the nine mouse lines investigated (see text for details). Adapted from [165]

of the reticular thalamic nucleus the thalamocortical axons fail to turn into the internal capsule and therefore are unable to meet the corticothalamic fibers. In addition, deficiencies of corticothalamic connections contribute to the hypoplasia of the internal capsule in polysialylation-deficient mice [80]. The same study revealed a striking degeneration of the reticular thalamic nucleus (Rt) in specifically the $II^{-/-}IV^{-/-}$ mice. Apoptotic loss of Rt neurons occurred right after birth in a narrow time-window, closely matching the onset of glutamatergic innervation by thalamocortical and corticothalamic fibers under healthy conditions. Apoptosis of Rt neurons could also be induced by lesioning corticothalamic fibers on whole-brain slice cultures, suggesting that defective afferent innervation leads to anterograde transneuronal degeneration. The loss of Rt neurons in polysialylation-deficient, NCAM-positive mice, therefore, seems to be caused by the defects of thalamocortical and corticothalamic axon development.

4.3 PolySia in Oligodendrocyte Maturation and Myelination

Surprisingly, the polysialylated form of the synaptic cell adhesion molecule SynCAM 1 was recently found to be expressed by a subpopulation of NG2 cells (polydendrocytes) in the perinatal mouse brain [33]. These multifunctional precursor cells serve as the primary source of myelinating oligodendrocytes during development and myelin repair but are also able to give rise to astrocytes and neurons [166, 167]. Possible functions of polySia as a modification of SynCAM 1 have not

yet been explored. The most prominent function of SynCAM 1, however, is its potency to induce neuronal synapse formation [168]. Interestingly, NG2 cells receive glutamatergic synaptic input [169, 170]. Thus, integration of NG2 cells into neural networks might be modulated by polysialylation of SynCAM 1 [33].

PolySia on NCAM is expressed by migrating oligodendrocyte precursor cells (OPC) but down-regulated during maturation into myelinating oligodendrocytes [72, 91, 92]. During the two phases of oligodendrocyte development polySia seems to play a dual role. On the one hand, the presence of polySia promotes OPC migration in response to chemoattractive guidance cues [171–173]. On the other hand, polySia helps to keep the precursors in an undifferentiated state, while downregulation of polySia enhances differentiation into mature oligodendrocytes as shown in vitro and under pathological conditions of precursor recruitment from the anterior subventricular zone after lysolecithin-induced demyelination of the corpus callosum [174, 175]. In a complementary approach, neural precursor cells overexpressing ST8SiaIV were transplanted into the brain of hypomyelinated shiverer mice. The engineered cells displayed widespread integration and myelination in the host, but differentiated more slowly than controls [176]. Involvement of polySia as a negative regulator in the process of myelination itself has been derived from co-cultures of oligodendrocytes and neurons. In this in vitro system, removal of polySia enhanced myelin formation, but, in contrast to the studies on OPCs discussed above, the negative regulation of myelination was attributed to the presence of polySia on axons, thought to prevent attachment of the myelin-forming oligodendrocyte processes [177]. The question whether down-regulation of polySia is required for the myelination process in vivo was addressed in transgenic mice expressing the polysialyltransferase ST8SiaIV under the control of the proteolipid protein promoter [178]. In these mice, postnatal down-regulation of polySia in oligodendrocytes was abolished. Similar to the transplantation study with polySia overexpressing precursors [176], the sustained polysialylation caused a delay of oligodendrocyte maturation and myelin formation. Furthermore, the transgenic mice exhibited structural abnormalities of their myelin and axonal degeneration. Thus, myelin formation per se does not necessarily require the loss of polySia from the oligodendrocyte membrane but down-regulation of polysialylation during oligodendrocyte differentiation is a prerequisite for efficient myelin formation and maintenance [178].

4.4 Cellular Models of Polysialic Acid-Controlled NCAM Signaling

In rodents and humans, polySia is part of the neurogenic niches in the anterior subventricular zone and in the subgranular layer of the dentate gyrus [102, 104, 106, 179, 180]. As shown by endosialidase treatment in vivo, loss of polySia causes premature differentiation of neuronal precursors in both systems [181, 182]. In the dentate gyrus of ST8SiaII-deficient mice, many of the immature granule neurons

display aberrant locations and morphology, suggesting a role of ST8SiaII in their terminal differentiation [139]. Reminiscent of the in vivo data, removal of polySia from cultured subventricular zone-derived neuroblasts promotes neurite induction and maturation into olfactory bulb interneurons [183]. Interestingly, both effects were independent from changes in cell migration and could be mimicked by exposure to polySia-free NCAM. The assumed gain of NCAM function in the absence of polySia is corroborated by the finding that the degree of differentiation in cultures obtained from polySia-negative, NCAM-positive $II^{-/-}IV^{-/-}$ mice was higher than in $Ncam^{-/-}$ neuroblasts [183]. This outcome is highly compatible with the proposed role of polySia as a key regulator of NCAM interactions in brain development (see Sect. 4.2). Further experiments revealed that the effect of polySia removal depends on cell–cell contacts and that NCAM-negative and polySia-NCAM-positive neuroblasts respond equally well to polySia-free NCAM. Thus, NCAM on the cell surface is not required for these effects, suggesting the existence of heterophilic signaling. In agreement with these observations, heterophilic NCAM binding has been shown to promote differentiation of hippocampal progenitors from the embryonic brain [184]. In this study, however, the influence of polySia was not addressed.

The potency of polySia as a regulator of particularly heterophilic NCAM interactions has been clearly demonstrated in a series of in vitro studies with tumor cells [21–23, 140]. The prevailing model of NCAM-induced signaling involves association with fibroblast growth factor (FGF) receptors and predicts their activation as well as downstream signaling through the mitogen-activated protein kinase ERK1/2 pathway [185, 189]. Consistent with this model, a crucial role of ERK1/2 in polySia-regulated, heterophilic NCAM signaling was identified, leading to cell differentiation, growth arrest, and increased cell survival (Fig. 4) [21, 22]. A recent study confirms that the activation of ERK1/2 in response to a loss of polySia indeed depends on FGF receptor activity [23]. Moreover, experimentally induced loss of polySia initiates NCAM-mediated signaling at cell–cell contact sites causing reduced motility and enhanced focal adhesion at the cell-substrate interface. Surprisingly, this response was independent from FGF receptor and ERK1/2 activation but involves association of the src-family kinase $p59^{fyn}$ (Fyn) with paxillin (Fig. 4). The analysis of a set of truncated NCAM variants revealed that induction of focal adhesion is triggered by NCAM domains distinct from the FGF receptor binding site. A fragment comprising the immunoglobulin-like domains Ig3 and Ig4 is sufficient to induce focal adhesion but lacks the ability to activate ERK1/2. By contrast, the fibronectin type III repeats containing the FGF receptor binding site are sufficient to induce activation of ERK1/2 but unable to promote focal adhesion [23].

Although these studies were performed in tumor cells, the mechanisms of polySia-controlled NCAM signaling may apply to other cell models. As described above, subventricular zone-derived neuroblasts and hippocampal progenitors respond with enhanced differentiation to loss of polySia or exposure to polySia-free NCAM [182–184], and OPCs differentiate significantly faster after enzymatic removal of polySia than in the absence of NCAM [174, 175]. It should also be

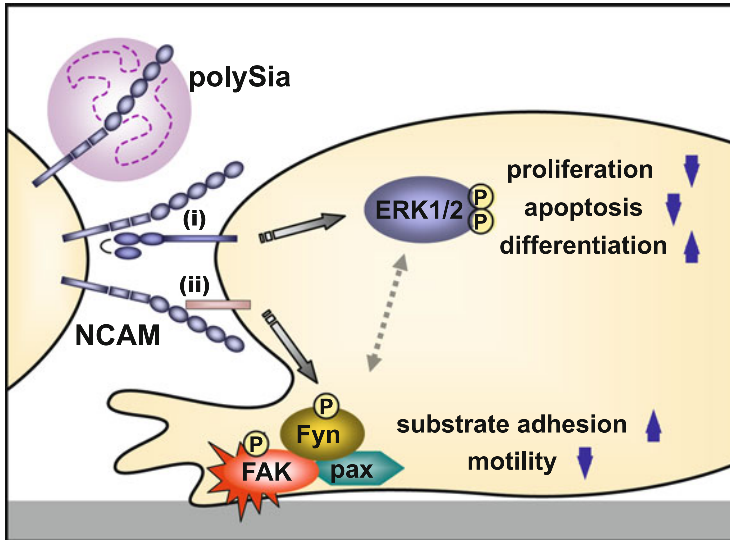

Fig. 4 Model of polySia as a negative regulator of heterophilic NCAM signaling. Removal of polySia unmasks NCAM and thereby initiates NCAM-mediated heterophilic interactions at cell–cell contacts. This involves (**i**) FGF receptor activation by the fibronectin type III repeats of NCAM leading to ERK1/2 dependent promotion of cell differentiation with reduced proliferation and enhanced survival and (**ii**) interactions of NCAM Ig3-4 domains with an unknown binding partner causing recruitment of the src family kinase Fyn to paxillin (pax) and focal adhesion kinase (FAK) to enhance focal adhesion at the cell-substrate interface. Kinase activation by phosphorylation is indicated by (P). Based on [21–23]

noted that focal adhesion kinase-dependent point contacts regulate growth cone motility [190]. Thus, polySia-dependent NCAM signaling from cell–cell to cell–substrate contacts may modulate growth cone adhesion and motility and this could contribute to the axon guidance deficits caused by the gain of NCAM functions in polysialylation deficient mice (see Sect. 4.2).

5 Polysialic Acid in Synapse Formation, Synaptic Plasticity, Learning and Memory

5.1 *Formation of Excitatory Synapses*

The role of NCAM in formation of excitatory hippocampal synapses is mediated by a polySia-dependent heterophilic mechanism [80]. As polySia and NCAM are expressed both pre- and postsynaptically, the original topic of investigation was to distinguish between pre- vs postsynaptic effects. This was done using so-called heterogenotypic co-cultures of *Ncam*$^{+/+}$ and *Ncam*$^{-/-}$ neurons. Comparison of the

mean amplitudes of excitatory postsynaptic currents in synaptic connections with different patterns of pre- vs postsynaptic NCAM expression revealed that the presence of NCAM presynaptically did not influence synaptic strength, whereas postsynaptic expression of NCAM increased the synaptic strength by a factor of 2. Analysis of synaptophysin immunoreactivity associated with NCAM-positive and NCAM-negative neurons revealed a twofold higher synaptic coverage of NCAM-positive cells. This was observed only in heterogenotypic cultures, i.e., under conditions when growing axons had a choice of which postsynaptic target to select, *Ncam*$^{+/+}$ or *Ncam*$^{-/-}$. There was no difference between NCAM-positive and NCAM-negative neurons in synaptic coverage in homogenotypic cultures. Thus, expression of NCAM dictates where to form synapses, but is not required for synapse formation. Since expression of NCAM and polySia in the CNS is regulated in an activity-dependent manner [191], an increase in NCAM/polySia-NCAM expression may promote experience-dependent excitatory synaptogenesis in stimulated neurons and/or dendritic subdomains [192].

Does NCAM act as a ligand or a receptor during formation of excitatory synapses? Transfection of NCAM-deficient neurons with any of three major NCAM isoforms, GPI-linked NCAM120, or transmembrane domain-containing NCAM140 or NCAM180 stimulated preferential synapse formation on all NCAM isoform-expressing neurons [82]. These experiments suggest that the extracellular domain of NCAM functions as a synaptogenic ligand. To investigate the involvement of polySia, cultures were treated with endosialidase. This treatment completely abolished preferential formation of synapses in NCAM-expressing cells. Enzymatic removal of heparan sulfates from cultured neurons, a mutation in the heparin-binding domain (HBD) of NCAM, and application of recombinant soluble extracellular domains of NCAM and polySia-NCAM similarly diminished synaptogenic activity of neuronally expressed polySia-NCAM, suggesting that interaction of NCAM with heparan sulfate proteoglycans is involved. PolySia-NCAM-driven synaptogenesis was also blocked by antagonists to FGF receptor and the NMDA subtype of glutamate receptors, but not by blockers of non-NMDA glutamate receptors or voltage-dependent Na^+ channels. Enzymatic removal of polySia and heparan sulfates also suppressed the increase in the number of perforated spine synapses associated with NMDA receptor-dependent long-term potentiation (LTP) in the CA1 region of organotypic hippocampal slice cultures [82]. Thus, neuronal polySia-NCAM in complex with heparan sulfate proteoglycans promotes synaptogenesis and activity-dependent remodeling of synapses.

In *St8siaII*-knockout mice, ectopic synapse formation of hippocampal mossy fibers has been detected together with axon mistargeting and abnormal extension of the infrapyramidal mossy fiber bundle [143]. In the mature brain, however, polySia expression on the mossy fibers depends on ST8SiaIV activity [141] and is maintained in adult *St8siaII*-knockout mice [143]. This indicates that ectopic formation of mossy fiber synapses originates from a lack of ST8SiaII during development, when both polysialyltransferases are co-localized in the dentate gyrus [83]. Recently, the role of ST8SiaII and polySia in synapse formation of hippocampal mossy fibers has been addressed by the use of a chemically modified

sialic acid precursor (*N*-propanoyl-D-mannosamine, ManNProp) [193]. ManNProp can be used by ST8SiaIV to produce unnatural propanoyl-polySia but inhibits ST8SiaII activity [194]. Treatment of hippocampal slice cultures derived from newborn mice with ManNProp resulted in aberrant mossy fiber projections forming functional glutamatergic terminals on CA1 pyramidal neurons with a typical mossy fiber synapse-like morphology [193]. Reminiscent of the phenotype of the *St8siaII*-knockout mice, in vivo application of ManNProp to newborn rats for 4 weeks yielded a significantly longer infrapyramidal mossy fiber bundle. However, unlike in *St8siaII*-knockouts, aberrant fibers were polySia-negative but NCAM-positive and entered into the CA1 pyramidal layer. Moreover, recurrent mossy fibers were observed in the ManNProp-treated rats, which aberrantly crossed the granule cell layer to terminate on neurons in the molecular layer [193]. Interestingly, this aberrant innervation pattern resembles the mossy fiber sprouting observed after kainate induced status epilepticus. Homeostatic regulation of polySia synthesis, therefore, is essential for correct outgrowth and synaptic targeting of hippocampal mossy fibers.

5.2 *Plasticity of Inhibitory Synapse Maturation*

In the visual cortex, polySia is downregulated shortly after eye opening. This decline is inversely correlated with the maturation of GABAergic innervation and hindered by visual deprivation, indicating a role of polySia in the critical period of ocular dominance plasticity [83]. Indeed, premature reduction of polySia promotes functional maturation of GABAergic synapses. Removal of polySia by application of endosialidase to cortical organotypic cultures causes precocious maturation of perisomatic GABAergic synapses as evidenced by enhanced branching of axon arbors and higher density of mature presynaptic boutons [83]. As shown by injection of endosialidase, a too early loss of polySia in the adolescent visual cortex also promotes the maturation of perisomatic GABAergic innervation in vivo and, consistent with a higher number of GABAergic synapses, increased the frequency of miniature inhibitory postsynaptic currents. In addition, a shift in occular dominance, which can normally be evoked by monocular deprivation during a critical period between P24 and P35, could be induced much earlier in endosialidase-treated mice [83]. The enhanced inhibition in response to the loss of polySia, therefore, seems to trigger precocious ocular dominance plasticity.

Preceding the decline of polySia in the mouse visual cortex, ST8SiaII and ST8SiaIV mRNA levels decrease around the time of eye opening. However, only the reduction of ST8SiaII is regulated by sensory experience [86]. Moreover, in organotypic slice cultures, developmental downregulation of ST8SiaII is reduced by blocking spike activity with tetrodotoxin or by AP5 as antagonist of NMDA receptors and enhanced by blocking $GABA_A$ receptors with bicuculline. This indicates that ST8SiaII gene expression is regulated by activity and in particular by NMDA-mediated excitation [86]. Interestingly, a similar regulation of polySia

by sensory input and activity through NMDA receptor-dependent mechanisms has been shown during postnatal development of the dorsal vagal complex in the brain stem [195]. Conversely, as detailed in the next section, polySia modulates extrasynaptic NMDA receptor signaling, pointing towards a possible feedback regulation.

Contrasting the precocious maturation of perisomatic innervation after an acute loss of polySia, mice over-expressing a soluble extracellular domain fragment of NCAM under the neuron-specific enolase promoter (NCAM-EC mice) display a reduction in perisomatic synaptic puncta formed by parvalbumin-positive cortical interneurons, indicating a decrease in the number of synaptic terminals of basket cells [198]. Further investigations of these mice revealed perturbed arborization of basket cells in the prefrontal cortex during early postnatal stages, when endogenous polysialylated NCAM is replaced by polySia-negative NCAM [85]. Consistent with the enhanced inhibition in the visual cortex after endosialidase treatment [83], a recent study demonstrates increased numbers and sizes of perisomatic basket cell synapses in *Ncam*-knockout mice [199]. Moreover, the study also provides evidence that polysialylated is required to promote ephrinA5-induced axon remodeling of basket interneurons in cortical slices. Together, these data impressively demonstrate that the balanced regulation of polySia and NCAM is essential for proper synapse development of basket cells.

5.3 Synaptic Plasticity in the Mature Nervous System

The first evidence that NCAM may play a role in synaptic plasticity was provided in 1994 by a seminal study that showed that perturbation of NCAM function significantly reduced LTP in the CA1 area of the hippocampus [200]. Polyclonal antibodies against NCAM, soluble oligomannosides that block interaction of NCAM with oligomannosidic carbohydrates carried by L1, and synthetic peptides from the fourth Ig-like domain of NCAM, which mediates interaction with L1, were used in these experiments. Further analysis of constitutive and conditionally NCAM deficient mice (NCAMff+), in which the NCAM gene was ablated in neurons after cessation of major developmental events by expression of Cre recombinase under the CaMKII promoter, showed impairment of CA1 LTP in both mutants [191, 196, 201], thus supporting the view that NCAM plays an acute role in synaptic plasticity in the CA1 region. Additionally, long-term depression (LTD) in the CA1 was impaired in constitutive and conditional *Ncam*-knockout mice [197, 201]. In the CA3 region, constitutive but not conditional NCAM-deficient mice were found to have abnormalities in lamination of mossy fiber projections and to be impaired in mossy fiber LTP, suggesting that NCAM is required for proper development of mossy fiber-CA3 synapses [201, 202]. Recording of LTP in the dentate gyrus of anesthetized mice confirmed the role of NCAM in synaptic plasticity in vivo [203] (Table 1). Overexpression of soluble extracellular domain of NCAM in NCAM-EC

Table 1 Effects of NCAM, polySia and polysialyltransferases on multiple forms of hippocampal synaptic plasticity

Condition	CA1 LTP	CA1 LTD	CA3 LTP	DG LTP
Ncam−/−	↓	↓	↓	↓
*Ncam*ff+	↓	↓	=	n.d.
Endosialidase	↓	↓	=	n.d.
*St8sia*IV−/−	↓	↓	=	=
*St8sia*II−/−	=	n.d.	=	=

↓ impaired, = normal, *n.d.* not determined

mice did not affect LTP in CA3-CA1 synapses but resulted in specific impairment of LTP in the prefrontal cortex [204].

The role of polySia in synaptic plasticity was initially studied using the enzymatic removal of polySia by endo-N, which inhibited LTP and LTD in CA1 [123, 191]. Experiments using mice deficient in ST8SiaII or ST8SiaIV provided the genetic evidence for the importance of polySia in synaptic plasticity in the CA1 [141, 143]. No involvement of these enzymes in mossy fiber LTP in the CA3 region or in the dentate gyrus LTP was revealed using either of two lines of polySia-deficient mice, despite abnormal lamination of mossy fiber projections in ST8SiaII deficient mutants [141, 143, 203].

Several early observations suggested that polySia may modulate activity of glutamate receptors and hence regulate induction of LTP. Peptides blocking interaction of proteins with the fourth immunoglobulin-like domain of NCAM reduced LTP when applied before induction of LTP but not afterwards [205], pointing to the importance of NCAM for LTP induction. Since impairment of LTP in NCAM deficient mice could be rescued by elevation of extracellular Ca^{2+} concentration, it has been speculated that NCAM influences Ca^{2+} signaling via NMDA receptors [201]. This idea was supported by the data showing a similar central location of NCAM180 and the GluN2A receptors within the postsynaptic density in untreated animals, and a similar redistribution of these molecules to the edges of postsynaptic density in animals after induction of LTP [206]. Because polySia may directly potentiate opening of AMPA subtype glutamate receptors [207], it was hypothesized that polySia may influence activity of LTP-mediating receptors via a direct interaction with the extracellular domain of receptors. This hypothesis was supported by a study [208] in which impaired CA1 LTP in hippocampal slices was rescued via application of soluble polySia or ectodomain of polySia-NCAM, but not of NCAM. A parallel in vitro study [209] reported that soluble polySia alone or attached to NCAM inhibited activation of GluN2B-containing NMDA receptors by low micromolar concentrations of glutamate in hippocampal cultures and artificial lipid bilayers (Fig. 5). These concentrations are characteristic for extrasynaptic space but much lower than synaptic concentrations of glutamate following transmitter release.

Two recent studies demonstrate that polySia-NCAM regulates synaptic plasticity by setting a balance in signaling via extrasynaptic GluN2B and synaptic GluN2A receptors [196, 197]. Consistent with the findings of Hammond and colleagues [209], isolation of NMDA receptor-mediated component in hippocampal slices

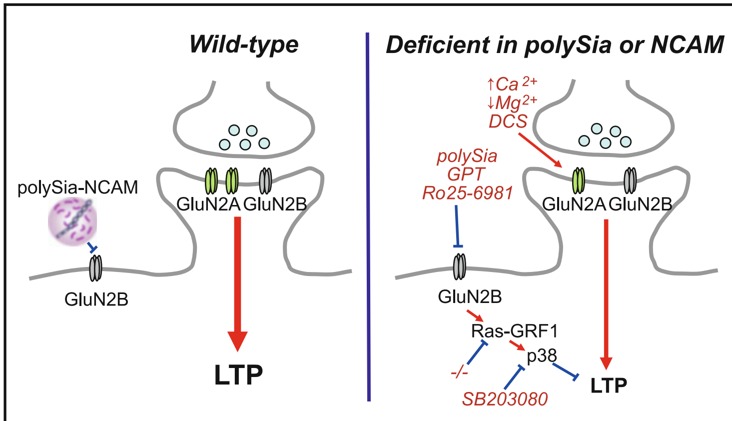

Fig. 5 Model for polySia-NCAM-mediated modulation of signaling via NMDA receptors. Endogenous and exogenous molecules are shown in *black* and *gray*, respectively. Stimulatory and inhibitory relationships are shown by *arrows with sharp and blunt ends*, respectively. In *Ncam*+/+ mice, polySia-NCAM inhibits extrasynaptic GluN2B-containing receptors and LTP is induced through activation of GluN2A receptors. In *Ncam*−/− mice, signal enhancement occurs via extrasynaptic GluN2B-containing receptors, whereas signal reduction occurs via GluN2A-containing receptors, which leads to impaired LTP. This model is supported by experiments with rescue of LTP in *Ncam*−/− mice by elevated extracellular Ca^{2+} and reduced extracellular Mg^{2+} concentrations, and application of NMDA receptor modulators DCS or Ro 25–6981, the glutamate scavenger GPT, polySia, or SB 203580 [196]. All these treatments change the signaling balance between GluN2A- and GluN2B-mediated pathways in favor of the GluN2A-mediated pathway and restore LTP in *Ncam*−/− mice. Similarly, LTP is restored in endosialidase-treated slices from *Ncam*+/+ mice by DCS, Ro 25–6981, and SB 203580, and by genetic ablation of Ras-GRF1. Furthermore, fear memory is restored by DCS and Ro 25–6981 in *Ncam*−/− mice, whereas LTD is rescued by DCS. Modified with permission from [197]

also revealed an increase in GluN2B-mediated transmission in NCAM-deficient mice and an increase in GluN2B-mediated Ca^{2+} signaling after removal of polySia [196]. In parallel, a decrease in GluN2A-mediated transmission was found. Strikingly, the suppression of extrasynaptic GluN2B signaling with Ro 25–6981 or by reduction of extrasynaptic glutamate concentrations using the glutamate scavenger GTP, or facilitation of GluN2A receptors by D-cycloserine fully restore levels of LTP in either NCAM or polySia deficient slices to wild-type levels.

The following data support the view that the mechanisms downstream of GluN2B in NCAM/polySia deficient slices involve signaling via Ras-GRF1 to the Rac effector p38 MAPK. The level of phosphorylated p38 MAPK is upregulated in NCAM-deficient mice and in endosialidase-treated slices, while it is reduced in Ras-GRF1−/− mice [210]. An inhibitor of p38 restores levels of LTP in polySia or NCAM-deficient slices to those seen in wild-type mice. The level of phosphorylated p44 is co-upregulated although to a smaller degree than p38, as expected in response to activation of Ras-GRF1, which has been shown to mediate synaptic depression through p38 MAPK [210]. PolySia deficiency does not lead to impaired LTP in

Ras-GRF1−/− mice, supporting the view that Ras-GRF1 is a transducer downstream of hyperactive GluN2B in polySia deficient neurons. Interestingly, activation of p38 MAPK signaling has also been shown to mediate impairment in LTP by tumor necrosis factor [211], by injection of the ectodomain of another cell adhesion molecule, neuroplastin [212], and by the Aβ peptide generated from the amyloid precursor protein, which is widely believed to underlie the pathophysiology of Alzheimer's disease [213]. In these cases, p38 MAPK enhances the removal of AMPA and NMDA receptors from the postsynaptic cell surface, which is likely to be the mechanism for impaired LTP in polySia/NCAM deficient mice. The mechanisms of impaired LTP in the prefrontal cortex of NCAM-EC mice are unknown. However, the study of tenascin-R deficient mice demonstrates that a deficit in perisomatic GABAergic inhibition in the hippocampus may induce metaplastic increase in the threshold for induction of LTP [214]. Whether this is also the case in the prefrontal cortex of NCAM-EC mice remains to be investigated.

5.4 Learning and Memory

The results showing the role of polySia and NCAM in hippocampal plasticity are nicely complemented by studies of hippocampus-dependent spatial and place (contextual) learning in the Morris water maze and fear conditioning paradigms. These studies demonstrated learning-associated changes in the expression of NCAM and polySia [213–218], and impaired memory after treatment with NCAM antibodies [219], in constitutively NCAM-deficient mice [152, 208, 220] and in conditionally NCAM-deficient mice [201, 221]. Temporal perturbations of NCAM or associated polySia at different phases of learning and memory lead to the same memory deficits in spatial navigation [123, 218] and contextual fear conditioning [208]. Furthermore, NCAM-EC mice are impaired in contextual and cued fear conditioning and working memory [198, 204], and genetic ablation of polysialyltransferases ST8SiaII or ST8SiaIV results in impaired fear conditioning [143, 208] or impaired spatial and reversal learning in the Morris water maze [222]. As the pre-training treatment with GluN2B antagonist Ro 25–6981 and GluN2A agonist D-cycloserine, which restored LTP in polySia/NCAM deficient hippocampal slices, also rescued hippocampus-dependent contextual fear memory in NCAM deficient mice [196, 197], acquisition of this form of learning appears to depend on polySia-NCAM mediated modulation of signaling through NMDA receptors. Another study suggests that polySia might also contribute to consolidation of memories, as mice which were injected with polySia cyclic mimetic peptide pr2 into the dorsal hippocampus (5 h after massed training in the spatial version of the water maze) showed higher levels of memory retention 24 h, 1 week and 4 weeks after the training [223].

6 Implications for Nervous System Disease

6.1 PolySia and Epilepsy

Because various plastic changes have been suggested to be functionally involved in the epileptogenesis, and polySia is the key molecule involved in plasticity, several studies addressed the polySia role in development and progression of epilepsy and associated comorbidities. There is increased expression of polySia in the hippocampus and the entorhinal cortex in human temporal lobe epilepsy [224]. Removal of polySia with endosialidase in rodents increased acute seizure susceptibility, as indicated by reduced seizure threshold [225], and lowered the number of newborn neurons following the status epilepticus (SE, induced by electrical stimulation of basolateral nucleus of the amygdala), as compared to vehicle-treated rats, thereby counteracting the SE-induced increase in neurogenesis [226]. Nevertheless, the SE induced increases in the total number of doublecortin expressing immature neurons and the fraction of doublecortin-positive cells with persistent basal dendrites was not affected by endosialidase treatment. There was also no effect of endosialidase on the number of seizures, their severity, and the duration of single seizures [226].

This is in contrast to the results obtained by intraperitoneal injection of kainate for induction of seizures, as mice deficient in ST8SiaIV showed a reduced latency to the first generalized seizure and higher lethality due to SE [227]. In the elevated plus maze paradigm, the loss of polySia in *St8siaIV*-knockout mice increased anxiety-associated behavior, suggesting a major implication of the polySia–NCAM system in the control of anxiety after SE [227]. In view of the fact that anxiety disorders represent a frequent clinical problem in epileptic patients [228], it will be of interest to evaluate further the potential of polySia–NCAM targeting for the treatment of these comorbidities.

Also in the model of mesial temporal lobe epilepsy with unilateral hippocampal injection of kainate, contralateral i.c.v. endosialidase infusion severely increased neurodegeneration in the KA-lesioned hippocampal formation [229]. A significant increase in cell death was evident in the lesioned CA3 pyramidal cell layer, accompanied by strong astrogliosis throughout the lesioned hippocampal formation. Neurodegeneration also extended to the dentate gyrus and led to early onset of focal seizures, in line with data obtained in ST8SiaIV-deficient mice and with a previous study showing that hyperthermic preconditioning-induced upregulation of polySia-NCAM has a neuroprotective effect against kainate [230]. The striking *trans*-hemispheric influence of endo-N suggests that polySia-NCAM might mediate transport of neuroprotective signals into the lesioned hippocampus. One of the signals appeared to be the binding partner of NCAM – GDNF – since injection of GDNF antibodies into the contralateral hippocampus of kainate-treated mice mimicked injection of endosialidase by enhancing neurodegeneration and decreasing expression of the GDNF family receptor α1 in the epileptic focus. Thus, polySia-NCAM-mediated modulation of GDNF signaling restrains neurodegeneration and delays onset of SE.

6.2 PolySia and Neurodegeneration

There are significantly fewer NCAM-positive neurons in the frontal cortex of Alzheimer's disease (AD) patients than in normally aging individuals, although there is little difference in the levels of NCAM in the occipital cortex and hippocampus of the two groups [231]. However, polySia is over-expressed in the outer two-thirds and the inner third of the molecular layer of the dentate gyrus in AD patients [232]. Furthermore, fiber and neuropil staining is increased in the strata oriens, radiatum, and pyramidale of the CA1 subfield. There are changes in polySia immunoreactivity in layer II and in the superficial portion of layer III of the entorhinal cortex. Thus, polySia is upregulated in hippocampal areas where neurofibrillary tangles, amyloid plaques, and neuronal loss appear, or where neurons suffer from a lack of inputs and undergo remodeling. Interestingly, acute administration of Aβ increased expression of polySia in the CA1 and DG subfields [233], but significantly decreased expression of another carbohydrate carried by NCAM, HNK-1 [234]. Moreover, HNK-1 immunoreactivity was decreased in brain tissue of a transgenic mouse model of AD.

In this context, it is remarkable that application of polySia mimetic and the combination of polySia and HNK-1 mimetics, but not the HNK-1 mimetic alone, improves functional recovery after spinal cord injury [235]. A better outcome in polySia mimetic-treated mice is associated with higher, as compared with control mice, numbers of cholinergic and glutamatergic terminals and monaminergic axons in the lumbar spinal cord, and better axonal myelination proximal to the injury site. These data suggest that polySia mimetic peptides can be efficient therapeutic tools augmenting plasticity and synaptogenesis. Furthermore, several NCAM-mimicking or -derived peptides have neuroprotective properties. For instance, systemic treatment with the FGL peptide (mimicking NCAM interaction with FGF receptors) reverses depression-like behavior in NCAM deficient mice, reduces neuroinflammation and neuroglial activation within the aged rat hippocampus and the age-related loss of synaptophysin immunoreactivity within CA3 and hilus, and attenuates Aβ induced neuropathology and cognitive impairment that are hallmarks of Alzheimer's disease [236]. The latter effects are mediated by inhibition of the GSK3beta kinase activity. The mechanisms of polySia mimetic peptides are less clear, but several putative polySia binding molecules have been identified, including BDNF, NT-3 and NT-4 [237], FGF2 [238], GluN2B-containing NMDA receptors [196], and the human-specific Siglec-11 [239], with prominent neuroprotective properties. It is particularly noteworthy that human Siglec-11 ectopically expressed on murine microglia and interacting with polySia on neurons reduces lipopolysaccharides-induced gene transcription of proinflammatory mediators, impairs phagocytosis, and alleviates microglial neurotoxicity [239].

6.3 PolySia in Demyelinating Disease and Remyelination

PolySia has been detected on chronically demyelinated axons in multiple sclerosis lesions, whereas remyelinated axons in so-called shadow plaques with partial repair were negative for polySia [240]. These data suggest that re-expression of polySia on axons could act as an inhibitor of remyelination. This is supported by the strong polySia immunoreactivity on axons of hypomyelinated white matter in a mouse model of Niemann–Pick disease type C [241] and by the persistence of polySia expression on unmyelinated fibers of the healthy rodent brain such as axons in the fimbria and in the mossy fiber tract of the hippocampal formation [77]. A hint that polySia expression interferes to at least some extent with myelin repair in vivo, was obtained by applying the mouse model of cuprizone-induced de- and remyelination to ST8SiaIV-deficient mice [242]. These mice have normal developmental myelination, and comply with the stereotyped pattern of white and gray matter demyelination described for mice fed with the neurotoxic copper chelator cuprizone [243, 244]. However, reexpression of several myelin markers and thus remyelination were accelerated in *St8siaIV*-knockout mice during the first week after withdrawal of the toxin. The effect has been assigned mainly to enhanced OPC differentiation and to a lesser extent to OPC recruitment [242]. The data are proof of the principle that targeting polysialyltransferases could be used to improve remyelination.

6.4 PolySia and Neuropsychiatric Disorders

6.4.1 Altered NCAM and PolySia Levels in Neuropsychiatric Disorders

A long standing record links dysregulation of NCAM to the pathophysiology of schizophrenia and other neuropsychiatric disorders. As reviewed in detail elsewhere [245, 246], a number of studies found increased concentrations of NCAM fragments in either serum or cerebrospinal fluid of schizophrenic patients or in post-mortem brain including samples from hippocampus and cingulate cortex. Elevated levels of NCAM fragments were also detected in cerebrospinal fluid and post-mortem brain samples of patients with bipolar disorder – for review see [245, 246]. In autism, an early study reported a decrease in a small, 70-kDa serum fragment of NCAM [247]. This finding was not reproduced by a later study, which instead found a trend towards an increase of a 105-kDa to 115-kDa NCAM immunoreactive band corresponding to the major form of NCAM typically detected in human serum samples [248]. In addition, the same study reported lower levels of specifically the 180-kDa isoform in post-mortem samples of the cerebellar cortex. In most of the studies, however, the exact nature as well as possible sources of the NCAM fragments remains elusive. Concerning polySia, one small, but prominent study found moderate to severe reductions of polySia-positive cell numbers in the hilus of the dentate gyrus in eight out of ten post-mortem brains of medicated schizophrenic patients as compared

to control brains [249]. Importantly, no significant difference was detected in the total numbers of cells in the hilus, and polySia immunoreactivity in the granular cell layer of the dentate gyrus was not apparently altered between schizophrenic and control brains. Furthermore, a recent immunohistochemical comparison of brain sections from psychiatric disorder patients indicates that polySia is not altered in the amygdala of schizophrenics but is reduced in depressed patients and increased in bipolar disorder [250].

6.4.2 Genetic Associations

Schizophrenia has a high heritability and genome-wide association studies indicate a polygenic origin with a shared genetic liability between schizophrenia and bipolar disorder [251, 252]. *NCAM1* and both polysialyltransferase genes map to chromosomal regions that harbor susceptibility loci for schizophrenia (11q23.1, 15q26, and 5q21 for *NCAM1*, *ST8SIA2*, and *ST8SIA4*, respectively) [253–255]. More telling, single nucleotide polymorphisms (SNPs) in *NCAM1* as well as in the promoter region of *ST8SIA2*, but not *ST8SIA4*, have been associated with schizophrenia [256–259]. Interestingly, risk haplotypes in the promoter region of *ST8SIA2* were identified by two independent studies in the Japanese and the Han Chinese population [256, 259]. In vitro promoter assays with one of the risk-associated variants point towards enhanced transcriptional activity [256]. Recently, analysis of a point mutation detected heterozygously in just one schizophrenic patient in the Japanese sample revealed that the E141K substitution near the sialylmotif *L* leads to reduced activity and production of shorter polySia chains [260]. Other SNPs within *NCAM1* have been found to contribute to the risk of bipolar disorder [257, 261] and a genome-wide association study in the Han Chinese population found a strong association of an SNP close to *ST8SIA2* [262]. A genome-wide scan in an Italian population indicates a common susceptibility locus for schizophrenia and bipolar disorder in 15q26 including *ST8SIA2* [263]. In this study, however, analysis of two SNPs in the promoter region of *ST8SIA2*, directly associated with schizophrenia in the Japanese sample [256], failed due to low allele frequencies in the European population and therefore, association with *ST8SIA2* was not confirmed or ruled out [263]. Moreover, an exploratory analysis of a genome-wide association study of SNPs in more than 1,500 families with autism spectrum disorders (ASD) identified a strong association signal for an intronic SNP in *ST8SIA2* in a subgroup of the ASD sample stratified by verbal status [264].

6.4.3 Animal Models

Compelling evidence suggests that schizophrenia is associated with disturbed neurodevelopment resulting in altered brain connectivity [265, 266]. In light of the particularly strong links between the polySia-NCAM system and schizophrenia

in humans, the remarkable parallels between the phenotype of NCAM- or polySia-deficient mice and pathophysiological findings in schizophrenia merit a short survey. First, ventricular enlargement, one of the most abundant abnormalities in schizophrenia [267], has been reported for mice with specific deletion of NCAM-180 [268] and variable degrees of ventricular dilatations including cases of severe hydrocephalus were observed in $II^{-/-}IV^{-/-}$ mice [154]. Second, smaller olfactory bulbs occur in schizophrenia [269] and $N^{-/-}$ or $II^{-/-}IV^{-/-}$ mice [153, 154]. Third, reductions of corpus callosum and internal capsule as found in schizophrenic patients [266, 270–273] correlate with deficits of these axon tracts in polysialylation-deficient mice [80, 165]. Importantly, the almost linear correlation of gross anatomical defects with the premature occurrence of polySia-free NCAM [165] (see Sect. 4.2 and Fig. 3) suggests that even minor imbalances of NCAM polysialylation during brain development lead to deficits of connectivity. Fourth, reminiscent of cognitive impairment in schizophrenia [274], $N^{-/-}$ as well as polysialyltransferase-deficient $II^{-/-}$ and $IV^{-/-}$ mice display deficits in synaptic plasticity, learning, or memory formation (see Sects. 5.3 and 5.4) and one study reported reduced prepulse inhibition of acoustic startle in NCAM-180 knockout mice ([268], but see [275]). These animal models therefore highlight that genetic interference with the complex coordination of NCAM polysialylation has the potential to cause a neurodevelopmental predisposition to schizophrenia and possibly other disorders.

Chronic stress is a well established model of anxiety and depressive-like behavior in rodents. Daily exposure of rats to restraint stress for 3 weeks causes an up-regulation of polySia levels in the hippocampus [276] and a transient increase of polySia-positive neurons in the dentate gyrus associated with suppression of proliferation and reduced numbers of granular cells [276, 277]. Interestingly, a recent study revealed increased vulnerability to restraint stress and depression-like behavior as well as impaired neurogenesis in the dentate gyrus of heat shock factor 1 (HSF1)-deficient mice associated with reduced polySia and polysialyltransferase mRNA levels in early postnatal stages [278]. Binding of HSF1 to the *St8siaII* and *St8siaIV* promoters suggests regulation through direct transcriptional control. Since enzymatic removal of polySia from the neonatal hippocampus also affects depression-like behavior, the data imply that polysialylation under the control of HSF1 is essential for hippocampal development and behavioral maturation [278].

A stress-induced increase of polySia-positive cells is also observed in the piriform cortex [279]. In contrast, chronic stress leads to reduced levels of polySia immunoreactivity in the amygdala [280]. This was confirmed by a recent study showing that these changes can be attributed to altered polySia levels of interneurons, and are paralleled by lower mRNA levels of ST8SiaII and reduced expression of the GABAergic marker GAD67 [113]. Conversely, polySia and GAD67 are significantly increased in the amygdala of rats subjected to social isolation rearing, indicating that this model, which causes a behavioral phenotype with schizophrenia-like traits, is not reproducing the decrease of inhibitory markers found in the amygdala of schizophrenic patients [281].

Dysfunction of inhibitory neurotransmission and connectivity in the prefrontal cortex (PFC) are involved in the pathogenesis of schizophrenia and major

depression [282–284]. The medial PFC is particularly affected and in depression shows remodeling, which may be corrected by antidepressants. As discussed before (see Sect. 3.2), a series of studies show that polySia is specifically expressed by interneurons of the adult neocortex, and this holds true for the PFC in rodents and men [107, 109]. Strikingly, chronic antidepressant treatment with the selective serotonin reuptake inhibitor fluoxetine (Prozac®) increases polySia levels within the rat PFC [285–287] but also in other cortical areas [288]. Similar results have been obtained with another antidepressant, imipramine [289]. Likewise, a dopamine D2 receptor agonist increased, but the D2 receptor antagonist and antipsychotic drug haloperidol reduced polySia in the PFC [290]. Furthermore, polySia-positive interneurons show low levels of synaptic connectivity [114] and polySia seems to be required for the dopamine D2 receptor-mediated increase in perisomatic inhibition of principal neurons in the PFC of adult rats [291]. Collectively, these findings point towards polySia as a trigger of structural plasticity of inhibitory networks in the mature PFC. As shown in mice, polySia of interneurons in the mature PFC is exclusively produced by ST8SiaIV [142]. Thus, complementary to the potential role of dysfunctional ST8SiaII expression during brain development, altered NCAM polysialylation of cortical interneurons by ST8SiaIV may contribute to the etiology of neuropsychiatric disorders at later stages.

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UDP-GlcNAc 2-Epimerase/ManNAc Kinase (GNE): A Master Regulator of Sialic Acid Synthesis

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Abstract UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase is the key enzyme of sialic acid biosynthesis in vertebrates. It catalyzes the first two steps of the cytosolic formation of CMP-*N*-acetylneuraminic acid from UDP-*N*-acetylglucosamine. In this review we give an overview of structure, biochemistry, and genetics of the bifunctional enzyme and its complex regulation. Furthermore, we will focus on diseases related to UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase.

Keywords Bifunctional enzyme · GNE myopathy · GNE-opathy · *N*-Acetylneuraminic acid biosynthesis · Sialuria · Vertebrates

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AT2R	Angiotensin 2 receptor
bp	Basepairs
CHO	Chinese hamster ovary
CMP	Cytidine monophosphate
CRMP1	Collapsin response mediator protein 1
DMRV	Distal myopathy with rimmed vacuoles
ES cell	Embryonic stem cell
GalNAc	*N*-Acetylgalactosamine
GlcNAc	*N*-Acetylglucosamine
GNE	UDP-GlcNAc 2-epimerase/ManNAc kinase
HEK	Human epithelial kidney
HIBM	Hereditary inclusion body myopathy
IBM2	Inclusion body myopathy 2
kDa	Kilodaltons
ManNAc	*N*-Acetylmannosamine
ManNGc	*N*-Glycolylmannosamine
MRI	Magnetic resonance imaging
NCAM	Neural cell adhesion molecule
Neu5Ac	*N*-Acetylneuraminic acid
Neu5Gc	*N*-Glycolylneuraminic acid
PEP	Phospho*enol*pyruvate
PKC	Protein kinase C
PLZF	Promyelocytic leukemia zinc finger protein
PTM	Posttranslational modification

RIF1	Receptor interacting factor 1
ROK family	Repressor/open reading frame/kinase protein family
SPR	Surface plasmon resonance
STD-NMR	Saturation-transfer difference – nuclear magnetic resonance spectroscopy
TUC family	Toad-64/Ulip/CRMP protein family
UDP	Uridine diphosphate

1 Introduction

The family of sialic acids comprises more than 50 members, particularly different regarding the hydroxyl substituents of the common precursor *N*-acetylneuraminic acid (Neu5Ac). The biosynthesis of sialic acids is a complex process, which can generally be divided into two parts. The first steps comprise the de novo synthesis of Neu5Ac and its activated nucleotide sugar CMP-Neu5Ac. The second part covers the introduction of the several substituents to Neu5Ac, including formation of *N*-glycolylneuraminic acid (Neu5Gc) at the stage of the nucleotide sugar and modifications of the hydroxyl groups of glycan-bound sialic acids. In the following review we will focus on the initial biosynthesis of CMP-Neu5Ac from UDP-*N*-acetylglucosamine (UDP-GlcNAc), and in particular on the key enzyme of this pathway in all vertebrates, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (UDP-GlcNAc 2-epimerase/ManNAc kinase; GNE).

First, we will give a brief overview of sialic acid biosynthesis (Sect. 2). Then we will summarize the research on GNE in a historical outline (Sect. 3). The following three sections will describe the structure, biochemistry, and genetics of GNE in detail. We will further place emphasis on the very complex regulation of GNE (Sect. 7), which will highlight the enzyme as a master regulator of sialic acid synthesis. The last section will focus on diseases related to GNE, the so-called GNE-opathies, a fast growing field of research with fascinating new insights into the roles of GNE and of sialic acids.

2 Sialic Acid Biosynthesis Pathway

The starting compound for the biosynthesis of sialic acids is UDP-GlcNAc. This nucleotide sugar is the key compound of the whole amino sugar metabolism, and it is formed by the hexosamine pathway, which splits off from glycolysis at the stage of fructose 6-phosphate. In a particular context the hexosamine pathway may also

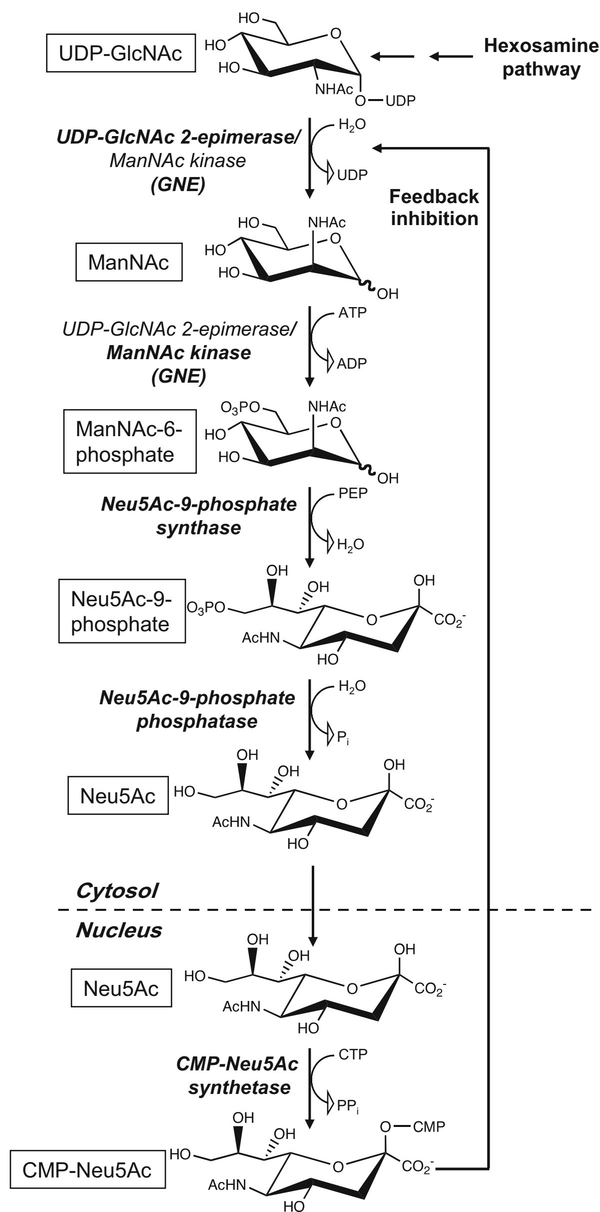

Fig. 1 Sialic acid biosynthesis in vertebrates. GNE catalyzes the first two steps of this pathway. The respective enzymatic reactions are indicated in *bold*. Note, that UDP-GlcNAc is supplied from fructose-6-phosphate by the hexosamine pathway, and that CMP-Neu5Ac is formed in the nucleus. Feedback inhibition of the UDP-GlcNAc 2-epimerase activity of GNE by CMP-Neu5Ac is further indicated

be included in the sialic acid biosynthetic pathway, but UDP-GlcNAc is a substrate for several reactions in glycan metabolism and is not exclusive for sialic acid formation.

The synthesis of CMP-Neu5Ac from UDP-GlcNAc requires five enzymatic steps (Fig. 1). Four of them are necessary to generate Neu5Ac, and they are

localized in the cytosol. The final formation of CMP-Neu5Ac takes place in the nucleus. Every step is combined with cleavage of one energy-rich bond, which directs the de novo pathway straight to product formation.

The biosynthesis of Neu5Ac begins with the formation of ManNAc from UDP-GlcNAc by simultaneous release of UDP. This initial step is catalyzed by UDP-GlcNAc 2-epimerase. ManNAc is then phosphorylated at C-6 by the specific ManNAc kinase. Other potential sources of ManNAc or ManNAc-6-phosphate would be GlcNAc and GlcNAc-6-phosphate, respectively. However, a GlcNAc-6-phosphate epimerase only exists in prokaryotes [1], and the formation of ManNAc from GlcNAc by GlcNAc 2-epimerase does not occur under physiological circumstances [2]. The only salvage pathway known is the introduction of ManNAc by direct phosphorylation via a kinase [3], e.g., from nutritional sources (which, however, contain only trace amounts of ManNAc) or from the degradation of Neu5Ac [4].

Neu5Ac is a condensation product of ManNAc and pyruvate. The activated form of pyruvate, phospho*enol*pyruvate (PEP), is derived from glycolysis. The Neu5Ac-9-phosphate synthase uses ManNAc-6-phosphate instead of ManNAc, and catalyzes an aldol addition with PEP for the formation of Neu5Ac-9-phosphate [5]. The reaction is thereby driven by the release of the phosphate group from PEP. This enzymatic reaction is followed by the release of the 9-phosphate group from Neu5Ac-9-phosphate by a specific phosphatase [6]. The final step of the de novo pathway is the formation of CMP-Neu5Ac in the nucleus of vertebrate cells by the CMP-Neu5Ac synthetase [7]. The nucleotide sugar is then transported to the Golgi apparatus by the CMP-sialic acid transporter [8] and is used there as a substrate for the different sialyltransferases.

A catabolic pathway of Neu5Ac metabolism also exists. As there is no direct involvement of GNE in this pathway, we only describe it briefly. Neu5Ac can be transported as a monosaccharide from the lysosome to the cytosol [9]. There it is cleaved by the Neu5Ac aldolase, which forms ManNAc and pyruvate [4]. Whereas pyruvate can reach the energy metabolism or gluconeogenesis, ManNAc is further converted to GlcNAc and introduced into the hexosamine pathway [2]. For CMP-Neu5Ac a degradation mechanism is not known, as no enzymatic activity for cleavage of the nucleotide sugar has been identified [10].

3 History of GNE Research

A historical view on GNE (Fig. 2) can be divided into two different eras. The first covers the description of the two functional parts of GNE, the UDP-GlcNAc 2-epimerase and the ManNAc kinase, as separate entities. The second started in 1997, when Reutter and co-workers discovered the bifunctionality of GNE [11, 12]. From then on all the following studies either directly investigated the bifunctional enzyme or at least kept this structure in mind.

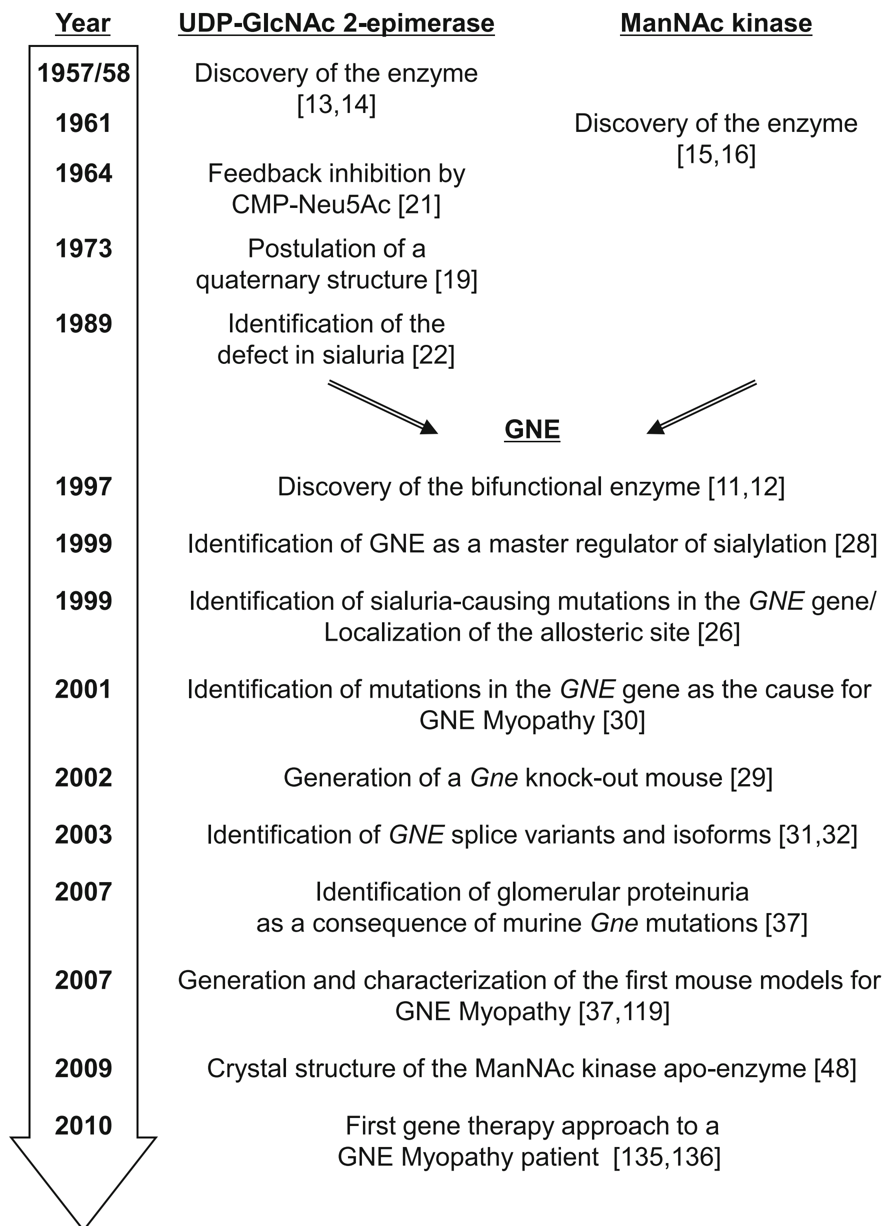

Fig. 2 Milestones in GNE research

UDP-GlcNAc 2-epimerase (E.C. 5.1.3.14) was first observed by Cardini and Leloir [13]. In 1958 Comb and Roseman [14] discovered that the products of the enzyme are ManNAc and UDP, which was not completely clarified in the previous report. In 1961, discovery of ManNAc kinase (E.C. 2.7.1.60) was reported

simultaneously by Gosh and Roseman [15] and Warren and Felsenfeld [16]. Initial biochemical characterizations of the enzymes were performed in the 1960s and early 1970s for the epimerase [17–19] and the kinase [20]. The ManNAc kinase was not an object of outstanding interest in the following years due to its biochemical features being comparable to several other sugar kinases. On the other hand, the UDP-GlcNAc 2-epimerase had already been discovered as a regulator in sialic acid biosynthesis due to its feedback inhibition by CMP-Neu5Ac in 1964 by Kornfeld et al. [21]. A complex quaternary structure involved in this regulation mechanism was postulated in 1973 by Kikuchi and Tsuiki [19], underlining the key role of UDP-GlcNAc 2-epimerase. At that time, the first speculations came out that a recently discovered disease, sialuria, might be due to malfunctioning of UDP-GlcNAc 2-epimerase. However, it took more than 10 years before Ashwell and co-workers proved a defect in the feedback inhibition mechanism of this enzyme as disease-causing [22].

In 1997 GNE was purified to homogeneity by independent isolation of the same polypeptide using procedures either focusing on UDP-GlcNAc 2-epimerase or on ManNAc kinase, accompanied by detection of both enzyme activities in all fractions [11]. In a parallel study the *GNE* cDNA was cloned from rat and functionally expressed in a heterologous system [12]. This experiment unequivocally showed the bifunctional character of GNE. From this time research on GNE started to speed up. The same gene was identified and studied in a variety of mammalian species [23–25], and later also in other vertebrate species by genome sequencing, leading to the rapid identification of the genetic defect in sialuria [26] and the determination of the allosteric site in the epimerase domain [27]. In the following years, biochemical characterization revealed an array of novel structural and enzymatic GNE features (see Sects. 5 and 6). The role of GNE as a master regulator for sialylation was proven by Keppler et al. [28] in a number of different cell types, and later by the lethal effect of targeted mutagenesis of the *Gne* gene in mice [29]. The latter study overlapped with the completely unexpected identification of a novel congenital disease caused by mutations in the *GNE* gene, hereditary inclusion body myopathy (HIBM; recently renamed "GNE myopathy") [30]. GNE now became the focus of researchers who were not glycobiologists before. They contributed with novel findings and approaches, including the discovery of *GNE* splice variants [31] and, as a consequence, additional protein isoforms [32–34]; GNE-dependent genome [35] and proteome analysis [36]; the discovery of other GNE-opathies [37]; the establishment of a set of mouse models, and, importantly, the development of metabolic and genetic therapies for GNE-related diseases (see Sect. 8).

4 The *GNE* Gene

The human *GNE* gene is localized on chromosome 9p12-13. It consists of 13 exons (Fig. 3a) in a gene stretching across a length of about 70,000 bp. Alternative splicing events of mainly the 5′-end *GNE* exons were recently described, resulting

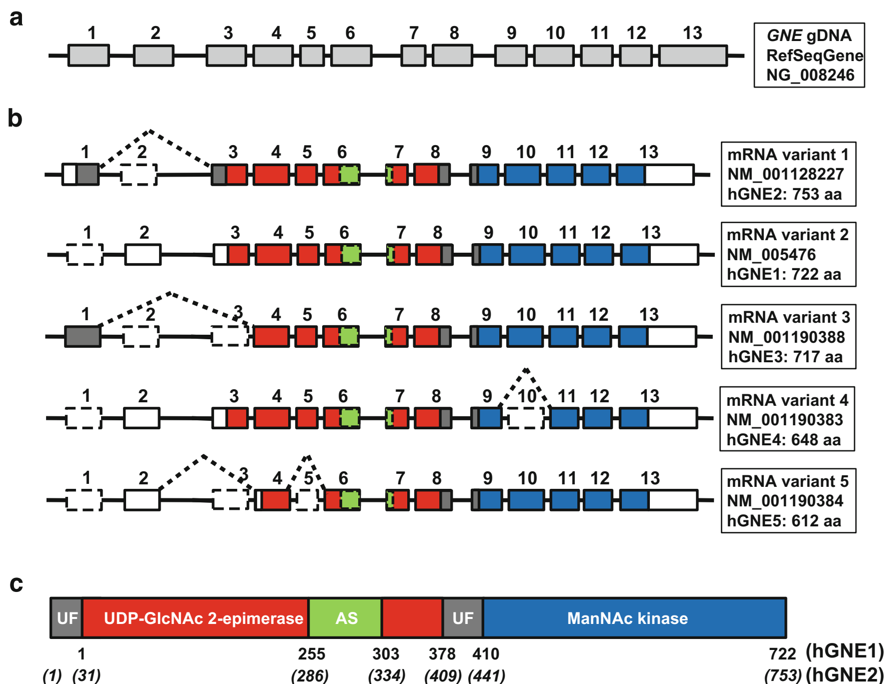

Fig. 3 Structure of the human *GNE* gene, mRNA and protein. (**a**) Schematic exon (*boxes*)- intron (*lines*) structure of the human *GNE* gene. (**b**) Exon-intron structures of the five human *GNE* mRNA transcripts, formed by alternative splicing listed in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). (Note that three additional alternatively spliced transcripts were described by Yardeni et al. [33]). mRNA variant 1 (the longest splice form) encodes the hGNE2 protein, while mRNA variant 2 encodes the hGNE1 protein (traditionally known and studied as the sole translated GNE protein). *Solid colored boxes* represent the open reading frame (*colors* correspond with translated protein domains in (**c**). *White boxes* represent untranslated regions (UTRs) and *white dotted lined boxes* the skipped exons of each transcript. (**c**) hGNE protein domain structure. Amino acid numbering of both hGNE1 and hGNE2 protein isoforms are indicated below the structure (AS, allosteric site; UF, unknown function)

in eight human mRNA transcripts, five of which are assigned GenBank accession numbers (Fig. 3b) [31, 33]. Whether all mRNA transcripts are translated into a GNE protein isoform in vivo remains unknown, partly because functional antibodies and/or extensive protein expression studies of GNE have been lacking [32, 33, 38].

In other species the total number of GNE splice variants is lower [34, 39]. Overall *GNE* transcription can be regulated by CpG methylation [40, 41] (see also Sect. 7). Although CpG methylation of the *GNE* gene was unequivocally shown only in lymphocytes, it is very likely that the same mechanism occurs in other tissues and contributes to tissue-specific adjustment of *GNE* expression. The non-coding 3′-end of human *GNE* exon 13 has an extraordinary length of more than 2,000 bp in human transcripts [24]. However, *GNE* transcripts of other mammalian species contain much shorter 3′-ends, as shown by northern blot analysis [12, 23]. It is therefore

likely that there is a recently unknown regulatory function of the 3′-end of *GNE* mRNA specific for humans.

5 The GNE Protein

5.1 Primary Structure

GNE is a very highly conserved protein. In all mammalian species with a known genome, GNE (actually hGNE1; see below and Fig. 3) consists of 722 amino acids (or in the case of giant panda 721 amino acids) with a sequence identity of >98%. Generally, homology of other proteins between several vertebrate species is lower. Moreover, even the amino acid sequence identities between human and chicken or frog GNE are still high, 92% and 86%, respectively. A sequence comparison of human GNE and that of different fish species also revealed a sequence identity of about 80%. In addition, all vertebrates possess the bifunctional GNE protein, and no evidence for separately expressed enzymes has been found within the available sequence information. The following detailed description of GNE features could therefore be applied to the enzyme from all vertebrate species.

GNE consists of two functional domains, an N-terminal part responsible for the UDP-GlcNAc 2-epimerase activity and a C-terminal part covering the ManNAc-kinase activity (Fig. 3c). The two parts are about equal in size, but the exact domain boundaries cannot be defined because one of the last challenges in GNE research, the resolution of the three-dimensional structure of the entire enzyme, has not been accomplished so far. However, due to sequence comparisons with other enzymes of homologous functions [42] and the construction of deletion mutants [43], the epimerase domain can be specified to the amino acids 1 to 378, and the kinase domain covers at least the amino acids 410–684. A further hinge domain has been postulated between amino acids 378 and 410, but this remains speculative without reliable structural data. The binding site of the feedback inhibitor CMP-Neu5Ac has been mapped first by sialuria patients' mutations at amino acids 263 and 266 [26]. Yarema et al. [27] identified further sialuria-like mutants in cultivated cells in between amino acids 255 and 275. Whether this area forms a separate regulatory subdomain within the epimerase domain, or "only" defines the allosteric binding site for CMP-Neu5Ac remains to be clarified.

The originally described GNE protein is also called hGNE1 (illogically, encoded by *GNE* mRNA transcript variant 2, NM_005476), which covers 722 amino acids with a calculated molecular mass of about 79 kDa. hGNE2 is an isoform with 31 additional amino acids at the N-terminus, which extends the N-terminal epimerase domain for about 3 kDa. hGNE2 is encoded by the longest *GNE* mRNA transcript (variant 1, NM_001128227). Human isoforms hGNE3-8 are encoded by shorter mRNA splice variants, and if these isoforms are expressed as proteins, they likely lack either epimerase (hGNE3, 6, 7, 8) or kinase (hGNE4) enzymatic

activities due to partial (splice) deletions within these respective domains, and they may function in fine-tuning of sialic acid synthesis [33, 39, 44].

The additional N-terminal sequence (and novel exon 1) encoding hGNE2, is potentially confusing since all previous biochemical and mutation analysis studies refer to the nomenclature of the hGNE1 isoform, while according to universally adapted gene/protein nomenclature rules the longest mRNA splice form ought to be used for annotating mutations and functional domains. However, to avoid confusion, we refer in this review to the hGNE1 isoform nomenclature. In future reports it is likely that amino acid numbering of previously reported GNE studies, including patient mutation reports, will be supplemented with 31 amino acids to yield a new (hGNE2) nomenclature.

5.2 Quaternary Structure

One of the extraordinary features of GNE is its ability to form different oligomeric structures. In early GNE research an oligomeric structure had already been postulated by Kikuchi and Tsuiki [19], who proposed a hexameric structure for rat UDP-GlcNAc 2-epimerase, derived from kinetic data of the allosteric enzyme inhibition by CMP-Neu5Ac. Hinderlich et al. [11] confirmed these data by gel filtration analysis of purified rat liver GNE, which revealed a native molecular mass for the fully active enzyme of 450 kDa, corresponding to a hexamer of 75 kDa subunits. However, determination of the oligomerization of purified rat liver GNE, by the identical kinetic calculation of a Hill plot from the CMP-Neu5Ac inhibition curve as performed by Kikuchi and Tsuiki, suggested a tetrameric protein [11]. Besides the fully active hexameric/tetrameric GNE, a second, dimeric state was also observed. This GNE dimer possesses only ManNAc kinase activity, but can be reassembled to the higher oligomeric state by incubation with the epimerase-substrate UDP-GlcNAc, revealing a potential regulation mechanism (see also Sect. 7). A more detailed analysis of GNE oligomerization using recombinant rat GNE and modern biophysical methods confirmed the existence of the two different oligomeric states whereby the higher one was unequivocally assigned to a tetramer [45]. Moreover, these experiments revealed a dynamic interplay between dimers and tetramers, also influenced by different GNE substrates. Interestingly, higher, but less defined, oligomers were also observed, which, at least in part, tend to precipitation. The latter observation might be due to the unphysiologically high concentration of GNE by its recombinant expression. But it may also explain the observation of hexamers of the native rat liver enzyme, which might reflect a mixed population of tetramers and higher oligomers, which appear as hexamers in size exclusion chromatography. Another potential explanation for GNE hexamers in that the physiological environment of the cell might be different posttranslational modifications (see also Sect. 7), which promote hexamer formation, but did not occur in the heterologous baculovirus expression system used for the study of Ghaderi et al. [45].

5.3 Three-Dimensional Structure

One of the unresolved questions of GNE research is the three-dimensional structure of the bifunctional enzyme. Due to the lack of a crystal structure, two different alternative approaches were made. First, models of the structure of the two different functional domains were generated based on similarities to related enzymes, mostly of prokaryote origin, and their known crystal structures [46, 47]. These studies were motivated in particular by the huge set of mutations causing GNE myopathy and its unclear influence on GNE function (see below). Second, the functional domains were expressed as separate recombinant proteins and exerted in crystallization experiments. Whereas the epimerase domain so far refused to form diffractable crystals, structures of human ManNAc kinase in its ligand-free state [48] and ligand-bound state [49] have been solved. We will therefore describe the ManNAc kinase structure mainly based on data of these studies, whereas our overview of the epimerase domain uses the model structures. However, the assembly of the two domains within the bifunctional enzyme or even the quaternary structure remains speculative and will not be a topic here.

The GNE kinase domain belongs to the ROK family (repressor/open reading frame/kinase family, PF00480). This protein family in particular consists of prokaryotic members, and the ManNAc kinase is its only human member, clearly differing from other sugar kinases with related functions. The crystal structure of the human ManNAc kinase domain revealed the typical bi-lobal structure with the postulated ATP-binding side in a deep cleft between the two lobes typical for all ROK kinases [48]. Both lobes consist of α/β folds with central β-sheets surrounded by α-helices. Furthermore, the ManNAc kinase contains an ROK-characteristic complex-bound zinc ion, integrated into an HC3-type zinc finger. As zinc fingers are often found as DNA-binding structures of transcription factors, speculation emerged that potential functions of GNE outside the sialic acid metabolism might be due to transcription regulation. However, the zinc ion seems to be more likely essential for substrate binding of ManNAc kinase [48, 49]. Recombinant ManNAc kinase assembles as a dimer, which was also observed in the crystal structure [48]. The dimerization site was localized within the C-terminal lobe of the protein. Interestingly, some hexameric structures were observed by gel-filtration analysis of the purified ManNAc kinase, and a crystallographic hexamer could also be postulated [48]. However, the authors could not completely rule out artifacts of recombinant protein expression or crystallization, so that the discussion about the oligomeric structure of GNE (see above) remains open.

The potential structure of the UDP-GlcNAc 2-epimerase domain of GNE was evaluated by models based on similarities to prokaryotic UDP-GlcNAc 2-epimerases. The prokaryotic enzymes cover non-hydrolyzing epimerases, which produce UDP-ManNAc and hydrolyzing epimerases resulting in UDP and ManNAc as products. However, the overall three-dimensional structures are similar and can be superimposed to the GNE epimerase domain due to a common secondary structure. Based on these data sets, an initial model was generated by Penner

et al. [46], and a strongly improved calculation was published by Kurochkina et al. [47], who also integrated the structures of phosphoglycosyltransferases. The GNE epimerase domain consists of two domains, which form a cleft between the domains harboring the active site, a structure which was also found for the ManNAc kinase domain. The N-terminal domain consists of seven β-sheets surrounded by seven α-helices, and the C-terminal domain contains a further six β-sheets and six α-helices. Both domains form structures similar to Rossman dinucleotide binding folds. Although some of the three-dimensional structures of the prokaryotic epimerases display dimers, conclusions about the contributions of the epimerase domain to GNE oligomerization were not possible from the models.

6 GNE Enzymatic Function

The two enzymatic functions of GNE could be considered independently. Comparable to the protein structure, which can be described as two independent functional domains, the physical separation of the two GNE domains results in enzymes with remaining activity [43]. Therefore, detailed descriptions about the two enzymatic mechanisms and active site architecture will be discussed separately in this section. The functional consequences of the two-domain structure are also discussed here, although some points can only be clarified by a still pending three-dimensional GNE structure. One of the basic features of bifunctional enzymes is the physical connection of two active sites for substrate channeling, when two successive steps of one pathway are catalyzed [50]. This mechanism avoids release of intermediates from the enzymes, which may either be unstable or should be prevented from further conversion by competing enzymes of other pathways. The latter point might be true for GNE, as ManNAc is also a substrate of GlcNAc 2-epimerase which would direct the sugar into amino sugar catabolism [2]. Furthermore, substrate channeling can accelerate the overall enzyme reaction, which is reasonable for GNE as the rate-limiting enzyme of sialic acid biosynthesis. Another important point of bifunctionality is the concerted regulation of two enzymatic steps. Although this may be the case mainly for bifunctional enzymes catalyzing opposed reactions, in the case of GNE a tight regulation mechanisms between the epimerase and the kinase function might be desirable (see also Sect. 7).

6.1 Enzymatic Properties of UDP-GlcNAc 2-Epimerase

UDP-GlcNAc is the physiological substrate of UDP-GlcNAc 2-epimerase. In an early study Salo and Fletcher [51] identified UDP-ManNAc as an additional in vitro substrate of the enzyme, resulting in hydrolysis of the nucleotide sugar. However, UDP-ManNAc occurs only in prokaryotes as a substrate in cell wall construction and is not a physiological substrate of GNE. Interestingly, Blume et al. [52] also

identified UDP-GalNAc as a GNE binding partner in STD-NMR analysis, but the binding mode of the substrate did not favor an enzymatic reaction. In the same study UDP was found as a substrate with an even higher affinity than UDP-GlcNAc to the epimerase site. UDP is therefore a competitive inhibitor of UDP-GlcNAc 2-epimerase. This is less important under physiological conditions, as the intracellular UDP-GlcNAc concentration usually exceeds the UDP concentration. However, in in vitro assays UDP is often used as a GNE stabilizing agent, as it maintains the fully active oligomeric state of the enzyme [11, 45] and its influence on activity assays has to be taken into account. Furthermore, a mechanism can be postulated where UDP remains in the active site after the enzymatic reaction and the release of ManNAc, and a new UDP-GlcNAc molecule enters the active site by an UDP/UDP-GlcNAc exchange.

The epimerization mechanism of GNE starts with the release of the non-acidic proton at C-2 [53]. A set of histidine residues comes into consideration as the acting base during this step, whereby the favorite one is His-220, which is homologous to the histidine residue essential for the activity of *E. coli* UDP-GlcNAc 2-epimerase [54]. The second step covers the elimination of the UDP moiety and the formation of 2-acetamidoglucal. Whereas the nucleotide most likely remains in the active site (see above), the sugar derivative is further converted to ManNAc by stereospecific addition of a water molecule to C-1 and C-2 [53]. Interestingly, 2-acetamidoglucal was already observed by Sommar and Ellis [55] as a byproduct of the epimerase reaction, and later as a minor, but significant, unusual component of the urine of sialuria patients [56] (see also Sect. 8).

The exact architecture of the epimerase active site remains to be clarified by a three-dimensional structure. However, several relevant amino acids could be postulated by the combination of mutation analyses and structural models [42, 46, 47], including H-220, which was already described as a possible general catalyst; H-45 also seems to be essential for the epimerase activity (S. Hinderlich, unpublished data). From the amino acids addressed by site-directed mutagenesis, the following showed reduced enzyme activities and are located at least in vicinity of the active site, suggesting a further role in the catalytic mechanism or in substrate binding: K-24, D-112, H-132, E-134, D-143, and C-303. Several other amino acids affect the epimerase activity by mutation, in particular in GNE myopathy patients, but an indirect effect via structural changes also seems to be likely and remains to be clarified. The epimerase domain models suggest additional amino acids such as R-19, D-225, S-301, R-306 and E-307 involved in substrate binding and are therefore worth investigating in the future.

The epimerase domain is able to bind the strong feedback inhibitor CMP-Neu5Ac (see also Sect. 7.2). The respective allosteric site at least covers the amino acids 255 to 275, as identified by a mutation screen for cells with high levels of sialic acid [27]. This area also includes the two recently identified mutated amino acids, R-263 and R-266, in the disease sialuria (see Sect. 8.1). In vitro site-directed mutagenesis studies identified amino acidC-303 as also contributing to the allosteric site, since C-303 mutations resulted in reduced feedback inhibition by CMP-Neu5Ac [46]. It is still unclear whether the allosteric site forms a discrete

subdomain or the binding area for CMP-Neu5Ac or is integrated within the structure of the epimerase domain. The three-dimensional models of the epimerase domain were unable to address these questions, as the bacterial counterparts, serving as a calculation basis, do not bind allosteric regulators. Nevertheless, we define the area of the amino acids 255–303 as the provisional allosteric binding site (see Fig. 3).

6.2 Enzymatic Properties of ManNAc Kinase

The enzymatic phosphorylation of ManNAc requires ATP as its phosphate donor. Kundig et al. [20] already excluded other nucleotide triphosphates as further substrates. Nevertheless, ADP is also able to bind to the active site of ManNAc kinase with an affinity in about the same range as ATP [57]. In in vitro assays ADP may act as a competitive inhibitor. However, under physiological conditions the cytosolic concentration of ADP is very low due to the action of the ubiquitous adenylate kinase. In addition to ManNAc, *N*-glycolylmannosamine (ManNGc) is a further sugar substrate of ManNAc kinase [20]. ManNGc is derived from the degradation of *N*-glycolylneuraminic acid and is salvaged by the sialic acid biosynthesis pathway. The metabolization of ManNGc suggests a promiscuity of ManNAc kinase and the following enzymes in this pathway for structural modifications of the *N*-acyl side chain. This feature was used for the introduction of unphysiological functional groups into sialic acids by metabolic oligosaccharide engineering [58]. Benie et al. [57] showed that two of the ManNAc derivatives often used for this method, *N*-propanoylmannosamine and *N*-butanoylmannosamine, are additional substrates of ManNAc kinase. In the same study, GlcNAc was also suggested to be phosphorylated by ManNAc kinase. However, affinity of GlcNAc to the enzyme and the presence of a specific GlcNAc kinase [3] most likely exclude a physiological function of ManNAc kinase in GlcNAc phosphorylation.

The enzymatic mechanism of ManNAc kinase has not been investigated directly. However, it can be assumed, that it is closely related to the mechanism of other 6-kinases such as hexokinase. This enzyme transfers the γ-phosphate from ATP to the 6-hydroxyl group of the sugar via a trigonal-bipyramidal intermediate. The reaction finally results in inversion of the phosphate configuration [59, 60]. The binding, and most likely also the transfer, of the phosphate group is supported by arginine and aspartate residues in 6-kinases. The mutation of the two highly conserved residues D-413 and R-420 of ManNAc kinase results in complete loss of enzymatic activity [42]. Besides these two amino acids, D-517 can be suggested as an additional essential amino acid, as it may act as a catalytic base in abstraction of the proton from the 6-hydroxyl group of ManNAc [47]. The sugar binding site of ManNAc kinase consists of the amino acids N-516, D-517, E-566, H-569, and E-588, whereby H-569, together with the three cysteine residues at positions 579, 581, and 586, is also involved in binding of the zinc ion of the active site.

7 Regulation of GNE

There are several possible mechanisms to interfere with and regulate an enzyme-catalyzed reaction. These include the expression level of the enzyme (quantity and/or isoform expression = enzyme induction), the control of the catalytic reaction by substrates or products (e.g., feedback control), posttranslational modification of specific amino acids (such as phosphorylation on serine, threonine, or tyrosine residues = enzyme interconversion), or alteration of chemical or physiological conditions. All these options are explored to control GNE enzymatic activities and will be discussed in detail in this section. The regulation of GNE seems to be of the same complexity as its structure and, interestingly, almost all described GNE regulation mechanisms specifically affect the UDP-GlcNAc 2-epimerase activity and not the ManNAc kinase activity. This might reflect the epimerization of UDP-GlcNAc to ManNAc as the key step of sialic acid biosynthesis.

A total knock-out of the *Gne* gene (and subsequently its enzymatic activities) results in complete loss of endogenous sialic acid production in cells or animals. Cultivated cells are able to tolerate a *GNE* gene knock-out because sialic acids seem not to be essential for survival and only certain sialic acid-dependent functions of these cultured cells appeared to be affected [28]. In a multicellular organism a GNE gene knock-out is lethal. A targeted mutagenesis of the Gne gene in mice results in early embryonal lethality at developmental day E8.5 [29]. Interestingly, heterozygous *Gne* knock-out mice displayed slightly reduced UDP-GlcNAc 2-epimerase activity and up to 25% reduced sialylation of glycans compared to wild-type mice, but no significant phenotype was observed [61]. This suggests a range of reduced sialylation that appears to be tolerated by an animal, and consequently a threshold of up- or down-regulation of GNE expression and sialylation status which has to be exceeded by the different regulation mechanisms.

7.1 Regulation by mRNA and Protein Expression

Sialylation occurs at all stages of mammalian development and even embryonic stem cells already express sialylated glycoconjugates [62–64]. The expression of *GNE* mRNA, encoding the key enzyme of sialic acid biosynthesis, strongly correlates with the occurrence of sialic acid. GNE mRNA is ubiquitously expressed in all organs and during all stages of development, as shown in northern blot and in situ-hybridization experiments. However, the major expression of GNE mRNA is found in liver [12, 23, 24], likely because liver is the organ with the highest biosynthesis rate of secreted sialylated glycoproteins (e.g., serum glycoproteins).

The detailed mechanisms of GNE mRNA transcription and GNE protein translation regulation have not been clarified so far. However, transcription of the GNE gene is unequivocally influenced by an epigenetic event. Methylation of CpG islets

in the GNE promoter region leads to down-regulation of *GNE* transcription in several cancer cell lines [40] and in HIV-infected lymphocytes [41]. GNE protein expression and enzyme activity are reduced below detectable levels in these cell lines. The same mechanism seems to be involved in down-regulation of *GNE* gene expression in a Morris hepatoma-derived cell line [40]. Morris hepatoma displayed less than 10% of the GNE activity of rat liver [65]. This is obviously due to reduced production of secretory sialoglycoproteins and a lower need for intracellular sialic acid production. A similar correlation was also found in regenerating rat liver after partial hepatectomy [66, 67], but without assigning this observation to CpG methylation.

It became obvious that GNE exists in several isoforms, which are generated by alternative splicing (for details see Sects. 4 and 5). However, the existence of each isoform in vivo has not been demonstrated yet on the protein level. Recombinant over-expression of the human isoforms revealed that hGNE1, which is the originally described 722 amino acids-containing isoform, has the highest epimerase activity, whereas the other two isoforms with additional N-terminal amino acids (hGNE2) or a different N-terminus (hGNE3) displayed strongly reduced epimerase activity [39]. Since the C-terminus of GNE is known to harbor the kinase domain, it was not surprising that ManNAc kinase activity was similar in all isoforms. It was therefore suggested, that the ubiquitous isoform hGNE1 serves as a general catalyst in sialic acid production, whereas hGNE2, hGNE3, and possibly other translated isoforms (Fig. 3b) may be involved in fine-tuning the sialic acid biosynthesis [33, 39]. This is supported by a tissue-specific expression of mRNA encoding hGNE2-8 [33, 39]. However, the regulation of isoform expression in vivo remains unknown.

As described earlier, the biosynthesis of sialic acids takes place in the cytosol. Therefore GNE is mainly located within the cytosol and standard purification of GNE starts from cytosolic preparations [11]. In one report, GNE was also found in the nucleus and further associated with the Golgi membrane [68]. Although the detection of endogenous GNE is difficult, since most available antibodies do not detect endogenous GNE due to its very low expression level, this observation suggests an additional regulation mechanism via differential subcellular localization. Direct detection of GNE in cellular compartments would also be possible by overexpression of tagged protein (e.g., via fluorescence), and is worth pursuing to clarify this point.

7.2 Regulation by Substrates and Multimerization

The main regulation mechanism of UDP-GlcNAc 2-epimerase activity is the feedback inhibition by CMP-Neu5Ac [21]. This allosteric inhibitor affects the epimerase activity in a concentration-dependent manner, which connects the production of sialic acids to glycan synthesis. A complete inhibition of the epimerase activity is reached at a CMP-Neu5Ac concentration of about 60 μM [22], which fits

well with the intracellular concentration of this nucleotide sugar in cells under normal circumstances [69]. This suggests a sophisticated mechanism of feedback inhibition, which responds even to minor variations of CMP-Neu5Ac concentration due to varying rates of biosynthesis of sialylated glycans and subsequent CMP-sialic acid use by the Golgi.

The feedback inhibition mechanism can be circumvented by extracellular application of ManNAc. ManNAc treatment of, for example, Madin Darby canine kidney cells results in an intracellular CMP-Neu5Ac concentration up to tenfold above the normal level [69]. These data indicate not only that the ManNAc kinase activity of GNE is not affected by CMP-Neu5Ac inhibition but also that no additional metabolic regulation mechanisms exist within the sialic acid biosynthesis pathway.

A second regulation mechanism using GNE substrates includes the epimerase educt UDP-GlcNAc and its product UDP. As mentioned above (Sect. 6.1), UDP binds with a high affinity to the active site of the epimerase enzyme and might act, at least in vitro, as a competitive inhibitor. Moreover, physiological regulation via UDP and UDP-GlcNAc is strongly connected to GNE oligomerization. Whereas the GNE dimer is only able to catalyze the phosphorylation of ManNAc, the epimerase activity requires the highest possible oligomeric state (tetramer or hexamer). This structure is maintained by UDP, which usually remains in the active site after one catalytic cycle, and can be stabilized in vitro by addition of 100 μM UDP to buffers. More interestingly, once GNE is decayed to a dimer, it can be reassociated into tetrameric states by addition of UDP-GlcNAc (but not UDP) to the system [11], suggesting a direct regulation of the ability of GNE to catalyze its first enzymatic step by the respective substrate. Intracellular UDP-GlcNAc concentrations are usually sufficient to maintain the tetrameric state. The interchange between the oligomeric states may therefore regulate the UDP-GlcNAc 2-epimerase activity only under certain conditions with low UDP-GlcNAc concentrations. This might be in pathological situations or in events which require a high amount of UDP-GlcNAc for other purposes, such as the synthesis of non-sialylated glycans or *O*-GlcNAcylation of proteins.

7.3 Regulation by Posttranslational Modification

Posttranslational modifications (PTM) are chemical modifications of proteins after its biosynthesis (translation). PTMs include glycosylation, phosphorylation, acetylation, and addition of lipids, ubiquitinylation, SUMOylation, or sulfation. PTMs often occur at specific residues of the primary sequence. Typically amino acids with additional amino- or hydroxyl groups such as lysine, asparagine, threonine, tyrosine, or serine bear PTMs. As mentioned earlier, GNE (hGNE1) consists of 722 amino acids and 144 of these are lysine (41), asparagine (29), threonine (33), or serine (41) residues. When analyzing the sequences around these amino acids in more detail it becomes obvious that hGNE1 contains 42 possible phosphorylation

Table 1 GNE myopathy-related mutations which introduce novel serine or threonine residues and their phosphorylation probability

GNE myopathy mutation[a]	Phosphorylation probability[b]
C13S	62% PKA
P27S	No
M29T	51% CkI
G89S	No
I106T	No
I142T	No
G206S	60% PKC
F233S	63% PKA
I241S	50% Cdc2
M265T	78% PKC
P283S	No
I298T	No
I472T	No
L508S	No
N519S	51% DNAPK
L556S	No
I557T	No
G568S	51% Cdc2
I587T	No
A591T	No
A600T	80% PKC
A630T	70% PKC
A631T	No
G708S	No
M712T	75% PKC

PKA protein kinase A, *PKC* protein kinase C, *CkI* casein kinase I, *Cdc2* cyclin-dependent kinase 2, *DNAPK* DNA protein kinase

[a]For mutation references, see Fig. 5

[b]Phosphorylation probabilities were calculated by NetPhosK [150]

sites (thereof 13 protein kinase C (PKC)-sites with the highest possible score using the tool NetPhosK). These analyses are supported by earlier studies where it was demonstrated that GNE is phosphorylated in vivo and interacts with several isoforms of PKC in mouse liver [70]. Consequently, PKC is one candidate enzyme acting on GNE and its phosphorylation of GNE results in an activation of the UDP-GlcNAc 2-epimerase activity. In this light it is remarkable that about 25 of the known mutations responsible for GNE myopathy (see Sect. 8.2) are mutations where a novel serine or threonine is introduced (Table 1), making GNE accessible for further phosphorylation and likely misregulation involved in the pathogenesis of these GNE myopathy-related *GNE* mutations.

A unique PTM is the modification of serine or threonine with *O*-GlcNAc [71]. There are five potential *O*-GlcNAc sites in hGNE1 (S-122, T-223, S-505, S-689, T-718; calculated by YinOYang_1.2). These residues are always very close to phosphorylation sites, but never represent the same residue. Therefore we tested

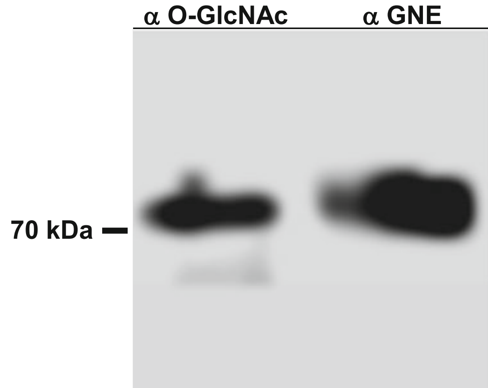

Fig. 4 GNE is modified by *O*-GlcNAcylation. GNE was immunoprecipitated from rat liver by a GNE-specific antibody and western-blotted. Immunodetection was performed with *O*-GlcNAc- and GNE-specific antibodies, indicating *O*-GlcNAcylation of the GNE protein

GNE for its content of *O*-GlcNAc by western blot analysis after immunoprecipitation from rat liver and demonstrated that GNE is modified by *O*-GlcNAc (Fig. 4). Together with a phosphorylation likely close to the *O*-GlcNAcylation site, this suggests another level of fine-tuning of the enzyme function in response to cellular changes, as observed for other proteins simultaneously modified by phosphorylation and *O*-GlcNAcylation [71]. Further analysis revealed that hGNE1 contains three potential sites for SUMOylation (K-33, K-427, K-618; calculated by SUMOsp_2.0), but no consensus sequence for ubiquitinylation (www.UbPred.org). A regulation via SUMOylation is therefore likely. Finally, hGNE1 possesses three potential *N*-glycosylation sites (N-300, N-395, N-661; calculated by NetNGlyc_1.0). However, *N*-glycosylation was already ruled out for the purified rat liver enzyme [12], and is in agreement with the usual absence of this PTM for intracellular proteins

7.4 Regulation Potential by Interacting with Other Proteins

Protein–protein interactions are well-documented events to regulate the function of a protein or enzyme. During the last 5 years several GNE-interacting proteins have been identified. Using 5,600 cDNAs from a human brain library in a yeast two-hybrid system, four proteins were identified as potential interacting partners: promyelocytic leukemia zinc finger protein (PLZF), collapsin response mediator protein 1 (CRMP1), receptor interacting factor 1 (RIF1), and KIAA 1549, a protein of unknown function [72]. The interaction of CRMP1 and GNE could be verified by co-immunoprecipitation from PC12 cell lysates, and the interaction of PLZF and GNE was confirmed by a pull-down assay. A fragment-based approach for identification of the GNE domain involved in these protein-protein interaction revealed that only the full length enzyme as well as the separately expressed ManNAc kinase

domain showed positive results, indicating the importance of the ManNAc kinase domain and likely also the oligomeric state of the full length enzyme for these interactions [72].

CRMP1 is a member of the TUC (Toad-64/Ulip/CRMP) protein family and is involved in growth cone collapse [73]. As a modulator of the Rho-A dependent signaling pathway it regulates the F-actin depolymerization [74]. CRMP1 analogs in humans are called dihydropyrimidinase-related proteins (DHPase) and their different isoforms are tissue specifically expressed [75]. A connection between sialic acids and members of the TUC family was shown by Büttner et al. [76]. They used unnatural sialic acid precursor ManNProp to induce neurite outgrowth in PC12 cells. During this process a protein was downregulated, which was identified as the TUC protein family member Ulip by MALDI-TOF MS analysis.

PLZF was identified as fusion partner of retinoic acid receptor α, causing acute promyelocytic leukemia (APL). PLZF is a transcription factor and regulates Cyclin A2 and Hox gene expression [77, 78]. Krause et al. [68] showed co-localization of GNE with nuclear markers and support the interaction of GNE and PLZF as a potential transcription regulator. The N-terminal BTB/POZ domain of PLZF also interacts with E3 ligase Cullin 3 mediating ubiquitinylation [79]. This interaction may link GNE with protein degradation via the proteasome pathway. A deregulation of protein degradation could also play a role in the formation of inclusion bodies in GNE myopathy. Furthermore, PLZF is found in the cytosol where it is associated with the angiotensin 2 receptor (AT2R) [80] and with the prorenin/renin-receptor [81]. AT2R and AT1R also play roles in mitochondrial biogenesis in skeletal muscle, and Eisenberg et al. [35] showed a potential connection by identifying impaired mitochondrial processes in GNE myopathy.

More recently, Amsili et al. [82] found a direct interaction between GNE and α-actinin. They used a Surface Plasmon Resonance (SPR)-based biosensor assay to search for potent GNE interacting proteins and verified the interaction by co-immunoprecipitation and immunohistochemistry. However, Amsili et al. [82] could not identify a difference in the binding kinetics of wild-type and mutant GNE to α-actinin using SPR biosensor analysis. It was therefore suggested that GNE has a specific function in muscle physiology, but its role in GNE myopathy pathology remains unclear.

Although it is convincingly demonstrated that GNE interacts with these binding proteins, there is no evidence that these interactions alter GNE enzymatic activity or subcellular distribution. Further work is needed to elucidate the function of these protein-protein interactions and their impact on sialic acid biosynthesis.

8 GNE-opathies

Mutations or aberrant expression of the *GNE* gene can underlie distinct diseases, collectively called GNE-opathies. Since GNE has a central role in sialic acid metabolism, most GNE-opathies are characterized by access or paucity of sialic

acid synthesis, resulting in hyper- or hyposialylation of glycoproteins or glycolipids. These processes may occur tissue-specific, glycan-specific, or globally (most glycans and/or most tissues affected). This section describes the intriguing group of human GNE-opathies, their molecular and cellular features, disease mechanisms, established cellular or animal models for these disorders, as well as proposed therapeutic options.

8.1 Sialuria

8.1.1 Clinical Features

Sialuria (also called "French type" sialuria; OMIM 269921) is a rare, autosomal dominant inherited inborn error of metabolism which was first described in 1968 by Montrieul et al. [83] and Fontaine et al. [84]. Sialuria patients clinically manifest with mild coarse facies and slight motor delay, with additional sporadic features of hepatosplenomegaly, delayed skeletal development, microcytic anemia, and mild intellectual impairment. They are diagnosed by the detection of gram quantities (>1 g/day) of free sialic acid in urine and significantly increased concentrations of free sialic acid in the cytoplasm of cultured fibroblasts [26, 85–87]

So far, only seven sialuria patients have been reported worldwide, summarized in Table 2. The prevalence of sialuria is probably underestimated due to the mildness of the disorder. Moreover, assaying urinary free sialic acid is not a routine laboratory procedure, but should be considered as part of the metabolic screening of young children with mild developmental delay and in patients with a phenotype suggestive of mucopolysaccharidosis or oligosaccharidosis [88]. Such free sialic acid screening would not only diagnose patients with sialuria but also with the lysosomal storage disorders Salla disease (MIM 604369) and Infantile Free Sialic Acid Storage Disorder (ISSD, MIM 269920), both caused by recessive mutations in the lysosomal sialic acid transporter SLC17A5 [89].

8.1.2 Molecular and Cellular Features

The biochemical defect of sialuria involves failure to regulate sialic acid synthesis due to impaired allosteric feedback inhibition of the epimerase domain of GNE by CMP-sialic acid as outlined in Sect. 5.1. Sialuria has an autosomal dominant mode of inheritance; a heterozygous missense mutation in the allosteric site of *GNE* leads to loss of feedback inhibition of GNE activity by CMP-sialic acid, resulting in cytoplasmic accumulation and urinary excretion of large quantities of free sialic acid [26, 85, 87]. All known patients are heterozygous for a missense mutation in one of two amino acids, arginine at position 263 (R263L) or arginine at position 266 (R266Q; R266W). The clustering of these mutations in the region of codons 263 to 266 marks this region as the allosteric site for CMP-sialic acid binding.

Table 2 Summary of ethnicity, molecular and cellular aspects of sialuria patients

Patient no.	hGNE1 mutation	Ethnicity	Urinary sialic acid content[a] (μmol/mmol creatinine)	Sialic acid content in fibroblasts[b] (nmol/mg protein)	Reference
1	R263L	Caucasian (French)	13–15 g/24 h[c]	NR	[83, 84]
2	R266W	Caucasian (Australian/ British)	8,500–8,900	NR	[26, 96]
3	R266Q	Caucasian	5,436	65.2	[22, 26, 88]
4	R263L	Caucasian	10,453	95.9 ± 54.3	[26, 151]
5	R266Q	Caucasian (Portuguese)	1,693	17.4 ± 7.4	[86]
6	R266Q	Caucasian (Belgium)	8,950–14,680	NR	[87]
7	R266Q	Caucasian (Belgium)	4,278	NR	[87]

NR, reported
[a]Urinary free sialic acid normal range: <74 μmol/mmol creatinine
[b]Fibroblast free sialic acid normal range: 0.2–3.2 nmol/mg protein
[c]Not reported per mmol creatinine

However, the full extent of the allosteric site has not been formally defined (see Sects. 5.1 and 6.1).

To our knowledge, there are no known animal models of sialuria (including rat, mouse, and zebrafish). However, sialuria cell lines have been employed for a variety of in vitro studies, including Jurkat cells [27], Chinese Hamster Ovary (CHO) cells [90, 91], *Spodoptera frugiperda* (*Sf* 9) insect cells [46], and human epithelial kidney (HEK) cells [92]. These mutant cell lines either arose through mutagenesis screens or were created by the expression of a *GNE* carrying a sialuria mutation in normal cells. The latter ones can potentially be used to produce highly sialylated proteins for biotechnological purposes [93].

8.1.3 Disease Mechanism and Therapy

Increased cytoplasmic free sialic acid in sialuria patients' cells may contribute to the clinical symptoms due to increased acidic and negatively charged properties. Moreover, it is likely that specific glycans or groups of glycoconjugates in sialuria cells are hypersialylated which may also explain some of the clinical features of sialuria. Iso-electric focusing studies of serum from three sialuria patients demonstrated significant hypersialylation of apoC-III (a marker for *O*-glycan sialylation) and slight hypersialylation of serum transferrin (a marker for *N*-glycan sialylation) [94]. Levels of lysosomal enzymes, plasma amino acids, and urine organic acids are normal in sialuria patients, and there is no microscopic evidence of stored material in fibroblasts, as seen in lysosomal storage disorders.

Dominant human disorders due to failed allosteric inhibition as sialuria are extremely rare [95]. Therapies for these disorders are lacking. Currently, only symptomatic therapy is available for sialuria patients. The lowering of sialic acid levels in fibroblasts by cytidine feeding has been suggested [85, 96]. However, recent in vitro studies have shown that allele-specific silencing of disease genes might provide treatment for dominant disorders [97]. Allele-specific silencing studies with siRNAs targeting a specific sialuria mutation in patient's fibroblasts (c.797G>A; p.R266Q) demonstrated successful down-regulation of the mutant allele, a significant decrease of free sialic acid (to the normal range), and recovery of feedback inhibition of UDP-GlcNAc 2-epimerase activity by CMP-sialic acid [98]. Even though these findings indicate that allele-specific silencing of the mutated allele in sialuria is a viable therapeutic strategy, further investigations are required for the use of siRNA-based therapies in humans [97, 99].

8.2 GNE Myopathy

8.2.1 Clinical Features

GNE myopathy, formerly called hereditary inclusion body myopathy (HIBM; OMIM 600737), is a rare autosomal recessive adult onset progressive neuromuscular disorder. The disease was originally described in Japanese patients as Nonaka Distal Myopathy [100], now commonly referred to Distal Myopathy with Rimmed Vacuoles (DMRV) (OMIM 605820). Later, a similar disorder was described as vacuolar myopathy sparing the quadriceps in Iranian-Jewish patients [101], now commonly referred to as Inclusion Body Myopathy 2 (IBM2) or HIBM. Only after the HIBM causing gene, *GNE*, was mapped and identified was it recognized that HIBM was allelic to DMRV [102]. The multiple historic names of the disorder could potentially be confusing for clinicians, patients, and researchers. Therefore, the disorder has recently been renamed, substituting all previous disease definitions, as the profound name "GNE myopathy," to which we henceforth refer.

Apart from the Persian–Jewish and Japanese isolates, GNE myopathy patients have now been described in a wide variety of ethnicities, including Caucasian, Indian, Thai, Mexican, and African [103]. The diagnosis of GNE myopathy is based upon clinical features, muscle pathology, and, ultimately, the presence of *GNE* gene mutations.

GNE myopathy is clinically characterized by progressive proximal and distal muscle weakness and atrophy of the upper and lower limbs, beginning in most patients after age 20. Following onset, progression of muscle weakness is relentless and continues over the next decades, most patients becoming wheelchair bound within 10–20 years after onset. A unique feature of GNE myopathy is sparing of the quadriceps muscle, partially or completely, even in advanced stages of the disease [101, 104, 105]. Eye, throat, and respiratory muscles seem not to be affected either. Patients also have normal cognition, sensation, and coordination. Serum creatine

kinase levels are normal to slightly elevated and myopathic and neuropathic patterns in electromyograms are diverse among patients. Magnetic resonance imaging (MRI) T1 weighted images of GNE myopathy patients generally show fatty or fibrous replacement of the hamstring muscles with sparing of the quadriceps muscle [104, 106]. GNE myopathy muscle tissue generally does not show inflammation, although a few patients are described with inflammatory symptoms [107]. Histologically, muscle fibers degenerate with fiber size changes and formation of central nuclei. Affected muscle fibers develop characteristic cytoplasmic or nuclear filamentous inclusions as well as cytoplasmic rimmed vacuoles which are immunoreactive to various proteins, and contain clusters of autophagic vacuoles [101, 104, 108, 109]. GNE myopathy degenerating muscle fibers contain abnormal accumulations of β-amyloid protein, and other Congo-red-positive pathologic markers are also found in brain specimens from neurodegenerative disorders, suggesting a common pathogenetic mechanism [101, 105, 110]. However, GNE myopathy patients do not develop central nervous system disease.

8.2.2 Molecular and Cellular Features

Homozygosity mapping in several GNE myopathy families of Persian–Jewish and Kurdish–Iranian–Jewish origin aided identification of *GNE* as the gene responsible for GNE myopathy [30]. All patients of Middle Eastern origin have since been found to harbor a p.M712T *GNE* founder mutation [30, 111]. Two *GNE* founder mutations are recognized within the Japanese population, p.V572L and p.D176V [102, 112]. At present, more than 100 other *GNE* mutations have been reported in GNE myopathy patients worldwide, as summarized in Fig. 5.

Out of the 109 reported human GNE myopathy-related *GNE* mutations, 88 are missense variants, 10 are nonsense mutations, 8 frame-shift mutations leading to a premature stop codon, 2 splice site variants, and 1 in-frame 3-bp deletion. *GNE* null mutations have never been identified on both alleles in a patient; this would most likely be lethal; a *Gne* "knock-out" mouse model did not survive past the embryonic stage [29]. GNE myopathy-related *GNE* mutations are scattered throughout the UDP-GlcNAc 2-epimerase and ManNAc kinase coding domains. Interestingly, there are three missense variants located in the putative nuclear export signal (Ep(N) in Fig. 5) which may play a role in nuclear localization of GNE [68]. There are no GNE myopathy mutations in the allosteric site defined by human sialuria mutations (amino acid 263–266); however, ten mutations are located in the "experimental" allosteric region (amino acid 255–303, AS in Fig. 5) and may need further research regarding their effect on allosteric feedback inhibition of CMP-sialic acid. For a large number of mutations, secondary structure predictions are described [46, 47].

The effects of selected *GNE* mutations on the enzymatic properties of both UDP-GlcNAc 2-epimerase and ManNAc kinase were assessed, which were reduced, but not absent, in cultured GNE myopathy patients' fibroblasts,

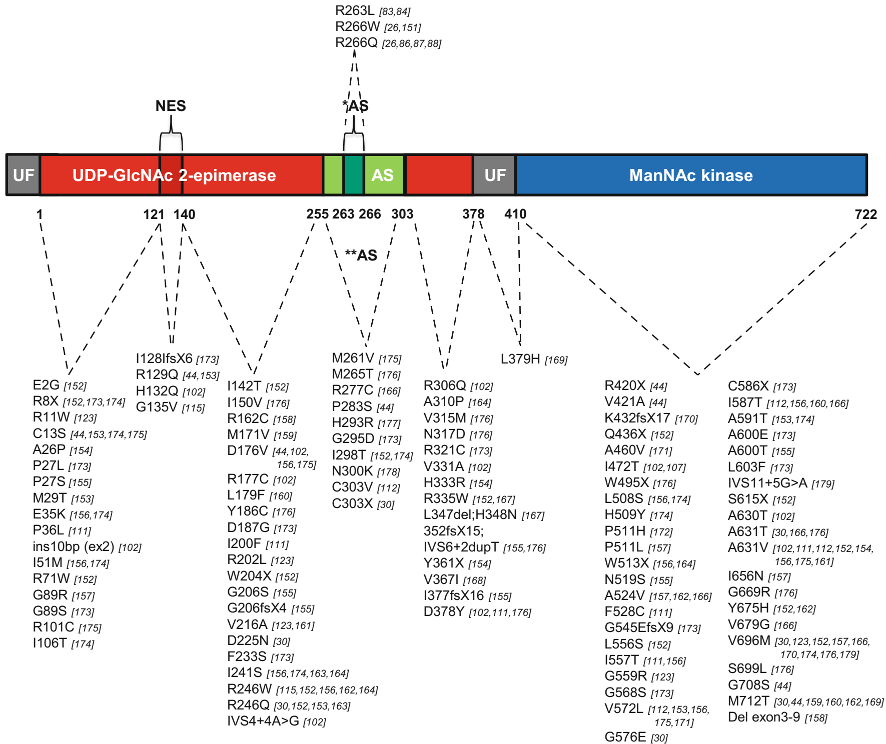

Fig. 5 *GNE* mutations associated with GNE myopathy and sialuria. Locations and characteristics of all reported (as of April 2013) human *GNE* mutations within the functional domains of the GNE protein (amino acid numbering according to hGNE1). Mutations associated with sialuria are printed above the protein structure, and GNE myopathy-associated mutations below, including their references. *Red bar*, UDP-GlcNAc 2-epimerase domain (GNE, Ep); *dark red bar*, the putative GNE nuclear export signal (NES); *green bar*, experimental allosteric region based on in vitro studies (**AS); *dark green bar*, allosteric site based on human sialuria mutations (*AS); *gray bar*, region of unknown function (UF); *blue bar*, ManNAc kinase domain (MNK, Kin). Note that [152–179] were only given in this figure

lymphoblasts, and myoblasts [113–116]. In addition, in vitro studies assessing enzymatic activities by expressing specific human *GNE* mutations in COS-7 cells [113], S*f*9 insect cells [46], or in a cell-free in vitro transcription–translation system [115] revealed that the reductions in UDP-GlcNAc 2-epimerase and ManNAc kinase enzymatic activities are mutation-dependent. Moreover, mutations in one enzymatic domain affect not only that domain's enzyme activity but also the activity of the other domain. Compared with enzyme activities in a cell-free system, fibroblasts exhibited higher residual activities of both UDP-GlcNAc 2-epimerase and ManNAc kinase, suggesting the presence in fibroblasts of additional sugar epimerases and kinases with overlapping substrate specificity [115].

8.2.3 Disease Models

Mouse and cellular models for GNE myopathy have been established. A complete knock-out mouse of the *Gne* gene displayed early embryonic lethality [29]. Heterozygous *Gne*-deficient mice were found to be vital and did not show a significant phenotype, although their overall sialylation was reduced by 25% [61]. Even though the *Gne* knock-out model could not be utilized to study GNE myopathy pathology in mice, the *Gne* deficient embryonic stem (ES) cells of this model have successfully been employed for additional studies. For example, polysialylation of the neural cell adhesion molecule (NCAM) was affected in *Gne* deficient ES-cells, which could be restored with supplementation of ManNAc to the growth medium [29]. Recent studies of *Gne* deficient ES-cells showed that GNE, apart from its role in sialic acid synthesis, may also play a role in cell proliferation, gene expression, and cell differentiation [64].

The first GNE myopathy *Gne* knock-in mouse model was created by Galeano et al. [37] by homologous recombination, introducing the Persian-Jews founder mutation p.M712T into the endogenous mouse *Gne* gene. Unexpectedly, mutant mice died within 3 days after birth of severe glomerular disease including proteinuria, hematuria, effacement of the podocyte foot processes, and segmental splitting of the glomerular basement membrane. Biochemical analysis of mutant mice kidneys revealed decreased UDP-GlcNAc 2-epimerase activity, deficient overall glomerular sialylation (Fig. 6a), and poor sialylation of the major podocyte sialoprotein, podocalyxin, suggesting that decreased renal sialic acid production led to lethality in these mice. Oral supplementation of ManNAc to pregnant and nursing mothers resulted in survival of 43% of mutant pups beyond 3 days of life. Mutant survivors displayed improved kidney histology, increased overall sialylation (Fig. 6a) as well as podocalyxin sialylation, increased GNE protein expression, and UDP-GlcNAc 2-epimerase activities. GNE myopathy patients, however, have no indications of renal abnormalities. The importance of sialic acid to the kidney may differ between humans and mice, and protein glycosylation patterns also vary. It is known that O- and N-linked glycosylation patterns of podocalyxin differ among species, including different types of sialic acids [117].

Mutant p.M712T knock-in pups did not live long enough to develop a muscle phenotype. However, in mutant pups rescued from neonatal lethality by ManNAc administration, and not receiving further ManNAc after weaning (onward of age 3 weeks), hyposialylation of muscle tissue can be detected by lectin staining at age 9 months (Fig. 6b)[118]. Further studies are pending regarding the muscle pathology. The findings in this knock-in mouse model not only established these mice as a genetic model for hyposialylation-related podocyte injury, but also supported evaluation of ManNAc as a therapy for GNE myopathy as well as for renal disorders involving proteinuria and hematuria due to podocytopathy and/or segmental splitting of the glomerular basement membrane [37].

A second GNE myopathy *Gne* mouse model was created in 2007 by Malicdan et al. [119]. This model was a transgenic mouse which expressed the human *GNE*

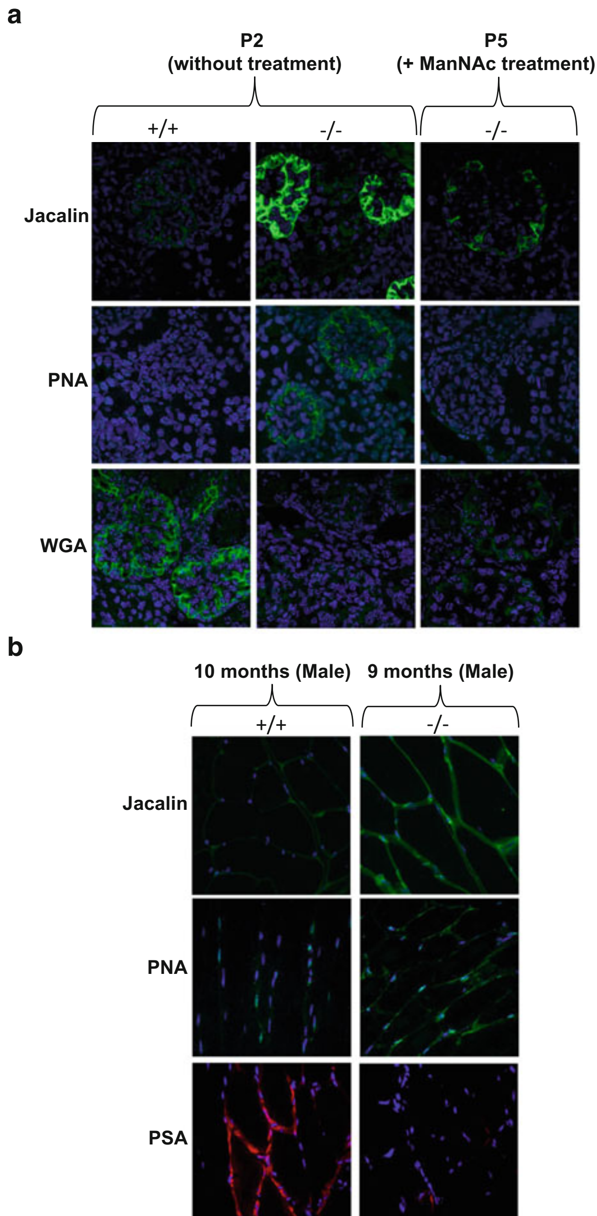

Fig. 6 Fluorescence lectin and antibody imaging demonstrating hyposialylation of kidney glomeruli and quadriceps muscle in mutant *Gne* M712T knock-in mice. (**a**) Representative images of kidney glomeruli and quadriceps muscle cells in paraffin embedded mouse slides stained with FITC-labeled lectins (*green*) Jacalin (*jackfruit agglutinin*) and PNA (*peanut agglutinin*), which both bind predominantly to terminal galactose residues, and WGA (*wheat germ agglutinin*), which predominantly recognizes terminal Neu5Ac and GlcNAc. Muscle samples were also

cDNA with the p.D176V epimerase domain mutation, common among Japanese patients, on a mouse background with a disrupted mouse *Gne* gene: *Gne*$^{(-/-)}$hGNED176V-Tg. Mutant offspring appeared normal at birth, with no apparent renal issues, but had decreased sialic acid levels in serum and in different organs. These mice recapitulated the clinical adult onset features of human GNE myopathy over time. They developed poor motor performance and signs of muscle weakness/atrophy at ~21 weeks of age, and by ~40 weeks the mice showed significant changes in muscle pathology with intracytoplasmic rimmed vacuoles which were immunoreactive to lysosomal markers, amyloid and phosphorylated tau and neurofilaments. Ultrastructural and immunohistochemical studies confirmed the presence of autophagosomes in affected mouse muscle [120]. Importantly, oral prophylactic treatment (starting at weeks 10–20) of ManNAc, sialic acid, or the sialic acid conjugate sialyllactose (containing ~45% of sialic acid) rescued the muscle phenotype in these mice. Compared to untreated mutant mice, mutant mice in all treatment groups at 54–57 weeks of age showed higher survival rates, increased body weight and muscle mass, increased sialic acid levels in serum, muscle, and other organs, decreased serum creatine kinase, better overall motor performance (treadmill and hanging wire tests), and a marked improvement in pathology (decreased number of rimmed vacuoles and congophilic, amyloid-positive, and tau-positive inclusions) [121]. These results strongly support consideration of ManNAc, sialic acid or sialic acid conjugates as a treatment for GNE myopathy. Further research is required to elucidate phenotypic differences between the transgenic *Gne*$^{(-/-)}$h*GNE*D176V-Tg and the knock-in p.M712T *Gne* mouse models.

To our knowledge, no other multiple cell organisms are reported as models for GNE myopathy. A few cell lines with *GNE* mutations or decreased *GNE* expression have been used for in vitro studies. Two Lec3 cell lines with either *GNE* nonsense mutation E35X or missense mutation p.G135E were shown to have a loss of UDP-GlcNAc 2-epimerase enzymatic activity and dramatically reduced sialic acid on glycans, including polysialylation of NCAM. The hyposialylation was rescued by exogenously added ManNAc or mannosamine [25]. Similar results were found in studies of GNE myopathy patients' cultured myotubes [113] or human lymphoid (B lymphoma cell line BJA-B) or hematopoietic (HL-60 myeloid

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Fig. 6 (continued) treated with antibodies to polysialic acid (PSA) (*red*). All slides were counterstained with the nuclear dye DAPI (*blue*). Confocal imaging intensity settings were the same across all ages and genotypes for each lectin or antibody. Glomeruli from *Gne* M712T knock-in pups at postnatal day 2 (P2) showed hyposialylation in mutant (−/−) compared to wild type (+/+) kidneys, as demonstrated by increased Jacalin and PNA signals and decreased WGA signal in −/− glomeruli compared to +/+. After ManNAc treatment, −/− glomeruli show at P5 decreased Jacalin and PNA signals and a more intense WGA signal compared to −/− glomeruli at P2 without treatment, suggestive of increased sialylation after ManNAc treatment. (**b**) Quadriceps muscles from adult male mice (+/+, 10 months; −/−, 9 months old) showed increased Jacalin and PNA signals and decreased PolySia staining in −/− compared to +/+ tissues, indicating hyposialylation of muscle glycans in adult GNE myopathy mice mimicking the human disorder

leukemia) cell lines with no detectable UDP-GlcNAc 2-epimerase activity [28]. Although ManNAc kinase activity was also severely decreased or absent in these mutant cells, it was demonstrated that ancillary kinases (e.g., GlcNAc kinase) exist that can convert ManNAc to ManNAc-6-P and aid sialic acid synthesis [3].

8.2.4 Disease Mechanism and Therapy

The exact pathophysiology of GNE myopathy remains unknown, but the *GNE* mutations associated with the disease suggest involvement of impaired sialylation. Since GNE myopathy is an adult onset disease, and patients have residual UDP-GlcNAc 2-epimerase and ManNAc kinase enzymatic activities, the effects of sialic acid deficiency may appear gradually. Some glycoconjugates (such as N-linked glycans) might be more readily sialylated than others, for example, O-linked or polysialylated glycans. Depending on the expression of sialyltransferases and the affinity of the enzymes to its substrate CMP-Neu5Ac, a preference for a specific glycosidic linkage (e.g., α2,3, α2,6, or α2,8) is likely. It was suggested that when a shortage of sialic acid occurs, specific proteins may be inadequately glycosylated, contributing to the pathology of GNE myopathy. Although overall sialylation of GNE myopathy patients' cells and tissues appears normal [114, 116, 122], specific glycoproteins and glycolipids were found to be hyposialylated in muscle tissue, including alpha-dystroglycan [123], the neural cell adhesion molecule [124], neprilysin together with a set of other *O*-glycans [125, 126], and the ganglioside GM3 [127]. The contributions of these findings to the pathophysiology of GNE myopathy remain under investigation. Additional evidence that hyposialylation could be a key factor in the pathomechanism of GNE myopathy came from the transgenic $Gne^{(-/-)}$h*GNE*D176V-Tg mouse model, in which muscle atrophy and weakness could be prevented by treatment with sialic acid metabolites [121].

Apart from hyposialylation, other hypotheses exist for a role of mutated GNE in the pathology of GNE myopathy. These include the unusual sub-cellular distribution (nuclear vs cytoplasmic) of the GNE protein in cells [68, 128], existence of different GNE isoforms with tissue-specific expression [32], involvement of mutated GNE in apoptotic pathways [129] and mitochondrial processes [35]. Other intriguing findings that may contribute to disease pathology are that GNE controls sialyltransferase expression, ganglioside production, and modulation of proliferation and apoptosis, independent of sialic acid production [92], and that GNE can interact with alpha-actinin 1, an actin binding and crosslinking protein [82], with the transcription factor PLZF [72], the collapsin response mediator protein CRMP1 which is involved in growth cone collapse and F-actin depolymerization [72], with receptor interacting factor 1 [72], and with KIAA 1549, a protein of unknown function [72].

No therapies are currently approved for GNE myopathy. Some dietary modifications were proposed, including administration or avoidance of some nutrients or trace elements which may influence GNE activity [130]. Significant progress toward GNE myopathy therapy was made during the last decade,

including development of clinical treatment protocols. Increasing sialic acid levels through exogenous means was tested in a pilot study on four affected GNE myopathy patients by administration of intravenous immunoglobulin G (IVIG), which has a large sialic acid content on IgG (~8 μmol of sialic acid/g) that could potentially be utilized to sialylate other glycoproteins (http://clinicaltrials.gov: Identifier NCT00195637). This study showed selective improvement of muscle strength, but no improvement of hyposialylation could be demonstrated biochemically [131]. Treatment studies of GNE myopathy mouse models convincingly showed that oral administration of sialic acid itself, the sialic acid conjugate sialyllactose, or the sialic acid precursor ManNAc can rescue hyposialylation [37, 121]. These studies served as a strong basis for the development of human clinical trials for this disease and Phases 1 and 2 studies are currently ongoing, including http://clinicaltrials.gov/ identifiers NCT01634750 ("Phase I Clinical Trial of ManNAc in Patients with GNE Myopathy or Hereditary Inclusion Body Myopathy (HIBM)"), NCT01236898 ("Pharmacokinetic Study on N-Acetylneuraminic Acid"), and NCT01517880 ("A Phase 2 Study to Evaluate the Dose and Pharmacodynamic Efficacy of Sialic Acid-Extended Release (SA-ER) Tablets in Patients with GNE Myopathy or Hereditary Inclusion Body Myopathy").

Apart from manipulating products and/or substrates in the sialic acid pathway, other GNE myopathy therapies could be envisaged. These are, for instance, gene therapeutic approaches [132] or enzyme replacement strategies. To reach the latter goal, cells, engineered to produce the active recombinant healthy GNE could be transplanted into affected muscles [133, 134]. A single patient with severe GNE myopathy was treated by gene therapy by intramuscular injections or by intravenous delivery of a healthy *GNE* cDNA embedded in liposomes (GNE-Lipoplex) [135, 136]. The results were encouraging but inconclusive and more patients with different severities of GNE myopathy muscle symptoms would need to be assessed. With GNE myopathy research and treatment trials quickly evolving, it is likely that elucidating the pathophysiology and therapeutic interventions of this devastating disorder will progress rapidly within the next few years.

8.3 Other GNE-opathies

Although not directly related to *GNE* mutations, some disorders and cell lines are reported with aberrant *GNE* expression, likely influenced by epigenetic [40] or other factors. Altered *GNE* expression can lead to altered sialylation patterns. Altered sialylation (whether or not GNE related) could increase the disposition for diseases including cancers, infectious diseases, and neurodegenerative and renal disorders [137]. We will address some disorders and cell lines for which a direct link to aberrant *GNE* expression (not due to *GNE* mutations) was demonstrated.

Some cancer cell lines were found with significantly reduced *GNE* mRNA and protein expression and UDP-GlcNAc 2-epimerase enzyme activities below detectable levels, resulting in hyposialylation of cell surface glycans. Such lines include a

human B lymphoma cell line and a human myeloid leukemia cell line. Both these cell lines showed abnormal hypermethylation of the CpG-rich *GNE* promoter, leading to silencing of *GNE* expression [28, 40]. In addition, Morris hepatoma cell lines were reported with decreased *GNE* expression [65], as did a chicken hepatoma cell line [138], although no direct links to the *GNE* promoter methylation status were reported in these latter cases. In HIV-1 infected T lymphocytes it was clearly demonstrated that hypomethylation of the *GNE* promoter underlie decreased UDP-GlcNAc 2-epimerase enzyme activity and hyposialylation. Interestingly, the changes in this cell line could be entirely corrected by supplementation of ManNAc to the growth medium [41]. Human Burkitt's lymphoma cell lines were demonstrated to exhibit sensitivity to ceramide-induced cell death regulated by *GNE* expression levels [139]. Regenerating rat hepatocytes were shown to be hyposialylated due to decreased UDP-GlcNAc 2-epimerase enzyme activity. It was postulated that an intracellular surge of calmodulin in these cells inhibited UDP-GlcNAc 2-epimerase activity [140].

Despite the above-mentioned hyposialylated lines, in most cancers increased sialic acid levels correlate with increased malignancy, tumorigenicity, and metastatic status [137]. This observation is in line with the earlier suggestion that cancer development goes along with a genome-wide hypomethylation [141]. A hypomethylated *GNE* promoter would result in an increased *GNE* expression and a concomitant increase in GNE protein and activity. Regardless of mechanistic details, the widely observed hypersialylation of metastatic cells favors the idea that cancer therapies may profit from decreasing sialylation in these cells. In fact, trials were undertaken to remove sialic acid enzymatically by neuraminidase and immunization with neuraminidase-treated tumor cells was tested with limited success [142, 143]. Therefore, inhibition of sialic acid synthesis might be more advantageous, which can be achieved through inhibition of *GNE* mRNA transcription, protein translation, or enzyme activity. Identification of sialic acid synthesis inhibitors has been pursued for several decades. It was shown that 2-deoxy-2-propionamido-D-glucose (GlcNProp) and, to a lesser extent, 2-deoxy-2-propionamido-D-mannose (ManNProp) were inhibitors of sialic acid synthesis in a cell-free system [144]. Periodate-oxidized UDP-*N*-acetylglucosamine, 2′,3′-dialdehydo-UDP-*N*-acetylglucosamine, was described as an irreversible inhibitor of UDP-GlcNAc-2-epimerase [145], and 3-*O*-methyl-GlcNAc was found to inhibit ManNAc kinase activity, resulting in decreased sialic acid synthesis [146]. After identification of the binding epitopes of the substrates UDP-GlcNAc and ManNAc to the GNE protein by NMR spectroscopy [52, 57], several substrate analogs were synthesized as inhibitors of GNE enzymatic activities, including exo-glycal- and thioglycoside-based UDP-sugar derivatives [147, 148], and deoxyiminosugars [149]. Further research, including testing on cellular and animal systems, is needed to evaluate the applicability of these inhibitors.

So far, no other disorders with aberrant sialylation have been reported to be associated with abnormal *GNE* expression. For disorders where the origin of sialylation defects remains unknown, investigations into *GNE* expression may be worthwhile, especially since therapeutic options are emerging, such as sialic acid

(or ManNAc or sialylated glycan) therapy for disorders involving hyposialylation, and *GNE* inhibitors for disorders involving hypersialylation.

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CMP-Sialic Acid Synthetase: The Point of Constriction in the Sialylation Pathway

Melanie Sellmeier, Birgit Weinhold, and Anja Münster-Kühnel

Abstract Sialoglycoconjugates form the outermost layer of animal cells and play a crucial role in cellular communication processes. An essential step in the biosynthesis of sialylated glycoconjugates is the activation of sialic acid to the monophosphate diester CMP-sialic acid. Only the activated sugar is transported into the Golgi apparatus and serves as a substrate for the linkage-specific sialyltransferases. Interference with sugar activation abolishes sialylation and is embryonic lethal in mammals. In this chapter we focus on the enzyme catalyzing the activation of sialic acid, the CMP-sialic acid synthetase (CMAS), and compare the enzymatic properties of CMASs isolated from different species. Information concerning the reaction mechanism and active site architecture is included. Moreover, the unusual nuclear localization of vertebrate CMASs as well as the biotechnological application of bacterial CMAS enzymes is addressed.

Keywords CMP-sialic acid synthetase · *N*-Acetyl-neuraminic acid · Sialic acid · Sialoside

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Abbreviations

BbeCMAS	CMAS from *Branchiostoma belcheri*
BtaCMAS	CMAS from *Bos taurus*
CKS	CMP-3-deoxy-D-manno-octulosonic acid synthetase
CMAS	Cytidine monophosphate *N*-acetylneuraminic acid synthetase
CthCMAS	CMAS from *Clostridium thermocellum*
DmeCMAS	CMAS from *Drosophila melanogaster*
DreCMAS	CMAS from *Danio rerio*
EcoCMAS	CMAS from *Escherichia coli*
HduCMAS	CMAS from *Haemophilus ducreyi*
KDN	Deaminated neuraminic acid
KDO	3-Deoxy-D-manno-octulosonic acid
MhaCMAS	CMAS from *Mannheimia haemolytica*
MmuCMAS	CMAS from *Mus musculus*
Neu5Ac	*N*-Acetyl-neuraminic acid
Neu5Gc	*N*-Glycolyl-neuraminic acid
NmeCMAS	CMAS from *Neisseria meningitidis*
OmyCMAS	CMAS from *Oncorhynchus mykiss*
PAF-AH	Platelet-activating factor acetylhydrolase
PmuCMAS	CMAS from *Pasteurella multocida*
SagCMAS	CMAS from *Streptococcus agalactiae*
Sia	Sialic acid
SsuCMAS	CMAS from *Streptococcus suis*

1 Introduction

Cytidine monophosphate *N*-acetylneuraminic acid synthetase (CMAS, EC 2.7.7.43), also known as CMP-Neu5Ac synthetase (CNS) or CMAS (CSS, CSAS, NeuA) is a highly conserved protein in pro- and eukaryotes, catalyzing the activation of sialic acids (Sia) to the cytidine-monophosphate diester. As illustrated in Fig. 1, CMAS catalyzes the nucleophilic attack of the anomeric oxygen of β-Neu5Ac on the alpha-phosphate of CTP, producing CMP-Neu5Ac and pyrophosphate. The presence of a divalent cation, usually Mg^{2+}, is obligate and the pH optimum mostly lies between pH 8 and 9.5 (reviewed in [1]; see also Sect. 2). CMAS appears to be a non-redundant enzyme, as indicated by mutagenized Chinese hamster ovary cells that lack sialylated cell surface glycoconjugates due to loss of endogenous CMAS activity [2, 3]. In addition, the *Escherichia coli* EV5 strain cannot synthesize a polysialic acid capsule as a consequence of a mutation in the CMAS gene [3, 4]. In multicellular organisms the crucial role of CMAS is demonstrated in a mouse model with reduced CMAS expression. Homozygous mice die within three days after birth due to kidney failure [5]. Regardless of whether Sia originate from cellular de novo synthesis pathways or glycoconjugate recycling, only the activated sugar CMP-Sia is an essential donor

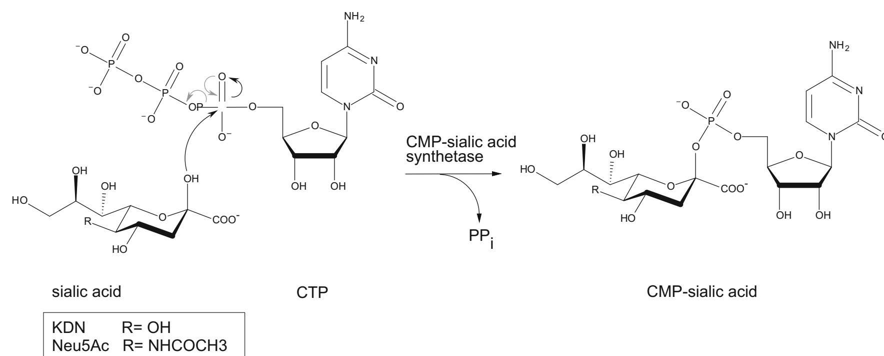


Fig. 1 Catalyzed reaction of CMP-sialic acid synthetase

substrate used by sialyltransferases for the addition of Sia to hydroxyl groups at the terminal end of glycoproteins, polysaccharides, and glycolipids. Proteins with this post-translational modification play an important role in the development, structure, and function of animal tissues [6–8] (see Hildebrand and Dityatev [9]).

The CMAS catalyzed reaction is unique in several respects. First, in vertebrates only Sia are activated by the nucleotide monophosphate CMP, with the majority of sugars activated in the form of uridine or guanine dinucleotides [10]. Second, compared to similar nucleotide-sugar forming reactions, the use of the non-phosphorylated sugar as substrate is unique. Third, the reaction product CMP-Neu5Ac is labile in acidic conditions, dissociating into Neu5Ac and CMP under acid hydrolysis [11]. However, CDP-Neu5Ac, chemically synthesized by Kajihara et al., has been found to be even more labile under both acidic and basic conditions due to anhydride formation between the carboxylic acid and the phosphate group. This observation explains why CDP-Neu5Ac is not found in biological organisms [12]. In eukaryotes, the CMAS reaction product CMP-Neu5Ac contributes to the tight regulation of Neu5Ac biosynthesis as a strong feedback inhibitor of the upstream acting UDP-GlcNAc 2-epimerase/*N*-acetylmannosamine kinase [13] (see Hinderlich et al. [14]). In contrast to eukaryotic cells, CMP-Neu5Ac does not function as an allosteric inhibitor of a committed step in the Sia pathway in *E. coli* [4]. Bacteria use Sia as nutrient, for environmental signaling, and as sources of amino sugars for cell wall biosynthesis (see review [15]). It has been shown that cell surface Sia from Gram-negative mucosal pathogens mimic host sialoglycoconjugates and thereby supply a protective shield for the microorganisms to evade detection by the host's innate immune system [16, 17]. The isolation of the CMAS reaction product CMP-Neu5Ac was first reported in 1959 from *E. coli* K-235 extracts [18]. This finding led to the isolation and extensive characterization of CMAS enzymes from various bacterial and animal sources. CMAS has first been partly purified from *Neisseria meningitidis* by Warren and Blacklow and from hog submandibular glands by Roseman in 1962 [19, 20]. Further purifications from diverse organs and species followed, like sheep brain [21], *Escherichia coli* [22], rat liver [23], frog liver [24], and *Photobacterium leiognathi* [25]. The first *cmas* gene, also termed *neuA*, was

cloned by Zapata et al. [26] in 1989 from *E. coli* K1 and different other bacterial *cmas* genes followed: those from *Neisseria meningitidis* serogroup B [27], *Streptococcus agalactiae* [28], *Haemophilus ducreyi* [29], *Haemophilus influenzae* [30], *Clostridium thermocellum* [31], *Streptococcus suis* [32], and *Pasteurella multocida* [33]. The first vertebrate *cmas* cDNA was isolated from the mouse [3] followed by *cmas* from *Oncorhynchus mykiss* [34] and *Homo sapiens* [35]. Interestingly, two *cmas* genes were identified in *Danio rerio*, which most likely originate from the third whole genome duplication, which occurred at the base of teleost radiation [36]. Four ortholog *cmas* genes are present in the lancelot *Branchiostoma belcheri* genome. However, enzymatic activity has so far only been analyzed for BbeCMAS1 [37]. The only insect *cmas* was cloned in 2006 from *Drosophila melanogaster* [38]. The gene product DmeCMAS shares 37% and 30% sequence identity with mouse and *E. coli* CMAS, respectively. In comparison, the highly related murine and rainbow trout CMASs share about 57% and the two zebrafish enzymes 56% sequence identity [36].

CMAS enzymes can be classified into smaller and larger enzymes, depending on their molecular weight. While most prokaryotic as well as *Drosophila melanogaster* CMASs belong to the group of the smaller enzymes of about 25 kDa, all so far known vertebrate *Cmas* genes encode proteins of approximately 430 amino acids with a predicted molecular weight of about 48 kDa. As illustrated in Fig. 2, five highly conserved primary sequence motifs have been identified in all CMAS enzymes (red boxes) [39]. The motifs are distributed over the whole sequence of short CMAS proteins (Fig. 2, lower part) but only over the N-terminal part of the long forms (Fig. 2, upper part). To date, bifunctionality has been shown for three long form bacterial CMASs that exhibit acetylhydrolase activity in addition to their sugar nucleotidyltransferase activity (Sect. 7).

2 Substrate-Specificity of CMAS

More than 50 naturally occurring Sia derivatives have been described that all originate from the parent compounds 2-keto-3-deoxy-5-acetamido-D-glycero-D-galacto-nonulosonic acid (*N*-acetylneuraminic acid, Neu5Ac) and 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (deaminated neuraminic acid, KDN, see Table 1), which are synthesized in two independent biosynthetic pathways [6, 40]. The Sia derivatives vary in the type of substituent(s) (acetyl-, sulfate-, methyl-, lactyl groups) added to the C4, C5, C7, C8, and C9 positions. One of the most common modifications is the hydroxylation of the *N*-acetyl group at carbon (C5), forming *N*-glycolylneuraminic acid (Neu5Gc, Table 1, [41] and see Davis and Varki [42]). *In vivo*, CMP-Neu5Gc is formed by hydroxylation of CMP-Neu5Ac, which is catalyzed by CMP-Neu5Ac hydroxylase (EC 1.14.18.2). The most abundant Sia species in mammals is Neu5Ac, followed by Neu5Gc [6, 43]. KDN was frequently found in lower vertebrates and Gram-negative bacteria as a component of glycoproteins, glycolipids, and capsular polysaccharides. Minor amounts have

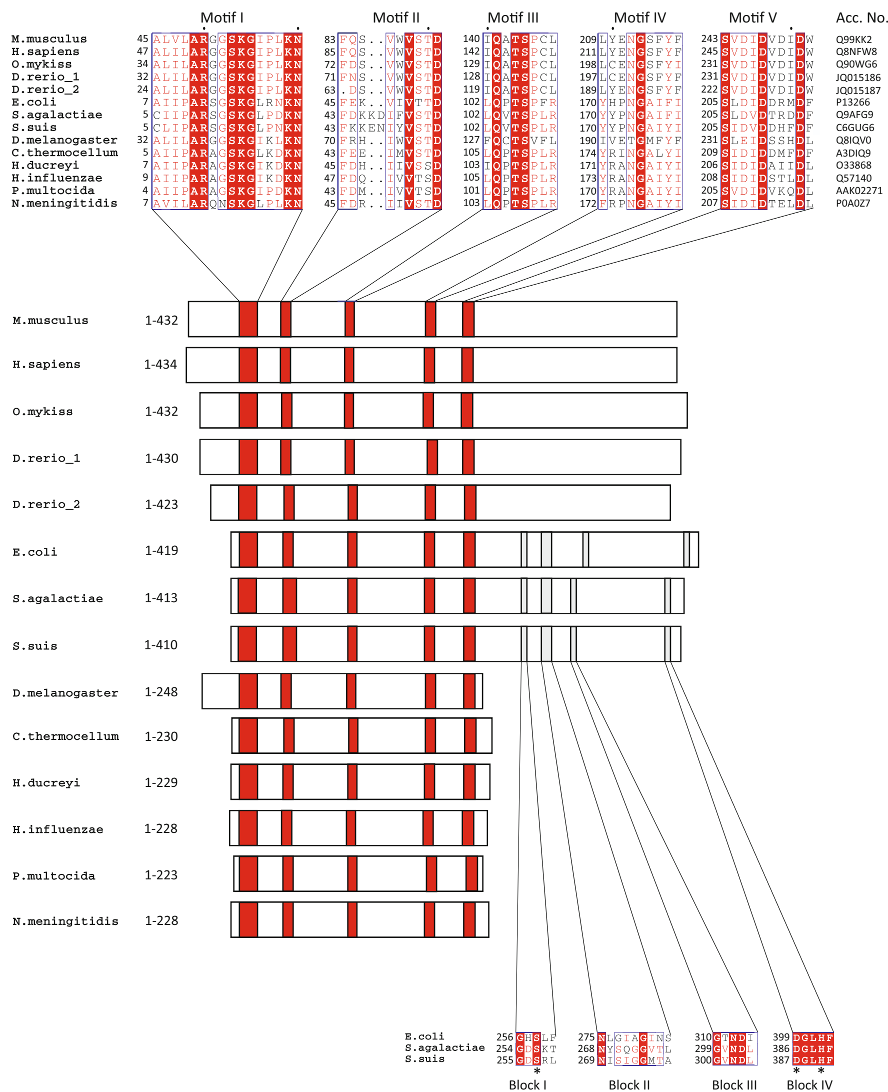
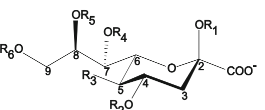


Fig. 2 Schematic representation of CMP-sialic acid synthetases from *Clostridium thermocellum*, *Danio rerio*, *Drosophila melanogaster*, *Escherichia coli*, *Haemophilus ducreyi*, *Haemophilus influenzae*, *Homo sapiens*, *Mus musculus*, *Neisseria meningitidis*, *Oncorhynchus mykiss*, *Pasteurella multocida*, *Streptococcus agalactiae*, *and Streptococcus suis*. Only characterized CMAS enzymes deposited in databases are considered. Highly conserved primary sequence motifs common to all CMASs are depicted as *red bars* and highlighted in a multi-sequence alignment (MULTALIN 5.4.1) [134]. Accession numbers from the databases UniProtKB or GenBank are given on the right. *Gray bars* and the corresponding multi-sequence alignment stretches (Block I–IV) indicate positioning and amino acid sequence of conserved motifs of the C-terminal domains of long bacterial CMAS enzymes. Catalytic triad residues are marked by *asterisks*. Fully conserved residues are shown as *white* characters with red background; similar residues are depicted with *red* characters. Numbers indicate the first amino acid residues of the displayed sequences

Table 1 Substrate specificity of CMP-sialic acid synthetases

Species	Natural substrates	Artificial substrates, modified at					Not accepted	References
		C4	C5	C7	C8	C9	Substrates	
B. belcheri CMAS1	Neu5Gc > Neu5Ac ≫ KDN	–	–	–	–	–	–	[37]
B. taurus	Neu5Ac and Neu5Gc ≫ KDN	4-Deoxy-Neu5Ac	5-*N*-Formyl-Neu, 5-*N*-trifluoroacetyl-Neu, 5-*N*-benzyloxycarbonyl-Neu, 5-*N*-aminoacetyl-Neu, Neu5SAc	–	–	9-Acetamido-Neu5Ac, 9-amino-Neu5Ac, 9-azido-Neu5Ac, 9-benzamido-Neu5Ac, 9-deoxy-9-thioacetamido-Neu5Ac, 9-hexanoylamido-Neu5Ac	Bulky C4-substituents	[55–60]
D. rerio CMAS1	Neu5Ac > Neu5Gc ≫ KDN	–	–	–	–	–	–	[36]
D. rerio CMAS2	KDN ≫ Neu5Ac and Neu5Gc	–	–	–	–	–	–	[36]
H. ducreyi	Neu5Ac, Neu5Gc	–	Neu5AcF > Neu5AcN3 > Neu5GcMe > Neu5CPg	–	Neu5Ac8OMe	Neu5Ac9N3	–	[33]
H. sapiens	Neu5Ac, KDN, Neu5Gc	–		–	–	–	–	[35]
M. musculus	Neu5Ac ≫ KDN	–		7-F-Neu5Ac	–	–	–	[34, 51]

O. mykiss	KDN > Neu5Ac, Neu5Gc	KDN4NH2	KDN5F, KDN5Cl, KDN5Br, KDN5Me, 5-*N*-formyl-Neu, 5-*N*-fluoroacetyl-Neu, 5-*N*-trifluoroacetyl-Neu, Neu5SAc	7-F-Neu5Ac	–	–	–	[34, 51, 55, 56]
E. coli	Neu5Ac, Neu5Gc	–	5-*N*-Carbomethoxy-Neu, Neu5AcN3	–	–	9-*O*-Acetyl-Neu5Ac, 9-azido-9-deoxy-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac, 9-*O*-lactyl-Neu5Ac	KDN, KDN5F, KDN5N3	[49, 53, 54]
N. meningitidis	Neu5Ac, KDN, Neu5Gc	–	Neu5Acryl, Neu5Alloc, Neu5Cbz, Neu5Prop, 5-deoxy-KDN, Neu5AcF > Neu5AcN3 > Neu5GcMe and Neu5CPg	7-F-Neu5Ac	Neu5Ac8OMe, 8-*H*-NeuNAc, 8-*O*-Me-NeuNAc 8-*F*-NeuNAc	9-Azido-9-deoxy-Neu5Ac, 9-azido-9-deoxy-KDN, 4,6-bis-epi-KDO, Neu5Ac9N3 Neu5,9Ac2	–	[33, 48–52, 133]
S. agalactiae	Neu5Ac, Neu5Gc ≫ KDN	–	Neu5AcN3, Neu5CbzGly	–	–	9-Azido-9-deoxy-KDN	KDN5N3, 4,6-bis-epi-KDO	[49]
P. multocida	Neu5Ac, Neu5Gc	–	Neu5AcF > Neu5AcN3 > Neu5GcMe > Neu5CPg	–	Neu5Ac8OMe	Neu5Ac9N3	–	[33]



KDN = deaminated sialic acid (R3 = OH; R1,2,4,5,6 = H)
Neu = neuraminic acid (R3 = NH_2; R1,2,4,5,6 = H)
Neu5Ac = *N*-acetylneuraminic acid (R3 = $NHCOCH_3$; R1,2,4,5,6 = H)
Neu5Gc = *N*-glycolylneuraminic acid (R3 = $NHCOCH_2OH$; R1,2,4,5,6 = H)
Neu5CPg = *N*-propagylyloxycarbonylneuraminic acid (R3 = $NHCOCH_2CCH$; R1,2,4,5,6 = H)

also been found in mammalian tissues [44, 45]. CMAS unifies the two Sia producing metabolic pathways by activating both Neu5Ac and KDN.

Table 1 gives an overview of the substrate specificities of the various CMAS enzymes. The recombinant CMASs from *N. meningitidis* (NmeCMAS), *S. agalactiae*, *H. ducreyi*, *H. influenzae*, *P. multocida*, *B. belcheri,* and *C. thermocellum* accept Neu5Ac as well as Neu5Gc as substrate [33, 37, 46]. Originally it has been reported that the CMASs from *N. meningitidis* and *E. coli* do not activate Neu5Gc [19, 47, 48]. However, Yu et al. disproved these findings for CMAS from both species by demonstrating the formation of CMP-Neu5Gc in coupled "one-pot two enzyme" approaches [49]. In fact, NmeCMAS seems to exhibit a broad substrate tolerance, particularly by accepting C5-modified Neu5Ac derivatives, including the natural sialic acids Neu5Ac, Neu5Gc, and KDN, as well as various synthetic sugar derivatives. 9-Azido-9-deoxy derivatives of Neu5Ac and KDN [48], as well as neuraminic acid *N*-carbamoylated with protective groups of different length and bulkiness (Neu5Propanosyl, Neu5Acryloyl, Neu5Cbz, Neu5Alloc), were activated by NmeCMAS. In addition, KDN derivatives, varied by deoxygenation, epimerization at C5 or at the terminal chain, and even the 8-carbon sugar 4,6-bis-epi-3-deoxy-D-manno-octulosonic acid (4,6-bis-epi-KDO) served as substrate for NmeCMAS [50]. The promiscuous substrate specificity of NmeCMAS also becomes apparent in the activation of 7-deoxy-7-fluoro sialic acid [51] and various C8-modified sialic acid derivatives [52]. In a comparative study with the enzymes from *P. multocida* and *H. ducreyi*, NmeCMAS has been observed to be highly active on Neu5Ac, Neu5AcF, Neu5Ac9N3, and Neu5Ac8OMe. In contrast, PmuCMAS and HduCMAS showed higher activity towards Neu5Gc, Neu5GcMe, and Neu5AcCPg [33]. Furthermore, based on the substrate specificity studies in combination with primary sequence alignments of *P. multocida*, *H. ducreyi*, and NmeCMAS, mutants of the latter enzyme with improved substrate promiscuity have been generated [33].

S. agalactiae CMAS (SagCMAS) showed a more restricted substrate tolerance than NmeCMAS. SagCMAS exhibited very good activities for Neu5Ac and its derivatives Neu5Gc, Neu5AcN3, and *N*-Cbz-glycine neuraminic acid (Neu5CbzGly). The KDN derivative KDN9N3 was converted to CMP-KDN9N3 with a yield of 80%, but KDN itself was a poor substrate (30%) and KDO was not accepted at all [49]. In comparison, the *E. coli* K1 CMAS (EcoCMAS) has narrow substrate tolerance concerning position C5. EcoCMAS recognized the natural substrates Neu5Ac and Neu5Gc, and only a few C5-derivatives like 5-*N*-azido-acetyl- and 5-*N*-carbomethoxy-neuraminic acid [49, 53]. KDN or other C5-modified Sia derivatives (5-azido-, 5-deoxy, and 5-deoxyfluoro-neuraminic acid) were not accepted at all [54]. In contrast, modifications at C9, like 9-*O*-acetyl-, 9-*O*-lactyl-, 9-deoxy-9-fluoro, and 9-azido-9-deoxy-Neu5Ac, were tolerated by EcoCMAS [54].

Human CMAS has significant CMP-sialic acid synthetic ability using Neu5Ac, Neu5Gc, or KDN as substrates with CTP [35]. For the recombinant murine CMAS KDN has been a poor substrate (Neu5Ac factor 15 over KDN [34]). In contrast, CMAS from rainbow trout (OmyCMAS) efficiently converted KDN, Neu5Ac, and Neu5Gc to the corresponding CMP-Sia compounds [55], and even exhibited

a 1.6-fold higher activity with KDN than with Neu5Ac [34]. Terada et al. [56] demonstrated broad substrate specificity of OmyCMAS in terms of substitutions at C4 or C5 position by the use of KDN analogues (5-fluoro, 5-chloro, 5-bromo, 5-methyl, and 4-amino) and Neu5Ac analogues (5-*N*-formyl, 5-*N*-fluoroacetyl, 5-*N*-trifluoroacetyl, and 5-*N*-thioacetyl) as acceptor substrates. In contrast to OmyCMAS, CMAS purified from calf brain (BtaCMAS) was highly active on Neu5Ac and Neu5Gc but was only slightly active on KDN [55]. Gross and Brossmer demonstrated that BtaCMAS is quite sensitive to C4 modifications of Sia, whereas a huge range of substituents is accepted at positions C5 and C9. *N*-Acetyl-4-deoxy-neuraminic acid was a good substrate, proving that BtaCMAS activity does not depend on the presence of the hydroxyl group. The size of the equatorial substituent at C4 strongly influenced the activation reaction: 4-*O*-methyI-Neu5Ac led to a decrease of about 80% of the V_{max} measured for Neu5Ac, but 4-*O*-acetyI-Neu5Ac was not accepted as a substrate at all. An axial substituent at C4 blocked activation by BtaCMAS [57]. Chemical modifications at C9 affected the affinity of the enzyme for the substrate as well as its catalytic rate but did not prevent activation. A lower affinity of the enzyme was observed for space-filling substituents at C9. Substrate affinity decreased in the following order: 9-acetamido-Neu5Ac, 9-hexanoylamido-Neu5Ac, 9-benzamido-Neu5Ac. The results indicate that the substituent at C9 of Neu5Ac is of minor importance for the correct coordination of Sia inside the active center of BtaCMAS [58]. Though the acetyl group at C5 of the Sia molecule is considered to be a crucial structural element for enzyme-substrate interactions, BtaCMAS also activated the C5 modified Sia analogues 5-*N*-formyl-, 5-*N*-trifluoroacetyl, 5-*N*-benzyloxycarbonyl-, and 5-*N*-aminoacetyl-neuraminic acid [59]. In addition, the sulfur derivatives 5-*N*-thioacetylneuraminic acid as well as *N*-acetyl-9-deoxy-9-thioacetamido neuraminic acid could be readily activated by BtaCMAS [60]. The two zebrafish CMAS enzymes differ in terms of the preferred Sia derivative. While DreCmas1 shows the highest activity toward Neu5Ac, lower activity toward Neu5Gc, and just basal activity toward KDN, DreCmas2 preferentially activates KDN and shows only basal activity toward Neu5Ac and Neu5Gc [36]. Recombinant lancelet BbeCMAS1 uses Neu5Ac, Neu5Gc, and KDN as substrates with highest activity for Neu5Gc and poorest for KDN [37].

Apart from sugar modifications, some CMAS also accept various nucleotides though CTP is the preferred substrate in all cases. UTP, CDP, and UDP were accepted by both *M. haemolytica* A2 CMAS (MhaCMAS) and NmeCMAS [47, 61]. In the case of MhaCMAS, the corresponding K_m values were similar to K_m (CTP), but V_{max} values have been significantly lower for all nucleotides tested, as compared with the physiological values. Thus, pyrimidine nucleotides seem to enter the active site of the enzyme, but only CTP efficiently yields the product CMP-Neu5Ac. No CMAS activity was detected when purine nucleotides were used as substrate [47]. Vertebrate CMASs as well as the CMASs from *C. thermocellum* and *S. agalactiae* appear to be highly specific for CTP since this nucleotide cannot be replaced by other tri-, di-, or monophosphate nucleosides [28, 31, 36, 62]. However, ATP, GTP, TTP, UTP, CDP, and CMP act as competitive inhibitors,

suggesting that the CTP-binding site in CMAS is very specific and, although other nucleotides may bind to it, only CTP can be used as a substrate [31, 62]. Moreover, the enzyme from rat liver is also inhibited by the reaction product CMP-Neu5Ac [62], and MhaCMAS and the purified enzymes from rat and bovine tissues were inhibited by high concentrations of the substrate CTP [47, 62, 63]. Recently Wong et al. [61] identified sulfonucleotide analogues as inhibitors of NmeCMAS.

CMAS enzymes operate under the absolute requirement of divalent metal ions, with Mg^{2+} favored most. It is reported for CMAS from *H. ducryi*, *M. haemolytica*, and *P. leiognathi* that other divalent cations, like Mn^{2+} or Ca^{2+}, can partially substitute Mg^{2+} [25, 46]. One exception is CMAS from *C. thermocellum* which even favors Mn^{2+} over Mg^{2+}, Co^{2+}, or Zn^{2+} [46]. For NmeCMAS it is suggested that two Mg^{2+} ions are positioned in the active site [64]. Both Mg^{2+} ions are thought to be responsible for the activation of the α-phosphate of CTP and the correct orientation of the substrates. Additionally, the catalytic Mg^{2+} ion is proposed to activate the sugar hydroxyl group [64].

3 Crystal Structures of CMP-Sialic Acid Synthetases

Crystal structures of CMASs have been solved from two different species (Table 2). First, Mosimann et al. crystallized the full-length NmeCMAS in the apo-form and in the presence of CDP [65]. Second, Krapp et al. solved the structure of the catalytic part of MmuCMAS (amino acids 39–267) in the product bound state (CMP-Neu5Ac) [66]. Although the overall sequence identity is only 27%, both enzymes exhibit a highly related overall fold and active site architecture. Both enzymes form functional homodimers with each monomer organized in a ~190 residue, globular domain, exhibiting an open α/β fold, linked to a small ~40 residue dimerization domain. The globular core domain is formed by a central mixed β-sheet composed of one antiparallel and six parallel strands, flanked by helices giving rise to an αβα three-layered sandwich structure. As illustrated by the surface presentation in Fig. 3a, b, the dimerization domains of both monomers are entwined around each other and stay in contact with the globular domain of the other monomer. The active sites are localized at these interfaces, and are formed by loop-structures that coincide with the above-mentioned five highly conserved sequence motifs common to all known CMAS enzymes (Fig. 2, red bars). In the surface presentation of the NmeCMAS and MmuCMAS crystal structures (Fig. 3a, b) the five sequence motifs are colored in red for one monomer each (yellow). The root-mean-square deviation of Cα atoms in structurally equivalent positions is 1.3 Å for 97 corresponding amino acid residues [66]. The highest sequence diversity among the CMAS enzymes is observed in the dimerization domains (MmuCMAS residues 170–210, NmeCMAS residues 136–171) with considerable conservation only in a short stretch of residues (MmuCMAS: Arg199–Trp205, NmeCMAS: Glu162-Leu168) that carries catalytically important amino acid residues [66]. NmeCMAS has been crystallized in an "open" conformation,

Table 2 Data statistics from the crystal structures obtained

	NmeCMAS	NmeCMAS	MmuCMAS	MmuCMAS	EcoCKS capsule specific	EcoCKS LPS specific
EC	2.7.7.43	2.7.7.43	2.7.7.43		2.7.7.38	2.7.7.38
Fragment	Full length	Full length	N-terminal domain	C-terminal domain	Full length	Full length
Residues	1–228	1–228	39–267	267–432	1–245	1–264
PDB ID	1EYR	1EZI	1QWJ	3EWI	1H7T[a]	3K8D[b]
Space group	P 21 21 21	P 21 21 21	P 21 21 21	C 1 2 1	P 1 21 1	$P3_1$
Resolution (Å)	2.2	2.0	2.8	1.9	2.48	1.9
Ligand	CDP	–	CMP-Neu5Ac	–	CMP-Neu5Ac	CTP, Mg^{2+} 2-deoxy-KDO
References	[65]	[65]	[66]	[72]	[71]	[70]

[a]See also 1H7E, 1H7F, 1H7G, 1H7H for unligated, CMP-, CDP-, and CTP-bound states, respectively
[b]See also 3K8E for unbound state

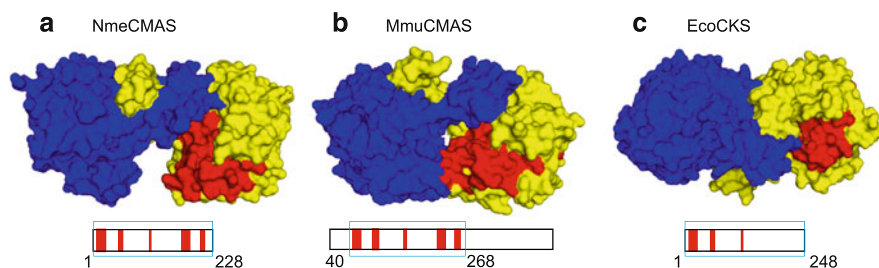


Fig. 3 Surface presentation of functional CMAS and CKS units. Surface presentation of crystal structures of NmeCMAS (**a**), the N-terminal domain of MmuCMAS (**b**), and EcoCKS (**c**). The *bars* illustrate the position of conserved primary sequence motifs (*red boxes*) and the crystallized protein part (framed in *blue*). Monomer A is shown in *blue*, Monomer B in *yellow*. Amino acid residues belonging to the conserved sequence motifs are depicted in *red*, and build-up the active site pocket. PDB-codes: 1eyr (NmeCMAS), 1qwj (MmuCMAS), 1k8d (EcoCKS)

which allows entry of the second substrate [65], whereas the MmuCMAS has been crystallized in a "closed" conformation with the product CMP-Neu5Ac in the active site [66]. Superposition of the two crystal structures reveals that the enzyme undergoes significant structural changes upon substrate binding. In the closed conformation of MmuCMAS, Arg202 is translocated by more than 5 Å toward the active site in comparison to the corresponding Arg165 of the NmeCMAS structure [66]. It has been shown for MmuCMAS that replacement of this residue by alanine abolishes the catalytic activity [67].

CMP-KDO synthetases (CKS, EC 2.7.7.38) exhibit high sequence similarities to CMAS enzymes (39–58%), and catalyze the addition of the negatively charged 8-carbon sugar 3-deoxy-D-manno-octulosonic acid (KDO) to CTP to form CMP-KDO. KDO is found in Gram-negative bacteria as an essential component of lipopolysaccharides (LPS) and it is expressed in higher plants and green algae as a part of cell-wall polysaccharides [68, 69]. Available crystal structures of capsule-specific and LPS-specific CKS from *E. coli*, respectively, show significant similarities to CMAS enzymes in the overall structure as well as in the nucleotide binding pocket (Table 2) [66, 70, 71]. As exemplarily shown for LPS-specific CKS in Fig. 3c, the enzymes are also built up of one globular domain linked to a smaller dimerization domain that entwines around the dimerization domain of the neighboring monomer. CKSs share three of the five primary sequence motifs that are conserved in CMASs (shown in red, Fig. 3c), thus exhibiting an equivalent assembly of the nucleotide binding pocket, which is basically formed by motif I residues (see review [39]). However, the dimerization interfaces and therefore the sugar binding pockets are different in both the primary sequence and the 3D-structure, explaining the strict substrate specificities of the two enzyme classes. LPS-specific CKS of *E. coli* (EcoCKS) was crystallized in both the “open,” unbound conformation and in the “closed” conformation as a complex with CTP and the substrate analogue 2β-deoxy-KDO [70] (Table 2). Open and closed conformations can be superimposed by rotating the CTP-binding domain by 13° around the hinge regions, leading to a maximal shift of the phosphate binding loop residue Arg15 of about 9 Å. As a direct consequence of the open to close transition the α-phosphate is brought into direct contact with the bound 2β-deoxy-KDO. Displacement of the associated Mg^{2+} ion accompanies this reorganization [70]. According to their close evolutionary relationship it is supposed that similar movements take place in CMAS enzymes [64].

The crystal structure of the separately expressed C-terminal domain of MmuCMAS (MmuCMAS-CT, residues 267–432) was solved in 2009 by Oschlies et al. [72]. In contrast to the C-terminal domains of bacterial CMAS, MmuCMAS-CT showed no enzymatic activity, but functions as tetramerization platform for the entire protein. The physiological role of this protein domain will be further discussed in Sect. 5.

4 Active Sites of CMP-Sialic Acid Synthetases

CMP-β-Neu5Ac is presumably made by direct transfer of the anomeric oxygen of β-Neu5Ac to the α-phosphate of CTP (Fig. 1) [73, 74]. The catalytic reaction is specific for the β-anomer of the Neu5Ac C2 hydroxyl group and is performed under retention of configuration. In contrast, Sia transfer to glycoconjugates occurs under inversion of configuration, and thus Neu5Ac is exclusively found α-linked in natural glycoconjugates [75]. It has been proposed that CMAS functions in terms of an ordered sequential Bi-Bi catalytic mechanism, meaning that CTP binds first,

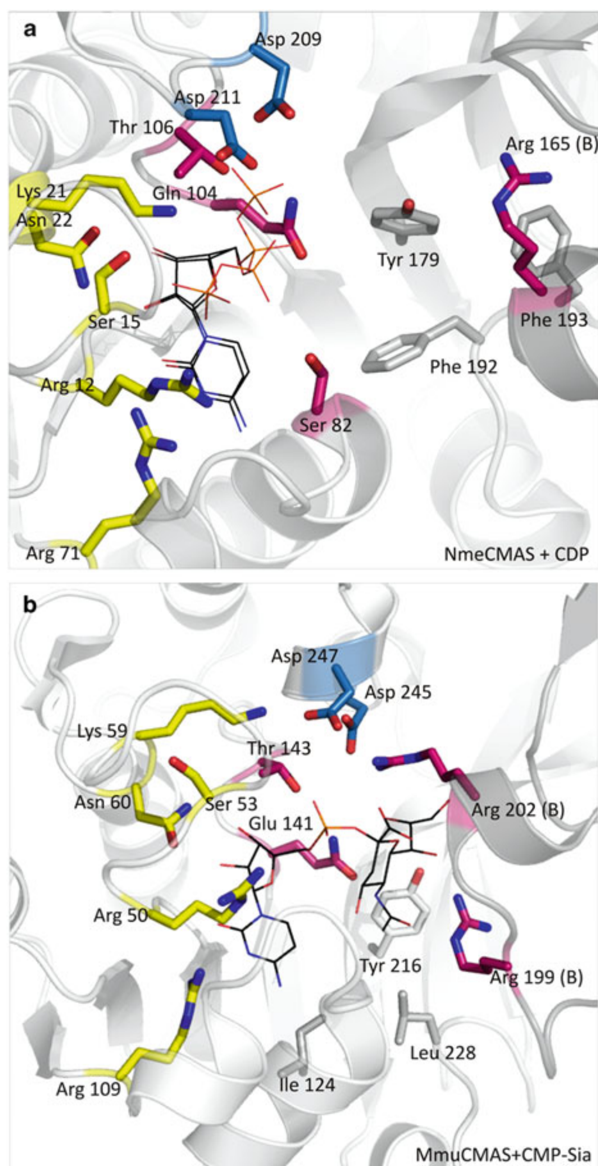


Fig. 4 Active site pockets of NmeCMAS (**a**) and MmuCMAS (**b**) shown in ribbon presentation with Cα-traces of monomer A and B colored in *light gray* and *dark gray*, respectively. The co-crystallized substrate analogue CDP (NmeCMAS) and the product CMP-Neu5Ac (MmuCMAS) are shown as *lines*. Amino acid residues important for nucleotide binding, sugar binding, and Mg^{2+} coordination are shown in *yellow*, *red*, and *blue*, respectively. Hydrophobic pocket residues are depicted in *gray*. The PDB codes are 1eyr (NmeCMAS) and 1qwj (MmuCMAS), respectively

followed by Sia binding and subsequent release of the products PP_i and CMP-Sia [76]. This assumption has been confirmed by isothermal titration calorimetry experiments with NmeCMAS showing that CTP-binding is a prerequisite for Sia binding [64]. Additionally, the kinetic parameters obtained for various CMAS enzymes frequently show a higher affinity for CTP than Neu5Ac as reflected by the K_m values obtained [46]. The first and last steps of catalysis have been resolved in more detail by crystallization of NmeCMAS with the substrate analogue CDP

Table 3 Residues important for catalysis of CMP-sialic acid synthetase reaction

Motif	Residue	MmuCMAS	NmeCMAS	Function	References
I	Arg	50	12	Nucleotide binding	[65, 66, 78]
I	Ser	53	15	Nucleotide binding	[64–66]
I	Gly	55	17	Nucleotide binding	[64, 65]
I	Lys	59	21	Nucleotide binding	[65, 66, 78][a][77][b]
I	Asn	60	22	Ribose binding	[66]
	Ser		82	Sia binding	[65]
	Arg	109	71	Base recognition (N3)	[65, 66]
	Ile	124		Sia binding: hydrophobic pocket	[66]
III	Gln	141	104	Sia-binding and Mg^{2+} re-orientation	[64–66]
III	Thr	143	106	Ribose and Sia binding	[64, 65]
dim. dom.	Arg	199		Coordinates CO of *N*-acetyl group	[66, 67]
dim. dom.	Arg	202	165	Coordinates COO^- of Neu5Ac	[65, 67]
dim. dom.	Gln	203	166	Quaternary enzyme organization	[65, 67]
IV	Asn	212	175	Backbone forms pocket for O9	[64]
IV	Tyr	216	179	Sia binding: hydrophobic pocket	[64, 65]
	Phe		192	Sia binding: hydrophobic pocket	[64, 65]
	Phe		193	Sia binding: hydrophobic pocket	[64, 65]
	Leu	228		Sia binding: hydrophobic pocket	[64, 66]
V	Asp	245	209	Metal-binding (bidental)	[64–66]
V	Asp	247	211	Metal-binding (monodental)	[64–66]

[a]Residue also identified for *E. coli* (*) CMAS
[b]Residue also identified for *H. ducreyi* CMAS

(open conformation), and the N-terminal domain of MmuCMAS in complex with the reaction product CMP-Sia (closed conformation) [65, 66]. A ribbon presentation of the similarly organized active sites of both structures is shown in Fig. 4. Amino acids crucial for nucleotide-binding are depicted in yellow, those for Sia binding in red (hydrophilic) and grey (hydrophobic), and residues involved in metal coordination in blue. Table 3 summarizes the intramolecular localization and function of residues important for catalysis of NmeCMAS and MmuCMAS, respectively. The CTP-binding pocket of CMAS proteins is formed by loops at the C-terminal ends of the central β-sheet of the globular core domain. Arg12 and residues 71–80 of NmeCMAS are responsible for base recognition [65]. Arg12 and Arg71 of NmeCMAS are highly conserved throughout all CMAS homologues and correspond to Arg50 and Arg109 of MmuCMAS (Figs. 2 and 4), respectively. The phosphate binding loop (NmeCMAS p-loop: residues

10–22, MmuCMAS loop L1: residues 50–69) primarily interacts with the ribose and phosphate moieties of the substrate analogue CDP and the product CMP-Sia and is supposed to stabilize the phosphate groups by positively charged residues (NmeCMAS: Arg12, Lys16, and Lys21, MmuCMAS: Arg50, Lys54, and Lys59) [65, 66]. Corresponding positively charged residues have also been identified in CMAS enzymes from *E. coli* and *H. ducreyi* and shown to be important for enzymatic activity [77, 78]. Furthermore, "Saturation Transfer Difference"- NMR experiments with the recombinant OmyCMAS supported these observations. The H1' protons of the ribose moiety of both CTP and CMP-Neu5Ac were found in close proximity to the protein surface [79]. Sia can enter the active site pocket subsequent to CTP binding. The Neu5Ac binding pocket is formed by residues of both monomers and is significantly less conserved than the CTP binding site. In NmeCMAS the polar side chains of residues Ser82, Gln104, Thr106, and Asp209 line the Neu5Ac pocket nearest to the bound substrate analogue CDP. The polar contact to the carboxylate-group at C1 is made by Arg165 (NmeCMAS) and Arg202 (MmuCMAS), respectively, which is part of the dimerization domain of the opposing monomer (shown in red, Fig. 4). This residue is also conserved in capsule-specific EcoCKS, where Arg155 coordinates the carboxyl function of KDO [80]. The highly conserved Gln-residue of motif III (Gln104 in NmeCMAS and Gln141 in MmuCMAS) is involved in the coordination of the O8 and N5 atoms of Neu5Ac as shown by both modeling (NmeCMAS) and structural (murine enzyme) data. Horsfall et al. introduced a series of mutations at Gln104 of NmeCMAS, and analyzed the kinetic properties of the mutants [64]. The Q104A mutant showed a ~40-fold increased K_m (Neu5Ac), while K_m (CTP) decreased ~2-fold. However, true k_{cat} and K_m values could not be identified for Q104E, Q104N, and Q104L mutants, because it was not possible to saturate the enzymes. Interpretation of the apparent kinetic parameters, obtained at fixed concentrations of the other substrate, suggest a role for Gln104 in catalysis as well as substrate binding. The authors furthermore propose that Gln104 plays a role in the re-orientation of Mg^{2+} during the re-opening of the active site after catalysis allowing product release [64], in analogy to results obtained for Gln98 of the related LPS-specific EcoCKS enzyme [70].

The active site of CMAS enzymes is generally very polar, but harbors a hydrophobic pocket. In the CMP-Neu5Ac bound MmuCMAS crystal structure, a number of hydrophobic interactions between residues Ile124, Tyr216 and Leu228 (gray residues, Fig. 4b) and the methyl group of the *N*-acetyl moiety of Neu5Ac have been observed [66]. "Saturation Transfer Difference"-NMR experiments with the recombinant OmyCMAS confirmed the close proximity of protons of the C5 *N*-acetyl moiety of CMP-Neu5Ac to the protein surface [79]. It has been proposed that the composition of the hydrophobic pocket is an important determinant for the discrimination of various Sia derivatives and prevents binding of the 8-carbon sugar KDO [64, 65]. The hydrophobic pocket is not present in the related EcoCKS, but is conserved in CMAS enzymes. KDN, carrying a hydroxyl-group at position C5, is the favored substrate of OmyCMAS, which has presumably a less pronounced hydrophobic cavity formed by only two amino acid residues (Ile113 and

Leu216). MmuCMAS, with three hydrophobic residues, prefers Neu5Ac over KDN as substrate [34, 66] (gray residues, Fig. 4b). The most distinct hydrophobic pocket in this line is that of NmeCMAS with Tyr179, Phe192, and Phe193 (Fig. 4a). Kinetic parameters obtained for the NmeCMAS mutants F192A and F193A support the role of these residues in binding Neu5Ac, because the mutants have k_{cat} values comparable to that of the wild-type enzyme but increased K_m values, indicating a lower affinity toward the sugar substrate [64]. Wild-type NmeCMAS also accepts KDN, but exhibits a k_{cat}/K_m value around 5,000 times lower than with the natural substrate Neu5Ac. Horsfall et al. therefore suggest the requirement of three hydrophobic residues in forming a binding pocket that leads to a preference for Neu5Ac over KDN. Furthermore, residues of the hydrophobic pocket, namely Tyr179 and Tyr216 of NmeCMAS and MmuCMAS, respectively, lie within hydrogen-bonding distance of O7 and O9 of the glyceryl moiety of Neu5Ac and are supposed to be part of a network of non-covalent bonds between substrate and enzyme [64, 65].

5 Localization of CMP-Sialic Acid Synthetases in Eukaryotes

Vertebrate CMAS enzymes are ubiquitously expressed in human and murine tissues as shown by Northern Blot analysis [3, 35], but exhibit an unusual localization within the cell. Sugar activating enzymes normally act inside the cytoplasm, but the major localization of the CMAS enzymes is the cell nucleus [81]. The first observation of nuclear localization was in 1969 by E.L. Kean in the hog eye lens [82]. The epithelial layer is the only region of the lens which contains nucleated cells. It can be separated from the residual tissue that is formed by enucleated cells. CMAS activity has only been found in fractions containing the epithelial layer. Subsequent studies revealed nuclear localization of CMAS activity in a variety of tissues, like hog retina [82], rat brain [83], and calf kidney [84] (for review see [1, 81]). In 1998 the first vertebrate CMAS encoding cDNA was cloned and nuclear localization has been confirmed for the murine enzyme by fluorescent labeling in mouse fibroblast cells [3]. Later, the recombinant human and rainbow trout CMAS were also found in the nucleus [35, 85]. Intriguingly, while zebrafish CMAS1 is – as expected – found in the nuclear compartment, DreCMAS2 – as the first and so far only vertebrate CMAS – is retained in the cytoplasm [36]. Vertebrate CMASs are tetrameric proteins with a calculated molecular mass of the monomers of about 48 kDa, indicating the need of active transport for passage through the nuclear pore complex [36, 85]. Distinct recognition sites that mediate the contact to import and export proteins have been identified and are called nuclear localization signals (NLS) and nuclear export signals (NES), respectively (reviewed in [87, 88]). NLSs can be separated into monopartite and bipartite motifs. Monopartite NLSs are generally characterized by 1 short stretch (4–8 residues) of positively charged amino acids with the consensus sequence K–K/R–X–K/R–R, whereas bipartite

NLS motifs contain 2 positively charged clusters separated by a linker region of 10–12 amino acids ($((K/R)_2–X_{10–12}–(K/R)_{3–5})$ [89]. Several clusters of basic amino acids have been identified for MmuCMAS, OmyCMAS, and DreCMAS1, and their functionalities have been tested using deletion mutants [3, 36, 85]. The MmuCMAS sequence harbors two stretches of basic amino acids that mediate nuclear import when fused to the green fluorescent protein. However, the crucial NLS turned out to be the monopartite sequence K^{198}RPRR that also contains amino acids important for activity. Complementation studies in mutant cells lacking endogenous CMAS activity have demonstrated that enzymatic activity and nuclear localization are two independent properties of MmuCMAS, which can be separated. Both R199A and R202A CMAS mutants showed a clearly reduced activity, but were still transported to the nuclear compartment [67]. In contrast, mutation of Lys198 and Arg201 to Ala, resulting in $\mathbf{A}^{198}$RP**A**R, led to a cytoplasmic but active enzyme, demonstrating that nuclear localization is no prerequisite for enzymatic activity [67]. Alternative and structurally different NLSs have been identified in the fish enzymes. Nuclear localization of the OmyCMAS depends on a free N-terminus with an accessible bipartite NLS $K^5KR(X)_{10}$RKAK [85], while the bipartite NLS ($K^9RAMK^{13}(X)_{11}K^{24}RRK^{27}$) directs nuclear import of DreCMAS1 [36]. The development of different NLS signals in vertebrate CMASs indicates the presence of evolutionary pressure. So far no explanation has been found for this unusual localization, especially because it is not needed for enzymatic activity in a cellular system. It has been speculated that nuclear localization is linked to a second as yet unknown function [81]. A residual cytoplasmic localization of MmuCMAS has been observed after transient overexpression in the cell culture system and led to the identification of two potential leucine-rich NESs ($P^{57}LKNIKRLAGVPLIGWV^{73}$ and $G^{408}RGAIREFAEHIFLLIE^{424}$ [90]). However, the identified NESs appear to be subordinate to the parallel existing NLS-motif because fusion to GFP has not been sufficient to retain GFP completely in the cytoplasm. The biological significance of a potential nucleo-cytoplasmic shuttling of the vertebrate CMAS remains to be elucidated. Unlike vertebrate CMAS the enzyme isolated from *Drosophila melanogaster* has been found to be Golgi targeted when expressed as recombinant protein in both mammalian and insect cells [38]. The DmeCMAS sequence begins with an N-terminal signal sequence rich in hydrophobic amino acids, anchoring the protein in the Golgi membrane. Localization in the Golgi apparatus seems to be important for its function, because replacement of the N-terminal signal sequence (27 amino acid residues) in the DmeCMAS cDNA with the first 40 residues from the human enzyme entailed nuclear localization of an enzymatically inactive fusion protein [38]. Expression of DmeCMAS has been detected in embryonic stages [38] that correlate with the development of the central nervous system. In addition, elevated expression levels in the head of adult flies indicate a role for Sia in the nervous systems of insects [91].

6 Biotechnological Applications of CMP-Sialic Acid Synthetases

Sia is a frequently occurring terminal modification of glycoconjugates presented at the cell surface. Sia increases the plasma half-life of underlying glycoproteins and carbohydrate-based therapeutics [92–94]. The synthesis of Sia containing oligosaccharides and corresponding derivatives is essential for deepening the understanding of biochemical processes and for developing novel carbohydrate-based therapeutic agents (for review see [95]). In general terms, two strategies can be pursued for synthesis of Sia and derivatives: purely chemical approaches and enzymatic or chemoenzymatic methods. Total synthesis of Neu5Ac from non-carbohydrate precursors was first reported by Danishefsky et al. in 1988 [96]. More recently, total synthesis of a fully protected Neu5Ac derivative has been described [97]. For chemical sialylation multiple protection and deprotection steps are required to control the stereo- and regiochemistry of *O*-glycosidic linkage formation (see reviews [98–100]). *Chemoenzymatic synthesis* appears to be the more powerful approach to obtain complex carbohydrates that combines both the flexibility of chemical synthesis and the highly efficient stereo- and regioselectivity of enzymatic reactions. *Whole cell-based approaches* take advantage of the microorganisms' own metabolic machinery for the synthesis of sialosides from inexpensive materials without adding nucleotides. The *living factory approach* functions on the basis of genetically engineered *E. coli* cells that overexpress the genes encoding enzymes for sugar-nucleotide biosynthesis and the appropriate glycosyltransferases. One recent review [101] describes much of the previous work concerning these approaches; therefore these issues will not be readdressed here. Chemical and chemoenzymatic synthesis, as well as cell-based approaches, have provided a large library of Sia derivatives that can now be used for structure–activity relationship studies. In the above-mentioned studies, NmeCMAS is the most favored enzyme because of its broad substrate specificity (see Sect. 2). Gilbert et al. used the genes from *Neisseria meningitidis* and constructed a fusion protein that has both CMAS and α-2,3-sialyltransferase activity [102]. The recombinant fusion protein exhibited turnover numbers comparable to the single enzymes and appears to be an attractive tool for large-scale biotechnological applications involving multiple enzymatic steps.

In a different study, NmeCMAS was immobilized at its C-terminus by attachment to cysteine-functionalized magnetic nanoparticles (MNPs) in a site-specific manner [103]. Covalent linkage to MNPs present the advantages of potential reuse and long-term stability. However, CMAS activity was reduced to 77% of native NmeCMAS activity, possibly caused by the bulky MNP, which may decrease the enzyme's dynamics [103]. In line with this hypothesis, Yu and coworkers were able to increase the activity of the immobilized NmeCMAS by increasing the length of a PEG linker between the enzyme and the MNP surface, thus providing more flexibility [104]. Another attractive alternative for the production of complex sialosides and therapeutically relevant proteins is the use of genetically engineered plants [105].

As impressively shown for the secretory immunoglobulin A, plants are able to express correctly folded and assembled complex proteins [106]. One advantage of using plants as an expression system is their ability to perform *N*-glycosylation similar to mammalian cells. However, besides a common core structure, plant *N*-glycans differ from their mammalian counterparts to some extent as they (1) carry plant-specific immunogenic β1,2-Xylose and α1,3-Fucose residues and (2) lack terminal β1,4-Gal and *N*-acetylneuraminic acid (Neu5Ac) residues [107, 108]. Engineering of the *N*-glycosylation pathway in plants by knockout and RNA interference approaches resulted in lines that synthesize complex *N*-glycan structures lacking the plant-specific glycan epitopes [109–113]. Furthermore, Castilho et al. [114] established Neu5Ac and CMP-Neu5Ac expression in *Arabidopsis* from endogenous precursors by simultaneous expression of the three enzymes required for the synthesis of CMP-Sia in mammals: the bifunctional UDP-GlcNAc 2-epimerase/*N*-acetylmannosamine kinase from mouse, human *N*-acetylneuraminic acid phosphate synthase, and human CMAS. To obtain activity an N-terminal truncated version of human CMAS lacking its 40 N-terminal amino acids had to be used. Taken together, glycoengineering of the appropriate plant species seems to be a promising tool for the production of therapeutic proteins with authentic mammalian glycans.

7 The C-Terminal Domain of Long CMP-Sialic Acid Synthetases

S. agalactiae, *S. suis*, and *E. coli* K1 carry capsular polysaccharides, mimicking sialylated host structures, and therefore limit the activation of an effective innate immune response. *S. agalactiae* and *S. suis* capsules are capped by terminal α2,3-linked and α2,6-linked Neu5Ac- residues, respectively, whereas *E. coli* K1 is protected by a thick polysialic acid layer formed by a homopolymer of α2,8-linked Neu5Ac [115–117]. Modification of the *E. coli* K1 capsule by Sia *O*-acetylation in positions C7 and C9 correlated with increased virulence in patients suffering from bacteremia [118–120]. The interaction between *S. agalactiae* and host siglecs is also affected by the degree of Sia *O*-acetylation [121] (see also Vlasak et al. [122]). Prokaryotic CMAS enzymes of the larger type play a role in the regulation of Sia *O*-acetylation: CMAS from *S. agalactiae*, *S. suis*, and *E. coli* K1 are bifunctional, combining the N-terminal CMP-Neu5Ac synthetase with a C-terminal esterase activity (Fig. 2). The esterase domains are composed of about 170 amino acids, and can be classified into the GSDL (Gly, Ser, Asp, Leu) family of SGNH (Ser, Gly, Asn, His) hydrolases [123, 124], with the active site formed by a catalytic triad [125]. In Fig. 2 the four conserved primary sequence motifs (Block I–IV) are shown as gray bars and catalytic triad residues are marked by asterisks. All prokaryotic CMASs are reported to form dimers [26, 28, 29, 65, 126] and dimerization has also been proposed for the separately expressed C-terminal domain of EcoCMAS

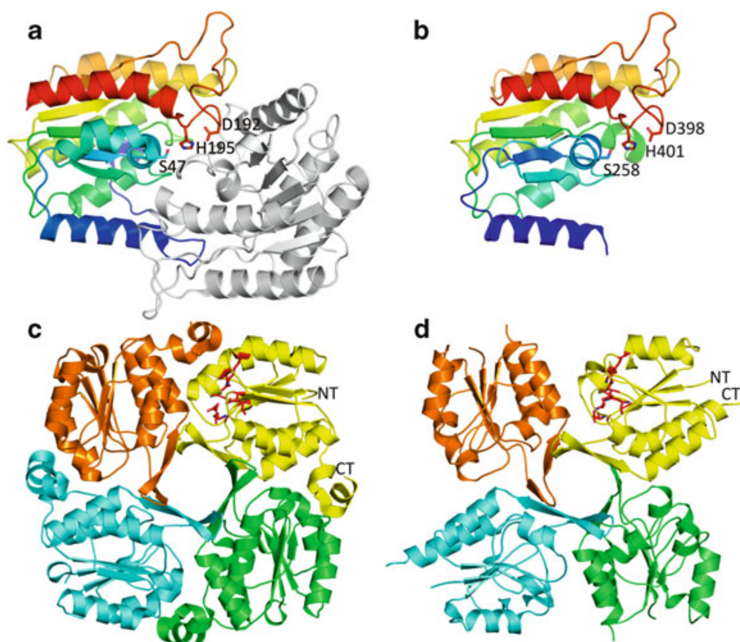


Fig. 5 C-terminal domains of CMP-sialic acid synthetases and their structural homologues. (**a**, **b**) Ribbon presentation of quaternary structure of bovine PAF-acetylhydrolase and predicted tertiary structure of EcoCMAS-CT (amino acids 228–418), respectively. PAF-acetylhydrolase α1-subunit and EcoCMAS-CT are colored from *blue* at the N-termini to *red* at the C-termini. PAF-acetylhydrolase α2-subunit is colored in *gray*. Catalytic triad residues are shown as sticks. (**c**, **d**) Quaternary structures of *H. influenzae* KDO8PPase and MmuCMAS-CT (amino acids 267–432) with the monomers colored in *orange*, *yellow*, *green*, and *blue*. Active site residues for the *yellow* monomer of KDO8PPase (Asp14, Asp16, Ser58, Lys84, Asp107, Asp111) are depicted as *red* sticks (**c**). The same color code was used for residues of the pseudo-active site of the *yellow* monomer of MmuCMAS-CT (Asn279, Asp281, Ser323, Lys348, Asn371, Asp375). PDB-codes for **a**, **c**, **d** are 1fxw, 1k1e, 3ewi, respectively

(residues 228–418) [125]. Initially the function of the CMAS C-terminal domain (CMAS-CT) resulted from a tertiary structure prediction study by Liu et al. in 2004 with the *E. coli* enzyme [125]. High structural homology was observed in the α1-subunit of bovine platelet-activating factor acetylhydrolase (PAF-AH). In Fig. 5 the 3D structure of bovine PAF-AH (Fig. 5a) is juxtaposed to a predicted 3D model of EcoCMAS-CT (Fig. 5b, prediction by the use of phyre [127]). Catalytic triad residues are shown as sticks. Eventually acetylhydrolase activity could be demonstrated in vitro for full-length EcoCMAS and EcoCMAS-CT with the substrates PAF and 4-nitrophenyl-acetate [125]. In vivo studies performed with SagCMAS revealed that overexpression decreases total Sia-*O*-acetylation while deletion of the *cmas*-gene increases the production of *O*-acetylated Sia. Similarly, the disruption of the SagCMAS esterase domain results in the formation of Sia-capsules with significantly increased levels of *O*-acetate [128]. Thus,

O-acetylated Sia is the native substrate for the SagCMAS-CT as further shown in vitro with the recombinant EcoCMAS-CT and SagCMAS-CT [124, 128]. CMAS purified from *S. agalactiae* was demonstrated to catalyze the de-*O*-acetylation of free Neu5,9Ac$_2$ and the activation of the resulting Neu5Ac to CMP-Neu5Ac as well as the activation of free Neu5,9Ac$_2$ followed by the de-*O*-acetylation of the activated sugar CMP-Neu5,9Ac$_2$ in consecutive steps [128]. Thus, for SagCMAS, CMP activation of Sia is not a prerequisite for the Sia-*O*-acetyl esterase reaction. In contrast, EcoCMAS prefers CMP-Neu5,9Ac$_2$ over *O*-Ac-Neu5Ac [32] and CMAS *O*-acetylesterase activity is required for synthesis of capsular Neu5Ac in *E. coli* [32]. Remarkably, CMAS enzymes from *E. coli* could be separated into two independently active protein domains [125]. In contrast, the Sia activating N-terminal domain of SsuCMAS was shown to be structurally dependent on the presence of the complete esterase domain, which in turn could act alone [32]. The SsuCMAS esterase de-*O*-acetylated the substrates CMP-Neu5,9Ac$_2$ and pNPAc, but did not accept Neu5,9Ac$_2$ [32]. Taken together, this data illustrate that the bacterial CMAS-CTs are involved in modulating the virulence and immunogenicity of the capsular polysaccharides by regulating the level of *O*-acetylation of Sia components in these structures.

In contrast to bacteria, where only some CMASs carry a C-terminal domain, all known vertebrate CMASs are long-form enzymes. However, vertebrate CMAS-CTs do not show any sequence homology to bacterial CMAS-CTs. According to the primary sequence, homology has been predicted with phosphatases of the *haloacid dehalogenase* (HAD) superfamily [129], but enzymatic proof has not yet been provided. MmuCMAS-CT was tested with various phosphorylated substrates but did not exhibit phosphatase activity [72]. The HAD superfamily embraces a variety of enzymes that catalyze carbon or phosphoryl group transfer reactions (for reviews see [130, 131]). Within this family sequence conservation is strikingly low and focuses on three HAD motifs that define the active site and the substrate-binding, as well as the cofactor-binding residues. Essential Asp residues of these motifs are changed to Asn in MmuCMAS-CT, explaining the lack of phosphatase activity in vitro. However, homology to HAD phosphatases is also reflected in the 3D-structure, solved for the 18 kDa MmuCMAS-CT (Table 2) [72]. Like functional HAD phosphatases, the separately expressed MmuCMAS-CT domain exhibits an α/β-type hydrolase fold with the center of a six-stranded parallel β-sheet, surrounded by six α-helices in right-handed β–α–β connectivity. An extended loop mediated tetramer formation by forming a central eight-stranded β-barrel. In Fig. 5 the 3D structure of MmuCMAS-CT (D) is compared to the structure of KDO8-phosphate phosphatase (KDO8PPase) from *H. influenzae* (C), also belonging to the HAD family (RMSD: 2 Å). Exemplarily, active site residues of the KDO8PPase and the corresponding residues of MmuCMAS-CT are highlighted in red (Fig. 5c, d, yellow monomers). Apart from the amino acid changes in the HAD-motifs mentioned above, the entry of the putative active site of MmuCMAS-CT is blocked by protruding amino acids [72]. Size exclusion chromatography data supports the assumption that the stable shamrock-like tetramer of murine CMAS-CT induces tetramer formation of the whole MmuCMAS and

provides a platform for two enzymatically active CMAS-NT dimers. While tetramerization is dispensable for Sia activation, the quaternary structure impacts the kinetic parameters of the physiologically active enzyme [72]. High molecular weight species of about 160 kDa have also been isolated from frog liver [24] and bovine anterior pituitary glands [132], respectively, indicating tetramer formation for other vertebrate CMASs also.

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Natural Antibodies Against Sialoglycans

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Abstract Natural antibodies, part of the innate immunity system, are produced at strictly regulated levels in normal sera without immunization and thus are part of the innate immune system. The best studied natural antibodies are those directed against blood group antigens A and B and xeno-antigens including glycolyl-neuraminic acid containing Hanganutziu–Deicher (HD) glycolipid. Abnormal levels of anti-glycan antibodies were found in a number of pathologies. In many cases pathological antibodies are known to bind gangliosides. The genesis of anti-glycan antibodies in healthy humans and the reasons for their changes in pathologies are poorly understood. With a growing interest in their diagnostic applications, it is important to determine the carbohydrate structures that are recognized by antibodies present in the circulation of healthy individuals. We tested a large number of healthy donors using a printed glycan array (PGA) in a microchip format. The PGA contained ~300 glycans, representing mostly normal mammalian structures of glycoproteins and glycolipids, and many of the structures presented are biologically relevant sialylated motifs. As revealed by PGA, the sera interacted with at least 70 normal human glycans.

With only few exceptions, antibodies recognizing sialosides have not been identified. Moderate levels of antibodies and moderate variability were observed

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in the case of SiaT$_n$ and its glycolyl variant. Unexpectedly, we found minimal antibody titer directed against Neu5Gcα and the trisaccharide Neu5Gcα2-6Galβ1-4GlcNAc, although this form of neuraminic acid does not occur naturally in humans. Antibodies recognizing sialosides in unnatural β-configuration have been detected and confirmed Springer's paradigm that circulating antibodies represent a reaction against bacteria. Gram-negative bacteria contain LPS with βKDN and/or βKDO which are very close analogs of Neu5Ac that are found in β-connected form. Antibodies against the biantennary *N*-glycan chain, (Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα)$_2$-3,6-Manβ1-4GlcNAcβ1-4GlcNAc were never observed and similarly we never saw antibodies directed against the SiaLe^a/SiaLe^x motifs. Anti-sialoglycan antibodies can be masked with gangliosides: for example, we observe about a five times higher level of anti-GD3 in purified total IgG compared to the same concentration of total Ig in the composition of native serum. For several antibodies we observed anomalous binding in diluted sera, namely, the signals towards sialylated glycans were increased in the PGA if diluted sera were used.

Keywords Anti-glycan antibodies · Autoantibodies · Glycans · Glycochip · Natural antibodies · Printed glycan array · Sialic acid

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Abbreviations

Ab	Antibodies
CFG	Consortium for functional glycomics
LPS	Lipopolysaccharide
MAD	Median absolute deviation
NAb	Natural antibodies
PAA	Polyacrylamide
PGA	Printed glycan array

PS Polysaccharide
RFU Relative fluorescence units
Sia Sialic acid
Su *O*-Sulfate residue

1 Introduction

A repertoire of antibodies (Ab) is known to be the first line of defense against pathogens, and remove dysfunctional or malignant cells and particles [1, 2]. These immunoglobulins (Ig) are called natural antibodies (NAb), since they are present in sera of all individuals in the absence of deliberate immunization [3], and are thus part of the innate immune system [4]. G.F. Springer postulated that anti-glycan Ab appear in response to stimulation of the immune system by bacterial O-antigens and lipopolysaccharides (LPS) of gastrointestinal bacteria [5, 6]. Many NAb are directed to carbohydrate antigens found in normal human tissues, and can be called autoantibodies [3]. The best known and studied NAb are directed to blood group antigens A and B [7], and to the xenoantigen Galα1-3Galβ1-4GlcNAc [8–10], Forssman (Fs) glycolipid antigen GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc [11, 12], Hanganutziu–Deicher (HD) antigen Neu5Gcα2-3Galβ1-4Glc [13], and anti-ganglioside Ab. In normal human serum low-affinity anti-GM1 NAb are found [14], caused probably by bacteria (not symbiotic); isolated Ab are shown to interact with LPS of *Campilobacter jejuni* [15]. Anti-glycan natural Ab appear in a number of pathologies, the group of particular attention including antibodies against tumor-associated carbohydrate antigens such as Neu5Acα2-6GalNAcα1-OSer/Thr (SiaT$_n$) [6]. Antibodies to ganglioside GT3 were found in patients with type 1 diabetes mellitus [16]. A number of neuropathies are associated with characteristic patterns of Ab directed against gangliosides [17].

Development of PGA, a multi-hundred glycan array printed on a microchip platform [10, 18–20], offers a possibility for systematic, large-population based research [21]. The present study is focused on natural antibodies against sialoglycans, the most abundant mammalian carbohydrate chains present on glycoproteins and a significant proportion of glycosphingolipids. This work reveals the presence of unexpected Ab in the serum of healthy donors as well as in persons that received pig islet cell transplants, and poses questions pointing to further investigations.

2 Experimental

2.1 Reagents

Horseradish peroxidase (HRPO) labeled goat anti-human Ig and monospecific anti-human IgM, IgG, and IgA were obtained from Southern Biotechnology Associates,

Inc. (USA). Unlabeled reference human IgG, IgM, and IgA, and bovine serum albumin (BSA) were obtained from Sigma (USA). All other chemicals were analytical grade from Fluka (Switzerland) or Merck (Germany). MaxiSorp 96-well microtiter immunoplates were obtained from Nunc (Denmark). Glycan-polyacrylamide conjugates Glycan-PAA, and affinity adsorbents were obtained from Lectinity (Moscow, Russia). Other affinity glycoadsorbents used were synthesized according to the same protocol [20]. Glycans were linked to PAA or to NHS-activated surfaces of glass slides in the printed glycan array (PGA) via $-O(CH_2)_3NH_2$ or $-O(CH_2)_2NH_2$ linkers.

2.2 PGA

PGA chips were printed in Cellexicon, Inc. (La Jolla, CA, USA) or Copenhagen University (Denmark). Glycans of 95–98% purity (Lectinity Holdings, Moscow, Russia) were printed as previously described [19], at 50 μM concentration, with 8 or 16 replicates each. The chips were incubated with human serum (1:15 dilution in PBS/3%BSA/1% Tween-20) with gentle rocking for 2 h at 37°C. Serum Ab bound to printed glycans were visualized simultaneously with the "combo" biotinylated secondary Ab against human IgG, IgM, and IgA (Pierce, Rockford, IL) followed by streptavidin-Alexa^{555} (Invitrogen/Molecular Probes, Carlsbad, CA). Fluorescence signal intensities corresponding to Ab bound to printed glycans were collected at 95% laser power, and quantified with BioDiscovery/ImaGene software (El Segundo, CA) or ScanArrayExpress v.3.0 using the "adapted circle" method. The background level of the assay was defined as in [21].

2.3 ELISA

Microtiter plates were coated with Glycan-PAA conjugates or glycan-free PAA (as background control), 10 μg/mL, 60 μL per well, in carbonate buffer (50 mM $Na_2CO_3/NaHCO_3$, pH 9.6) overnight at 4°C. Plates were blocked with 1% BSA in PBS, 60 μL per well, for 45 min at 37°C. Pooled serum (initial dilution 1:20) or affinity-purified antibodies (initial concentration 1 μg/mL) were diluted two-fold with PBS containing 0.3% BSA, 50 μL was added per well to the plate and incubated for 60 min at 37°C. Plates were washed four times with PBS-T20 (PBS containing 0.1% Tween-20). HRPO-labeled anti-human Ig (diluted 1/4,000 with 0.3% BSA in PBS) was added, 50 μL per well, for 60 min at 37°C. Plates were washed four times with PBS-T20. Color was developed by 20 min incubation at RT in 0.1 M sodium phosphate/0.1 M citrate buffer, containing 0.04% *o*-phenylenediamine and 0.03% H_2O_2. The color reaction was stopped by the addition of 1 M H_2SO_4. Absorbance was read at 492 nm with a Wallac1420 Victor^2 (Perkin Elmer, USA). The background level of binding with glycan-free polyacrylamide was subtracted from the binding with Glycan-PAA.

3 Results and Discussion

The library of ligands arrayed onto the chip used in this study was composed of ~300 glycans and included the most frequent terminal oligosaccharides, as well as backbones and cores of mammalian N- and O-linked glycoproteins and glycolipids, with a total number of sialoglycans of ~40. Numerous structures, including oligomers of GlcNAcβ1-4 (chitobiose and higher chito-glycans), GMDP-Lys, GlcNAc-Mur, and of other common components of bacterial polysaccharides and LPS were also present. The list of sialoglycans also included structures with "forbidden" for mammals Neu5Acβ2-connected motif, because related KDNβ- and KDOβ- motifs are well known for bacterial LPS.

Thus, we could conclude that biologically significant and not random response to at least 50% of the glycans had been observed in the blood serum of the examined healthy donors. Since about half of these glycans are typical for human cells, we could further conclude that at least 50–70 species of the carbohydrate-binding auto-antibodies were present in the blood serum.

We identified only a limited number of normal human glycans to which no antibody binding was observed in all 106 donors' sera, or low overall median binding except for a few subjects. These structures showing none to minimal binding included: (1) 6′-sialyllactosamine (6′SLN) and bi-antennary sialylated 11-OS; (2) Sia_2 and Sia_3; (3) type 2 backbone oligosaccharides: Le^y, Le^x, $SiaLe^x$, and $Su\text{-}Le^x$; (4) mannose and manno-oligosaccharides; and (5) oligolactosamines. The binding to a "negative control" glucitol was indeed very low and uniform, not exceeding the level of 0.5×10^5 RFU, and represented the "minimal to none" binding pattern.

3.1 Negatively Charged Non-Sia Glycans

As sialoglycans are negatively charged and IgM binding is definitely multivalent, we were interested to evaluate the non-specific impact of charge in the overall binding if it really exists. NAb are typically characterized by the presence of many arginine and lysine residues in their hypervariable regions [22] and many polyreactive NAb in fact tend to bind negatively charged antigens [23]. In our PGA several glycans possess two or three negative charges (oligosialyl–motifs or the combination of Sia and sulfate), so we investigated the possibility of non-specific binding but found that the charge of the glycan was not relevant [21]. With two exceptions, no antibodies directed against sulfated glycans have been observed. One of them, disaccharide $Su\text{-}Le^C$, i.e., 3-O-Su-Galβ1-3GlcNAc, is an "all-round champion" between all 300 glycans of the PGA. Fine specificity of these Ab is discussed below. Another sulfated motif, i.e., 4-O-Su-Galβ1-4GlcNAc, is also at the highest rank. Specificity of the Ab seems to be high, this following from the lack of binding to isomeric sulfated glycans.

The next group of charged glycans were molecules containing glucuronic acid, GlcA [21], and first of all the GlcAβ1-3Gal motif known to be a fragment of the

HNK-1 antigen, 3-O-Su-GlcAβ1-3Gal. On the HNK-1 antigen this epitope is always masked with sulfate group in humans, so, antibodies to GlcAβ1-3Gal should not interact with HNK-1. The found Ab against βGlcA containing glycans could well be explained in the frame of intestinal bacteria paradigm: glucuronic acid is an abundant component of bacterial LPS [www.glyco.ac.ru/bcsdb/cgi-csdb/search.cgi].

Summing up, we investigated the possibility that non-specific factors influence the outcome of our array data but found that glycan charge (a number of glycans possess two or three negative charges) did not impact the results with statistical relevance.

3.2 Sialoglycans

As expected, most α-sialosides (Tables 1 and 2) do not bind human serum antibodies. An exception is tumor-associated Neu5Acα2-6GalNAcα (SiaT$_n$) antigen (Table 3), having a drastic structure difference from all the other sialo-OS, namely, αGalNAc unit instead of regular βGal and, additionally, proximity to peptide backbone. The second top rank sialoglycan was 3′-SiaLe^c, discussed in detail below. Interestingly, high levels of Ab were detected against all sialosides in β-configuration (Table 3). As Neu5Ac is present only as α-anomer in nature, the existence of antibodies directed against βNeu5Ac looks strange at first glance. However, we can explain their occurrence on the basis of Springer' hypothesis [5], meaning that these Ab are the result of intestinal immunization by Gram-negative bacteria, containing LPS with βKDN and/or βKDO – very close analogs of Neu5Ac but capable of existing in β-configuration in glycoconjugates [www.glyco.ac.ru/bcsdb/cgi-csdb/search.cgi].

Unexpectedly, there were no (in sporadic cases low) levels of Ab directed against the monosaccharide Neu5Gc and the trisaccharide Neu5Gcα2-6Galβ1-4GlcNAc. This finding was surprising as this form of neuraminic acid is foreign in humans [24]. There are many reports on the presence of antibodies against Neu5Gc in form of HD-antigen, with 2-3 bound glycolylneuraminic acid, Neu5Gcα2-3Galβ1-4Glc or Neu5Gcα2-3Galβ1-4GlcNAc [25, 26] in humans. These glycans were absent in our array, but both of them were present in the CFG array [www.functionalglycomics.org]. Of note, the CFG data confirm the low level of Ab against Neu5Gc containing glycans. We identified only Ab directed against Neu5Gcα2-6GalNAcα (Neu5Gc-T$_n$) in high titers, although binding to Neu5Gc-T$_n$ proved to correlate statistically with binding to Neu5Ac-T$_n$. Thus, these Ab actually seems to be mostly or exclusively anti-Neu5<u>Ac</u>-T$_n$.

Table 1 Sialoglycans demonstrated absence or sporadic low level binding to healthy donor sera as probed with PGA

Glycan	Short name
Neu5Acα2-6(Neu5Acα2-3Galβ1-3)GalNAcα-sp	Sia_2-3′,6-TF
Neu5Acα2-6Galβ1-4GlcNAcβ-sp	6′SLN
Neu5Gcα2-6Galβ1-4GlcNAcβ-sp	6′SLN(Gc)
Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ-sp	$SiaLe^x$
$(Neu5Ac\alpha 2\text{-}8)_3\alpha$-sp	$(Sia)_3$
Neu5Acα2-8Neu5Acα2-sp	$(Sia)_2$
$(Neu5Ac\alpha 2\text{-}6Gal\beta 1\text{-}4GlcNAc\beta 1\text{-}2Man\alpha)_2$-3,6-Manβ1-4GlcNAcβ1-4GlcNAcβ-sp1	11-OS
$(Neu5Ac\alpha 2\text{-}3Gal\beta 1)_2$-3,4-GlcNAcβ-sp	
Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ-sp	$SiaLe^a$
Neu5Acα2-3Galβ1-4(Fucα1-3) 6-*O*-Su-GlcNAcβ-sp	6-*O*-Su-$SiaLe^x$
Neu5Acα2-3Galβ1-3-(6-Su)GalNAcα-sp	6-Su-3′SiaTF
Neu5Acα-sp	Neu5Ac-sp
Neu5Acα2-3(6-*O*-Su)Galβ1-4(Fucα1-3)GlcNAcβ-sp	6-*O*-Su-$SiaLe^x$
Neu5Acα2-6Galβ1-4-(6-Su)GlcNAcβ-sp	6-Su-6′SLN
Neu5Acα2-3Galβ1-4-(6-Su)GlcNAcβ-sp	6-Su-3′SLN
Neu5Acα2-8Neu5Acα-$OCH_2C_6H_4$-p-sp1	Sia_2Bn
(9-NAc-Neu5Acα2-6)Galβ1-4GlcNAcβ-sp	
Neu5Acα2-3Galβ1-3GalNAcα-sp	3′-Sia-TF
Neu5Acα2-3Galβ1-4GlcNAcβ-sp	3′SLN
Neu5Acα2-3Galβ1-4Glcβ-sp	3′SL
Neu5Acα2-3Galβ-sp	GM4
Neu5Gcα-sp	Neu5Gc
$(Neu5Ac\alpha 2\text{-}8)_3\beta$-sp	$(Sia)_3\beta$
Neu5Acα2-6Galβ1-4Glcβ-sp1	6′SL
Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ-sp	$SiaLe^x$-penta
Neu5Gcβ2-6Galβ1-4GlcNAcβ-sp	β-6′SLN(Gc)
9-NAc-Neu5Acα-sp	9-NAc-Neu5Ac
Neu5Acα2-3GalNAcα-sp	3-$SiaT_n$
Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ-sp	$SiaLe^a$
Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ-sp1	GD3
Neu5Acα2-6(Galβ1-3)GalNAcα-sp	6-SiaTF
Neu5Acα2-6(Galβ1-3)GlcNAcβ1-4Galβ1-4Glcβ-sp1	LSTb

"sp" means aminoethyl or aminopropyl spacer, "sp1" – glycine spacer

3.3 Lewis and Sialylated Lewis Glycans

The Lewis blood group system is represented by two antigens with type 1 backbone structure, namely Fucα1-4(Galβ1-3)GlcNAc (Le^a) and Fucα1-4(Fucα1-2Galβ1-3) GlcNAc (Le^b). The biosynthetic precursor of these structures, Galβ1-3GlcNAc, is known as Le^c and is not significant for blood typing [27]. We observed very low median binding to both the Le^a trisaccharide and the Le^b tetrasaccharide. Generally, type 1 core glycans, i.e., Galβ1-3GlcNAc, are less frequent in humans than type 2 analogs, whereas Su-Le^a and $SiaLe^a$ are well-known tumor-associated antigens,

Table 2 Sialoglycans demonstrated moderate or high level binding to healthy donor sera as probed with PGA

Glycan	Short name
Neu5Acα2-8Neu5Acβ-OCH$_2$C$_6$H$_4$-p-sp1	Sia$_2$βBn
Neu5Acα2-3Galβ1-4Glcβ-sp1	3′SL
Neu5Acα2-6Galβ-sp	
Neu5Gcα2-6GalNAcα-sp	Neu5Gc-T$_n$
Neu5Acα2-6GalNAcα-sp	SiaT$_n$
Neu5Acβ-OCH$_2$C$_6$H$_4$-p-sp1	β-Neu5Ac
Neu5Acβ2-6GalNAcα-sp	β-SiaT$_n$
Neu5Acβ2-6(Galβb1-3)GalNAcα-sp	6-SiaβTF
Neu5Acα2-3Galβ1-3GlcNAcβ-sp	3′-SiaLe^c

RFU*10^{-6} 0.0 20.0 40.0 60.0

The bars at the right part of the Table illustrate relative intensity of binding; scale of the intensity corresponds to the scale of positively binding glycans on Table 1
"sp" means aminoethyl or aminopropyl spacer, "sp1" – glycine spacer

Table 3 Glycans in composition of PGA, showing elevated levels of binding in the case of successfully transplanted pig islet cells comparing to patients with rejected cells; note that three of nine glycans are β-Sia containing ones

Glycan
Neu5Acβ-sp
Neu5Acβ-OCH$_2$C$_6$H$_4$-p-spl
3-*O*-Su-Galβ-4(6-*O*-Su)Glcβ-sp
Fucα1-2Galβ1-3GalNAcα-sp
Neu5Acα2-3Galβ1-4Glcβ-sp1
(Neu5Acα2-8)$_3$β-sp
Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ-sp1
(GlcNAcβ1-4)$_5$β-sp1
Neu5Acα2-6(Galβ1-3)GlcNAcβ1-3Galβ1-4Glcβ-sp1

RFU * 10^{-6} 0 5 10 15 20

successful non succesful 1 non succesful 2

For abbreviations for spacers see Table 1

allowing us to suppose a high probability for the existence of auto-antibodies directed against type 1 structures. However, we never or very rarely observed Ab to both SiaLe^x and its type 1 isomer, the tumor-associated SiaLe^a tetrasaccharide. At the same time, as mentioned above, there were high levels of Ab directed against Le^c and very high levels directed against Su-Le^c. This fact is intriguing taking into consideration the abundance of the Su-Le^c epitope on many epithelial cells [28].

In order to fine characterize the epitope specificity in this group of antibodies, we affinity isolated immunoglobulin using an affinity column with Le^c disaccharide as ligand. Interestingly, the isolated material bound equally to all three glycans, neutral disaccharide Le^c and charged Su-Le^c and SiaLe^c (Fig. 1). Statistical analyses on a large

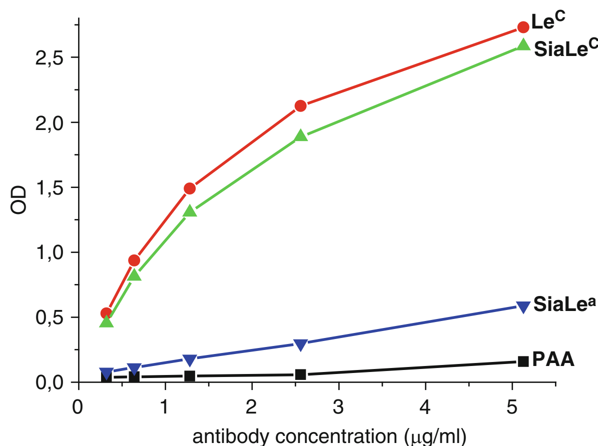

Fig. 1 ELISA of Ab affinity isolated with help of sialic acid free glycan Le^{C} coupled to Sepharose. The ELISA plate is coated with Glycan-PAA conjugates of Le^{C}, SiaLe^{C}, and SiaLe^{a} glycans

group of donors [21] also indicated a very high correlation between all three pairs of glycans. Thus, human blood contains antibodies recognizing the inner part of Le^{c} disaccharide. The substituent at position 3 of the galactose moiety does not contribute in the binding (see Fig. 1 – rather high interaction with GlcNAcβ). This means that binding of serum to SiaLe^{c} visible on PGA is actually a "false positive," and there are no antibodies specific to the sialylated form of the Lewis C antigen. This fact was confirmed by an experiment (not shown) where a SiaLe^{c} column was used for Ab isolation: isolated material was identical to that obtained with the help of the Le^{c} affinity media.

3.4 *Neu5Acα2-3Galβ1-4Glc and Neu5Acα2-6Galβ*

According to Table 3 the title glycans are positive ones in PGA when normal human sera are probed. However, when statistical analyses [21] similar to that described above for the Le^{c}/SiaLe^{c} pair were performed, we revealed good correlation between Neu5Acα2-3Galβ1-4Glc (3′SL) and other glycans containing inner lactose motif, i.e., there are no specific antibodies against the sialoglycan Neu5Acα2-3Galβ1-4Glc; similarly, with the Le^{c}/SiaLe^{c} pair, the binding seems to be false positive and, actually, antibodies are sialic acid independent. The situation with Neu5Acα2-6Galβ is not so obvious because the number of analogs with other substituents at position 6 of the Gal moiety at the reducing end was limited on the PGA. Nevertheless, we believe that the visual positive signal of this glycan is also false positive because we do not observe binding to Neu5Acα2-6Galβ1-4GlcNAc, the more complex Neu5Acα2-6Galβ containing glycans. In contrast, we observe binding to the Galβ monosaccharide.

In summary, the data presented in Table 3 show that it is important to relocate 3′SL, Neu5Acα2-6Galβ, and SiaLe^{c} in Tables 1 and 2 and lead to the conclusion that serum of healthy donors contains only one sort of auto-antibody against sialoglycans, namely, anti-SiaT$_{n}$/Neu5(Gc)α2-6GalNAcα.

3.5 Anti-sialoglycan Antibodies in Intravenous Immunoglobulin Preparations

IgG purified from pools of at least 1,000 healthy individuals is determined as intravenous immunoglobulin preparations (IvIg) and represents a statistically reliable repertoire of human antibodies. At the same time, regular IvIg preparations do not contain IgM nor a multitude of other components of human serum. This means that in principle results of PGA binding could be different for native serum and IvIg preparation. We compared data obtained with sera of the healthy donor cohort with data obtained with IvIg using the same PGA and the same experimental conditions. The results obtained demonstrated similarity of the two profiles (with an amendment taking into consideration the absence of the IgM fraction in IvIg) but four prominent exceptions – see Fig. 2. Formally, the glycans revealed are sialooligosaccharides. However, as discussed above, antibodies against 3′SL and Neu5Acα2-6Galβ seem to be Sia-independent, and against Neu5Acα2-6(Galβ1-3)GlcNAcβ1-3Galβ1-4Glc (LSTb) as well. The single Sia-dependent activity appeared to be associated with anti-GD3: the signal in IvIg was one order of magnitude higher than in serum demonstrating one order of magnitude difference in favor of IvIg compared to serum. We explain this dramatic difference by masking anti-GD3 antibodies with GD3 ganglioside in the serum. An obligatory stage in IvIg fabrication is ethanol precipitation of IgG, and we assume the dissociation of the anti-GD3/GD3 complex and the liberation of antibodies from soluble GD3 take place during this process. Thus the repertoire of antibodies against sialoglycans could not be as restricted as discussed above due to the masking effect of serum components. Another mechanism of masking, where entire sialoglycans of human serum glycoproteins are involved, was proposed for biantennary *N*-glycan chains in glycoproteins [21].

3.6 The Effect of Serum Dilution

Some serum samples, e.g., material from newborns, were accessible in very limited amounts, so it was necessary to dilute them one order of magnitude more than usual. To work with such samples we increased the incubation time in order to get approximately the same PGA signal values as under standard conditions. A comparison of healthy donor sera in these two modes, however, showed insignificant differences. Interestingly, the list of glycans with the most pronounced differences included the structures common for LPS and bacterial peptidoglycans, first of all β-sialosides (Fig. 3). The reason why dilution increases the affinity of this particular group of antibodies will be matter for future study.

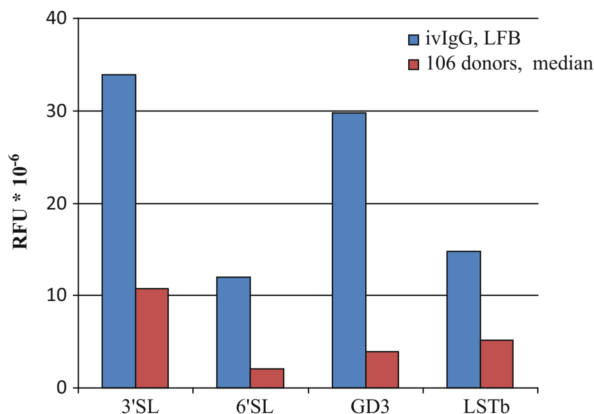

Fig. 2 Comparison of PGA data for intravenous immunoglobulins (LFB, France), *blue bars*, and for sera of >100 healthy donors (see Tables 1–3), *red bars*. Only glycans demonstrating a difference of more than three times were selected

3.7 Islet Cells Transplantation Data

We compared PGA data for patients after transplantation of pig islet cells, i.e., xenotransplantation. In one patient the transplantation was successful, whereas in two other patients the transplanted xenografts were rejected. We were interested in finding out whether antibodies may serve as potential prognostic markers. In our PGA test the signals for nine glycans were significantly lower for the two patients with rejection if compared with the patient with successful transplantation. Surprisingly, three of nine antibodies with significantly elevated levels were directed against β-Sia containing glycans (Table 3).

Although we are aware that this single example cannot give more than a hint, this important observation cannot be ignored in the light of our other previously mentioned findings that human antibodies exist that recognize β-sialosides.

4 Concluding Remarks

The modern pharmaceutical industry gives us more and more examples of small molecule drugs based on sialoglycans or sialomimetics, as well as therapeutic glycoproteins (especially monoclonal Abs) and synthetic vaccines glycosylated with complex glycans in native sialylated form. Next in the pipeline seems to be cell therapy where engineered sialylated cells have to be in extended contact with the patient's serum. Using the PGA platform, we provide here first data on the existence of cryptic antibodies which can be risk factors in the application of the above-mentioned therapeutics. With this initial study we demonstrate that detailed investigations on the existence of natural human antibodies directed against sialoglycans, and especially on their cryptic forms, are necessary.

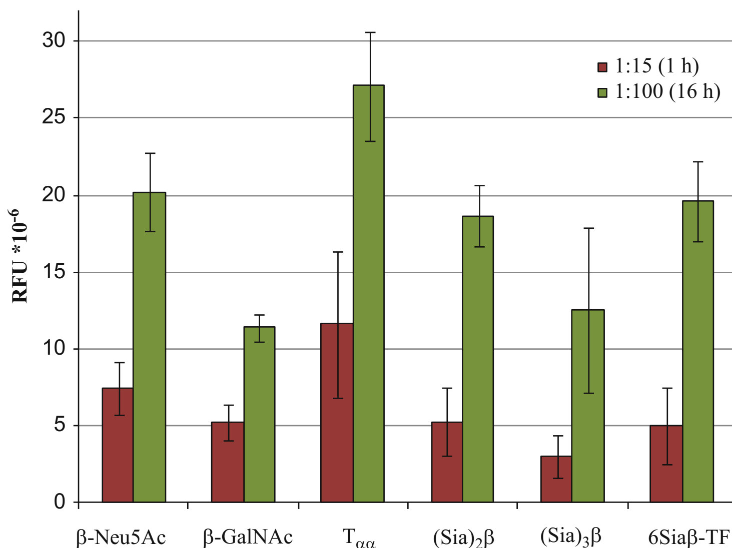

Fig. 3 Comparison of two conditions of PGA for the same sera 1:15 dilution for 1 h vs 1:100 dilution for 16 h. Only six glycans (from list of the Tables 1–3) demonstrating a difference more than two times were selected; four of them are β-Sia containing glycans

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Structure and Function of Mammalian Sialidases

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Abstract The removal of sialic acids, catalyzed by sialidase, is the initial step in degradation of oligosaccharides, glycoproteins, and glycolipids. The catalytic reaction may greatly influence biological processes through changing the conformation of glycoproteins and create or mask binding sites of functional molecules. Recent progress in sialidase research has clarified that mammalian sialidases indeed contribute to the regulation of various cellular functions as well as lysosomal catabolism, unlike the sialidases of microbial origin that probably play roles limited to nutrition and pathogenesis. However, the mammalian enzymes contain consensus sequences in the six-blade β-propeller structural organization typical of microbial sialidases, despite the low degree of similarity to the amino acid sequences of the microbial enzymes. The present review briefly summarizes structural and functional features of mammalian sialidases.

Keywords Ganglioside · Glycoprotein · Sialic acid · Sialidase · Transmembrane signaling

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1 Introduction

Sialidases or neuraminidases [EC 3.2.1.18] are glycosidases that catalyze the removal of α-glycosidically linked sialic acid residues from carbohydrate groups of glycoproteins and glycolipids. Sialidases commonly exist in metazoan animals, from echinoderms to mammals, and also in various viruses and microorganisms including fungi, protozoa, and bacteria even mostly lacking sialic acids. In higher organisms they were described for the first time in 1960 by Warren and Spearing [1], but it long remained uncertain whether activity was due to one or multiple types of sialidase. In microorganisms, sialidases are likely to function for nutritional purposes and in the processes of adhesion to and invasion of host cells. Unlike those of microbial origin, mammalian sialidases have been implicated not only in lysosomal catabolism but also in modulation of functional molecules involved in many biological processes. Four types have been identified and characterized to date, designated as NEU1, NEU2, NEU3, and NEU4. They are encoded by different genes, and differ in major subcellular localization and enzymatic properties, including substrate specificity. The first three are localized predominantly in the lysosomes, cytosol, and plasma membranes, respectively, and the fourth sialidase, NEU4, has been suggested to exist in lysosomes, or in mitochondria and endoplasmic reticulum. However, very recent observations have revealed that the subcellular localization can vary with particular cell stimuli. For example, NEU1 and NEU4 may mobilize to the cell surface under certain conditions as described below. There is also evidence of NEU1 existing in the outer membrane and NEU3 in the inner membrane of the nuclear envelope [2], and NEU2 in the nucleoplasm of muscle fibers, rather than in cytosol, as assessed by immuno-histochemical analysis on electron microscopy, probably due to the presence of a nuclear localization signal near the N-terminus [3]. The properties of the four forms are briefly summarized and compared in Table 1. Although many functional aspects are not fully understood, recent progress in gene cloning has facilitated elucidation of important biological roles such as involvement in events contributing to cell differentiation, cell growth, and apoptosis. From the structural viewpoint, the three-dimensional structure has been determined only for human NEU2 by X-ray crystallography [4] and compared

Table 1 Comparison of mammalian sialidases

	NEU1	NEU2	NEU3	NEU4
Major subcellular localization	Lysosomes	Cytosol	Plasma membrane	Lysosomes[a] Mitochondria[a] and ER
Good substrates	Oligosaccharides Glycopeptides	Oligosaccharides Glycoproteins Gangliosides	Gangliosides	Oligosaccharides Glycoproteins Gangliosides
Optimal pH (in vitro)	4.4–4.6	5.6–6.5	4.6–4.8	3.2–4.5
Total amino acids				
Human	415	380	428	496 (484)[b]
Mouse	409	379	418	497 (413)[b]
Chromosomal location				
Human	6p21.3	2q37	11q13.5	2q37.3
Mouse	17	1	7	10

[a]For the subcellular localization of human NEU4, two different results have been reported
[b]NEU4 exists as two isoforms

with that of *Salmonella typhimurium* sialidase, allowing prediction of the structure of the other human sialidases NEU1, NEU3, and NEU4, using a homology modeling approach [5]. A recent literature review has also summarized the earlier reports on sialidase research [6, 7].

2 Structural Features of Sialidases

2.1 General Survey

A simple search of the Protein Data Bank (PDB; http://www.pdb.org/pdb/home/home.do), the archive that contains information about the experimentally determined protein structures, using sialidase as a text search term allowed the identification of 150 structure hits. Using only the query refinements options more information is immediately retrievable. The taxonomy option gives the taxonomic group of an entry: in our case the 40%, 30%, and 30% of the hits are from viruses, eukaryotes, and bacteria, respectively. Among the structure hits it is possible to remove similar sequences by filtering search results based on sequence similarity to provide only "unique" structures that could otherwise appear several times because, for example, the same protein has been studied with several ligands and thus appears with a number of separate PDB files. In this case the hits diminish to 11, and only 1, the human cytosolic sialidase NEU2, represent the group of the mammalian sialidases. Another very interesting and useful resource available on the web is the CAZy site (http://www.cazy.org/) [8]. The acronym CAZy stands for Carbohydrate-Active enZYme database and since 1998 CAZy has collected information on genomic organization and biochemical and structural features of the so-called CAZymes (Carbohydrate-Active enZYMES). Sialidases or neuraminidases

(systematic name: *N*-acetylneuraminyl hydrolase) are glycoside hydrolases (GHs) grouped into the family 33. This group contains GHs characterized by sixfold β-propeller structural organization (GH-E clan) and, again, 11 different enzymes have their structures reported here (http://www.cazy.org/GH33_structure.html). Of those, six are sialidases from bacteria and the remaining five from eukaryotic organisms, namely the KDN sialidase from *Aspergillus fumigatus*, a fungus, the sialidase L from *Macrobdella decora*, a leech, the sialidase and trans-sialidase from the parasite flagellate protozoans *Trypanosoma rangeli* and *T. cruzi*, respectively, and, finally, the human cytosolic sialidase NEU2. A huge amount of data on microbial and trypanosomal enzymes has been collected so far and, together with the detailed studies carried out on viral neuraminidases, this knowledge can be used to shed light onto the biology of mammalian sialidase as well [9]. In this section the available papers on mammalian sialidase dealing with their structural features will be reviewed. Furthermore, the mechanism of catalysis that can be proposed based on the experimental evidence produced so far, as well as the behavior of these enzymes in the presence of neuraminidase-targeted anti-influenza drugs and newly synthesized possible competitive inhibitors will be discussed.

2.2 Structural Features of the Human Cytosolic Sialidase NEU2

The NEU2 structure was published in 2005 [4] and, despite the low degree of similarity detectable between the amino acid sequences of bacterial enzymes, such as those of *Micromonospora viridifaciens* and *S. typhimurium* and the human enzyme [10], the cytosolic sialidase has the six-blade β-propeller structural organization typical of bacterial sialidases and viral neuraminidases [11]. Thus, NEU2 (Fig. 1) shows the six blades (roman numbers I–VI) of the propeller composed of four antiparallel β-strands (each indicated by capital letters A–D). The propeller is closed by the interaction between strands C and D/blade VI, with the element of the blade belonging to the N-terminus (aa 8–14) of the polypeptide. The N- and C-termini are outside the β-propeller and closely located with 7 and 12 residues, respectively. There are three so-called Asp-boxes, consensus motifs (**S**x**D**x**G**xx**W/F**) typical of sialidases located in topologically equivalent position between the strands C and D/blades II–IV. Asp boxes have been detected in a number of protein families from bacterial ribonucleases to reelin, netrins, sulfite oxidases, and some lipoprotein receptors, as well as in a series of GHs [12]. The function of these conserved motifs is still unclear although a comprehensive analysis of Asp boxes indicates a structural role for these well-defined β-hairpins in β-propellers [13]. In NEU2 the three Asp boxes are grouped far away from the active site, closer to the acidic cleft opening on the opposite part of the β-propeller [4].

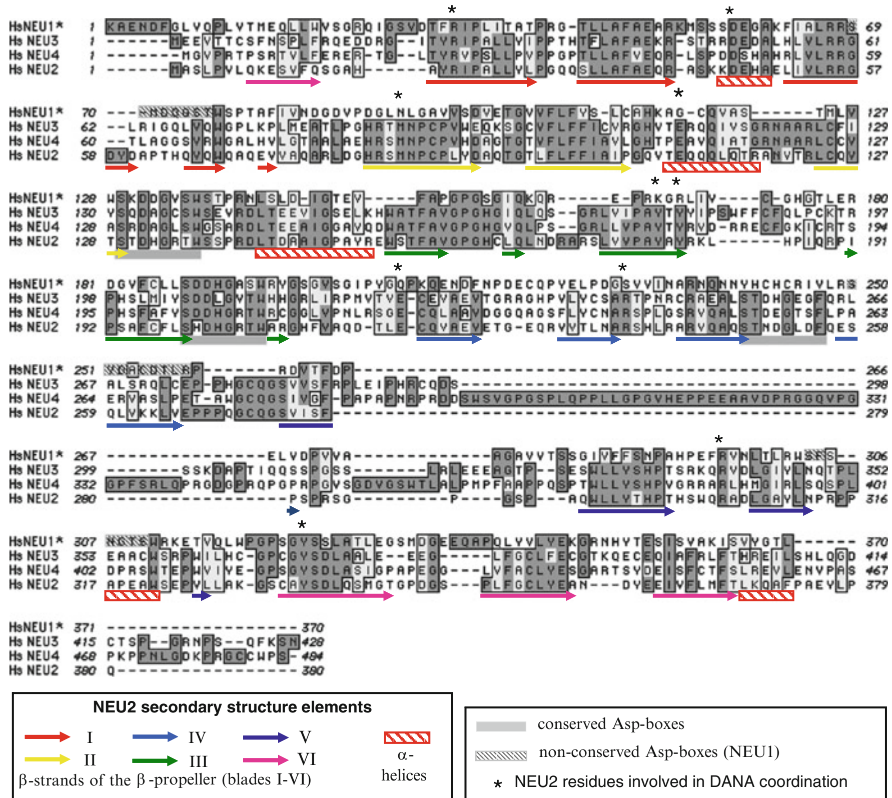

Fig. 1 Multiple alignment of human sialidases. NEU1 (Accession AAB96774 – without the 45 residues of the signal peptide at the N-terminus), NEU2 (Accession NP_005374), NEU3 (Accession Q9UQ49 – short isoform of 428 residues), and NEU4 (Accession A8K056 – short isoform of 484 residues) have been aligned using ClustalW algorithm. *Arrows* below NEU2 sequence indicate β strands. The color code of the β-strands is the same reported in the paper of Chavas et al. [4]. *Red striped boxes* and *gray boxes*, always below NEU2 sequence, indicate alpha helices and conserved Asp-boxes, respectively. *Dashed gray boxes* directly within NEU1 sequence indicate non-conserved Asp-boxes present only in the lysosomal sialidase. *Asterisks* above the sequence alignment indicate residues involved in DANA coordination in NEU2 crystal structure [4]

The most relevant structural feature of NEU2 is the presence of two mobile loops between the strands B and C/blades I and II of the β-propeller (Figs. 1 and 2). These connecting stretches of residues are disordered in NEU2 apo form (with the active site free, PDB ID 1SNT), and soaking of apo form crystals with a monosaccharide such as galactose, glucose, or maltose orders the loop of the second blade (PDB ID 1SO7). Finally, diffraction studies on NEU2–DANA complex obtained by co-crystallization techniques (molar ratio in solution of enzyme to the competitive inhibitor DANA was 1:50) show not only a rearrangement of the second blade loop that now is bent close to the *N*-acetyl and glycerol portion of DANA but also the loop of the first blade becomes

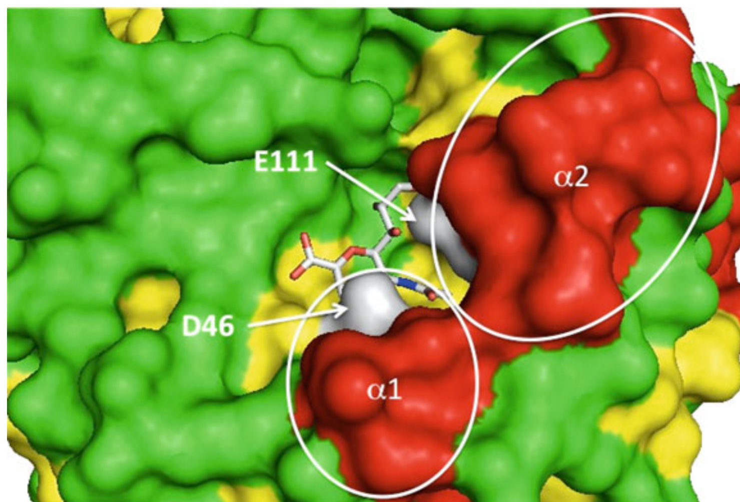

Fig. 2 NEU2 active site in complex with DANA (PDB ID 1VCU). The surface is colored according to the secondary structure: α helices in *red*, β strands in *yellow*, and loops connecting the β strands of the six bladed beta propeller in *green*. Two white ovals surround α helix 1 and 2, and the residues D45 and E111, involved in DANA coordination and belonging to these mobile portion of the enzyme, are colored in *gray*. For details see the text

ordered and is positioned near the C4 hydroxyl group of the competitive inhibitor (PDB ID 1VCU). Noteworthy, both of these loops contain one of the seven short α-helices found in NEU2, namely α1 and α2, and the acidic residues (D46 and E111, respectively) involved in DANA coordination. Thus, upon DANA binding in the active site, the two loops containing D46 and E111 partially cover the inhibitors (Fig. 2), demonstrating the dynamic nature of substrate recognition [4].

Concerning the residues involved in DANA coordination, the overall architecture of catalytic sites of *exo*-sialidases is conserved [9]. Among the highly conserved residues there are the arginine triad (R21, R237, and R304) that binds the carboxyl group of all sialic acids and others residues directly involved in the catalysis, a tyrosine/glutamic acid nucleophile pair below the inhibitor ring (Y334/E?), and an aspartic acid on the opposite side (possibly the "mobile" D46) that behaves as the acid/base catalyst. The second member of the nucleophile pair is missed in the paper describing NEU2 3D structure [4] and a detailed comparison between the active site architecture of the human enzyme with those of *M. viridifaciens* and H1N1 influenza A virus reveals, as a possible candidate, the glutamic acid in position 218 [14]. These highly conserved residues typical of sialidases recognize just one portion of DANA, from the carboxyl group C1 to the hydroxyl group of the C4 atom, whereas the other portions of the transition state analog, namely the *N*-acetyl group linked to C5 and the glycerol moiety linked to C6, are recognized by residues not shared by bacterial and viral enzymes [4].

2.3 Mechanism of Catalysis

The catalytic mechanism of microbial *exo*-sialidases has been studied and the general mechanism proposed for glycosyl hydrolases [15] and viral neuraminidases [16] has recently been further clarified [16]. A detailed discussion of the relationships between structure and reaction mechanism of sialidases is given in the contribution of Andrew Bennet and Garry Taylor in this issue. Just one study has been published dealing with NEU2 catalytic mechanism, using the partially purified recombinant form of the enzyme from *Rattus norvegicus* [17]. The hydrolysis of the fluorescent substrate analog 4-methylumbelliferyl *N*-acetylneuraminide (4MU-NANA) has been studied using NMR techniques demonstrating that the reaction catalyzed by NEU2 proceeds with an overall retention of anomeric configuration. Although no specific experiments have been carried out so far using NEU2 as a model enzyme, the high degree of conservation of the residues involved in the catalytic process strongly suggests a unique catalytic mechanism for *exo*-sialidases. Briefly, in the case of NEU2, the general mechanism should be the following: (1) as a consequence of the binding of the substrate into the active site, the terminal sialic acid, positioned through the interaction with the arginine triad (R34, R237, and R304), changes its conformation and is distorted from a chair to a boat configuration; (2) D46, after its positioning upon the C2 of sialic acid at the top of the catalytic crevice, acts, directly or through a water molecule, as acid catalyst and leads to the release of the reducing end glycan; (3) the resulting oxocarbonium intermediate is stabilized by the Tyr/Glu catalytic nucleophile pair located at the bottom of the catalytic crevice (Tyr acts as nucleophile after deprotonation induced by the terminal carboxylate group of Glu side chain), forming a covalent adduct with Y334; (4) the enzyme-sialic acid adduct is hydrolyzed through a second engagement of the acidic catalyst D49 and a water molecule, and finally the sialic acid is released from the active site with retention of configuration.

2.4 Structural Prediction of NEU1, NEU3 and NEU4 Based on NEU2 Crystal Structure

The availability of the NEU2 atomic coordinates allowed the prediction of the three-dimensional structure of NEU1, NEU3, and NEU4 using a homology modeling approach [5]. As expected, the models show the six-bladed β-propeller of NEU2 and are useful tools to study similarities and differences between the different members of the sialidase enzyme family in mammals. First of all, the active site architecture of NEU2 is highly conserved, with a striking conservation of the residues putatively involved in the coordination the substrate in the case of NEU3 and NEU4, whereas in the case of the lysosomal sialidase NEU1 a relatively minor degree of conservation is detectable (Fig. 3). It is interesting to note that the first mobile loop containing D46 residue in the short α1 helix appears to be most likely present in

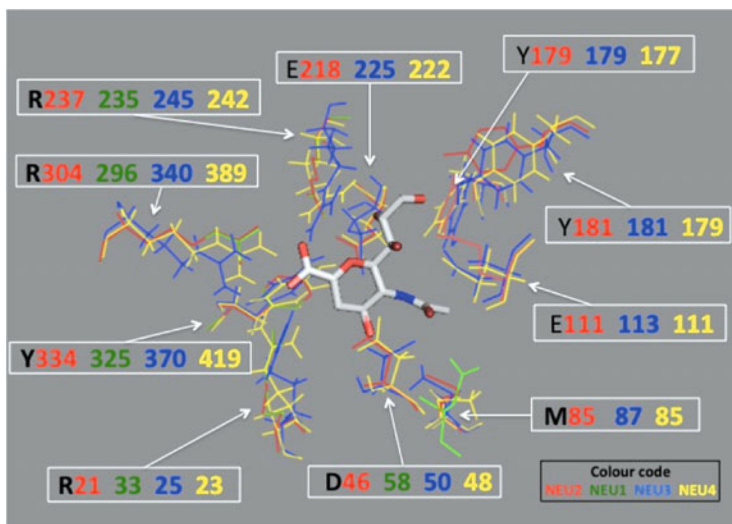

Fig. 3 Overlay of the crystal structure of NEU2/DANA complex (PDB ID 1VCU) with the predicted 3D structures of the other member of the family, obtained through homology modeling. The active site residues of NEU1, NEU2, NEU3 and NEU4 are colored in *green*, *red*, *blue*, and *yellow*, respectively (or mixed in case of a complete superimposition). For details see the text

NEU1, NEU3, and NEU4 as well, with the aspartic acid residues conserved in all the proteins, corresponding to D58, D50, and D48, respectively (Figs. 1 and 3). Conversely, the second NEU2 mobile loop appears to be conserved only in NEU3 and NEU4 (Figs. 1 and 3). Actually, the E111 residue at the beginning of the longer α2 helix that coordinates the glycerol portion of the competitive inhibitor in NEU2/DANA crystal [4] corresponds to E113 and E111 in NEU3 and NEU4, respectively, whereas in NEU1 the connecting loop between the strands C and D/blade III is shorter and does not contain acidic residues (aa 114–123). It should be noted that the identification in NEU2 of E218 as the second member of the nucleophile pair with Y334 has been further supported by the conservation of the residue in NEU3 and NEU4 (E225 and E222, respectively). Magesh et al. report a detailed picture of the putative residues involved in DANA coordination using hydrogen bonds and hydrophobic contacts, deduced from the comparison of NEU2 crystal structure and the predicted 3D models of NEU1, NEU3, and NEU4 [5]. In summary, the differences are detectable in the case of NEU1 and are concentrated in residues involved in the coordination of the glycerol and *N*-acetyl moiety (distal portion) of DANA (Fig. 3). Another interesting feature of mammalian sialidases can be inferred by considering the position of Asp-boxes in NEU models. As already mentioned, NEU2 has three canonical Asp-boxes located in topologically equivalent positions in blades II–IV of the β-propeller. In NEU2 crystal structure, Asp boxes start with a Ser that represents the last residue of strand C and are located within the loop that connects strand C and D [4]. It should be noted that in NEUs only the Asp-boxes of blades II and III appear to be highly conserved, being the third of blade IV conserved only in NEU3 and

NEU4. In NEU1 this Asp box has a less conserved sequence, characterized by the lack of glycine and of the terminal aromatic residue (**S**Y**D**ACDTL, aa 250–257). Moreover, the lysosomal sialidase NEU1 has two additional Asp-boxes (Fig. 1): the first, located in blade I, has a canonical, highly conserved sequence (**S**M**D**Q**G**ST**W**, aa 69–76), whereas the second, located in blade V, has lost an aspartic residue (**S**FSN**G**TS**W**, aa 304–311).

2.5 Site-Directed Mutagenesis Studies

A site-directed mutagenesis approach has been used so far only in the case of NEU3. In one paper [18], published several years before the resolution of NEU2 crystal structure [4], the residues to be mutated have been chosen based on multiple alignment of the human enzyme primary structure [19, 20] with NEU1 [21], NEU2 [22], and the microbial enzyme from *S. typhimurium* [23], whose 3D structure was available [11]. More recently, the same experimental approach has been carried out using the more precise information available on mammalian sialidase 3D structure [24]. Although the overall homology between NEU2 and NEU3 of human origin is 45.6% (33.8% sequence identity), as already mentioned all of the residues that coordinate DANA in NEU2 are conserved in a topologically equivalent position in the NEU3 primary structure. Consequently, a reliable 3D model of the enzyme can be obtained using a homology modeling approach based on the atomic coordinates of NEU2 [5]. Overall these reports give a comprehensive picture of the role played by the active site residues, grouped on the basis of the portion of DANA recognized. Starting with the argininyl triad and the residues involved in the hydrolysis of the sialosyl linkage, R25H, D50S, E225C, R245A, R340A, and Y370F/C mutants have been analyzed. Surprisingly, both R25H and Y370C mutants retain a low but appreciable enzyme activity using both 4MU-NANA and gangliosides [18]. In the case of R25, one of the three Arg involved in the coordination of the sugar carboxyl group, it seems that the presence of the two other members of the triad still allows a low efficiency positioning of sialic acid within the active site, resulting in a massive decrease of the Vmax and, surprisingly, a hardly detectable variation of the apparent Km using ganglioside G_{D1a} as substrate. Conversely, mutants on arginine 245 and 304 abolish the enzyme activity assayed with 4MU-NANA [24]. The residual enzyme activity of Y370C mutant remains without explanation, this residue playing a pivotal role in the catalysis as a member of the nucleophile pair positioned below the C2 of the sialic acid ring [9] as demonstrated by the complete lack of activity detectable in Y370F mutant as well as in E225S, the second member of the pair [24]. The D50S mutant is inactive as well, further demonstrating the role of this residue as a proton donor in the catalytic cycle. Interestingly, R45V mutant maintain less than 2% of the catalytic activity detectable in the wild type enzyme, thus supporting the role of this highly conserved residue in the recognition of DANA 4-hydroxyl group [24]. Moving to the pocket involved in the recognition of the *N*-acetyl portion on C5 of sialic acid, N88D [18], M87G, and I105G [24]

show a marked reduction of activity (<10%). In the description of NEU2 3D structure [4], only M85, corresponding to M87 in NEU3, has been identified as a residue involved in hydrophobic contact with the two carbons of the acetyl moiety, whereas these results demonstrate the participation of other amino acids. Another portion of the acidic sugar recognized by several highly conserved residues is the glycerol chain on C6 of the sugar ring. All of the mutants produced, namely E113A, Y179F, and Y181F, show a great decrease of enzyme activity (<10%) measured using the artificial fluorescent substrate 4MU-NANA, thus demonstrating their relevance in the recognition of sialic acid by the active site [24]. Finally, the role of the residues located at the periphery of the active site has been tested. The E51S [24] and E51D [18] mutants do not show any relevant variation of enzyme activity compared to the wild-type protein, suggesting that such mutations do not affect the relevant role played in the catalysis by the D50 residue and the dynamics of the loop encompassing the α1 helix. R114 and H277 are expected to interact with the reducing-end glucose of G_{M3} [24] and again, using 4MU-NANA as a substrate, no great variation of enzyme activity are detectable in R114A [18] and H277F mutants. Conversely, R114Q mutant shows roughly a 50% decrease of the enzyme activity measured using gangliosides [18], suggesting a role of this residue in substrate recognition and/or in the functionality of the loop containing the α2 helix.

2.6 Competitive Inhibitors of Sialidases: Effect of Influenza Virus Neuraminidase Inhibitors and Synthesis of New Compounds

Sialidase competitive inhibitors have been synthesized as drugs to fight influenza virus infections [25]. The presence, in higher organisms, of different forms of sialidases [6] and the conservation of the overall active site architecture along the evolutionary scale open the debate about possible side effects of the administration of these anti-influenza drugs due to their effects on enzyme activity of endogenous sialidases [26]. The most severe complications, such as neuropsychiatric events and mortality, have been reported upon administration of Tamiflu (Oseltamivir) in pediatric cases in Japan [27–29]. A Chinese group identified in NEU2 a non-synonymous SNP (single nucleotide polymorphism) occurring in 9.29% of Asian population and absent in European and African American population, leading to R41Q missense replacement [30]. Based on the NEU2 crystal structure, R41 is the last residue of strand B, blade I of the propeller, from which the mobile loop that contains the α1 helix departs [4]. Intriguingly, R41 is highly conserved in all members of the mammalian sialidase family; it corresponds to R45 in NEU3 and, as already mentioned, is expected to interact with the C4 hydroxyl group of DANA. In NEU3, the R45V mutant shows about 2% of the activity detectable in the wild-type protein using 4MU-NANA as substrate [24]. In the case of NEU2 R41Q variant, the enzyme shows a lower sialidase activity, with a 25% increase of the apparent Km and a 55% decrease of the Vmax, together with an increased affinity towards the active form of Tamiflu [30]. In this perspective, the authors propose

that individuals carrying this SNP likely in homozygous form, when treated with Tamiflu, could be affected by certain severe complications that resemble the symptoms of human sialidase related disorders. As already mentioned, among sialidases only NEU1 is implicated in a severe human inherited disease [31]; thus it seems unlikely that an inhibition of NEU2 can exert such dramatic effects. In order to shed light on this subject, the effect of Tamiflu and zanamivir (Relenza) has been tested on all human sialidases expressed as recombinant enzymes in human kidney 293 T cells [32]. Surprisingly, and in contrast with the results of Li and colleagues [30], Tamiflu, even if administered at 1 mM, seems ineffective on all sialidases, whereas zanamivir acts on NEU2 and NEU3 in the micromolar range of concentrations. Recently, these results have been further confirmed and extended, with the evaluation of other anti-influenza drugs and three DANA mimetics, by solving the crystal structure of NEU2 in complex with these compounds [33]. Again, no NEU2 inhibition is detectable using Tamiflu, even at 5 mM, and soaking experiments of NEU2 crystals with 1–20 mM inhibitor containing solutions result in no electron density for the drug or crack/disrupt in the crystals. Conversely, the complexes of NEU2 with zanamivir, peramivir, and the DANA mimetics show a number of hydrogen bonds and hydrophobic interactions within the enzyme active site which correlates with the corresponding measured Ki values.

Intriguingly, and despite the recently cited experimental evidence, Tamiflu is used to inhibit endogenous sialidase(s) in several papers. For example, the treatment of PC12 cells and primary cortical neurons with Tamiflu concentrations up to 500 mM leads to a block of the sialidase activity induced by nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), respectively [34]. More recent studies identify NEU1 as a preferential target for Tamiflu in neuronal cells [35], as well as in macrophage and dendritic cells [36], with an IC_{50} ranging from 1.2 to 3.9 mM. Finally, the inhibitory effect of Tamiflu has been observed on NEU4 sialidase as well, using bone marrow macrophages [37]. Thus, the possible (side) effects of viral neuraminidase inhibitor(s) on the different mammalian sialidases remain an open and interesting field of study. Nevertheless, sialidase inhibitors could represent a valuable tool in order to switch-off mammalian sialidases selectively. In this perspective, the homology modeling of mammalian sialidase based on the crystal structure of the cytosolic sialidase NEU2 represents a valuable starting point [5]. The first systematic study has been carried out on a series of amide linked C9 modified NANA, tested on all the members of the mammalian sialidase family [38]. Overall, these particular DANA derivatives show a remarkable selectivity toward NEU1 and some of them are characterized by IC_{50} values roughly ten times lower than DANA. The same group has studied the inhibitory effect of 4-acetamido-5-acylamido-2-fluoro benzoic acids characterized by a variety of chemical substituents bound at position 5 [39]. These compounds do not show any inhibition on NEU1 and only NEU2 is slightly affected by one of them, bearing a bulky biphenyl group roughly in the position of the glycerol moiety in sialic acid. Using the active form of Tamiflu (oseltamivir carboxylate) as a starting point, another study on the inhibitory effect of a number of derivatives bearing chemical substituents of increasing complexity in position 4 has been performed [40]. Just two

of these derivatives showed a mild inhibitory effect on the membrane-associated sialidases NEU3 and NEU4, the two enzymes tested; these results further confirm, at least using purified recombinant protein(s) or crude extracts from transfected cells as enzyme source, the specificity of Tamiflu for influenza virus neuraminidases. Finally, in a survey on recombinant NEU2 substrate specificity carried out using various *p*-nitrophenol-tagged sialyl galactosides containing different terminal sialic acids (Neu5Ac; Neu5Gc and Kdn), the corresponding 2-deoxy-2,3-dehydro derivatives has been synthesized and assayed as possible inhibitors [41]. Compared to zanamivir, used as reference competitive inhibitor, all of these derivatives, except Kdn, act as inhibitors with an IC_{50} in the micromolar range of concentration. Overall, the results summarized here validate the relevance of a combined approach based on structural biology, computational modeling, organic chemistry, and biochemistry of sugars in order to have a comprehensive picture of the relationships between structure and functions of these glycohydrolases.

3 Functional Features of Sialidase

3.1 Sialidase NEU1

The *NEU1* gene was identified in humans [21, 42, 43] and mice [44–46] as a major histocompatibility complex (MHC) related sialidase gene. Taking advantage of an SM/J mouse strain carrying a defective sialidase allele, the mouse gene was mapped by linkage analysis near the H-2D end of the MHC on chromosome 17, a region which is syntenic to human MHC on chromosome 6. The human lysosomal sialidase NEU1 is associated with a protective protein (carboxypeptidase A) and β-galactosidase as a complex in lysosomes, dissociation of the complex leading to sialidase inactivation [47]. It further features a lysosomal C-terminal targeting motif and evidence has been generated of a protective protein responsible for transport lysosomal compartmentalization and maturation as well as activation of NEU1.This is achieved by formation of an NEU1-protective protein heterodimer [48]. The N-terminal *N*-glycans of NEU1 is important for maintaining stability or catalytic activity, and the protective protein compensates for *N*-glycan defects [49]. In addition, recent observations revealed an intracellular distribution of this sialidase in plasma membranes as well as within lysosomes under conditions of cell stimulation, apparently connected to the new physiological function of NEU1.

NEU1 is linked to two neurodegenerative lysosomal storage disorders: sialidosis and galactosialidosis. The former is caused by mutations in the genomic DNA including frameshift insertions and missense mutations of the gene, and the latter results from a combined deficiency of NEU1 and β-galactosidase due to the absence of functional protective protein, leading to defective or deficient enzyme activity. Transfection of NEU1 cDNA has been shown to restore normal levels of sialidase activity toward 4MU-NANA in human sialidosis fibroblasts, indicating that it is a target gene for sialidosis [21, 43]. The sialidase possesses narrow substrate

specificity, with oligosaccharides and glycopeptides serving as good substrates, increased sialyloligosaccharides being found in sialidosis patients' urine and fibroblasts. In NEU1 knock-out mice, hearing loss [50] and muscle degeneration [51] have been observed as consequences of sialidosis. The available data suggest that marked increase of the expression and apical localization of lysosomal membrane protein LAMP-1 in marginal cells of the stria vascularis causes dysfunction of transduction in sensory hair cells, and that infiltration of muscle fibers by connective tissue results in muscle degeneration. Findings with NEU1 knock-out mice also indicate important roles of NEU1 in the regulation of phagocytosis by macrophages and dendritic cells through desialylation of surface receptors [36] and in negative control of lysosomal exocytosis by processing sialic acids on LAMP-1, as evidenced by oversialylation of LAMP-1 enhancing lysosomal exocytosis in macrophages [52].

In addition to its roles in glycoconjugate catabolism in lysosomes, NEU1 is involved in various cellular signaling pathways, contributing to immune responses, as well as elastic fiber assembly through transportation to plasma membranes. Sialidase activity in activated T lymphocytes results in hyposialylation of specific cell surface glycoconjugates and production of IFN-gamma [53]. In human T-cells both NEU1 and NEU3 mRNAs are significantly induced by T-cell-receptor stimulation, and several cytokines, including IL-2 and IL-13, are expressed upon up-regulation of these sialidases [54], probably playing important immunoregulatory roles. During PMA-induced monocyte differentiation, NEU1 is up-regulated and targeted together with its protective protein to MHC II-positive vesicles that merge later with the plasma membrane [55]. Desialylation of sialyl α-2,3-linked β-galactosyl residues of TOLL-like receptor 4 by NEU1 may be essential for receptor activation and cellular signaling via NFkB activation [36]. Consistent with negative regulation by sialic acid residues of CD44 on its hyaluronic acid receptor function, hyaluronic acid receptor activity of CD44 on $CD4^+$ T cells and acute asthmatic reactions, including Th2-mediated airway inflammation and airway hyperresponsiveness, are dependent upon NEU1 enzymatic activity [56]. Facilitation of elastic fiber assembly is probably through desialylation of microfibrillar glycoproteins by NEU1 in aortic smooth muscle cells [57], whereby the elastin-binding protein (EBP), identical to the spliced variant of β-galactosidase, forms a cell surface-targeted complex with the protective protein and NEU1 [58]. The carboxypeptidase activity of the protective protein is required for this EBP complex formation, participating in regulation of blood pressure as an endothelin-1 inactivating enzyme [59], although it is thought to be unnecessary for complex formation with NEU1 and β-galactosidase in lysosomes. Furthermore, NEU1 inhibits proliferation of aortic smooth muscle cells and fibroblasts by desialylation of both PDGF and IGF-1 receptors, and also negatively regulates proliferative responses of skeletal myoblasts to insulin by desialylation of insulin and IGF-1 receptors [60], leading to diminution of signaling through the AKT and ERK1/2 pathways. NEU1 has also been reported to participate in NGF-induced Trk tyrosine kinase receptor activation and cellular signaling, probably by making complexes with matrix metalloproteinase-9 (MMP-9) at cell surfaces [35]. These observations suggest that, in addition to lysosomal catabolism,

NEU1 regulates various important cellular phenomena through desialylation of cell surface molecules, presumably after mobilization to the membrane.

Various cancers show general tendencies for decreased NEU1 expression. Interestingly, there is a good inverse relationship between the NEU1 expression level and metastatic ability, also evident with NEU1 reduction in rat 3Y1 fibroblasts after Src-transformation. Furthermore, v-*fos* transfer to transformed cells caused an even more severe decrease in sialidase activity and acquisition of higher lung metastatic potential [61]. In mouse adenocarcinoma colon 26 cells, compared to low metastatic NL4 and NL44 cell lines, highly metastatic NL17 and NL 22 cells exhibit lower expression of NEU1 sialidase, accompanied by higher levels of sialyl Le^x and GM3 [62]. As expected, introduction of NEU1 sialidase into Bl6 melanoma cells resulted in suppression of experimental pulmonary metastasis and tumor progression, with reduction of anchorage-independent growth and increased sensitivity to inducers of apoptosis [63]. Overexpression of human NEU1 with its protective protein brought about similar alterations of human cancer cells to those observed in murine colon adenocarcinoma cells, such as suppressed cell migration and invasion in colon adenocarcinoma HT-29 cell, whereas its knock-down resulted in the opposite effects. When NEU1-overexpressing cells were injected transsplenically into mice, the in vivo liver metastatic potential was significantly reduced. One of the target molecules for NEU1 was found to be integrin β4, undergoing desialylation and decreased phosphorylation followed by attenuation of the FAK and ERK1/2 pathway and down-regulation of matrix metalloproteinase-7 [64], as shown in Fig. 4 (right panel).

3.2 *Sialidase NEU2*

Cytosolic sialidase NEU2 was the first example of a mammalian sialidase for which cDNA cloning was achieved with samples from rat skeletal muscle [22]. Homologs were subsequently cloned from cDNA libraries of CHO [65], mouse brain [66, 67], and mouse thymus [68], and from a genomic library of human skeletal muscle [69]. In all cases high amino acid identity (70–98%) was found to the rat gene. Unlike the NEU1 sialidase, NEU2 is able to hydrolyze a wide range of glycoproteins, oligosaccharides, and gangliosides at near neutral pH. Regarding comparative expression levels of human sialidases, human NEU2 is extremely low and is at best only four- to ten-thousandths of the NEU1 value in a wide range of tissues, as assessed by quantitative real time RT-PCR using a standard curve for each cDNA [32], although these profiles differ among the human, rat, and mouse.

NEU2 participates in muscle cell and neuronal differentiation, and the rat NEU2 is highly expressed in rat skeletal muscle. It contains two E-box pairs, known to be consensus-binding sites for muscle-specific transcription factors, in the 5′-flanking enhancer/promoter region. This region exhibits transcriptional activity in rat L6 [70] and murine C2C12 [71] myogenic cells, and this activity, as well as the mRNA level, is increased during myotube formation, indicating that the enzyme plays a critical role in muscle cell differentiation. In this context, it is of interest that the

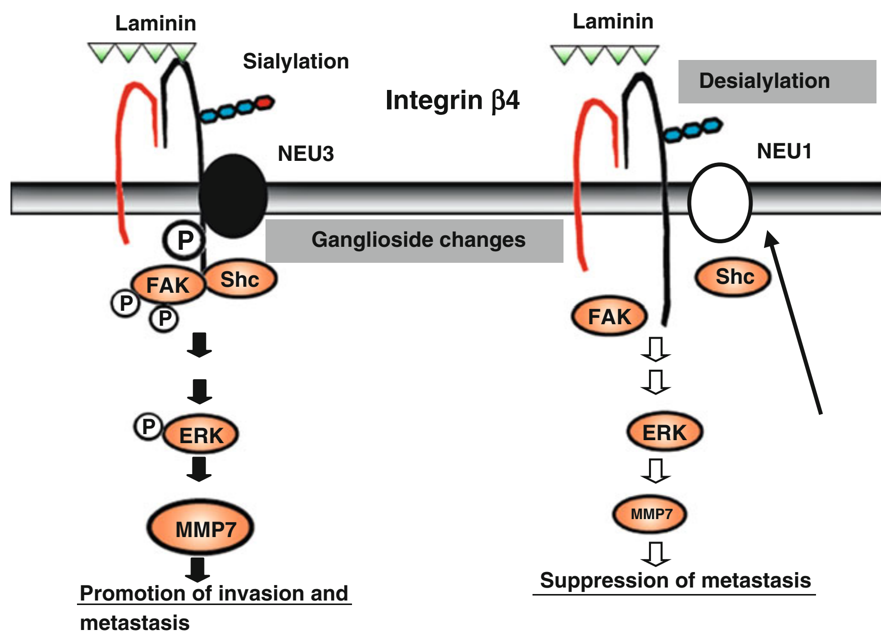

Fig. 4 Opposite roles of two sialidases in interim β4-mediated signaling on laminin-5 [64, 94]. NEU1 inhibits signaling through desialylating the integrin probably at cell surface followed by decreased tyrosine phosphorylation and subsequent attenuation of FAK and ERK1/2 pathway, whereas NEU3 promotes phosphorylation of the integrin with recruitment of Shc and Grb-2 through ganglioside modulation

SJL mouse, a model for human dysferlinopathy, shows down-regulation of NEU2, probably being involved in impaired muscle regeneration [72]. Further support for this sialidase participating in myogenic function is provided by human rhabdomyosarcoma cells that display defective differentiation, which is accompanied by suppression of NEU2 expression [72]. However, it is uncertain whether NEU2 up-regulation might abrogate these effects. In PC12 cells, NEU2 may participate in neuronal differentiation on nerve-growth factor-induced transcriptional activation [73]. Human NEU2 has been reported to degrade complex-type *N*-glycans in the cytosol of MKN7 and MKN45 stomach cancer cells, in which accumulation of free complex-type *N*-glycans occurs [74].

Several studies have focused on whether NEU2 up-regulation affects cancer cell behavior. When the rat *NEU2* gene was transfected into highly metastatic mouse colon 26 adenocarcinoma cells [62], marked inhibition of lung metastasis, invasion, and cell motility after intravenous injection into syngeneic mice resulted, with a concomitant decrease in sialyl Le^x and G_{M3} levels, in line with spontaneously low metastatic sub-lines having a relatively high level of endogenous sialidase. Treatment of the cells with antibodies against sialyl Le^x and G_{M3} affected cell adhesion and/or cell motility, providing evidence that desialylation of these molecules, as

natural substrates of sialidase, is involved in the suppression of metastasis. Introduction of the same gene into a B16-BL6 mouse melanoma cell line [63], highly invasive and metastatic, also caused a marked decrease in pulmonary metastasis, with reduction in the ganglioside G_{M3} and an increase in lactosylceramide, but no change in either cell growth or cell attachment. The highly metastatic cells exhibited rather decreased sialic acid content, both total and at the cell surface, as compared to the low metastatic cells, consistent with sialidase activity. The results when taken together indicate that the sialidase level may be a determining factor for metastatic ability, independent of the cell type, and irrespective of the sialic acid content.

Another line of experiments with Chinese hamster ovary *NEU2*, featuring high amino acid identity with the rat gene, reported by Meuillet et al. [75] demonstrated that *NEU2* gene transfection into the A431 human epidermoid carcinoma cell line reduced the G_{M3} level and enhanced cell growth and tyrosine autophosphorylation of EGF receptors at low EGF concentration. Furthermore, human NEU2 has been reported to participate in cell apoptosis. Tringali et al. [76] found that its introduction into leukemic K562 cells brought about a marked decrease of anti-apoptotic factors Bcl-XL and Bcl-2, resulting in increased sensitivity to apoptotic stimuli. *NEU2* overexpression in the cells reduced gene expression and activity of Bcr-Abl, together with a decrease in Bcr-Abl-dependent Src and Lyn kinase activity, probably due to desialylation of some cytosolic glycoproteins.

However, as mentioned above, endogenous human NEU2 expression was found to be extremely low, or hardly detectable, in human several non-cancerous and cancerous tissues [32]. It should be noted here that because of its extremely low expression, it is uncertain whether NEU2 actually functions in human cells. The biological and phylogenic significance of the markedly low NEU2 expression in humans as compared to other mammals remains to be elucidated.

3.3 Sialidase NEU3

The plasma membrane-associated NEU3 sialidase was first cloned from a bovine brain library [77], based on peptide sequence information from the purified enzyme protein, and later from a human skeletal muscle cDNA library [19] and the human genome data base [20]. The primary sequences covering the entire coding region of the corresponding human, mouse, and rat genes display approximately 80% overall identity with the bovine gene. The bovine and human enzymes specifically hydrolyze gangliosides, while the murine enzyme acts on oligosaccharides, 4MU-NANA, and glycoproteins to a certain extent. Gangliosides G_{D3}, G_{M3}, G_{D1a}, and even G_{D1b}, but not G_{M1} and G_{M2}, are good substrates in the presence of Triton X-100. Unlike the bovine and murine enzymes with only one activity peak at a pH near 4.6, the human enzyme, NEU3, shows two peaks at pH 4.5–4.8 and at pH 6.0–6.5 [19]. The major subcellular localization of the bovine and murine sialidases proved to be plasma membranes as determined by Percoll density gradient centrifugation of cell

homogenates and by immunofluorescence staining. The human NEU3, however, is not always detected on the cell surface but may exist in other cellular membrane components, and can mobilize to membrane ruffles together with Rac-1 in response to growth stimuli such as EGF, enhancing cell movement [78]. An investigation of membrane topology suggested the enzyme to be localized partially on the cell surface as a peripheral membrane protein and also in endosomal structures [79]. With administration of the radiolabeled ganglioside G_{D1a} to murine NEU3-transfected cells, NEU3 was shown to hydrolyze ganglioside substrates in intact living cells at a neutral pH through cell-to-cell interactions [80].

Because of its unique substrate specificity and association with plasma membranes, NEU3 is presumed to be an important molecule for various cell surface events. Murine NEU3 can also regulate cell apoptosis in human fibroblasts by producing ceramide from G_{M3} in plasma membranes through further degradation of the sugar unit [81]. In line with previous biochemical evidence suggesting an essential role of ganglioside sialidase in the processes of proliferation control and differentiation in neuroblastoma cells [82], using cDNA clone, NEU3 was indeed confirmed to participate positively in neurite formation in mice [67] and in human neuroblastoma cells [83], and in the regulation and regeneration of rat hippocampus neurons [84, 85]. Consistent with these results, NEU3 sialidase activities remained high with differentiation and aging in culture of rat cerebellar granule cells, accompanied by increased β-galactosidase and β-glucosidase activities and ceramide levels of plasma membranes in parallel [86]. An involvement of NEU3 in skeletal muscle differentiation and apoptosis is evidenced by the fact that NEU3 knock-down in murine C2C12 cells causes inhibition of myotube formation and sensitization to apoptotic stimuli through accumulation of G_{M3} subsequent to epidermal growth factor receptor blockage [86]. NEU3 gene expression leads to reduced TNF-alpha-induced MMP-9 expression in vascular smooth muscle cells by decreasing MMP-9 promoter activity in response to TNF-alpha, suggesting a contribution to plaque instability in atherosclerosis as a physiological modulator of smooth muscle cell physiology [87]. It is located in rafts of neuroblastoma cells [88] and in caveolae of HeLa cells [89], closely associated with caveolin-1, probably through interaction with the caveolin binding region. In caveolae, NEU3 has been proposed to control PDGF-induced Src mitogenic signaling and DNA synthesis by modification of cell surface GM1 level. PAG, a member of the transmembrane adaptor protein family, induces NEU3 sialidase activity and G_{M1} accumulation, and subsequently excludes PDGF receptors from caveolae and inhibits Src mitogenic signaling, although the mechanisms remain unclear [90]. Depletion of ganglioside G_{M3} by NEU3 causes increased EGFR phosphorylation and inhibition of the EGF-induced tyrosine phosphorylation of caveolin-1, leading to activation of EGFR signaling by retention of caveolin-1 in caveolae [89]. As with NEU1 described earlier, human NEU3 has been found to contribute to development of adaptive immune responses. It is up-regulated together with NEU1 during differentiation of monocytes into dendritic cells, and enhances LPS-induced production of cytokine including IL-6, IL-12p40, and TNF-alpha through alteration of the sialic acid content of specific cell surface glycoconjugates [91].

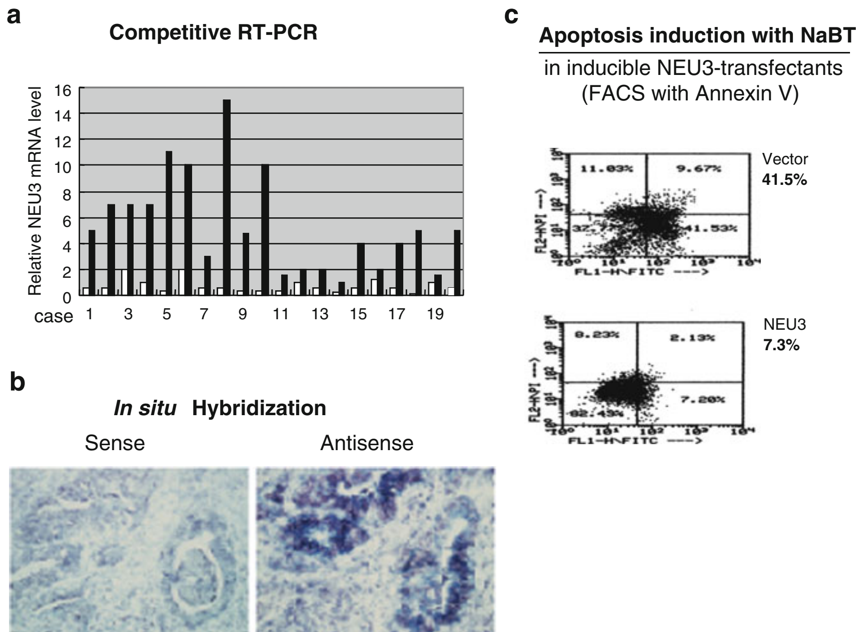

Fig. 5 Up-regulation of sialidase NEU3 and its involvement in apoptosis suppression in colon cancer [93]. (**a**) NEU3 mRNA level in colon cancer tissues and noncancerous mucosa (*closed* and *open columns*, respectively). (**b**) In situ hybridization of NEU3 in colon cancer tissues. (**c**) Flowcytometric analysis with annexin V in NEU3-transfected cells after sodium butyrate-induced apoptosis

Up-regulation of NEU3 is observed in various neoplasms including colon, renal, ovarian, and prostate cancers, although down-regulation has been noted in acute lymphoblastic leukemia in relation to disease progression [92]. In human colon cancers, *NEU3* mRNA levels were found to be increased 3- to 100-fold as compared to adjacent non-tumor mucosa [93] (Fig. 5a). Moreover, in situ hybridization analysis with antisense probes demonstrated positive signals to be localized to carcinoma cells, rather than surrounding stromal cells, with no clear signals using the sense probe (Fig. 5b). During sodium butyrate-induced apoptosis, human colon cancer cells show down-regulation of NEU3 expression, in contrast to up-regulation of NEU1. Transfection of an *NEU3* gene into cancer cells was found to inhibit sodium butyrate-induced apoptosis (Fig. 5c), accompanied by increased Bcl-2 protein and decreased caspase expression. Colon cancer tissues exhibit marked accumulation of lactosylceramide, a possible NEU3 product, and addition of this glycolipid to cultures reduced apoptotic cells during sodium butyrate treatment. In colon cancer cells, NEU3 was noted to regulate differentially cell proliferation through integrin-mediated signaling depending on the extracellular matrix [94], causing increased adhesion to laminins and consequent cell division, but rather decreased cell adhesion to fibronectin and collagens I and IV. Triggered by laminins, NEU3 markedly enhanced tyrosine

phosphorylation of integrin β4, with recruitment of Shc and Grb-2, stimulating phosphorylation of focal adhesion kinase and ERK1/2 (Fig. 4, left panel). Interestingly, in contrast to NEU3, NEU1 attenuated this integrin-mediated signaling by reducing the phosphorylation, as illustrated earlier in Fig. 4 (right panel).

NEU3 mRNA levels have also been found to be significantly increased in renal cell carcinomas (RCCs) [95], correlating with elevation of interleukin (IL)-6, a pleiotropic cytokine. NEU3 activation by IL-6 directs IL-6-mediated signaling via the PI3K/Akt cascade in a positive feedback manner and thus contributes to the malignant phenotype, including suppression of apoptosis and promotion of cell motility in RCCs. Up-regulation of NEU3 was also detected in prostate cancer, showing a significant correlation with malignancy as assessed by the Gleason score [96]. In androgen-sensitive LNCaP cells, forced overexpression of *NEU3* significantly induced expression of EGR-1, a progression-related transcription factor, as well as androgen receptors and PSA, both with and without androgen, the cells becoming hormone-sensitive. This NEU3-mediated induction was abrogated by inhibitors of PI-3 kinase and MAPK, confirmed by increased phosphorylation of AKT and ERK1/2 in *NEU3*-overexpressing cells. NEU3 siRNA introduction resulted in reduced growth of androgen-independent PC-3 cells in culture and of transplanted tumors in nude mice. These data suggest that NEU3 regulates tumor progression of prostate cancer through androgen receptor signaling.

To define further the molecular mechanisms of NEU3 influence and its possible targets, the encoding gene has been silenced with siRNA or overexpressed in various human cancer cells [97]. Silencing caused apoptosis without specific stimuli, accompanied by decreased Bcl-xL and increased mda7 and G_{M3} synthase mRNA levels in HeLa cells, whereas overexpression resulted in the opposite effect. Human colon and breast carcinoma cell lines, HT-29 and MCF-7 cells, appeared to be similarly affected by treatment with the NEU3 siRNA but, interestingly, non-cancerous human WI-38 and NHDF fibroblasts and NHEK keratinocytes showed no significant changes. NEU3 siRNA was found to inhibit and NEU3 overexpression to stimulate Ras activation with consequent influence on ERK and Akt. Ras activation by NEU3 was largely abrogated by PP2 (an Src inhibitor) or AG1478 (an EGFR inhibitor), and, in fact, siRNA introduction reduced phosphorylation of EGFR while overexpression promoted its phosphorylation in response to EGF. NEU3 co-immunoprecipitated with EGFR yielded a higher amount of immunoprecipitable NEU3 upon EGF stimulation. These results indicate that NEU3 suppresses apoptosis of cancer cells by promoting EGFR phosphorylation, probably through its association with EGF receptors and consequent activation of Ras cascades, especially via the Ras/ERK pathway.

To summarize the significance of NEU3 up-regulation in cancer, the sialidase activates molecules including EGFR, FAK, ILK, Shc, and integrin β4, often up-regulated in carcinogenesis, and may thus cause accelerated development of malignant phenotypes. Up-regulation of NEU3 has also been established to have importance for the promotion stage of colorectal carcinogenesis in vivo, from experiments using NEU3 transgenic mice [98]. Thus NEU3 was found to increase azoxymethane-induced aberrant crypt foci formation in colon mucosa by

suppression of apoptosis, possibly via activation of EGF signaling. Taking into account all of the evidence, NEU3 is certainly involved in regulation of transmembrane signaling at the cell surface, possibly through both modulation of gangliosides as an enzyme and by interaction with other signal molecules like caveolin-1, Rac-1, integrin β4, Grb-2, and EGFR [99].

Interestingly, NEU3 transgenic mice have been shown to develop impaired insulin signaling and insulin-resistant diabetes mellitus by 18–22 weeks [100], associated with hyper-insulinemia, islet hyperplasia, and increase in the β-cell mass. As compared to the wild type, insulin-stimulated phosphorylation of the insulin receptor and insulin receptor substrate I was significantly reduced, and activities of phosphatidylinositol 3-kinase and glycogen synthase were also decreased. In muscle extracts, association of tyrosine-phosphorylated NEU3 with Grb2 was observed in response to insulin, together with accumulation of G_{M1} and G_{M2}. The involvement of NEU3 in both cancer progression and development of diabetes suggests that these diseases might be closely related to each other in pathogenesis, this being in line with recent epidemiological reports of higher risk of certain type of cancers in diabetic patients than in controls [101, 102].

A recent study [103] on *NEU3* gene expression has shown evidence that NEU3 expression is diversely regulated by Sp1/Sp3 transcription factors binding to alternative promoters, which might account for multiple modulation of gene expression. The transcriptional control by these factors may also account for the up-regulation of NEU3 in cancer, because Sp1 and Sp3 were initially considered as constitutive activators of housekeeping genes but have recently been documented to play critical roles in regulating the transcription of genes involved in cell growth control and tumorigenesis. In fact, expression level of NEU3 and that of Sp1 or Sp3 exhibited good correlations in cancer, implying that Sp1 and Sp3 play a promoting role in NEU3 gene transcription.

3.4 Sialidase NEU4

The fourth sialidase, NEU4, was identified based on cDNA sequences in public databases [104–107]. With regard to the subcellular localization of the human NEU4, two different descriptions have been reported based on gene transfection studies, one demonstrated targeting to the lysosomal lumen [106] and the other to mitochondria and endoplastic reticulum [107, 108]. NEU4 consists of isoforms that differ in their possession of 12 N-terminal amino acid residues acting for the mitochondrial targeting. The isoforms are differentially expressed in a tissue-specific manner. The brain, muscle, and kidney contain both, while the liver and colon possess predominantly the short form, as assessed by RT-PCR [107]. The isoforms possess broad substrate specificity, including activity towards mucin. Murine NEU4 is expressed predominantly in brain [104], and also exists as two splicing isoforms [109], different from those of human NEU4. Expression of human NEU4 in cells of sialidosis patients results in clearance of storage materials from lysosomes and thus

NEU4 may be useful for novel therapeutic purposes [106]. Although NEU4 deficient mice lack gross morphological abnormalities, their ganglioside pattern in the brain features increased G_{D1a} and decreased G_{M1} [110]. Furthermore, mice with a double deficiency of NEU4 and β-hexosaminidase A exhibit epileptic seizures and rapid neuronal loss accompanied with G_{M2} accumulation. Unlike human Tay-Sachs disease, hexosaminidase A deficiency in mice has been characterized as remaining asymptomatic for over 1 year, G_{M2} catabolism being expected. In this context, the results for double deficient mice are of interest and indicate that NEU4 is a ganglioside-metabolizing sialidase in vivo [111]. There is a recent report suggesting that NEU4 is activated by thymoquinone from *Nigella sativa* plant in various cells including macrophages and that the induced NEU4 activity is related to activation of NF kB and proinflammatory cytokine production [37].

Human NEU4 may be involved in cell apoptosis or neural differentiation, based on the observation that the long form including the mitochondrial targeting signal probably impacts on the level of G_{D3}, known to be an apoptosis-related ganglioside [112]. Contrasting with NEU3, murine NEU4 has been demonstrated to regulate negatively neurite formation [109]. During retinoic acid-induced differentiation, NEU4 expression was found to be down-regulated in Neuro2a cells. Furthermore, overexpression of NEU4 resulted in suppression and its knock-down in acceleration of neurite formation.

When NEU4 mRNA levels were examined in human colon cancers, a marked decrease was noted [113], in clear contrast to the NEU3 case. Levels were not significantly correlated with the histological differentiation or the pathological stage, but the T/NT(tumor to non-tumor expression ratio) value remained significant at $p = 0.025$ with venous invasion between v0 ($n = 28$) and v1–3 ($n = 13$). In cultured human colon cancer cells, the enzyme was up-regulated in the early stage of apoptosis induced by either the death ligand TRAIL or serum-depletion. Transfection of *NEU4* into DLD-1 and HT-15 colon adenocarcinoma cells resulted in acceleration of apoptosis and decreased invasiveness and cellular motility. siRNA-mediated NEU4 targeting, on the other hand, caused significant inhibition of apoptosis and promotion of malignant characteristics. To elucidate the significance of NEU4 down-regulation in colon cancer, we investigated sialyl-Lewis antigens, sialyl-Le^a and sialyl-Le^x, as endogenous substrates for the sialidase [114], because they are utilized as tumor markers, and showed their increase in cancer to be associated with tumor progression. NEU4 was found to hydrolyze the antigens in vitro and decrease cell surface levels much more effectively than other sialidases. Western blot, thin layer chromatography, and metabolic inhibition studies of desialylation products further revealed NEU4 to catalyze preferentially sialyl-Lewis antigens expressed on *O*-glycans. Cell adhesion to and motility and growth on E-selectin were significantly reduced by NEU4. E-selectin stimulation of colon cancer cells enhanced cell motility through activation of the p38/Hsp27/actin reorganization pathway, whereas NEU4 attenuated the signaling. It is interesting to note here that the sialidase does not change the level of a normal glycan, disialyl-Le^a, generally expressed in non-malignant epithelial cells. Although it has been proposed that glycosyltransferases are responsible for synthesis of these antigens,

expression levels of the encoding genes have not always been found to correlate with sialyl-Lewis antigen contents, with even contradictory expression noted in various cells. It is feasible that desialylation by NEU4 may occur specifically with cancer related sialyl-Lewis antigens and thus maintenance of the normal glycan level can be achieved in colon mucosa highly expressing NEU4.

4 Future Perspectives

Recent progress in mammalian sialidase research has provided accumulating evidence of the great importance of these enzymes for cellular functions, with clarification of the structural and molecular basis and significance of physiological and pathological desialylation. Each sialidase has been found to play a unique role depending on particular properties and subcellular localization, but often functional variation or novel activities are associated with particular cell stimuli. For example, NEU1 contributes to immune function at cell surfaces, as well as lysosomal catabolism, in line with the gene locus in MHC, and NEU2 participates in muscle cell differentiation, whereas NEU3 and NEU4 regulate neuronal differentiation. With the observations described above, it is interesting to note that the two sialidases NEU1 and NEU3 appear to have unique functions opposing each other under various conditions, through preferential desialylation of glycoproteins and gangliosides, respectively, even when targeting the same molecule in the same cells, as illustrated in Fig. 4. Numerous studies have disclosed molecular mechanisms of pathological desialylation in sialidosis caused by NEU1 deficiency, and abnormal up-regulation of NEU3 has been demonstrated to give rise to progression to greater malignancy in various types of carcinomas. Remodeling of sialic acid residues in glycoconjugates by modulating expression of these sialidase genes should provide important clues to controlling the degree of sialylation of functional molecules in cells. In particular, it is of importance to investigate the detailed molecular phenomena underlying NEU1 involvement in immune responses to contribute to elucidation of infectious and autoimmune diseases. Discovery of specific tools for NEU3 down-regulation will hopefully light the pathway to development of novel cures for cancer. Further investigations of mammalian sialidases and pathological consequences of aberrant sialylation are clearly warranted.

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