

Advances in Neurobiology 29

Cara-Lynne Schengrund  
Robert K. Yu *Editors*

# Glycobiology of the Nervous System

*Second Edition*

 Springer

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Cara-Lynne Schengrund • Robert K. Yu  
Editors

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# Preface

According to PubMed, between 2013, when much of the work that appeared in the first edition of this book was done, and the start of 2022, over 4200 manuscripts were published that dealt with carbohydrates and neural function, with about 28% of them appearing during the SARS-CoV-2 pandemic years of 2019–2022 (March). Of the approximately 490,000 publications listed by PubMed on carbohydrates *per se* between 2013 and 2022, about 36% appeared between 2019 and 2022 (March). The numbers just emphasize the growing interest in glycobiology and the prescience of the 2012 publication of the National Academy of Science–sponsored committee report that discussed the importance of glycomics and glycosciences and was titled “Transforming glycoscience, a roadmap for the future” (The National Academies Press 2012). It is the growing interest in the potential roles of carbohydrates in neural function that presumably accounts for the interest the first edition of this book generated and which led Springer to request an update.

While the initial chapters have been updated or provided from a fresh perspective, they are included for people relatively new to the field of neural cell anatomy and/or carbohydrate chemistry/biochemistry as they provide an introduction to cells that comprise the nervous system and a basic understanding of the complexity of oligosaccharide synthesis. Glycoconjugates are formed by the addition of one sugar to another on either a lipid or protein as described in Chap. 1. Recent evidence indicates that small, noncoding RNAs can also be glycosylated via N-glycan biosynthesis and are primarily associated with cell membranes (<https://doi.org/10.1016/j.cell.2021.04.023>). Future reports are needed to provide more information about their potential roles in cells.

The lack of a defined template for oligosaccharide synthesis such as there is for protein synthesis means that there can be significant variability in the structures formed, and it is well known that errors in glycosylation can lead to significant disruptions in neural function. An introduction to cells found in the central nervous system (CNS) and how all of the cells function together to accomplish perception, integration, memory, and the generation of behaviors are provided in Chap. 2. Interruption of those interactions results in various disruptions of normal neural function. Chapters 3, 4, and 5 discuss N-linked glycoproteins, O-linked

glycoconjugates, and glycosaminoglycans, respectively. Each reviews methods of synthesis of glycan moieties and includes examples of their roles in diseases affecting the nervous system. It is also apparent that the number of possible products formed upon linking two or more sugars together is much greater than that formed when a similar number of amino acids form a polypeptide, a fact that makes their structural analysis more difficult.

A discussion about the structural analysis of oligosaccharides and glycoconjugates using NMR is provided in Chap. 6, and updated information about utilization of mass spectrometry in glycomics is available at <https://doi.org/10.1042/BST20190861>. As pointed out in discussion of glycoconjugates and their roles in axon growth/guidance as well as voltage-gated channels in Chap. 7, it is only because of recent technological advances in bio-physicochemical and imaging analyses that researchers have been able to define the structures of glycoconjugates and changes that either cause or occur during disease.

Subsequent chapters become more focused, pointing out specific errors in proteins responsible for specific steps in glycosylation/deglycosylation and how they can affect individuals in whom they are expressed. The sheer number of these errors can be seen in the discussion of congenital disorders of glycosylation presented in Chap. 8, while Chap. 9 focuses on brain O-GlcNAcylation, discussing molecular mechanisms and clinical phenotypes affected if it goes awry. Chapters 10, 11, 12, and 13 focus on the remarkable progress made in identifying the role(s) of gangliosides in neural function, starting with their roles in determining the fate of neuronal precursor cells (Chap. 10). This is followed by a discussion in Chap. 11 of how the structure of cell surface gangliosides, whose intracellular synthesis is completed in the Golgi, can be modified by plasma membrane-associated glycohydrolases. The authors discuss how these changes can affect membrane microdomain curvature which in turn can modify behavior of proteins in that area. Chapter 12 presents details about inborn errors of metabolism affecting neuronal gangliosides and glycosphingolipids and their attendant pathologies, while Chap. 13 focuses on findings of ganglioside changes/interactions associated with neurodegenerative disorders such as Parkinson's, Alzheimer's, and Huntington's diseases. Chapter 14 reviews glycosphingolipid deficiencies and broadens the concept of taking advantage of our growing knowledge of their ability to affect behavior of a variety of molecules by using glycosphingolipid replacement therapy to ameliorate deficiencies affecting the CNS.

Errors in carbohydrate metabolism aren't the only ways by which carbohydrates can affect the central nervous system. The role of carbohydrates in the action of pathogens that affect the CNS is discussed in Chap. 15, which points out that oligosaccharides on the target cell can function as receptors for carbohydrate-binding proteins on the pathogen or carbohydrates on the pathogen may be bound by carbohydrate-binding proteins on the target cell. As these molecules become identified, methods are being developed to block such interactions using specific antibodies, inhibitors of carbohydrate binding, or molecules to block release of nascent virus from the cells. A potential problem can occur when an infection that stimulates an immune response results in production of antibodies that recognize not just

carbohydrates on the pathogen but molecules on human cells as well. Referred to as “molecular mimicry,” this potential problem is discussed in Chap. 16.

As stated at the start, progress in the study of glycoconjugates and their roles in the CNS has benefited from the development of (1) animal models, (2) methods for carbohydrate characterization, and (3) imaging. As details accrue regarding the molecular effects of specific changes caused by defective expression of specific glycoconjugates, it is anticipated that those findings will lead to development of therapies able to ameliorate the attendant pathologies.

Finally, we want to thank the authors that either updated or provided us with new chapters for this volume. Some of them dealt with disruptions caused by SARS-CoV-2, while others dealt not only with the pandemic but also politically induced problems, and in some cases serious health conditions. To all, our sincere thank you for all your work. To readers, we hope that you find chapters that interest you, and if you are just starting to do research in this area, we hope you discover an unanswered question that stimulates you to try to find the answer.

Hershey, PA, USA  
Augusta, GA, USA

Cara-Lynne Schengrund  
Robert K. Yu



## Postscript: *In Memoriam*



It was with sadness that I learned that my co-editor, Dr. Robert K. Yu, lost his on-going battle with Parkinson's disease on May 18, 2022. Without him, this book, in either the first edition or this one, would not exist. It was Bob, whom I have known for over 50 years as both a colleague and good friend, who asked me to be his co-chair for a symposium on "glycobiology of the nervous system" that he was proposing for the 2012 meeting of the American Society for Neurochemistry. Springer noticed it and thought it a timely topic for a book. We agreed. The book was written, well-received, and an update requested. Despite knowing that he had

Parkinson's, Bob agreed that we should do it and indicated that he would write two chapters. Notwithstanding his declining health, Bob, in conjunction with his coauthors, completed them and sent them to me. I knew Bob was relieved when they were finished as he told me he had met his obligations. It was just a short time later that he was hospitalized, but even then, he kept in touch and was happy when I told him all chapters had been sent to Springer. I relate this to indicate the dedication Bob had to his research, to his excitement when his hypotheses were confirmed by experimentation, and to his following through on commitments that he made. Personally, I will miss his friendship, enthusiasm about his work, and enjoyment of life and his family.

For those interested in learning about Bob's scientific life, honors, and publications, see <https://doi.org/10.1007/s11064-011-0445-y>.

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# Chapter 1

## Introduction to the Complexity of Cell Surface and Tissue Matrix Glycoconjugates



Veer P. Bhavanandan and D. Channe Gowda

**Abstract** This chapter provides an overview of structures and functions of complex carbohydrates (commonly called glycans) that are covalently linked to proteins or lipids to form glycoconjugates known as glycoproteins, glycolipids, and proteoglycans. To understand the complexity of the glycan structures, the nature of their monosaccharide building blocks, how the monomeric units are covalently linked to each other, and how the resulting glycans are attached to proteins or lipids are discussed. Then, the classification, nomenclature, structural features, and functions of the glycan moieties of animal glycoconjugates are briefly described. All three classes of glycoconjugates are constituents of plasma membranes of all animal cells, including those of the nervous system. Glycoproteins and proteoglycans are also found abundantly as constituents of tissue matrices. Additionally, glycan-rich mucin glycoproteins are the major constituents of mucus secretions of epithelia of various organs. Furthermore, the chapter draws attention to the incredible structural complexity and diversity of the glycan moieties of cell surface and extracellular glycoconjugates. Finally, the involvement of glycans as informational molecules in a wide range of essential functions in almost all known biological processes, which are crucial for development, differentiation, and normal functioning of animals, is discussed.

**Keywords** Complex carbohydrates · *N*-Glycans · *O*-Glycans · Glycosaminoglycans · Glycoconjugates · Glycoproteins · Glycolipids · GPI anchors · Proteoglycans · Structure and functions of glycoconjugates

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## Abbreviations

C2,6diS	chondroitin 2,6-sulfate (CSD), C4,6diS, chondroitin 4,6-sulfate (CSE)
C4S	chondroitin 4-sulfate (CSA)
C6S	chondroitin 6-sulfate (CSC)
Cer	ceramide
CS	chondroitin sulfate;
DS	dermatan sulfate (CSB)
ER	endoplasmic reticulum
GAGs	glycosaminoglycans
GBPs	glycan-binding proteins
GPI	glycosylphosphatidylinositol
GSLs	glycosphingolipids
HA	hyaluronic acid
HNK-1	human natural killer-1
HS	heparan sulfate
KS	keratan sulfate
NCAM	neuronal cell adhesion molecule
PGs	proteoglycans

## 1 Introduction

Carbohydrates are the most abundant, structurally complex, and functionally diverse organic compounds found on earth. They comprise monosaccharides (e.g., glucose), oligosaccharides (e.g., sucrose, lactose, components of glycoproteins, and glycolipids; see below), and polysaccharides (e.g., starch, cellulose, plant and microbial cell wall polysaccharides, arthropod chitin, and animal glycosaminoglycans). Carbohydrates are important dietary components for animals, including humans, and play crucial roles in energy metabolism as exemplified by glucose homeostasis. In addition to their occurrence as free molecules, when conjugated to proteins and lipids, they form glycoconjugates (glycoproteins, proteoglycans, and glycolipids) (Brockhausen and Stanley 2017; Brooks et al. 2002; Ferguson et al. 2017; Gabius 2009; Haltiwanger et al. 2017; Prestegard et al. 2017; Schnaar and Kinoshita 2017; Stanley et al. 2017; Stanley and Cummings 2017; Taylor and Drickamer 2011; Varki and Kornfeld 2017; Varki et al. 2017a). The oligo- and polysaccharides covalently conjugated to proteins and lipids are referred to as complex carbohydrates or glycans (Prestegard et al. 2017; Varki and Kornfeld 2017). Recently a new class of glycoconjugates, in which glycans conjugated to small noncoding RNAs, that are displayed on cell surfaces were discovered (Flynn et al. 2021). These glycan RNA conjugates are termed glycoRNAs. However, several aspects of glycoRNAs remain to be understood. The glycan moieties of glycoconjugates are structurally complex and are involved in a myriad of functions, which are crucial for differentiation, development, and all aspects of normal functioning of animals. For

instance, they constitute the major blood group and other antigens, serve as informational molecules in cell–cell and cell–molecule interactions, function as receptors for biological processes, and assist in protein folding, targeting, and secretion. The glycan moieties of glycoconjugates are also involved in tissue organization, trafficking of lymphocytes, cell signaling, and immune regulation. In many cases, the true functions of glycan moieties are still not fully understood. Research during the past several decades and recent technological developments have deciphered the structures of thousands of glycans and substantially increased our knowledge of their biological roles. However, it is not exaggerating to state that what we know about the functions of these molecules represents only the tip of an iceberg.

Glycoconjugates occur extensively in all cells and tissues of animals, including those of the nervous system (Brockhausen and Stanley 2017; Brooks et al. 2002; Flynn et al. 2021; Ferguson et al. 2017; Gabius 2009; Haltiwanger et al. 2017; Mutalik and Gupton 2021; Prestegard et al. 2017; Schnaar and Kinoshita 2017; Stanley et al. 2017; Stanley and Cummings 2017; Taylor and Drickamer 2011; Varki and Kornfeld 2017; Varki et al. 2017a). They are found in tissue matrices and extracellular fluids such as serum, spinal fluid, saliva, and are highly abundant in respiratory, gastrointestinal, and urogenital mucus. However, the main reason for the increased interest in these molecules is that they occur ubiquitously on cell surfaces, where they play important roles in many biological processes. The intrinsic plasma membrane glycoconjugates of cells have their glycan moieties projecting outward far and wide and thus are available for functional interactions. The anionic sugar residues of glycans, which impart hydrophilicity and bestow negative charges to the cell surface, are important determinants in the social behavior of cells.

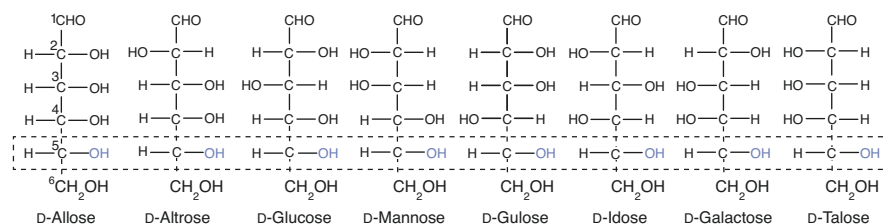
In this introductory chapter, we will briefly discuss (1) the nature of the monomeric building blocks of glycans, (2) how they are linked to one another to form glycans, (3) how glycans are conjugated to proteins, lipids, and small RNAs to form different glycoconjugates, and (4) provide an overview of the classification, key structural features, and functions of glycans. Since it is impossible to cover all aspects of glycan biology in this brief overview, readers are referred to more detailed books, monographs, and reviews for additional information (Allen and Kisailus 1992; Bertozzi and Rabuka 2008; Brockhausen and Stanley 2017; Brooks et al. 2002; Ferguson et al. 2017; Gabius 2009; Haltiwanger et al. 2017; Miljkovic 2010; Prestegard et al. 2017; Schnaar and Kinoshita 2017; Stanley et al. 2017; Stanley and Cummings 2017; Taylor and Drickamer 2011; Varki 2017; Varki and Gagneux 2017; Varki and Kornfeld 2017; Varki et al. 2017a, b, 2022; Voet and Voet 2010; Yuriev and Ramsland 2012).

## 2 Monosaccharides: Building Blocks of Glycans

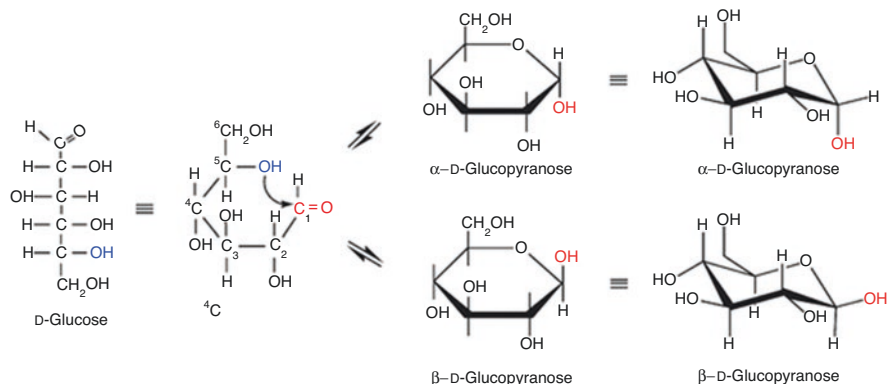
The structural complexity of glycans arises from the fact that monosaccharides, the monomeric units or building blocks of these molecules, have multiple functional groups and exhibit stereoisomerism (Allen and Kisailus 1992; Bertozzi and Rabuka

2008; Brooks et al. 2002; Gabius 2009; Miljkovic 2010; Seeberger 2017; Taylor and Drickamer 2011; Varki and Kornfeld 2017; Voet and Voet 2010; Yuriev and Ramsland 2012). Monosaccharides (generally called sugars) are polyhydroxy aldehydes or ketones with the general formula  $(\text{CH}_2\text{O})_n$  and are referred to as aldoses or ketoses, respectively. Both aldoses and ketoses are classified into different groups based on the number of carbon atoms they contain; for example, tetroses, pentoses, and hexoses are aldoses containing 4, 5, and 6 carbon atoms, respectively. Each of these groups is further classified into D- and L-series. All D-sugars have the same stereochemistry (i.e., identical absolute configuration) as D-glyceraldehyde at the asymmetric carbon atom that is most remote (e.g., C-5 in hexoses; Fig. 1.1) from the carbonyl group. As mentioned above, a detailed discussion of the structures and stereochemistry of various sugars is beyond the scope of this chapter as it is available in the introductory chapters of biochemistry textbooks and in specialty glyco-biology books (Bertozzi and Rabuka 2008; Brooks et al. 2002; Gabius 2009; Miljkovic 2010; Seeberger 2017; Taylor and Drickamer 2011; Varki and Kornfeld 2017; Voet and Voet 2010). The predominant constituents of the glycan moieties of animal glycoconjugates are hexoaldoses and their derivatives. The structures and stereochemistry of the eight possible D-series hexoses in open-chain Fischer projection formula are depicted in Fig. 1.1; the L-series sugars are the mirror images of D-sugars. Of the eight hexoses shown in Fig. 1.1, D-galactose and D-mannose are found widely in glycoproteins. D-Glucose is present in glycolipids but is rarely found in glycoproteins. Glucose is also the biosynthetic precursor for all other sugars, which occur in nature. Similarly, there are four each of the D-series and L-series pentoses, namely, ribose, arabinose, xylose, and lyxose. Of these, xylose occurs in animals as a constituent of proteoglycans.

Because of the tetrahedral bond angle requirement for carbon, sugars are not present as highly strained open-chain linear structures. They are folded in such a way that either C-4 or C-5 hydroxyl groups come in close proximity to the



**Fig. 1.1** Structures of eight possible D-aldohexoses are shown as open-chain Fischer projection formulas. They all have the same stereochemistry as that of D-glyceraldehyde at C-5, the asymmetric carbon that is most remote from the carbonyl group. The eight L-aldohexoses are the mirror images of these D-sugars. Please see refs. (Bertozzi and Rabuka 2008; Brooks et al. 2002; Seeberger 2017; Taylor and Drickamer 2011; Varki and Kornfeld 2017; Voet and Voet 2010) for the structures of aldopentoses and other monosaccharides. Ketopentoses and ketohexoses are not present as constituents of the glycan moieties of animal glycoconjugates



**Fig. 1.2** The folding of open-chain monosaccharides (e.g., D-glucose shown here) to relieve carbon bond angle strain facilitates intramolecular nucleophilic condensation of the C-5 hydroxyl group to the carbonyl group. This intramolecular reaction results in the formation of cyclic hemiacetal structures and the creation of a chiral center at carbon atom 1 (C-1). Therefore, the majority of sugars exist primarily as two thermodynamically stable six-membered cyclic structures called  $\alpha$ - and  $\beta$ -anomers. Haworth projection formulas and the chair conformational structures of  $\alpha$ -D-glucopyranose and  $\beta$ -D-glucopyranose are also shown. Some pentoses and hexoses can exist as five-membered cyclic structures see refs. (Bertozzi and Rabuka 2008; Brooks et al. 2002; Seeberger 2017; Taylor and Drickamer 2011; Varki and Kornfeld 2017; Voet and Voet 2010)

electrophilic carbonyl group and react to form intramolecular hemiacetal bonds; Fig. 1.2 shows the cyclic structure of D-glucose. Thus, five or more carbon atom-containing monosaccharides exist as thermodynamically stable six-membered or five-membered cyclic structures, designated as pyranose or furanose, respectively. The formation of ring structures, which are depicted by Haworth projection formulas, results in the creation of an additional chiral center at C-1, and thus, each sugar exists as two isomers. These isomers are referred to as  $\alpha$ - and  $\beta$ -anomers, and the carbonyl carbon of sugar in the ring form is the anomeric carbon. By convention, hexopyranoses in which the  $-\text{OH}$  group at C-1 and the  $-\text{CH}_2\text{OH}$  group at C-5 have a *trans* or *cis* configuration when depicted by Haworth formulas are named  $\alpha$ - and  $\beta$ -anomers, respectively (Fig. 1.2). In aqueous solutions the cyclic anomers are in equilibrium with open chain forms having either a free aldehyde or a keto group. This allows sugars to display the characteristic properties of aldehydes and ketones by shifting the equilibrium toward the open chain forms. For example, all aldoses exhibit reducing property. If the anomeric hydroxyl group of a sugar in cyclic structure is locked in covalent bond formation, for example, with alcohols forming glycosides, the reducing property of the sugar is lost.

Two sugars differing in configuration at a specific carbon, other than C-1, are referred to as epimers. Thus, glucose and mannose, which differ in configuration only at C-2, are C-2 epimers. Similarly, glucose and galactose, which differ in configuration only at C-4, are C-4 epimers, and D-glucuronic acid and L-iduronic acid

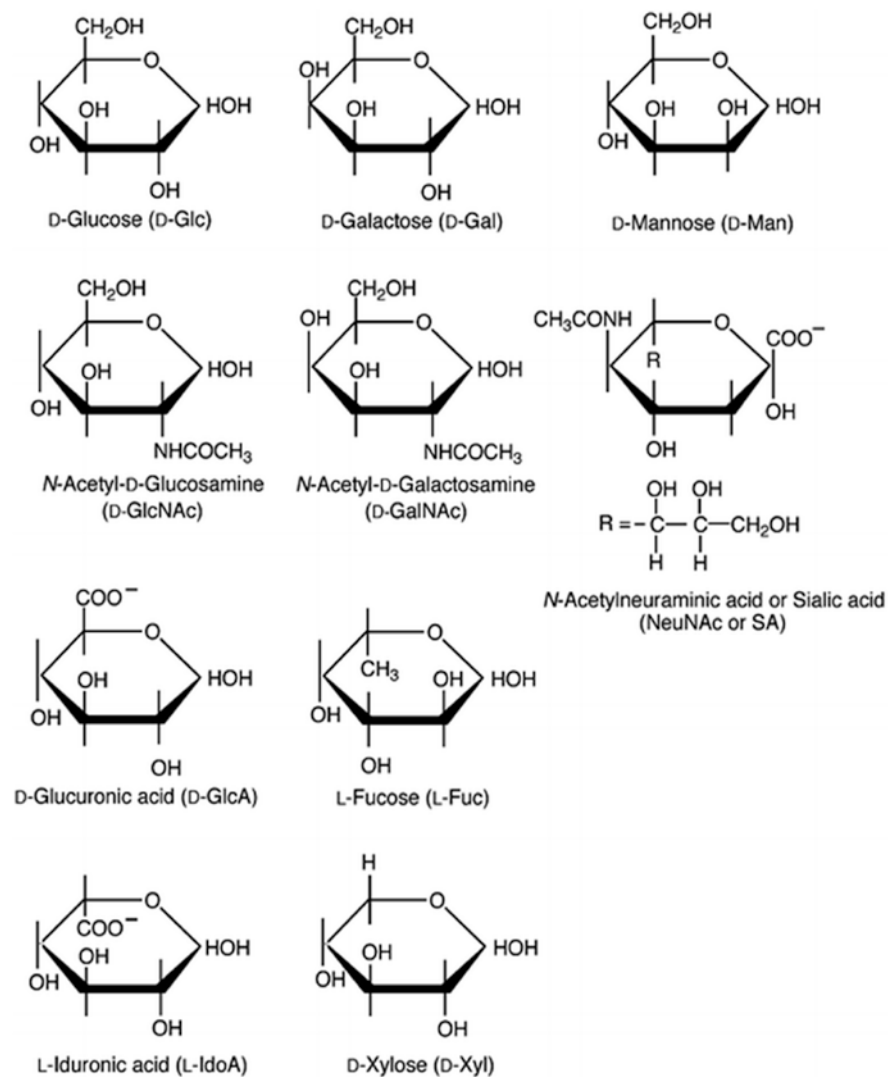


(see below) are C-5 epimers. Because mannose and galactose differ in configuration at more than one position, they are not epimers; they are isomers.

The ring carbon and oxygen atoms of six-membered pyranose structures of aldohexoses and their derivatives, including those that constitute glycoconjugate glycans, do not adopt coplanar structures as depicted in Haworth projection formulas (Fig. 1.2). To relieve bond angle strain and steric interactions between bulky functional groups, in most cases, sugars assume “chair” conformations with a large number of different arrangements of their various functional groups in space. The conformations in which bulky substituents occupy equatorial positions (lying in parallel to plane of the ring) are the more stable ones. The stable chair conformations of  $\alpha$ - and  $\beta$ -anomers of D-glucose are illustrated in Fig. 1.2.

As mentioned above, D-galactose (D-Gal), D-mannose (D-Man), D-glucose (D-Glc), and D-xylose (D-Xyl) are the unmodified sugars that occur as constituents of glycans in animal glycoconjugates (Bertozzi and Rabuka 2008; Brooks et al. 2002; Gabius 2009; Seeberger 2017; Taylor and Drickamer 2011). In addition, several modified forms of sugars are also common constituents of glycans. These include: 6-deoxy-L-galactose known as L-fucose (L-Fuc); the C-6 carboxyl derivative of D-Glc and L-idose (L-Ido), called D-glucuronic acid (D-GlcA) and L-iduronic acid (L-IdoA), respectively; and the *N*-acetylated forms of 2-amino-2-deoxy-D-glucose (D-GlcN) and 2-amino-2-deoxygalactose (D-GalN), called *N*-acetyl-D-glucosamine (D-GlcNAc) and *N*-acetyl-D-galactosamine (D-GalNAc), respectively. Other types of modified sugars found in animal glycans have certain hydroxyl groups acetylated, sulfated, or phosphorylated. An unusual 9-carbon monosaccharide acid widely distributed in animals is D-neuraminic acid (5-amino-3,5-dideoxy-D-nonulosonic acid) (Yu and Ledeen 1969), which has  $-\text{COOH}$ ,  $-\text{C}=\text{O}$ , and  $-\text{NH}_2$  functional groups (Schauer 2004; Varki 1992; Varki et al. 2017a). The amino group is either acetylated ( $-\text{NHCOCH}_3$ ) or glycolylated ( $-\text{NHCOCH}_2\text{OH}$ ) resulting in *N*-acetylneuraminic acid (NANA, NeuAc) or *N*-glycolylneuraminic acid (NeuGc); humans synthesize only NeuAc, but traces of NeuGc are found as a result of eating meat (Bardor et al. 2002). Some of the hydroxyl groups of these neuraminic acids are either acetylated, methylated, or sulfated forming a family of more than 20 derivatives, which are collectively called sialic acid (Schauer 2004; Varki 1992; Varki et al. 2017a). Sialic acid occurs abundantly in brain as a constituent of glycolipids (Nagai and Iwamori 1995; Wang et al. 1998).

All these modifications of monosaccharides contribute to the incredible structural diversity of glycans. The monosaccharides and their derivatives commonly found as constituents of glycans of animal glycoconjugates are shown in Fig. 1.3; note that, of these sugars, L-iduronic acid occurs only in proteoglycans. The abbreviations and symbols of monosaccharides commonly used to depict structures of *N*-glycans, *O*-glycans and glycosaminoglycans, and the glycan moieties of glycolipids are shown in Table 1.1 (Neelamegham et al. 2019). The symbol nomenclature for glycans (SNFG), hosted by the National Center for Biotechnology Information (NCBI), is available at [www.ncbi.nlm.nih.gov/glycans/snfg.html](http://www.ncbi.nlm.nih.gov/glycans/snfg.html).





















**Fig. 1.3** Haworth projection formulas of monosaccharides and their derivatives found in glycans of animal glycoconjugates. Note that of these ten sugars, eight are D-sugars and two are L-sugars

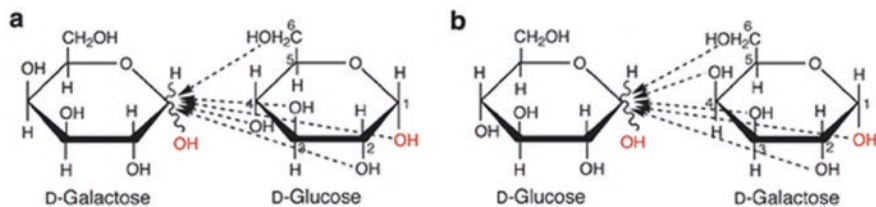
### 3 Formation of Oligosaccharides

The anomeric hydroxyl group of monosaccharides is more reactive than the other hydroxyl groups and undergoes protonation, becoming a relatively good leaving group (Bertozzi and Rabuka 2008; Brooks et al. 2002; Miljkovic 2010; Seeberger 2017). This makes C-1 relatively electrophilic and susceptible to attack by negatively charged or electron-rich atoms such as the oxygen of hydroxyl groups. Thus,

**Table 1.1** Letter abbreviations and symbols of monosaccharides used in writing the structures of glycans of glycoconjugates

Monosaccharide	Abbreviation	Symbol
Glucose	Glc	
<i>N</i> -Acetylglucosamine	GlcNAc	
<i>N</i> -Glucosamine	GlcN	
Galactose	Gal	
<i>N</i> -Acetylgalactosamine	GalNAc	
Galactosamine	GalN	
Mannose	Man	
<i>N</i> -Acetylmannosamine	ManNAc	
Mannosamine	ManN	
Fucose	Fuc	
Xylose	Xyl	
<i>N</i> -Acetylneuraminic acid	Neu5Ac	
<i>N</i> -Glycolylneuraminic acid	Neu5Gc	
2-Keto-3-deoxynanonic acid	Kdn	
Glucuronic acid	GlcA	
Iduronic acid	IdoA	
Galaturonic acid	GalA	
Mannuronic acid	ManA	

alcohols and other compounds containing hydroxyl groups can condense with the carbonyl group of monosaccharides, and the bond formed by this condensation is called a glycosidic bond or a glycosidic linkage (Bertozzi and Rabuka 2008; Brooks et al. 2002; Gabius 2009; Seeberger 2017; Taylor and Drickamer 2011). If the attacking nucleophile is a hydroxyl group of another monosaccharide, then the two monosaccharides are linked to each other, forming a disaccharide (Fig. 1.4). When a glycosidic bond is formed by involving the anomeric hydroxyl of one sugar with a non-anomeric hydroxyl of another sugar, the anomeric hydroxyl of the latter sugar is free. Therefore, this sugar retains its reducing property and is called the reducing end of the resulting disaccharide. The other monosaccharide moiety whose anomeric group is involved in the stable glycosidic bond has no reducing power and is called the nonreducing end sugar. The nonreducing and reducing ends of linear oligo- and polysaccharide chains are analogous to the N- and C-terminal amino acids of polypeptides. The  $\alpha$ - or  $\beta$ -anomer of one sugar can form different glycosidic bonds with multiple hydroxyl groups (five in the case of hexopyranoses) of a second sugar to form several different disaccharides. Thus, two molecules of the same sugar, for example, glucose, can form a total of 11 disaccharides, and two different monosaccharides can form 20 disaccharides (Fig. 1.4).



**Fig. 1.4** Glycosidic bond formation between the  $\alpha$ - or  $\beta$ -anomeric hydroxyl group of D-galactopyranose and the hydroxyl groups on carbons 2, 3, 4, and 6 of D-glucopyranose results in eight different disaccharides with glucose at the reducing end (a). When the positions of the sugars are reversed as in (b), eight disaccharides with galactose at the reducing end are formed. Four additional nonreducing disaccharides are formed when the anomeric hydroxyls of both sugars are involved in the interaction

An additional complexity is involved when more than two sugars are attached to form larger oligosaccharides, and polysaccharides (Bertozzi and Rabuka 2008; Brooks et al. 2002; Prestegard et al. 2017). For example, in the case of trisaccharides that are formed from three molecules of the same sugar, D-Glc, the third molecule can be attached to either the nonreducing glucose of disaccharides, Glc–Glc, to form linear trisaccharides [Glc–Glc–Glc] or the reducing end glucose to form branched trisaccharides [Glc–(Glc)–Glc]. Thus, oligosaccharides have three ways of generating structural diversity: one is by using different hydroxyl groups, the second is by the formation of  $\alpha$ - and  $\beta$ -anomeric linkages, and the third is by branching. Thus, three molecules of the same sugar can form 176 trisaccharides, and three different sugars can form 1056 trisaccharides. Four or more different sugars can form several thousands of tetra- or larger oligosaccharides. Note that linear oligosaccharides and polysaccharides will have one each of reducing and nonreducing sugar ends. Whereas, branched oligosaccharides and polysaccharides will have one reducing and multiple nonreducing sugar ends and the number of nonreducing ends indicate the degree of branching.

Since several sugars (see Fig. 1.3) and their acetylated, methylated, and sulfated derivatives constitute the glycan moieties of glycoconjugates, the combinatorial diversity of glycan structures is incredibly large and literally thousands of glycans occur in glycoconjugates (Bertozzi and Rabuka 2008; Brooks et al. 2002; Gabius 2009; Seeberger 2017; Taylor and Drickamer 2011; Varki and Kornfeld 2017). Since a single glycoprotein molecule may contain hundreds of different glycans at different glycosylation sites and even one glycosylation site may contain different glycan chains, determining the structures and understanding functional roles of glycans is challenging. However, recent technological advancements, including glycomics and glycoproteomics using mass spectrometry, glycan arrays, glycoinformatics (Campbell et al. 2017; Cummings et al. 2017; Esko et al. 2017; Mulloy et al. 2017; Leymarie and Zaia 2012; Li et al. 2009; Nishimura 2011; North et al. 2009; Orlando 2013; Rudd et al. 2017; Schiel 2012; Taylor and Drickamer 2011; Zaia 2010), have greatly eased structural determination. The structures of tens of thousands of glycans have been determined (Taylor and Drickamer 2011;

Consortium for Functional Glycomics: <http://www.functionalglycomics.org/glycomics/common/jsp/firstpage.jsp>). However, as mentioned above, our understanding of the functional roles of glycans is still limited.

## 4 Classification of Glycans

The glycan moieties of glycoconjugates (glycoproteins, glycolipids, proteoglycans, and glycoRNAs) are classified into two major groups: (1) *N*-glycans and (2) *O*-glycans (Bhavanandan and Furukawa 1995; Brockhausen and Stanley 2017; Brooks et al. 2002; Fukuda 2000; Haltiwanger et al. 2017; Patsos and Corfield 2009; Stanley et al. 2017; Stanley and Cummings 2017; Taylor and Drickamer 2011; Varki and Kornfeld 2017; Zuber and Roth 2009). *N*-Glycans are linked via an *N*-glycosidic bond formed between the reducing terminal of GlcNAc and the amide nitrogen atom of asparagine (Asn) residues of proteins that are found in the sequon Asn-Xaa-Ser/Thr, where Xaa is any amino acid other than proline. Although serum glycoproteins such as alpha-1-acid glycoprotein contain exclusively *N*-linked glycans, the vast majority of glycoproteins contain both *N*- and *O*-glycans. Some examples of these are fetuin, immunoglobulin A, human chorionic gonadotropin, and many cell surface glycoproteins. *O*-Glycans of glycoproteins are linked via an *O*-glycosidic bond formed between anomeric hydroxyl groups of sugars and the hydroxyl group of serine (Ser) or threonine (Thr) residues of proteins. The sugars involved in *O*-glycosidic linkages are predominantly GalNAc and to lesser extents Man, Fuc, Glc, and Gal. In the case of mucins and the mucin-type glycoproteins of the plasma membrane and secreted glycoproteins, the glycan substituents are linked via GalNAc. In mannan-type yeast glycoproteins, and in certain neuronal glycoproteins and proteoglycans and in a few animal muscle glycoproteins, *O*-glycans are linked through  $\alpha$ -Man to Ser/Thr (Brooks et al. 2002; Kleene and Schachner 2004; Krusius et al. 1986; Nakamura et al. 2010; Patsos and Corfield 2009; Stanley and Cummings 2017). In proteoglycans, the glycosaminoglycan polysaccharide chains are *O*-linked via  $\beta$ -D-Xyl to Ser residues of proteins (see below). In proteins such as Notch, coagulation factors, and urokinase-type and tissue-type plasminogen activator, *O*-glycans are linked through  $\alpha$ -L-Fuc to Ser/Thr (Brooks et al. 2002; Gebauer et al. 2008; Haltiwanger et al. 2017; Luther and Haltiwanger 2009; Patsos and Corfield 2009; Stanley and Cummings 2017). In collagens, Gal $\beta$ 1- monosaccharides and Glc $\alpha$ 1-2Gal $\beta$ 1- disaccharides are *O*-linked to the hydroxyl group of hydroxylysines. In various nuclear and cytoplasmic proteins, a special type of glycosylation involving single  $\beta$ -GlcNAc *O*-linked to Ser/Thr is found in almost all eukaryotes and believed to be involved in the regulation of signaling, transcription, and various other biological processes. This special type of glycan modification is discussed by Lagerlof in Chap. 9. The glycans of glycolipids are *O*-linked through their terminal  $\beta$ -Glc or  $\beta$ -Gal residues to C-1 of ceramides.

As mentioned earlier, recently a new class of glycoconjugates consisting of sialylated *N*-glycans and small noncoding RNAs was reported (Flynn et al. 2021).

The authors found these glycoRNAs on the surface of multiple cultured as well as *in vivo* mammalian cell types. Clearly, further research is needed to understand several aspects of these, apparently a new class of glycoconjugates. Important questions to be answered about these glycoconjugates include the nature of glycosidic linkage between the glycan and RNA, the mode of association with the plasma membrane and their functions.

## 5 Structural Features of *N*-Glycans

All *N*-glycans have a common pentasaccharide “inner core,” consisting of three Man and two GlcNAc (residues shown in blue/bold in Fig. 1.5). The occurrence of this conserved core structure is due to the involvement of a common biosynthetic pathway for all types of *N*-glycan chains found in glycoproteins (Brooks et al. 2002; Fukuda 2000; Stanley et al. 2017; Stanley and Cummings 2017; Taylor and Drickamer 2011; Zuber and Roth 2009). The *N*-glycans are classified into three subgroups based on the types of modifications peripheral to the inner core structures and are referred to as the (1) oligomannose or high mannose type, (2) complex type, and (3) hybrid type (Fig. 1.5). All these subgroups show enormous structural variations due to different types of peripheral modifications, including variation in chain lengths, attachment of different terminal sugars, incomplete addition of terminal sugars, and incomplete chain formation.

Oligomannose-type *N*-glycans contain only Man and GlcNAc residues (Fig. 1.5a), and the heterogeneity in this type of *N*-glycans is due to variations in the numbers and locations of outer Man residues linked to the two  $\alpha$ -Man of the inner core. Complex-type *N*-glycans (Fig. 1.5b–d) exhibit a far wider spectrum of structural variation compared to high-mannose-type *N*-glycans (Brooks et al. 2002; Fukuda 2000; Stanley et al. 2017; Stanley and Cummings 2017; Taylor and Drickamer 2011; Zuber and Roth 2009). Typically, each of the two  $\alpha$ -Man residues of the inner core is substituted with one or more *N*-acetylglucosamine (Gal $\beta$ 1-4GlcNAc $\beta$ 1-) moieties, which form outer chains. Those *N*-glycans that carry two *N*-acetylglucosamine substituents—one chain on each of the  $\alpha$ -linked Man are called complex-type di-antennary oligosaccharides (Fig. 1.5b). The glycans that contain more than one *N*-acetylglucosamine substituent on either one or both  $\alpha$ -Man are called multi-antennary structures and are referred to as tri-, tetra-, penta-antennary based on the total number of antennas present; an example of a complex tetra-antennary structure is shown in Fig. 1.5d. In *N*-glycans of many animal glycoproteins, the *N*-acetylglucosamine moieties are elongated with repetitive sequential additions of  $\beta$ -GlcNAc and  $\beta$ -Gal, resulting in poly-*N*-acetylglucosamine chains containing from two to as many as fifty or more of the repeating disaccharide, -3Gal $\beta$ 1-4GlcNAc $\beta$ 1- (type 2 *N*-acetylglucosamine), or -3Gal $\beta$ 1-3GlcNAc $\beta$ 1- (type 1 *N*-acetylglucosamine) units; for example, Fig. 1.5d in which  $n = 2$  to  $\sim 50$ . The sugar chains are terminated by the substitution of  $\beta$ -Gal with  $\alpha$ (2-3)- and/or  $\alpha$ (2-6)-linked SA,  $\alpha$ (1-2)-linked Fuc,  $\alpha$ (1-3)-linked Gal,  $\alpha$ (1-3)-linked GalNAc or sulfate

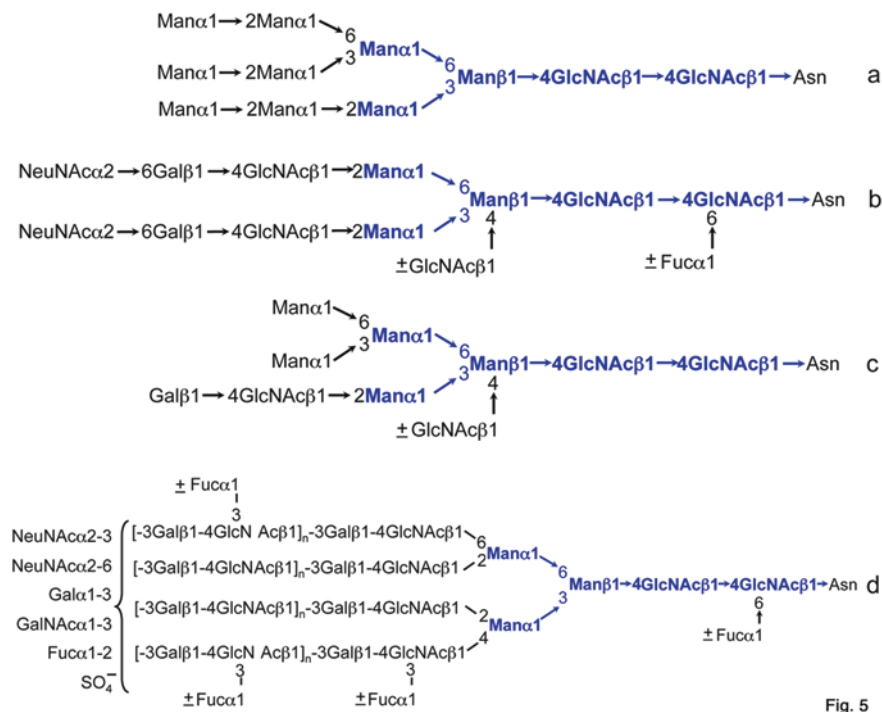


Fig. 5

**Fig. 1.5** The structural features of *N*-glycans of animal glycoproteins. Because of the involvement of a common biosynthetic lipid-linked oligosaccharide precursor, all *N*-glycans contain a common inner core structure, consisting of three D-mannose (Man) and two *N*-acetyl-D-glucosamine (GlcNAc) residues (shown in *blue/bold*). After transferring to proteins, the common oligosaccharide precursor is modified differently, while retaining the pentasaccharide core, to yield three subclasses of *N*-glycans referred to as high-mannose- or oligomannose-type (**a**), complex- or *N*-acetylglucosamine-type (**b**), and hybrid-type (**c**) *N*-glycans. The complex di-antennary *N*-glycans (**b**) may be further modified to form tri-antennary, tetra-antennary (e.g., **d**), and penta-antennary structures;  $n = 2$  to  $\sim 50$

groups. The terminal residues within one *N*-glycan may be the same sugar or two or more different  $\alpha$ -linked sugars (SA, Fuc, Gal or GalNAc). In some *N*-glycans, terminal  $\beta$ -Gal residues are unsubstituted or even absent, exposing  $\beta$ -GlcNAc as the terminal sugar. Additionally, a wide range of different types of substitutions on the inner and subterminal type 2 *N*-acetylglucosamine moieties exist, including substitution of inner GlcNAc with  $\alpha(1-3)$ -linked Fuc and that of  $\beta$ -Gal with  $\beta(1-6)$ -linked GlcNAc on which *N*-acetylglucosamine chains can be formed and elongated (Stanley et al. 2017; Stanley and Cummings 2017). Type 1 *N*-acetylglucosamine ( $-3\text{Gal}\beta 1-3\text{GlcNAc}\beta 1-$ ) structures carrying an  $\alpha(1-4)$ -linked Fuc substitution on GlcNAc also occur (Brockhausen and Stanley 2017; Brooks et al. 2002; Fukuda 2000; Stanley et al. 2017; Stanley and Cummings 2017). Moreover,  $\beta$ -Man of the core structure can be substituted with a single GlcNAc, forming bisecting structures (Fig. 1.5b, c). The *N*-glycans of secretory glycoproteins and erythrocyte surface

proteins exhibit blood A, B, H, Lewis<sup>a</sup>, Lewis<sup>b</sup>, and other blood group antigenic structures (Brooks et al. 2002; Schachter and Brockhausen 1992; Cummings 1992; Stanley and Cummings 2017). Many *N*-glycans of animal cells carry sialyl Lewis<sup>x</sup> and related antigens, and their expression is regulated during development and differentiation; highly expressed in fetuses but rarely in adults. Sialyl Lewis<sup>x</sup> and related structures are also highly expressed by cancer cells and therefore, are referred to as onco-fetal antigens. The hybrid-type *N*-glycans contain structural features of both oligomannose-type and complex-type oligosaccharides (see Fig. 1.5c). These glycans also show variations in the number of Man substitutions and length and substitutions of the complex-type chain, resulting in multiple structures. Because of all the different modifications mentioned above, literally tens of thousands of *N*-glycans occur as constituents of various glycoproteins.

In the central nervous system, in addition to many of the *N*-glycans described above, some unique structures are present (Bruses and Rutishauser 2000; Fukuda 2000; Gascon et al. 2007; Hildebrandt and Dityatev 2013; Kleene and Schachner 2004; Leeden and Wu 2009; Stanley et al. 2017; Stanley and Cummings 2017). For example, the neuronal cell adhesion molecules (NCAMs) of developing brain contain  $\alpha(2-8)$ -linked polysialic acid chains made up of as many as 50 or more sialic acid residues that are attached to  $\beta$ -Gal of one or more outer chains of the *N*-glycans. This unique modification is implicated in cell migration, neurite outgrowth, and the development of the nervous system. Another example of an uncommon glycan present in neural cells is the HNK-1 antigen, sulfate-3GlcA $\beta$ 1-3Gal $\beta$ 1-4GlcNAc-, found as a terminal structure in glycan chains (Fukuda 2000; Haltiwanger et al. 2017; Gebauer et al. 2008; Kleene and Schachner 2004; Luther and Haltiwanger 2009; Schachter and Brockhausen 1992; Stanley and Cummings 2017; Sytnyk and Leshchynska 2021). HNK-1 epitope specificity is provided by the terminal sulfate-3GlcA $\beta$ 1-3Gal $\beta$ 1- moiety (Tokuda et al. 1998). The HNK1 antigen was originally identified in human natural killer cells and subsequently found as an antigen involved in the autoimmune disease, peripheral demyelinating neuropathy (Ariga et al. 1987). The antigen is regulated both temporally and spatially in the developing nervous system (Schwartz et al. 1987) and is found in several neuronal cell adhesion molecules, including NCAM, myelin-associated protein, contactin, L1, and P0. HNK-1 mediates cell–cell and cell–substrate interactions (Ariga 2011; Fukuda 2000; Kizuka and Oka 2012; Kleene and Schachner 2004; Morita et al. 2008; Stanley and Cummings 2017).

## 6 Structural Features of *O*-Glycans

As discussed above, mucin-type glycans that are linked via  $\alpha$ -GalNAc to Ser/Thr of proteins are the most abundant *O*-linked glycans in eukaryotic cells, including cells of the central nervous system (Bhavanandan and Furukawa 1995; Brockhausen and Stanley 2017; Brooks et al. 2002; Patsos and Corfield 2009; Schachter and Brockhausen 1992; Taylor and Drickamer 2011). The  $\alpha$ -GalNAc *O*-linked to Ser/



Thr can be substituted with  $\beta$ -Gal and/or  $\beta$ -GlcNAc or with an additional  $\alpha$ -GalNAc residue, forming eight distinct core structures (Fig. 1.6). The wide variety of *O*-glycans found in animal cells is formed by the addition of different sugars to the core structures. *O*-Glycans having core structures 1–4 are widely distributed in animal cells and tissues, and those with core structures 5–8 are rarely found (Brockhausen and Stanley 2017; Brooks et al. 2002; Fukuda 2000; Schachter and Brockhausen 1992; Taylor and Drickamer 2011). The unsubstituted GalNAc $\alpha$ 1 linked to Ser/Thr constitutes the Tn antigen (Brockhausen and Stanley 2017; Brooks et al. 2002). The substitution of  $\alpha$ -GalNAc $\alpha$ 1 linked to Ser/Thr with sialic acid forms NeuAc $\alpha$ 2-6GalNAc $\alpha$ 1, the simplest sialylated disaccharide called the sialyl Tn antigen. The core 1 structure, Gal $\beta$ -1-3GalNAc $\alpha$ 1, is known as the Thomsen–Friedenreich antigen or T antigen. The Tn and T antigens are found at low levels in normal tissues, but are highly expressed in certain tumors (Brockhausen and Stanley 2017; Brooks et al. 2002). The  $\beta$ -Gal residue of core 1 structure Gal $\beta$ -1-3GalNAc $\alpha$ 1-Ser/Thr can be substituted with  $\alpha$ (2-3)-linked NeuAc, and the  $\alpha$ -GalNAc can be substituted with  $\alpha$ (2-6)-linked NeuAc. Further, the  $\beta$ -Gal residue of the core 2

Core Type	Core Structure	Representative Examples
1	Gal $\beta$ 1-3GalNAc $\alpha$ -Ser/Thr	NeuNAc $\alpha$ 2-3Gal $\beta$ 1-3GalNAc $\alpha$ -Ser/Thr NeuNAc $\alpha$ 2-3Gal $\beta$ 1- <sub>3</sub> GalNAc $\alpha$ -Ser/Thr NeuNAc $\alpha$ 2- <sub>6</sub> GalNAc $\alpha$ -Ser/Thr
2	Gal $\beta$ 1- <sub>3</sub> GalNAc $\alpha$ -Ser/Thr GlcNAc $\beta$ 1- <sub>6</sub> GalNAc $\alpha$ -Ser/Thr	NeuNAc $\alpha$ 2-3Gal $\beta$ 1- <sub>3</sub> GalNAc $\alpha$ -Ser/Thr NeuNAc $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1- <sub>6</sub> GalNAc $\alpha$ -Ser/Thr GlcNAc $\beta$ 1-3Gal $\beta$ 1- <sub>3</sub> GalNAc $\alpha$ -Ser/Thr Fuca1-2Gal $\beta$ 1-4GlcNAc $\beta$ 1- <sub>6</sub> GalNAc $\alpha$ -Ser/Thr NeuNAc $\alpha$ 2-3Gal $\beta$ 1- <sub>3</sub> GalNAc $\alpha$ -Ser/Thr -O <sub>3</sub> S-O-3Gal $\beta$ 1-4GlcNAc $\beta$ 1- <sub>6</sub> GalNAc $\alpha$ -Ser/Thr $\pm$ Fuca1
3	GlcNAc $\beta$ 1-3GalNAc $\alpha$ -Ser/Thr	Fuca1-2Gal $\beta$ 1-3GlcNAc $\beta$ 1-3GalNAc $\alpha$ -Ser/Thr
4	GlcNAc $\beta$ 1- <sub>3</sub> GalNAc $\alpha$ -Ser/Thr GlcNAc $\beta$ 1- <sub>6</sub> GalNAc $\alpha$ -Ser/Thr	NeuNAc $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1- <sub>3</sub> GalNAc $\alpha$ -Ser/Thr NeuNAc $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1- <sub>6</sub> GalNAc $\alpha$ -Ser/Thr $\pm$ Fuca1
5	GalNAc $\alpha$ 1-3GalNAc $\alpha$ -Ser/Thr	
6	GlcNAc $\beta$ 1-6GalNAc $\alpha$ -Ser/Thr	
7	GalNAc $\alpha$ 1-6GalNAc $\alpha$ -Ser/Thr	
8	Gal $\alpha$ 1-3GalNAc $\alpha$ -Ser/Thr	

**Fig. 1.6** The structures of core sugar moieties of *O*-glycans and structures of representative *O*-glycans found in animal cells and tissues

structure can be substituted with  $\alpha(2-3)$ -linked NeuAc, and  $\beta$ -GlcNAc can be substituted with  $\beta(1-4)$ -linked Gal to form a type 2 *N*-acetylglucosamine structure (Fig. 1.6). Similarly, both  $\beta$ -GlcNAc of core 4 can be substituted with  $\beta(1,3)$ -linked Gal to form two branches having type 1 *N*-acetylglucosamine structure. As in the case of *N*-glycans, in both core 2 and core 4 structures, the *N*-acetylglucosamine is either terminated by the substitution of  $\alpha$ -linked NeuAc, Fuc, Gal, and GalNAc or elongated to form polymeric *N*-acetylglucosamine chains, which are then terminated with  $\alpha$ -linked sugars. The *O*-linked glycans of some animal mucins are also sulfated, typically at the terminal ends and/or on internal Gal and GlcNAc. Many *O*-glycans of secretory proteins also contain the repeating units of type 1 *N*-acetylglucosamine -3Gal $\beta$ 1-3GlcNAc $\beta$ 1- or type 2 *N*-acetylglucosamine -3Gal $\beta$ 1-4GlcNAc $\beta$ 1- structure (Brockhausen and Stanley 2017; Brooks et al. 2002; Patsos and Corfield 2009; Schachter and Brockhausen 1992; Taylor and Drickamer 2011). As in *N*-glycans, the inner  $\beta$ -Gal can be substituted with  $\beta(1-6)$ -linked GlcNAc to form a branched structure, which can also be elongated with *N*-acetylglucosamine moieties. In addition, the  $\beta$ -GlcNAc residues of type 1 and type 2 *N*-acetylglucosamine can be substituted with, respectively,  $\alpha(1-4)$ -linked and  $\alpha(1-3)$ -linked Fuc. These and the substitution of subterminal  $\beta$ -Gal with  $\alpha$ -linked sugars results in the formation of *O*-glycans carrying the blood group A, B, H, and Lewis antigens, and development- and differentiation-specific sialyl Lewis antigens (Brockhausen and Stanley 2017; Brooks et al. 2002; Patsos and Corfield 2009; Schachter and Brockhausen 1992; Taylor and Drickamer 2011). All the above mentioned and various other type of modifications give rise to numerous distinct *O*-glycans found in animal glycoproteins.

Although *O*-glycans linked via  $\alpha$ -GalNAc $\alpha$ 1-Ser/Thr are found in most glycoproteins, they are abundantly present in two groups of glycoproteins: (1) mucins produced by epithelia of salivary glands, and the respiratory, gastrointestinal and urogenital tracts, and (2) membrane-associated mucins (Bhavanandan and Furukawa 1995; Brockhausen and Stanley 2017; Brooks et al. 2002; Patsos and Corfield 2009; Schachter and Brockhausen 1992; Taylor and Drickamer 2011). Epithelial mucin glycoproteins contain hundreds of  $\alpha$ -GalNAc-linked *O*-glycans, which account for as much as 50–80% of the mass of mucin molecules. In these mucin molecules, the *O*-glycans are found clustered at certain regions of the protein backbones. Because of the high levels of *O*-glycan substitution, mucin molecules can hold large amount of water and assume extended structures. In addition, because of the high net negative charge imparted by sialic acid and sulfate groups, mucin molecules in solution are randomly oriented, exhibiting high viscosity. This property allows mucin to serve as a protective barrier for epithelia against physical abrasion and inhibit infection by functioning as decoys for the adherence of pathogens. The latter is a double-edged sword since pathogens such as influenza virus and *Helicobacter pylori* exploit these molecules for their invasion/attachments and infect the respiratory system and stomach, respectively. Abnormality in mucin structure/function is implicated in the pathobiology of several human diseases (Brooks et al. 2002; Hennes 2009; Haukedal and Freude 2020; Reily et al. 2019; Taylor and Drickamer 2011). These include

cystic fibrosis, chronic bronchitis, Crohn's disease, duodenal ulceration, colonic adenocarcinomas, infertility problems, and inflammatory ulcerative colitis.

Compared to secretory epithelial mucins, the carbohydrate content in many membrane-associated cell surface mucins is relatively less because they have fewer and shorter glycan chains. Examples of such glycoproteins are the erythrocyte membrane glycophorins, and most of the cell membrane and tissue matrix glycoproteins of animal tissues, including the central nervous system. Other examples of membrane-associated, mucins are human white blood cell-associated leukosialin (CD43, sialophorin), red blood cell membrane decay-accelerating factor, low-density lipoprotein receptor, platelet membrane-associated CD42b (glycocalicin), and human milk fat granule membrane glycoproteins. These glycoproteins contain hundreds of shorter *O*-glycans and are present as highly extended structures, projecting far beyond the cell surface (Bhavanandan and Furukawa 1995; Brooks et al. 2002; Fukuda 2000; Taylor and Drickamer 2011). As in the case of *N*-glycans, expression of the membrane-associated cell surface *O*-glycans is markedly increased or altered in malignantly transformed cells. Because of their important role in the pathobiology of cancer there has been extensive research on this subclass of *O*-glycans. These glycans are also widely implicated as ligands for selectins, which are involved in thrombosis, inflammation, allergy, auto immunity, and cancer metastasis. For example, the endothelial cell-associated mucin-type glycoproteins such as glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), CD34, and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) function as ligands for L-selectin, and the leukocyte membrane mucin-type glycoprotein called P-selectin glycoprotein ligand-1 (PSGL-1) has been shown to be a ligand for P-selectin and E-selectin. Thus, these mucins are involved in recognition, binding and recruitment of immune cells to the site of injury, and in homing of the circulating lymphocytes to lymph nodes (Homeister and Lowe 2000).

It has been shown that Fuc *O*-linked to the Ser/Thr residues of EGF-like repeat-containing proteins is substituted to form GlcNAc $\beta$ 1-3Fuc $\alpha$ 1-, Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Fuc $\alpha$ 1-, Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Fuc $\alpha$ 1, NeuAc $\alpha$ 2-6 Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Fuc $\alpha$ 1-oligosaccharides, which play important roles in intracellular signaling (Brooks et al. 2002; Gebauer et al. 2008; Haltiwanger et al. 2017; Kleene and Schachner 2004; Luther and Haltiwanger 2009; Stanley and Cummings 2017; Taylor and Drickamer 2011). Some glycoproteins and proteoglycans of the nervous system, and neuronal and muscle  $\alpha$ -dystroglycan, in addition to having the GalNAc-linked *O*-glycans, are modified with significant levels of *O*-glycans linked via  $\alpha$ -Man to Ser/Thr (Chai et al. 1999; Krusius et al. 1986; Nakamura et al. 2010). The  $\alpha$ -Man that is *O*-linked to Ser/Thr is substituted with either  $\beta$ 1-2-linked GlcNAc residues or  $\beta$ 1-2- and  $\beta$ 1-6-linked GlcNAc residues. These GlcNAc residues are further substituted by sequential addition of  $\beta$ (1-4)-linked Gal and terminated with NeuAc or 3-sulfated glucuronic acid; the latter forming the HNK-1 epitope (Ariga 2011; Fukuda 2000; Kleene and Schachner 2004; Kizuka and Oka 2012; Morita et al. 2008; Stanley and Cummings 2017). Deficiency in the *O*-Man-linked oligosaccharide modification in nervous tissues is associated with several types of severe brain and eye abnormalities, mental retardation, severely impaired mobility, muscle

weakness, reduced muscle bulk, and dystrophic muscle (Kleene and Schachner 2004; Nakamura et al. 2010).

## 7 Biosynthesis of *N*- and *O*-Glycans

One important difference in the biosynthesis of glycans compared to nucleic acids and proteins is that the synthesis of glycans is not template dependent. Instead the genetic control is exerted by the expression of enzymes called glycosyltransferases, which catalyze the biosynthesis of glycans (Brockhausen and Stanley 2017; Brooks et al. 2002; Cummings 1992; Schachter and Brockhausen 1992; Stanley et al. 2017; Taylor and Drickamer 2011; Zuber and Roth 2009). The glycosidic bond formation involves the transfer of sugars from activated donors (nucleotide sugars) to acceptor monosaccharide or oligosaccharide substrates, which exist either as free molecules or as moieties linked to proteins/lipids. The activated sugars are derivatives of either uridine or guanine diphosphate and of cytidine monophosphate in the case of sialic acids. The biosynthesis occurs at several locations, cytoplasm, lumen of the endoplasmic reticulum (ER), and lumen of the Golgi. The nucleotide sugars are formed in the cytoplasm and, for glycan biosynthesis, are transported to the Golgi by membrane transporters. In contrast, the ER membrane has no nucleotide-sugar transporters; sugar donors for glycan synthesis in the ER lumen are dolichol phosphate sugars formed by the transfer of sugars from nucleotide sugars in the cytoplasm to dolichol phosphate in the membrane. The dolichol phosphate sugars in which the sugar residues face the cytoplasmic side are then flipped to the luminal side and thus can donate sugar residues to acceptor molecules. *N*-Glycans are initially synthesized in the ER as dolichol diphosphate-linked high-mannose-type oligosaccharides containing three terminal Glc, eight Man, and two internal GlcNAc residues, and the oligosaccharide is transferred en bloc to the Asn residues of Asn-X-Ser/Thr motifs of polypeptide chains while the latter are still being synthesized. The oligosaccharides on proteins are then processed beginning in the ER but mostly in the Golgi to complex-type or hybrid oligosaccharides (Fig. 1.5). The *O*-glycans and glycosaminoglycans are synthesized in the Golgi by the addition of sugars sequentially after the first sugar is added to the Ser/Thr residues of proteins.

The general reaction for glycan biosynthesis is represented as the following: nucleotide-sugar donors + acceptor substrates  $\rightarrow$  products + nucleotide diphosphate (Freeze et al. 2017). The reaction is driven by the energy released by the hydrolysis of nucleoside diphosphate to yield nucleoside monophosphate and phosphate. Sugars are transferred from donor nucleotide sugars to different hydroxyl groups of acceptor monosaccharides, oligosaccharides, proteins, or lipids. The glycosidic linkages thus formed vary with respect to their positions and anomeric configurations. The glycosyltransferases determine the types of glycosidic linkages formed by exhibiting specificity toward the donor sugars and acceptor mono- or oligosaccharide substrates (Colley et al. 2017; Rini and Esko 2017). Therefore, it is the specificity of glycosyltransferases that primarily directs and controls the formation

of a particular glycan. In general, one glycosyltransferase is required for the formation of each type of glycosidic linkage between two sugars in glycans, although a few exceptions exist. The glycosyltransferases involved in *N*- and *O*-glycan biosynthesis are membrane-bound enzymes and are involved in an assembly line-like process in the synthesis of specific glycans. The biosynthesis of *N*- and *O*-glycans is discussed in detail in Chaps. 3 and 4, respectively.

## 8 Glycosphingolipids

Glycosphingolipids (GSLs) are a class of glycolipids that are conjugates of glycans and ceramide-containing lipids and are distributed widely in all organisms (Brooks et al. 2002; Hanafusa et al. 2020; Kopitz 2009; Kundu 1992; Leeden and Wu 2009; Schnaar and Kinoshita 2017; Taylor and Drickamer 2011). They are ubiquitous plasma membrane components and are primarily localized in the outer leaflet with their hydrophilic sugar moieties projecting outside of the cell. GSLs are abundantly found in the brain. The glycan moieties on the cell surface function as specific receptors for certain pituitary hormones, growth factors, viruses, and cholera, tetanus, and botulinum bacterial toxins. They also serve as specific determinants of cell–cell interactions and as tumor-associated antigens. Disorders of GSL catabolism are responsible for several genetic diseases, which are referred to as glycosphingolipid storage diseases. These diseases are caused by defects in specific lysosomal glycosidases, involved in the degradation of the glycan chains or in sphingolipid activator proteins. For example, Tay–Sachs and Gaucher’s diseases are caused by defects in *N*-acetyl- $\beta$ -hexosaminidase and  $\beta$ -glucosidase, respectively. The inborn errors of GSL catabolism are discussed in detail by Sandhoff and Sandhoff in Chap. 12.

## 9 Structural Features of the Glycan Moieties of Glycosphingolipids

In the majority of glycolipids, glycans are conjugated to the terminal (C-1) primary hydroxyl group of ceramides (Cer), which are *N*-acyl fatty acid derivatives of sphingosine. Both the fatty acid (acyl chain) and sphingosine moieties of ceramides are heterogeneous (Brooks et al. 2002; Hakomori 2003; Kopitz 2009; Kundu 1992; Leeden and Wu 2009; Schnaar and Kinoshita 2017; Taylor and Drickamer 2011). The acyl chain can be 14–26 carbons in length, saturated, unsaturated, or 2-hydroxylated. The sphingosine can be 14–20 carbons in length. The carbohydrate moieties of the GSLs consist of the following sugars: D-Glc, D-Gal, L-Fuc, D-GlcNAc, D-GalNAc, and NeuAc. The simplest GSLs are the monohexosylcerebrosides, glucocerebroside (Glc $\beta$ 1-1Cer), and galactocerebroside (Gal $\beta$ 1-1Cer). While the Glc residue of Glc $\beta$ 1-1Cer is the link sugar in complex GSLs having a

wide range of short to large glycan chains, the Gal residue of Gal $\beta$ 1-1Cer is elaborated only to a limited degree to form GSLs with only short glycan chains and the sulfatide, 3-*O*-sulfo-Gal $\beta$ 1-ceramide (Kundu 1992). Thus, the glycan chains of different compositions, linkage types, and chain lengths are formed by the sequential addition of other sugars to the core Glc residue linked to Cer. Further, like in the case of glycoprotein glycans, the glycan chain lengths of glycolipids in certain cells such as erythrocytes and granulocytes are highly extended with repeating *N*-acetylglucosamine units; fucosylation of the inner GlcNAc forms Lewis antigens (Fukuda et al. 1986; Fukuda and Hakomori 1982; Müthing 1996; Stanley and Cummings 2017). In addition, these chains may be terminated by one or more of  $\alpha$ -linked NeuAc, Fuc, Gal, or GalNAc to yield various blood group and sialyl Lewis antigens (Stanley and Cummings 2017). Based on the core structures of glycan moieties that are attached to the Cer moiety, GSLs are classified into different subclasses: lacto-, lactoneo-, globo-, isoglobo-, ganglio-, muco-, and gal-series (Table 1.2). Lactosylceramide (Gal $\beta$ 1-4Glc $\beta$ 1-1Cer) serves as the precursor for the synthesis of five families of GSLs. The GSLs that contain sialic acid are called gangliosides. As in glycoproteins, the structures of GSLs are defined by the specificity of glycosyltransferases and their relative levels and distribution in cells.

GSLs are present ubiquitously in cells, and the subclasses are differentially distributed in different tissues (Cuttillo et al. 2020; Hanafusa et al. 2020; Kundu 1992; Zhang et al. 2019). For example, gangliosides are found at significant levels in many animal cells, including red blood and immune cells. However, they are present at high levels in neural tissues, where they perform several important functions, including determining the fate of neural cells (Cuttillo et al. 2020; Kolte 2012; Yohe et al. 2001; also see Chap. 10). Among the non-sialylated GSLs, only Gal-Cer and sulfatide, SO<sub>3</sub>-3Gal-Cer, but not Glc-Cer are present in brain and other neural tissues. However, fetal and neonatal brains contain almost equal amounts of Gal-Cer and Glc-Cer. Erythrocytes contain mainly Glc-Cer and complex GSLs having large glycan chains, whereas the kidney and intestine have substantial levels of both Glc-Cer and Gal-Cer (Kundu 1992).

**Table 1.2** The core structures of major glycolipids found in animals

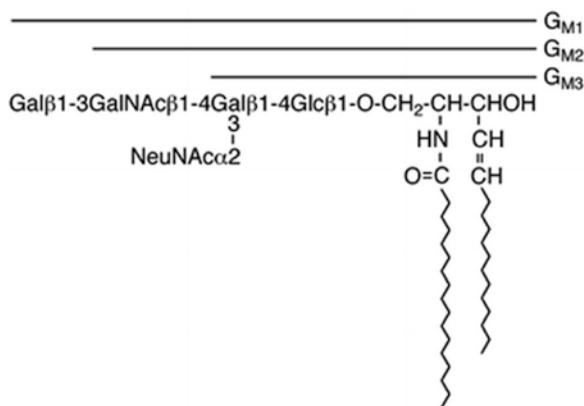
Type	Structure
Lacto	Gal $\beta$ 1-3GlcNAc $\beta$ 1-3 <b>Gal<math>\beta</math>1-4Glc<math>\beta</math>1-1-ceramide</b>
Lactoneo	Gal $\beta$ 1-4GlcNAc $\beta$ 1-3 <b>Gal<math>\beta</math>1-4Glc<math>\beta</math>1-1-ceramide</b>
Globo	GalNAc $\beta$ 1-3Gal $\alpha$ 1-4 <b>Gal<math>\beta</math>1-4Glc<math>\beta</math>1-1-ceramide</b>
Globoneo	GalNAc $\beta$ 1-4Gal $\alpha$ 1-3 <b>Gal<math>\beta</math>1-4Glc<math>\beta</math>1-1-ceramide</b>
Isoglobo	GalNAc $\beta$ 1-3Gal $\alpha$ 1-3 <b>Gal<math>\beta</math>1-4Glc<math>\beta</math>1-1-ceramide</b>
Ganglio	Gal $\beta$ 1-3GalNAc $\beta$ 1-4 <b>Gal<math>\beta</math>1-4Glc<math>\beta</math>1-1-ceramide</b>
Muco	Gal $\beta$ 1-3Gal $\beta$ 1-3 <b>Gal<math>\beta</math>1-4Glc<math>\beta</math>1-1-ceramide</b>
Galacto	Gal $\alpha$ 1-4Gal $\beta$ 1-1-ceramide
Sulfatides	3- <i>O</i> -Sulfo-Gal $\beta$ 1-ceramide

The sugars highlighted in bold represent common core residues

Sialic acid-containing GSLs were first isolated in high yield from the brain and were thought to be exclusively present in ganglia and hence named gangliosides. However, gangliosides were subsequently found to be present throughout the body, albeit at relatively lower levels (Cuttillo et al. 2020; Kolte 2012; Yohe et al. 2001). NeuAc is the almost exclusive sialic acid in gangliosides of humans, whereas animal gangliosides contain both NeuAc and NeuGc and their acetylated and/or methylated derivatives (Brooks et al. 2002; Schauer 2004; Varki 1992; Varki et al. 2017a). The gangliosides of various animal brains contain one to as many as five sialic acid residues. Gangliosides are named according to the rules proposed by Dr. Lars Svennerholm (1994).  $G_M$ ,  $G_D$ ,  $G_T$ ,  $G_Q$ , and  $G_P$ , where G stands for ganglio, and the subscript letters define the total number of sialyl residues, indicating mono-, di- tri-, tetra- and penta-sialylated gangliosides, respectively. The numerical numbers 1, 2, 3, and 4 following these letters define 5 minus the number of neutral sugars in the molecule. The lowercase letters, *a*, *b*, or *c* after the numerical number define, respectively, one, two, or three SA residues on the inner Gal. For example, GT1b refers to a trisialosyl ganglioside having SA $\alpha$ 2-8SA linked through  $\alpha$ (2-3) glycosidic bond to the inner Gal and one SA linked via  $\alpha$ (2-3) to the outer Gal of the ganglio backbone. The structures of the lacto-ceramide  $G_M$  gangliosides are shown in Fig. 1.7 and examples of other gangliosides are available elsewhere (Cuttillo et al. 2020; Hakomori 2003; Kolte 2012; Kopitz 2009; Kundu 1992; Schnaar and Kinoshita 2017).

An important difference in GSLs compared to glycoproteins and proteoglycans is the absence of heterogeneity at specific glycosylation sites. Glycan microheterogeneity is an inherent factor in glycoproteins and proteoglycans. Not only does the number of glycan chains attached to core proteins vary widely, there can also be heterogeneity in the glycan at a single site. For example, the single *N*-linked oligomannose chain attached to a specific Asn residue in ribonuclease varies widely in its number of mannose residues. In contrast, in GSLs such as  $G_{D1a}$  ganglioside (NeuAc $\alpha$ 2-3Gal $\beta$ 1-3GalNAc $\beta$ 1-4(NeuAc $\alpha$ 2-3)Gal $\beta$ 1-4Glc $\beta$ 1-ceramide) and  $G_{M3}$  ganglioside (NeuAc $\alpha$ 2-3Gal $\beta$ 1-4Glc $\beta$ 1-ceramide), the glycan chains are well defined and homogenous. While there is heterogeneity in the length of fatty acyl chain and

**Fig. 1.7** The structures of monosialogangliosides,  $G_{M1}$ ,  $G_{M2}$ , and  $G_{M3}$ , containing four, three, and two neutral monosaccharides, respectively.  $G_{M4}$  is NeuAc $\alpha$ 2-3Gal $\beta$ 1-1Cer



sphingosine moiety of ceramide as explained earlier, there is no glycan chain heterogeneity. In fact, if one monosaccharide is missing in the carbohydrate chain, the molecule becomes a different glycolipid.

## 10 Synthesis and Functions of the Glycan Moieties of Glycolipids

As in the case of glycoprotein *O*-glycans, glycolipids are synthesized in the Golgi following addition of either  $\beta$ -Glc or  $\beta$ -Gal to the C-1 hydroxyl group of ceramide in the endoplasmic reticulum prior to transport into the Golgi for subsequent sequential addition of other sugars. The newly synthesized glycolipids are then transported to plasma membranes (Brooks et al. 2002; Kolte 2012; Kopitz 2009; Kundu 1992; Schnaar and Kinoshita 2017).

Like *N*- and *O*-glycans of glycoproteins, GSLs perform numerous physiological functions, including cell–cell and cell–molecule interactions that are critical for biological processes such as development, differentiation, defining cell–cell communications, cell social behavior, antigenicity, modulation of immune responses, and cell signaling. As in the case of glycoproteins, the glycan moieties of GSLs can also function as various blood group- and development- and differentiation-specific antigens such as sialyl Lewis antigen (Stanley and Cummings 2017). In the central nervous system, GSLs are involved in many tissue-specific functions, including neurogenesis, normal neural development and function, nerve repair, inhibition of neurite outgrowth, neuromuscular formation, cell social behavior, and various other functions (Hakomori 2003; Kopitz 2009; Kundu 1992; Schengrund 2015; Schnaar and Kinoshita 2017).

Alterations in GSL composition, levels of expression, and distribution appear to contribute to tumor growth and spreading. For example, high levels of  $G_{M2}$  and  $G_{D2}$  gangliosides are found in melanoma and neuroectodermal tumors, and  $G_{D3}$  ganglioside containing 9-*O*-acetyl neuraminic acid is highly expressed by melanoma tumors (Kundu 1992). GD2 ganglioside is present at high levels in neuroblastoma tumors and anti-GD2 antibodies are used for diagnosis of neuroblastoma. Anti-GD2 antibodies are also used for treatment of neuroblastoma in children whose tumors are refractory to other therapies (Nguyen et al. 2018; Schengrund 2020). Various sialyl Lewis antigen-containing GSLs are also highly expressed by teratocarcinoma, colorectal adenocarcinoma, pancreatic cancer, ovarian cancer, and other cancers. A number of monoclonal antibodies such as CA50, N-19-9, OFA-1, and OFA-2 that specifically recognize GSL glycan antigens are being used for diagnosis of cancers, including breast cancer, brain tumors, and colon cancer (Kundu 1992).

As noted above, gangliosides are present at very high levels in the brain. They play important roles in various cellular functions, such as modulation of ion channels and transporters in the nervous system, neuronal interactions and recognition, Ranvier node stability and synaptic transmission (Cuttillo et al. 2020). Galgliosides

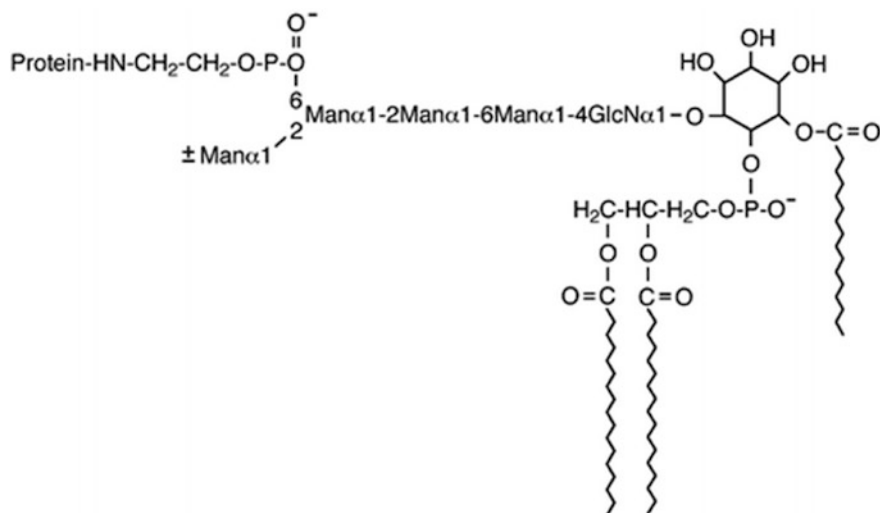


also function as a barrier against complement attack. Altered metabolism of gangliosides results in various diseases, including inflammatory neuropathies and autoimmune diseases. Examples of such diseases are infantile epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease and multiple sclerosis. Autoantibodies against gangliosides are linked to acquired demyelinating immune-mediated neuropathies, such as Guillain–Barré syndrome and Miller–Fisher syndrome (Cutillo et al. 2020; Simpson et al. 2004). In addition, gangliosides serve as targets for various microbes, including influenza viruses, cholera toxin, *Salmonella typhi* toxin, and malaria parasites, leading to microbial induced pathogenesis (Cutillo et al. 2020).

## 11 Glycosylphosphatidylinositol Anchors: A Special Group of Glycolipids

Glycosylphosphatidylinositol (GPI) anchors represent a distinct class of glycolipids and consist of glycan moieties attached to the C-6 position of the *myo*-inositol residue of phosphatidylinositol (PI) (Brooks et al. 2002; Ferguson et al. 2017; Paulick and Bertozzi 2008; Shams-Eldin et al. 2009; Taylor and Drickamer 2011). The glycan moieties of GPIs consist of a conserved trimannosyl core substituted with ethanolamine at the C-6 hydroxyl group of the terminal mannose, ethanolamine-phosphate-6Man $\alpha$ 1-2Man $\alpha$ 1-6Man $\alpha$ 1-4GlcN $\alpha$ 1-. The trimannosyl core moiety in GPIs from many sources is modified with additional sugars and one or more ethanolamine phosphate residues (Fig. 1.8). The lipid portion contains saturated or unsaturated fatty acyl/alkyl residues of 16–24 carbon atoms in length at the C-1 and C-2 positions of glycerol. In addition, the C-2 position of the *myo*-inositol residue is either substituted with fatty acid or unsubstituted. GPIs from different cells and species exhibit broad structural diversity due to heterogeneity in the structures of the glycan and lipid moieties (Ferguson et al. 2017).

GPIs are expressed ubiquitously by all eukaryotic cells and are found in all animal tissues. GPIs are particularly abundant in parasites, including species of the genus *Trypanosoma*, *Leishmania*, and *Plasmodium* (Ferguson et al. 2017; Shams-Eldin et al. 2009). The primary role of GPIs is to anchor certain proteins, glycoproteins, and proteoglycans of cells, including those in the brain, to the plasma membrane via formation of an amide bond between the amino group of the ethanolamine residue and the C-terminal carboxyl group of the protein. In addition to anchoring proteins to the plasma membrane, GPIs seem to be involved in many biological functions (Ferguson et al. 2017; Paulick and Bertozzi 2008; Shams-Eldin et al. 2009). GPI modification is not limited to a specific class of proteins/glycoproteins. Proteins including enzymes, receptors, cell surface antigens, cell adhesion molecules, transporters, and other functional proteins are anchored to plasma membranes via GPIs. Specific examples of GPI-anchored proteins include acetylcholine esterase and decay-accelerating factor present on the



**Fig. 1.8** Representative structure of a GPI anchor. The inner trimannosyl moiety is usually modified with additional sugars and ethanolamine phosphate residue(s). The lipid chains on glycerol are either acyl/acyl or acyl/alkyl chains. The chain lengths of both acyl and alkyl residues are variable and may contain unsaturated bonds (Ferguson et al. 2017). GPIs from some sources carry an acyl substituent, usually C16:0 at C-2 of *myo*-inositol

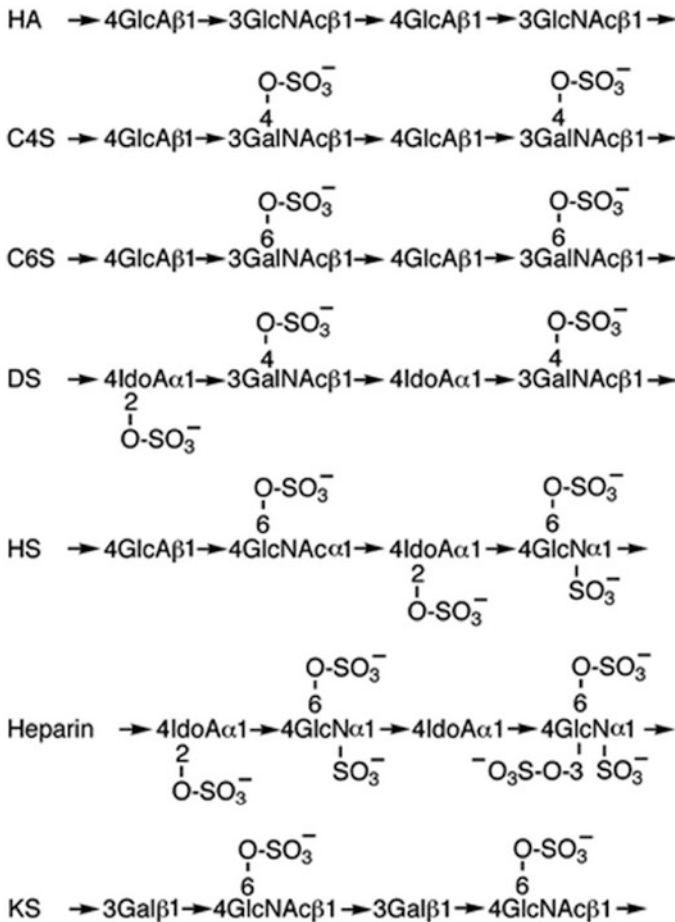
erythrocyte membrane, placental membrane alkaline phosphatase, the Thy-1 antigen of lymphocytes, the neural cell adhesion molecule and brevican proteoglycan.

Biosynthesis of GPIs occurs exclusively in the ER, although some modifications to the glycan moieties may occur after the GPI-anchored proteins move to the Golgi. In the ER, first GlcNAc is added to the membrane PI moiety on the cytoplasmic side, and after *N*-deacetylation of GlcNAc and acylation of *myo*-inositol residue at C-2, the GlcN-PI intermediate flips over to the luminal side of the ER, where Man residues donated by dolichol phosphate-Man are sequentially added. The fully assembled GPI is then transferred to the carboxyl group of the C-terminal amino acid of the acceptor protein to form an amide bond by the action of a transamidase, thereby anchoring the protein to the membrane.

## 12 Glycosaminoglycans and Proteoglycans

Glycosaminoglycans (GAGs) are linear, anionic polysaccharides consisting of alternating residues of an uronic acid or galactose and an *N*-acetylhexosamine (Bhavanandan and Davidson 1992; Buddecke 2009; Hascall and Esko 2017; Lindahl et al. 2017; Sasisekharan et al. 2008; Volpi 2006). An additional structural feature is that all GAGs except hyaluronic acid (see below) contain sulfate. GAGs are made up of repeating disaccharide units with or without sulfate; two such repeats of each

GAG are illustrated in Fig. 1.9. Several types of GAGs occur in animals and are classified into two broad groups based on the type of *N*-acetylhexosamine present: (1) Glucosaminoglycans, containing either GlcNAc or *N*-sulfated glucosamine ( $\text{GlcNSO}_3^-$ ), include hyaluronic acid (HA), heparan sulfate (HS), heparin, and keratan sulfate (KS). (2) Galactosaminoglycans, containing GalNAc, are chondroitin sulfates (CS). In early studies, three types of galactosaminoglycans were discovered and initially named CSA, CSB, and CSC. After the structures were elucidated, these GAGs were renamed chondroitin 4-sulfate (C4S), dermatan sulfate (DS), and chondroitin 6-sulfate (C6S), respectively. DS differs from C4S and C6S in having predominantly IdoA instead of GlcA. There are several other chondroitin sulfates that can be distinguished based on the position and number of sulfate groups present, for



**Fig. 1.9** The structural features of glycosaminoglycans (GAGs) that are commonly found in animal cells and tissues. Two repeating disaccharide units each consisting of alternating residues of an uronic acid or galactose and an *N*-acetylated or *N*-sulfated hexosamine are shown. Note that all GAGs except hyaluronic acid (HA) are sulfated

example, chondroitin 2,6-sulfate (C2,6diS; CSD) and chondroitin 4,6-sulfate (C4,6diS or CSE) (Malavaki et al. 2008; Nandini and Sugahara 2006). In C4S, C6S, and DS, the sulfate groups are mainly present on GalNAc residues. However, in some DS, and in C4,6diS and C2,6diS, sulfate groups are also present at C-2 of certain IdoA and GlcA residues.

The nonsulfated GAG, HA, consists exclusively of uniformly defined repeating disaccharide units (see Fig. 1.9). In contrast, the sulfated GAGs from various tissues of different animals are highly heterogeneous with respect to uronic acid composition and sulfate content. In addition, the disaccharide moieties with different compositions are variously distributed in the polymer chains. Thus, not all repeating disaccharide units of C4S and C6S from different animal tissues are exclusively and uniformly sulfated at C-4 and C-6, respectively. C4S from many sources contains significant amounts of nonsulfated and 6-sulfated disaccharide units. For example, bovine cartilage C4S consists of ~53% 4-sulfated, ~39% 6-sulfated, and ~8% nonsulfated disaccharide units. CS from some sources, including those of the brain, have, in addition to significant levels of both C4S and C6S repeating disaccharide units, one or more elements of 2,4-di-, 3,4-di-, 3,6-di-, and 3,4,6-trisulfated disaccharide moieties (Malavaki et al. 2008; Nandini and Sugahara 2006). Some tissues such as the cornea and placenta contain uniquely low sulfated CS with 80–95% of the disaccharides not sulfated. In placental CS, only 5–10% of the disaccharides are sulfated exclusively at C-4, and in corneal CS, the sulfation is mainly at C-4 and to certain extent at C-6 (Achur et al. 2000, 2004). DS from different tissues, including those from nervous tissues, while containing mainly IdoA, have low to moderate levels of GlcA. As in the case of oversulfated CS, 2,4-di-, 3,6-di-, 4,6-di-, and 2,4,6-trisulfated disaccharide units are also found in some DS (Nandini and Sugahara 2006). The variations in uronic acid composition and the position and degree of sulfation lead to incredible structural diversity and enormous microheterogeneity in the structures of GAGs. Interestingly, as in the case of *N*- and *O*-glycans, it is this structural variation and microheterogeneity that confer the ability for GAGs to perform a wide range of biological functions. The structure and functions of GAGs of the nervous system are discussed in detail in Chap. 5.

Heparan sulfate (HS) and heparin vary substantially in the proportions of GlcA and IdoA as well as sulfate content (Bhavanandan and Davidson 1992; Buddecke 2009; Lindahl et al. 2017). Usually, HS contains nearly equal levels of GlcA and IdoA, whereas in heparin, >80% of the uronic acid is IdoA and the remainder is GlcA. HS has 0.5–1.5 sulfate groups per disaccharide unit, but the average sulfate content of heparin varies from 1.5 to 2.5 sulfate groups per disaccharide moiety. In heparin, the majority of glucosamine is, in addition to having sulfate at C-6, *N*-sulfated, and some *N*-sulfated glucosamine residues are sulfated at both C-3 and C-6. Because of variations in GlcA and IdoA contents, the level of sulfate, and sulfation at different positions, including *N*-sulfation and variations in modifications at different regions of the polymer chains, both HS and heparin exhibit enormous microheterogeneity. The microheterogeneity in HS and heparin is far higher than that of CS and DS.

GAGs are ubiquitous constituents of all animal tissues and are expressed by almost all cell types of eukaryotes (Achur et al. 2000, 2004; Bernfield et al. 1999;

Bhavanandan and Davidson 1992; Bishop et al. 2007; Brooks et al. 2002; Buddecke 2009; Hascall and Esko 2017; Lindahl et al. 2017; Malavaki et al. 2008; Nandini and Sugahara 2006; Sasisekharan et al. 2008; Taylor and Drickamer 2011; Volpi 2006). CS, DS, and HA occur abundantly as gel-like ground substances in the extracellular matrix of connective tissues such as cartilage, tendon, skin, cornea, blood vessels, and umbilical cord. They also occur at substantial levels in matrices of almost all other tissues, including those of the central nervous system, and at moderate levels in the form of proteoglycans in plasma membrane. Although present at significant levels in the matrices of connective and skeletal tissues and brain (Funderburgh 2000; Krusius et al. 1986; Zhang et al. 2006), keratan sulfate (KS) is particularly abundant in the cornea (Funderburgh et al. 1987). HS is ubiquitously found as a cell surface component and also occurs as an extracellular component in blood vessels, in the brain, and in basement membranes, particularly in the kidney, where it is involved in filtration (Bhavanandan and Davidson 1992). Heparin, on the other hand, is exclusively found in intracellular granules of mast cells that line the arterial walls. It is secreted in response to injury and functions as an anticoagulant to regulate the blood-clotting cascade (Bhavanandan and Davidson 1992).

Because of the presence of numerous hydrophilic hydroxyl groups and the high net negative charge imparted by carboxyl and sulfate groups, GAGs have the capacity to hold large amounts of water and stay as extended molecules in solution. They form highly viscous and slimy mucus-like solutions and hence their older name, mucopolysaccharides, which is still used occasionally. The ability to form gel-like substance enables GAGs to function as shock absorbers and lubricants in joints and umbilical cord and to impart resilience to tissues. The ability of GAGs to absorb and release extracellular fluid in the absence and presence of mechanical shear force enables them to provide nutrients to cartilage and other connective tissues lacking blood vessels and to regulate tissue calcification (Bhavanandan and Davidson 1992). More importantly, GAGs are involved in cell–cell and cell–molecule interactions, cell–pathogen binding, cell signaling, binding and mobilizing growth factors, chemokines and cytokines, promoting growth, and regulating immune responses (Achur et al. 2000, 2004; Bernfield et al. 1999; Bishop et al. 2007; Bhavanandan and Davidson 1992; Brooks et al. 2002; Buddecke 2009; Funderburgh 2000; Funderburgh et al. 1987; Hascall and Esko 2017; Iozzo and Schaefer 2010; Lindahl et al. 2017; Malavaki et al. 2008; Nandini and Sugahara 2006; Sasisekharan et al. 2008; Taylor and Drickamer 2011; Volpi 2006; Zhang et al. 2006). Through these interactions, GAGs play important roles in biological processes such as development, differentiation, cell migration, tissue organization, cartilage and bone formation, wound healing, and in disease processes, including cancer and atherosclerosis. Furthermore, deficiencies in GAG catabolism due to lysosomal enzyme deficiencies lead to many diseases called mucopolysaccharidoses, which are discussed in detail in Chap. 5.

All GAGs, except HA, regardless of whether they are the components of various extracellular matrices or cell membranes, occur as moieties conjugated to proteins to form proteoglycans (PGs). A common tetrasaccharide core covalently attaches GAG (CS, DS, HS, and heparin) chains via xylose to the hydroxyl groups of Ser

residues: [HexNR-HexA]<sub>n</sub>-**GlcAβ1-4Galβ1-3Galβ1-4Xylβ1**-Ser, where HexNR is variously sulfated GlcNAc or GalNAc, HexA is nonsulfated or sulfated GlcA or IdoA, and n is number of repeating disaccharide units in GAGs; the tetrasaccharide core attached to Ser is shown in bold.

In proteoglycan biosynthesis, the addition of GlcNAc to the protein-linked tetrasaccharide core commits to the formation of heparan sulfate and heparin chains, whereas the addition of GalNAc leads to the formation of CS or DS chains. Once commitment to synthesize either CS or HS is made, presumably by the specific recognition of core proteins by the glycosyltransferase, the GAG chains are synthesized by the sequential addition of HexNAc and GlcA. Then, the sulfate residues are added, and in the case of DS, HS, and heparin, IdoA residues are formed by the epimerization of D-GlcA to L-IdoA residues. The Ser-Gly motifs of proteins are sites for the attachment of GAG chains. If several Ser-Gly are present, the proteins are modified with multiple GAG chains. For example, the core proteins of serglycin and aggrecan contain 49 and >100 tandem repeats of SerGly motifs, respectively, and all or most of these motifs are substituted with CS chains. The majority of PGs contain one type of GAG chain, CS, DS, or HS, and are designated as chondroitin sulfate proteoglycans (CSPGs), dermatan sulfate proteoglycans (DSPGs), and heparan sulfate proteoglycans (HSPGs), respectively. However, some PGs contain two types of GAG chains such as CS plus HS on the same core protein molecule and thus are hybrid PGs. For example, syndecan, a cell surface PG expressed by a number of cell types, contains both CS and HS chains. KS also is found in PGs, and examples of KS containing PGs include lumican, keratocan, fibromodulin, mimecan, and aggrecan. In the case of KS, the disaccharide-containing polymer chains are built on the core residues of either *N*-linked or *O*-linked glycans in a manner similar to those of poly-*N*-acetylglucosamine chains. The KS are classified into three types, KS-I, KS-II, and KS-III (Funderburgh 2000). KS-I chains are mainly found in the cornea and are linked to proteins via the core glycan moiety of *N*-glycans to Asn residues. KS-II chains are present mainly in skeletal tissues and are linked through α-GalNAc to Ser/Thr of proteins. KS-III is present in the brain and is linked via *O*-linked β-Man residues to Ser/Thr of proteins (Krusius et al. 1986).

In many PGs, including those of the central nervous system, the GAG chains define their functions, and hence the functions of PGs are the same as those mentioned above for GAGs. In addition, the core proteins of many PGs contain functional domains, including the C-type lectin domain that interacts with HA, fibroblast growth factor (FGF), fibronectin III, laminin-G, and fibronectin. As such, PGs interact with cell surface and extracellular matrix proteins and these interactions define their function.

### 13 Glycan-Protein Interactions

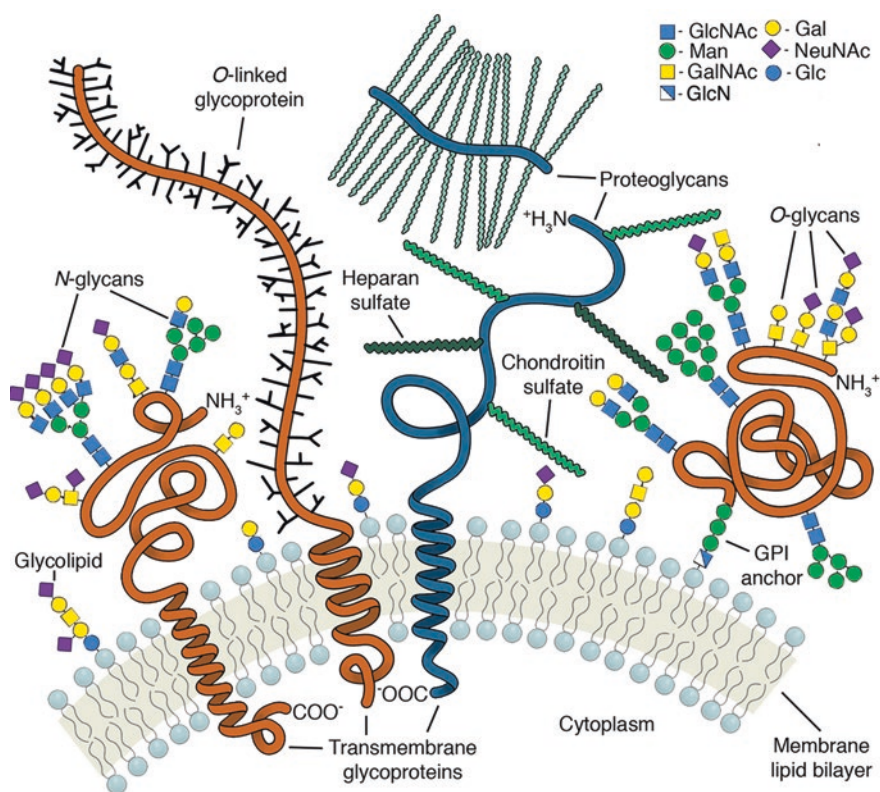
Glycan moieties of glycoproteins, glycolipids and proteoglycans interact with numerous proteins in almost all eukaryote cells. Protein-glycan interactions play crucial roles in most biological processes and physiological functions including

development and differentiation. Lack of protein-glycan interactions because of alterations in glycan structure, or protein structure due to mutation, results in pathological conditions including oncogenesis. The glycan-binding proteins (GBPs) are broadly classified into two groups, namely lectins and glycosaminoglycan-binding proteins. Lectins occur in all organisms, where they interact with glycans through their well-defined binding pockets called carbohydrate-recognition domains (CRDs). The CRDs of lectins recognize specific structural features of glycans, typically one or more peripheral sugars, including sulfated and phosphorylated sugars. Several classes of lectins are found in animals, including Galectins (Gal-binding proteins), Siglecs (sialic acid-binding proteins), mannose-binding proteins (MBPs), and Selectins (E-, L- and P-selectins that recognize sulfated or nonsulfated sialyl Lewis antigens). The glycosaminoglycan-binding proteins interact stereospecifically through clusters of positively charged amino acids with carboxylic and sulfate groups of the glycosaminoglycans (heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate). Generally, each glycan-protein interaction leads to a specific biological function. As such, given the wide range of glycan structures that occur in animals, interactions of glycans with GBPs initiate numerous physiological and pathophysiological processes (see Sect. 14 below). In fact, the recognition of glycans by GBPs allows for the information present in glycan structures to be translated to biological functions. GBPs are also widely distributed in microorganisms, where they interact with glycan moieties allowing microorganisms to adhere, invade, and colonize host cells. For example, *Escherichia coli* that causes urinary tract infection binds to epithelial cells of urinary tract epithelia via interaction of their lectins to mannose residues of cell surface glycans. Many viruses, including influenza virus, attach via their GBPs to terminal sialic acids residues linked  $\alpha$ 2-6 or  $\alpha$ 2-3 to Gal on host cell surface glycans to enter cells and deliver their genomic contents. Many pathogenic microorganisms use cell surface heparan sulfate and chondroitin sulfate chains for attachment and invasion of host cells (Rostand and Esko 1997; Wadstrom and Ljungh 1999). For comprehensive discussion of GBP-glycan binding specificity playing key roles in many physiological and pathological processes, the readers are referred to abundance of information available in book chapters (Cummings et al. 2017; Esko et al. 2017; Imberty and Prestegard 2017; Nizet et al. 2017; Szymanski et al. 2017; Taylor et al. 2017; Varki et al. 2022).

## 14 Glycans of Cell Surface Glycoconjugates Perform a Variety of Functions

The majority of animal cell surface proteins, including those on cells of the central nervous system, are modified with variable amounts of *N*- and *O*-linked glycans and/or glycosaminoglycan chains (Brooks et al. 2002; Collins and Paulson 2005; Fukuda 2000; Hatstrup and Gendler 2008; Iozzo and Schaefer 2010; Schauer 2009; Springer and Gagneux 2013; Taylor and Drickamer 2011; Varki 1992; Varki 2017;

Varki and Gagneux 2017). In some instances, such as in mucin-type glycoproteins, the mass of glycans exceeds that of proteins to which they are conjugated. In addition, a significant portion of the outer head group of cell surface ceramide is modified with glycans. Thus, the *N*- and *O*-glycans of glycoproteins, the glycan moieties of glycolipids, and the chondroitin sulfate and heparan sulfate chains of proteoglycans are prominently displayed on the outer leaflet of the plasma membrane (Fig. 1.10). As mentioned earlier, because of high hydrophilicity, glycans have a large capacity to hold water and exist as bulky and highly extended molecules. The eukaryotic cell surface is thus covered with a glycan-rich zone, referred to as the glycocalyx (Weinbaum et al. 2007; Salmon and Satchell 2012). Moreover, many glycan chains of glycoproteins and glycolipids carry terminal sialic acid residues and proteoglycans carry anionic chondroitin and heparan sulfate chains. Therefore, the glycocalyx is negatively charged and can be seen by electron microscopy after



**Fig. 1.10** Schematic illustration of a section of animal cell plasma membrane showing the placement of glycan moieties of glycoconjugates in the outer leaflet of the lipid bilayer. The *N*- and *O*-glycans of glycoproteins, the glycan moieties of glycolipids, and the GAG chains of integral membrane and secreted proteoglycans that together form the glycocalyx are shown. Also shown is a highly glycosylated mucin-type glycoprotein present as an extended molecule on the cell surface



staining cells with a dye such as ruthenium red. The thickness of the glycocalyx is typically greater than that of the plasma membrane itself.

Because of their strategic locations, the cell surface glycans that constitute the glycocalyx play several nonspecific yet crucial physical roles. By holding large amounts of water, they help to maintain cell shape, assist tissues in their function (e.g., keeping the lungs and airways moist and open), protect the epithelial barrier against mechanical damage and proteolysis, and provide an aqueous environment for biochemical interactions. They also function to stabilize protein conformation and provide the aqueous environment essential for cell–cell and cell–matrix interactions and communication. More importantly, cell surface glycans perform numerous specific, biological functions through interactions with proteins and glycans of other cells and tissue matrices (Brooks et al. 2002; Cummings et al. 2017; Freeze et al. 2017a; Gabius 2009; Imberty and Prestegard 2017; Taylor et al. 2017; Taylor and Drickamer 2011; Varki 1992, 2017; Varki and Gagneux 2017); also see Chap. 7. These functions are dependent on specific structural features of glycans and also on their clustering patterns and densities. The wide range of structural diversity, microheterogeneity, and distributions along protein chains enable glycans to serve as specific recognition and information molecules. Thus, they play essential and specific roles in almost all biological processes involved in fertilization and embryogenesis through the sperm–egg interactions, differentiation and development, and survival. Other biological processes in which glycans play important roles include cell migration and recruitment of cells to specific sites; turnover of cells and proteins; removal of hormones, receptors, and aged erythrocytes from the circulation; cell signaling; and immune modulation.

Glycan moieties of glycoconjugates also play many crucial roles in the development, diseases, and damage-response of the central nervous system (Higuero et al. 2017; Krug et al. 1991; Mutalik and Gupton 2021; Smalla et al. 2000; Smith et al. 2015; Waite et al. 2012). For example, the terminal fucose residues linked to Gal in *N*- and *O*-glycans,  $\text{Fuc}\alpha(1-2)\text{Gal}$ , have been implicated in cognitive processes including long-term memory (Bullock et al. 1992). The  $\text{Fuc}\alpha(1-2)\text{Gal}$  moiety expressed by synapsins Ia and Ib plays a role in the regulation of synaptic proteins and neuronal morphology, as revealed by the fact that defucosylation leads to stunted neurite growth and delayed synapse formation (Murrey et al. 2006). Sialic acid, abundantly present in brain as a component of gangliosides and glycoproteins, and as polysialic acid chains in neuronal cell adhesion molecules (NCAM), is essential for brain development and cognition (Wang et al. 1998; Wang 2009). The polysialyl chains in NCAM are known to play crucial roles in neuronal development, cell survival, and migration of neural precursors, neuronal guidance, and synapse formation (Smalla et al. 2000; Sytnyk and Leshchynska 2021; Wang et al. 2008). Glycan moieties of glycoproteins and glycolipids play crucial roles in neuronal development, neuronal physiology, and axon guidance (Masu 2016; Mutalik and Gupton 2021; Sytnyk and Leshchynska 2021). Finally, the chondroitin sulfate/dermatan sulfate and heparan sulfate chains of proteoglycans which, are abundantly expressed in the central nervous system also play key roles in brain development and axonal guidance (Jin et al. 2021; Maeda et al. 2011; Masu 2016; Schwartz and

Domowicz 2018; Smith et al. 2015; Sugahara and Mikami 2007; Wang et al. 2008; Waite et al. 2012). Besides their key roles in neuronal development and functions, the glycan moieties play protective roles in neurodegenerative diseases and Alzheimer's disease (Haukedal and Freude 2020; Jin et al. 2021; Wielgat and Braszko 2012a; Wielgat and Braszko 2012b; Zhang et al. 2014).

The glycans also play important roles in the pathogenesis of various diseases (Brooks et al. 2002; Freeze et al. 2017b; Gabius 2009; Taylor and Drickamer 2011; Varki 1992; Varki et al. 2017a; Varki and Gagneux 2017). For example, when cells become transformed to a malignant state, the cell surface glycan structures and profiles are drastically altered, promoting tumor growth and metastasis. Glycoproteins expressed by most tumors have altered glycan structures and are shed into the circulation; thus, they serve as cancer biomarkers (Brooks et al. 2002; Gabius 2009; Moskal et al. 2009; Silsirivanit 2019; Taylor and Drickamer 2011; Varki et al. 2017b). Monoclonal antibodies recognizing the cancer-associated glycan antigens are used clinically for cancer diagnosis and management. For example, neuroblastomas express high levels of ganglioside  $G_{D2}$ , and antibodies to the glycan portion of  $G_{D2}$  are currently being used experimentally to target drugs to these tumors (Tivnan et al. 2012; Ladenstein et al. 2018; Voeller and Sondel 2019). Furthermore, glycans are involved in bacterial, viral, and parasitic infections. Infectious diseases such as influenza, stomach ulcer caused by *H. pylori*, and several airway and urinary tract infections arise by the glycan-dependent recognition and binding of viruses and bacteria to host target cells and tissues (Brooks et al. 2002; Cummings and van Die 2017; Gabius 2009; Szymanski et al. 2017; Taylor and Drickamer 2011). For example, influenza infection involves the viral hemagglutinin-mediated attachment to cell surface sialic acid residues. The surface of the causative agent of AIDS, the human immunodeficiency virus (HIV-1), is studded with a major envelope glycoprotein (gp120). About 50% of the molecular mass of gp120 is carbohydrate, and it is involved in the viral invasion of lymphocytes.

## 15 Summary

Carbohydrates in the form of oligosaccharides and polysaccharides (called glycans) occur widely in almost all animal cells as moieties linked to proteins or lipids to form glycoproteins, glycolipids, and proteoglycans, which are collectively called glycoconjugates. The glycan moieties are structurally highly complex, and their complexity is attributed to the multiple ways the constituent sugar residues are linked to one another. More importantly, the glycan structural complexity arises from the variations in their sugar composition and size. The biosynthesis of these complex glycan structures is not template dependent as in the case of proteins, instead it is determined by the specificity of glycosyltransferases that catalyze their formation and the orderly manner these enzymes are organized in the biosynthetic compartments. The glycans function as informational molecules through varied and specific interactions with partner proteins and complementary glycans. As such, the

wide range of glycan structural complexity is translated to a myriad of biological roles. The glycoconjugates are abundantly found in extracellular matrix and on cell surfaces, where the glycan moieties participate in numerous biological interactions, which are crucial for differentiation and development and life of the organism. Therefore, abnormal glycan metabolism leads to disease pathogenesis. Ironically, many pathogens and toxins produced by them exploit the cell surface glycans to attach and invade cells, causing debilitating and fatal disease.

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# Chapter 2

## Cells of the Central Nervous System: An Overview of Their Structure and Function



**John W. Bigbee**

**Abstract** The central nervous system is the last major organ system in the vertebrate body to yield its cellular structure, due to the complexity of its cells and their interactions. The fundamental unit of the nervous system is the neuron, which forms complex circuits that receive and integrate information and generate adaptive responses. Each neuron is composed of an input domain consisting of multiple dendrites along with the cell body, which is also responsible for the majority of macromolecule synthesis for the cell. The output domain is the axon which is a singular extension from the cell body that propagates the action potential to the synapse, where signals pass from one neuron to another. Facilitating these functions are cohorts of supporting cells consisting of astrocytes, oligodendrocytes and microglia along with NG2 cells and ependymal cells. Astrocytes have a dazzling array of functions including physical support, maintenance of homeostasis, development and integration of synaptic activity. Oligodendrocytes form the myelin sheath which surrounds axons and enables rapid conduction of the nerve impulse. Microglia are the resident immune cells, providing immune surveillance and remodeling of neuronal circuits during development and trauma. All these cells function in concert with each other, producing the remarkably diverse functions of the nervous system.

**Keywords** Neuron · Astrocyte · Oligodendrocyte · Microglia · Myelin

### Abbreviations

ATP      Adenosine-5'-triphosphate  
BDNF     Brain-derived neurotrophic factor

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CNPase	2',3'-Cyclic nucleotide 3' phosphodiesterase
CNS	Central nervous system
CS	Chondroitin sulfate
CSF	Cerebrospinal fluid
GABA	Gamma aminobutyric acid
GFAP	Glial fibrillary acidic protein
GTP	Guanosine-5'-triphosphate
HS	Heparin sulfate
IBA1	Ionized calcium-binding adapter molecule 1
IL-12	Interleukin 12
IL-1 $\beta$	Interleukin 1 beta
MAG	Myelin-associated glycoprotein
MAPs	Microtubule-associated proteins
MBP	Myelin basic protein
mRNA	Messenger RNA
NG2	Neuron-glia antigen 2
NRS	Nitrogen reactive species
P <sub>0</sub>	P zero
PAEZ	Pre-axonal exclusion zone
PLP	Proteolipid protein
PNN	Perineuronal net
PNS	Peripheral nervous system
PSD	Postsynaptic density
PSD95	Postsynaptic density 95 protein
RER	Rough endoplasmic reticulum
ROS	Reactive oxygen species
SER	Smooth endoplasmic reticulum
TGF $\beta$	Transforming growth factor beta

## 1 Introduction

The nervous system is composed of arguably the most complex tissues in the vertebrate body with its cells forming intricate, unique and long lasting structural and physiological associations with each other, between different areas of the nervous system, and with multiple body organs. Remarkably, many of these cell associations are highly plastic, allowing for structural and functional tissue changes in response to a diversity of inputs, including those triggered by learning, stress, and different life experiences. This chapter will introduce the variety of cells that comprise the vertebrate nervous system which are broadly grouped into neurons, the functional units of the nervous system, and supporting cells (neuroglia) that perform a variety of functions that are required for proper neuronal function.

Due to the complexity of nervous tissue, determining the precise structure of its cells was a much more daunting task compared with that of other organs. Key to elucidating the cytoarchitecture, was the introduction of metal stains, most notably

by Camillo Golgi and Santiago Ramon y Cajal around the turn of the nineteenth century. Their methods, still in use today but not yet fully understood, provided much more highly detailed images of both neurons and neuroglia than can be revealed by more conventional methods like the Nissl stain. The use of immunocytochemical staining methods has further refined our knowledge of cellular subtypes, but ultimately, descriptions of the fine cellular details of the nervous system awaited the invention of the electron microscope, and its application in the mid-twentieth century.

This chapter will focus on the cells of the brain and spinal cord which comprise the central nervous system (CNS). It begins with a discussion of the structure and function of nerve cells (neurons), followed by a description of the supporting cells (astrocytes, oligodendrocytes, microglia, NG2 cells and ependymal cells) and a brief discussion of the extracellular matrix that surrounds all these cells. All these cells and structures function together and thus understanding the unique rolls of each will provide insights for understanding the implications of how interfering with the function of one cell type can affect those with which it interacts.

## 2 Neurons

Neurons are electrically excitable cells that maintain and adjust their membrane potentials in order to process and propagate signals within themselves and as part of highly complex circuits, communicating with each other through the use of a diverse array of neurotransmitter molecules. These circuits receive external and internal sensory signals, integrate that information with stored experiential data and generate adaptive behavioral responses. Additionally, together with the endocrine system, the nervous system maintains overall body homeostasis.

The human brain is estimated to contain over 100 billion neurons, each capable of forming thousands of synaptic connections with other cells. There are many different types of neurons, each structurally and functionally unique reflecting their diverse roles in receiving, integrating and responding to information. Neurons are highly polarized cells, which refers to the asymmetric segregation of their receiving and transmitting domains. Despite their diversity, neurons possess four morphologically and functionally distinct regions, the cell body, dendrites, axon and presynaptic terminals. Due to their functional similarities, the soma and dendrites are collectively referred to as the somatodendritic compartment.

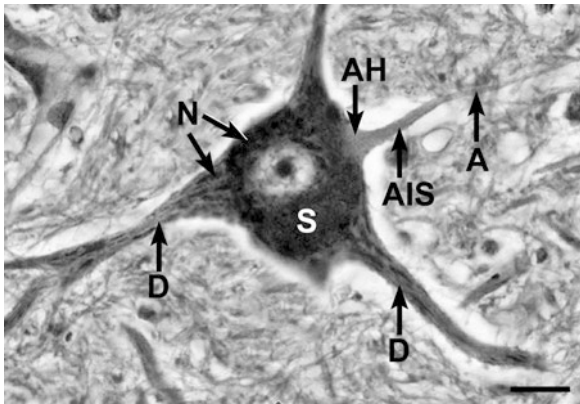
### 2.1 Neuronal Cell Body

Histologically, the cell body (soma, perikaryon) is the most conspicuous component of a neuron. Ranging in size from 5 microns (granule cells in the cerebellum) to 100 microns or more (spinal motor neurons) in diameter, the cell body may be round, oval or polygonal (Ross and Pawlina 2016). Based on the morphology of their processes, neurons are classified as multipolar, bipolar or pseudounipolar, with each of

these shapes closely correlated with function. As the name indicates, multipolar neurons have numerous and complex dendrites extending from the body, thus allowing for rich synaptic input. Multipolar neurons (Fig. 2.1) function in highly complex circuits that subservise motor behaviors and integrative tasks. These cells constitute the vast majority of neurons in the CNS and their shapes can be highly variable. Both bipolar and pseudounipolar neurons have a simpler morphology and are exclusively sensory in function. Bipolar neurons transmit information of the special senses, e.g., vision and olfaction, whereas pseudounipolar neurons transmit information of general somatic sense, e.g., pain, temperature and joint position. Bipolar neurons have a single dendrite and a single axon, emanating from opposite poles of the cell body. In contrast, pseudounipolar neurons have a single short process that immediately divides into a peripheral process that extends into the body tissues and a central process that enters the CNS. The relatively simple morphology of these sensory neurons insures fidelity of the sensory signals.

The cell body is the main synthetic region of a neuron and contains the nucleus, which is uniquely pale staining (euchromatic) reflecting dispersed chromatin and high transcriptional activity. In the cytoplasm, the most prominent organelles are protein-synthesizing Nissl bodies, composed of stacks of rough endoplasmic reticulum (RER) with numerous polyribosomes between them, an association that is unique to neurons. While absent in axons, Nissl bodies are present throughout the cell body and extend into the proximal regions of larger dendrites.

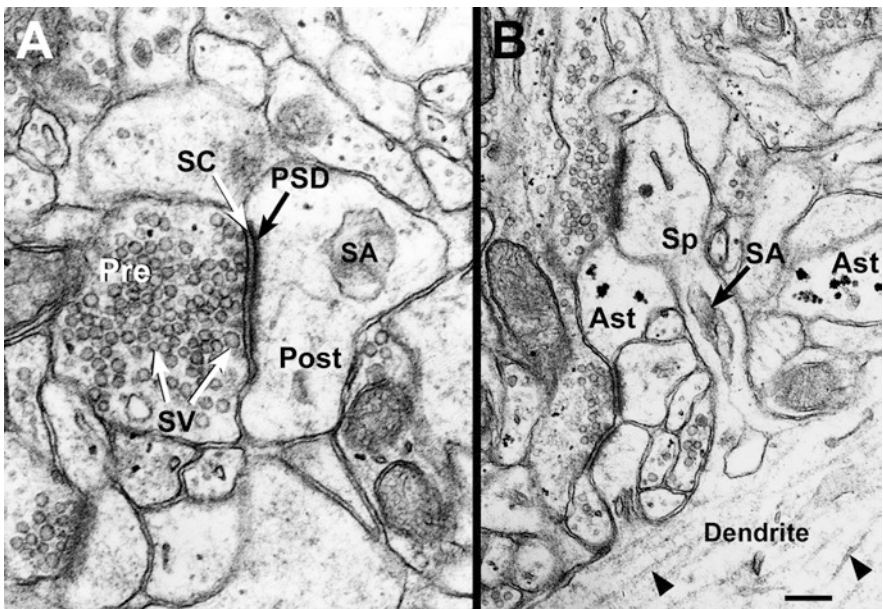
The cell body also possesses well developed and often numerous Golgi complexes. As in other cell types, the Golgi complex receives proteins from the RER,



**Fig. 2.1** Neuron. Multipolar neuron in the anterior horn of the spinal cord with major processes. Three dendrites (D) originating from the cell body or soma (S) are visible. The soma contains a large, pale-staining, transcriptionally active nucleus. Also seen are Nissl bodies (N) which are present in both the dendrites and the soma. On the right side of the soma is the axon hillock (AH), a portion of the cell body with a paucity of organelles. Originating from the hillock is the axon, beginning with the initial segment (AIS) and then continuing as the shaft of the axon (A). Surrounding the neuron are smaller nuclei of supporting cells and myelin sheaths. Cresyl violet, luxol fast blue stain; Scale bar = 50 microns

post-translationally modifies them and packages them into vesicles for transport to the plasma membrane. Like Nissl, Golgi bodies extend into proximal dendrites where they are referred to as Golgi outposts (Wang et al. 2020). This dendritic colocalization of Golgi outposts and Nissl bodies supports the functional integration of local protein synthesis, transport and trafficking.

The smooth endoplasmic reticulum (SER) forms a continuous, mostly tubular organelle that is present throughout the neuron, extending to the distalmost tips of both dendrites and axons. The SER participates in lipid synthesis and functions as a calcium-storage reservoir. A unique extension of the SER is the spine apparatus (Fig. 2.2b), found mostly in mushroom-shaped (mature) spines. Here, it serves as a sink for calcium, a key regulator of synaptic function (Bell et al. 2019). In agreement with the high energy demands of all neurons, their cell bodies also contain a large number of ATP-generating mitochondria that also provide a calcium sink along with the SER. Mitochondria are also distributed throughout dendrites and axons, including presynaptic terminals. Lysosomes are present throughout the neuron, predominating in the cell body with less in dendrites and few in axons. They



**Fig. 2.2** Synapses. Electron micrographs of excitatory, chemical synapses. In Panel (a), the presynaptic terminal (Pre) with its neurotransmitter vesicles (SV) is seen in close apposition to a postsynaptic dendritic spine (Post) containing a spine apparatus (SA). The pre- and postsynaptic structures are separated by a narrow gap, the synaptic cleft (SC). The prominent thickening of the postsynaptic membrane is the postsynaptic density (PSD) which contains neurotransmitter receptors. Panel (b) shows a presynaptic terminal ending on the head of a prominent mushroom-shaped spine (Sp) with a spine apparatus (SA). Adjacent to the spine are astrocyte processes (Ast) that contain electron dense, glycogen granules. Microtubules (MT) are visible in the shaft of the dendrite (arrowheads) below. Scale bar = 200 nm in A; 300 nm in b

provide degradation and recycling of macromolecules, processes that are particularly active during CNS development, and when genetically altered, can result in severe inherited lysosomal storage diseases (Platt et al. 2018). Nevertheless, and normally throughout life, undegraded material accumulates as lipofuscin pigment, a common feature in neurons which are long-lived and non-mitotic. As in other cells, the plasma membrane of neurons forms a semipermeable barrier between the cytoplasm and the extracellular space. However, in agreement with the functional complexity of neurons, their plasma membrane displays great local variability with specialized regions like the nodes of Ranvier, axonal initial segment, and pre- and post-synaptic membranes.

The region of the cell body where the axon emerges is called the axon hillock (Fig. 2.1), a conical region marked by the absence of basophilic material (Nissl substance) and other organelles. The hillock has recently been proposed to serve as a pre-axonal exclusion zone (PAEZ), where somatodendritic organelles are prevented from entering the axon (Britt et al. 2016; Farías et al. 2015). The sorting of organelles is thought to occur through their selective association with direction-specific molecular motors that move along polarized microtubules. The axon hillock along with the initial segment, which restricts membrane protein diffusion, preserve the functional polarity of the neuron by maintaining the separation of the receptive somatodendritic domain from the transmitting axonal domain.

## 2.2 Cytoskeleton

Neurons are the largest and most morphologically complex cells in the body. As a result, they have high structural and transport demands that are provided by an extensive cytoskeletal network. Their cytoskeleton forms a complex matrix throughout all regions of the cell and consists of three elements, microfilaments, neurofilaments (a class of intermediate filament) and microtubules. (Pigino et al. 2012). Cytoskeletal elements are also present in supporting cells, where they provide similar functions, though cell-type variations are present.

**Microfilaments** Microfilaments are the thinnest of the cytoskeletal elements (5–7 nm diameter) and consist of two strands of relatively short, helically wound polymers of globular actin (Stevens et al. 2021). Their assembly is ATP dependent. Microfilaments are mostly concentrated immediately beneath the plasma membrane forming a meshwork similar to the cortical web in epithelial cells. In this location, they regulate cell shape and movement and provide anchorage for channel and receptor proteins. Microfilaments are also enriched in presynaptic terminals and dendritic spines, where they regulate synaptic vesicle and spine dynamics, respectively. Microfilaments polymerize and depolymerize rapidly in response to a variety of extracellular signals that activate actin-associated proteins including profilin, gelsolin, cofilin and Arp 2/3 (Schaks et al. 2019). This remodeling of the microfila-



ments provides for cell migration, process extension and guidance during development.

**Microtubules** Microtubules consist of 13 protofilaments that are composed of alpha-beta tubulin dimers. Protofilaments associate to form a hollow tube with an outer diameter of 20–25 nm and their assembly is GTP dependent. Microtubules have a structural role, but are primarily responsible for process outgrowth during development and for providing the substrate for intracellular transport (Kapitein and Hoogenraad 2015). This general transport process was originally termed axonal transport, based on where it was first observed, but also occurs in dendrites and the cell body as well. Microtubules assemble at the microtubule organizing center (centrosome) of the cell, with a distal “plus” (+) or growing end and a proximal “minus” (–) end. This polarity provides directional cues for the molecular motors, kinesin and dynein that carry the cargo along microtubules. The polarity of microtubules is uniform in axons, with their plus ends oriented distally. Microtubules in dendrites have mixed polarity. The stability and spacing of microtubules are regulated by a family of proteins called microtubule-associated proteins (MAPs). One of these, tau, is axon-specific and associated with neurodegenerative disease, like Alzheimer’s disease, where it becomes hyperphosphorylated and assembles into paired helical filaments.

**Neurofilaments** Neurofilaments are the intermediate filaments of neurons (Pigino et al. 2012). There are six classes of intermediate filaments, some of which are cell type-specific: Class I (acidic keratins), Class II (basic keratins), Class III (vimentin, desmin, peripherin, and glial fibrillary acidic protein [GFAP, specifically found in astrocytes]), Class IV (neurofilament proteins), Class V (nuclear lamins), and Class VI (nestin). Neurofilaments provide rigid support for the neuron and unlike microfilaments and microtubules, are quite stable. In the CNS, they are heteropolymers consisting of four structurally similar proteins, the neurofilament triplet, NF-L, NF-M and NF-H, and alpha-internexin (Bott and Winckler 2020). Neurofilaments are present throughout the neuron, though they tend to predominate in axons over dendrites where they primarily function to maintain axonal size and caliber. In larger axons, neurofilaments are phosphorylated which provides additional support. The intermediate filaments of astrocytes are composed of glial fibrillary acidic protein (GFAP), which is often used for immunocytochemical detection of astrocytes in tissue sections.

### 2.3 Dendrites

Dendrites are extensions from the cell body and collectively serve as the “antenna” for the neuron, receiving the vast majority of synaptic inputs (Reviewed in Dendrites, Stuart et al. 2016). Depending on the type of neuron, the cell body extends between 1–10 primary dendrites that emanate from a broad base and gradually taper towards

their tips. (Fig. 2.1). Dendrites are generally much shorter than axons and mostly terminate close to the cell body. However, in some cases, such as in pyramidal cells in the cerebral cortex, dendrites may extend hundreds of microns. The shape of the dendritic arbor is often stereotypic for specific neuron types, for example Purkinje cells in the cerebellum or pyramidal cells in the cerebral cortex. These arbors are optimized for the unique circuitry of each neuron. Dendrites are highly branched, which along their multiple numbers, increases the surface area for receipt of synaptic input. For example, pyramidal cells in the visual cortex have a total dendritic arbor length of nearly 50,000  $\mu\text{m}$  (50 mm) and can receive 50,000–100,000 synapses. (van Elburg and van Ooyen 2010). This remarkable complexity allows neurons to function in highly integrated circuits.

Dendrites, along with the cell body, constitute the somatodendritic compartment of a neuron and share most of the same cytoplasmic organelles, including Nissl and Golgi. SER is present throughout dendrites where it forms a continuous reticulum that extends into dendritic spines where it is referred to as the spine apparatus (Stevens et al. 2021). The SER is involved in regulation of calcium. Mitochondria are present throughout the dendrite, though they tend to concentrate in the more distal tips, suggestive of their role in buffering calcium and preventing neurotoxicity from synaptic activity. Dendrites also share electrophysiological properties with cell bodies, supporting graded membrane potential changes (gradient or receptor potentials), in contrast to the all-or-nothing, depolarization (action potential) that occurs in axons.

An additional feature of dendrites of principal (projecting) neurons is the presence of dendritic spines (Fig. 2.2b). Spines are short protrusions, 2–3 microns in length and serve as the principal site for excitatory synaptic transmission. Pyramidal neurons in the visual cortex can have up to 15,000 spines (Larkman 1991), with each receiving multiple presynaptic contacts. The most mature, mushroom spines, have a bulbous head and a thin neck. Spine morphology is highly labile, rapidly changing due to alterations in their microfilament support network in response to synaptic activity (Oertner and Matus 2005). These morphological changes are facilitated by the presence of protein synthetic components in the dendrite, including free ribosomes and mRNA. Importantly, changes in spine morphology and number underlie the process of learning and memory formation by modifying the effectiveness of specific circuits. Activation of the NMDA receptor by glutamate leads to a calcium influx into the spine, which modulates the activity of actin-regulatory proteins such as gelsolin and profilin. Synapses also occur on the shaft of the dendrites where they are mostly inhibitory. In projection neurons, shaft synapses account for only a small proportion of the total synapses.

## 2.4 Axon

The axon is a single extension from the cell body and is responsible for transmitting the nervous impulse to other neurons. Axons are morphologically and physiologically distinct from dendrites. They are more slender, of uniform diameter and often terminate at great distance from the cell body (Fig. 2.1). Axons propagate the action potential which is a brief, “all-or-nothing”, spike-like depolarization of the axonal plasma membrane that travels down the axon without decrement and at a high, constant velocity (Bean and Koester 2021).

Depending on the circuit pathway and the type of neuron, axonal branching can be highly variable and extensive. For example, in humans, individual neurons of the substantia nigra pars compacta are calculated to give rise to 4.5 m of axon, once all the branches are summed (Matsuda et al. 2009). Axonal branching allows a neuron to connect to several, often widely divergent targets. These branches consist of axon collaterals, that arise along the length of the axon. Notably absent from the axon are Nissl bodies and Golgi which originally led to the conclusion that axons lacked the ability to synthesize proteins. More recent evidence however, has revealed that axons contain both mRNA and organelle-like RNA granules, consistent with local protein synthesis (Dalla Costa et al. 2021).

Axons have a very prominent cytoskeleton. Neurofilaments and microtubules are both highly enriched and provide structural support and pathways for intracellular transport (axonal transport), respectively. The neurofilaments in larger myelinated axons are mostly phosphorylated, a posttranslational modification that provides additional support for the axon as well as regulating their spacing and axonal diameter. As mentioned previously, microtubules in axons show uniform polarity with their “plus” (+) ends directed distally and provide the tracks for bi-directional, fast axonal transport of organelles (Morfini et al. 2012). Transport in the anterograde direction (toward the terminals) is accomplished by the kinesin family of molecular motors, while retrograde transport (toward the cell body) utilizes the motor, dynein. Movement in both directions occurs at 200–400 mm/day.

The first portion of the axon is the initial segment (Fig. 2.1), a unique region where the membrane possesses high concentrations of voltage-gated sodium and potassium channels. The channels are stabilized by ankyrin G which, along with spectrin, organizes a layered scaffold that anchors the proteins to the microfilament and microtubule cytoskeleton (Leterrier 2016). Often referred to as the “trigger zone”, the initial segment is the site where the action potential originates. In response to synaptic input, membrane potentials in the somatodendritic compartment are summated at the initial segment to initiate the action potential. In addition, as mentioned in 2.2.1, along with the axon hillock, the initial segment maintains polarity of the neuron by preventing diffusion of somatodendritic membrane proteins into the axon (Leterrier 2018). This role as the “gatekeeper of axonal identity” maintains the molecular identity of the axon.

Axons may be surrounded by a concentrically wrapped myelin sheath (myelinated axons) which is produced by oligodendrocytes (see below), or they may lack

this ensheathment (unmyelinated axons). In the CNS, unmyelinated axons are generally about 0.1–2 microns in diameter and have a uniform distribution of sodium channels along their membrane. Depolarization proceeds along the entire axonal membrane and thus conduction rates are slower than in myelinated axons (Rasband and Macklin 2012). Myelinated axons range from 1 to 20 microns in diameter, with the largest being in the PNS. Myelinated axons have a discontinuous distribution of sodium channels, restricted to nodes of Ranvier which are located between myelin segments (internodes). Myelinated axons conduct at a faster rate, by a process called saltatory conduction, where depolarization skips from node to node. Nodes possess a similar subplasmalemmal protein scaffold as that present at the initial segment.

The distal end of the axon and its collaterals branch repeatedly to form the terminal arborization. At the tips of each branch there is a small enlargement called the terminal bouton (presynaptic terminal) that forms the first part of the synapse.

## 2.5 Synapse

Nervous transmission from one neuron to another occurs at the synapse, a highly specialized cell junction between the presynaptic terminal and the postsynaptic cell (Fig. 2.2). The two basic types of synapses are chemical, which are the most numerous, and electrical (Siegelbaum and Fischbach 2021). In chemical synapses, the presynaptic terminal releases neurotransmitters that diffuse across a narrow gap, the synaptic cleft, and bind to their specific receptors on the postsynaptic neuron. Chemical synapses are classified as excitatory or inhibitory based on the type of neurotransmitter they contain. Neurotransmitters are stored in small synaptic vesicles which, upon arrival of the action potential, move to the presynaptic membrane where they fuse and release their transmitter content (Purves et al. 2018). This neurosecretion is stimulated by an influx of calcium into the terminal, triggered by the arrival of the action potential. The region of the membrane where the vesicles bind is called the active zone and consists of a variety of proteins that facilitate the capture and fusion of the vesicles. Fused vesicles are recycled back into the terminal where they are refilled with transmitter. Immediately opposite to the active zone is the postsynaptic density (PSD), a specialized region of the postsynaptic neuron where receptors for the neurotransmitters are concentrated (Fig. 2.2a). The high concentration of these proteins, along with additional scaffolding proteins, like PSD95, produces a distinctly dense band along the cytoplasmic surface of the postsynaptic membrane, hence the name, postsynaptic density. The PSD of an excitatory synapse is very prominent and is organized by the scaffolding protein, PSD95. The PSD of inhibitory synapses is less prominent and is organized by gephyrin. The list of neurotransmitters is extensive, chemically diverse and growing. There are between 40–60 known transmitters, though the excitatory glutamate and the

inhibitory gamma aminobutyric acid (GABA) are by far the most abundant neurotransmitters.

The second type of synapse is the electrical synapse. Electrical synapses are essentially gap junctions where communication occurs by the direct passage of ions through pores formed by connexons on apposing membranes (Siegelbaum and Fischbach 2021). The presynaptic terminal lacks vesicles, a synaptic cleft is absent and no PSD is present. Transmission across an electrical synapse is much faster than that at chemical synapses which requires vesicle trafficking to the active zone, fusion and release of transmitter, and transmitter diffusion across the synaptic cleft. Due to the speed of electrical synapses, they are able to synchronize the electrical activity among populations of neurons.

### 3 Supporting Cells

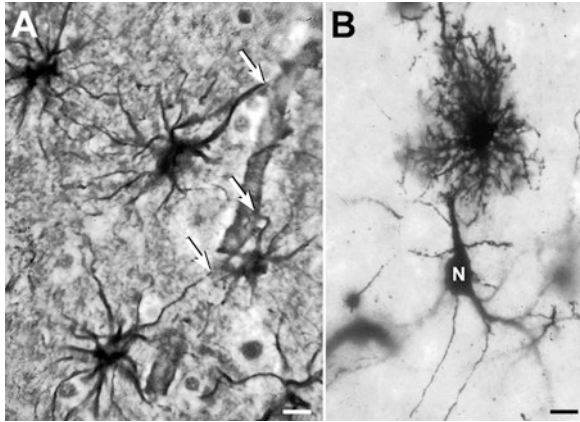
The complex functioning of neurons requires their intimate association with a cohort of non-neuronal, supporting cells. Originally described by Rudolph Virchow in 1856, as “nervenkitt”, referring to them as forming a “putty” or “glue” since he believed that they just simply held neurons together. Supporting cells or glial cells are now known to perform a large number of critical functions that will be discussed in more detail in the following descriptions of each cell type.

Supporting cells generally outnumber neurons in a ratio that, depending on the specific brain region, can vary from 10:1 to 1:1. Reflecting their critical role in higher order neural functioning, the diversity of supporting cells has increased during phylogeny (Verkhatsky et al. 2019). In the CNS, supporting cells consist of astrocytes, oligodendrocytes, NG2 cells or polydendrocytes and ependymal cells, all of which are of neuroectodermal origin. The fourth type, microglia, are the “brain macrophages” which, like peripheral macrophages are of hemopoietic origin.

A unique class of non-neuronal cells are radial glial cells. These cells are neural stem cells that are present throughout the developing CNS (Falk and Götz 2017). They are bipolar cells that span the developing neural tube and divide asymmetrically to generate neurons, oligodendrocytes and astrocytes. In addition their long processes provides guidance for migrating neurons. The majority of radial glial cells disappear by transforming into various forms of astrocytes, however some persist into adulthood.

#### 3.1 Astrocytes

Astrocytes are named based on their star-shaped morphology and comprise the largest population of glial cells. Astrocytes were originally classified based on their appearance in Golgi silver-impregnation preparations, as either fibrous, located primarily in regions of fiber tracts (white matter), or protoplasmic, located near



**Fig. 2.3** Astrocytes. Comparison of the two major categories of astrocytes, fibrous astrocytes (Panel **a**) and a protoplasmic astrocyte (Panel **b**). Fibrous astrocytes extend long, narrow, unbranched processes and are mostly present in fiber tracts (white matter). Astrocytes also extend process that contact small blood vessels (arrows). These perivascular endfeet provide for direct metabolic exchange between intracerebral vessels and neurons. Panel (**b**) shows a protoplasmic astrocyte adjacent to a neuron (N). Protoplasmic astrocytes extend short, broad, highly branched processes and are mostly present in areas with neuronal cell bodies (grey matter). Golgi silver stain. Scale bar = 50 microns

neuronal cell bodies (grey matter) (Fig. 2.3). Protoplasmic astrocytes constitute a larger proportion of total astrocytes and have short, highly branched processes (Reinchenbach and Wolburg 2005). Fibrous astrocytes have numerous, long narrow processes. This simple dichotomous classification has since been greatly expanded due to more sophisticated physiological studies and immunocytochemical staining techniques. Astrocytes differ according to their developmental origin (Chaboub and Deneen 2012) and there are also region-specific astrocytes including Bergmann glia in the cerebellum and Müller cells in the retina. Astrocytes express a unique Class III intermediate filament, termed glial filaments, that are composed of glial fibrillary acidic protein (GFAP). Antibodies to GFAP are routinely used to identify astrocytes in tissue sections, however the expression of GFAP is not uniform among astrocytes (Liddelow and Barres 2017).

Research in recent years demonstrated that the functions of astrocytes are extensive and growing (Butt and Verkhratsky 2018; Santello et al. 2019). Astrocyte processes occupy much of the intercellular space in the CNS and make contact with all parts of the neuron. They also form perivascular endfeet that establish and maintain the blood-brain-barrier as well as provide direct metabolic exchange between intracerebral vessels and neurons. Along with physical support of the neural parenchyma, astrocytes provide metabolic support for neurons through their glycogen reserves and lactate secretion (Alberini et al. 2018). Contributing to the overall homeostasis in the brain, astrocytes also buffer extracellular ions like potassium and sodium and modulate blood flow via their perivascular endfeet. Astrocytes secrete a variety of proteins that regulate

synapse formation, including thrombospondins, which are large extracellular matrix proteins that promote synapse formation and remodeling (Christopherson et al. 2005). Additionally, astrocytes participate in synaptic function where, along the pre- and postsynaptic elements, they form the “tripartite synapse”. Here, they take up excess glutamate, thereby terminating transmission and preventing glutamate excitotoxicity. In addition, that glutamate is converted to glutamine, which is transferred back to the presynaptic terminal where it is converted to glutamate for pre-loading of synaptic vesicles. Excess of other neurotransmitters, including acetylcholine, ATP/adenosine, dopamine, glycine and catecholamines is also known to be controlled by astroglial neurotransmitter inactivating systems (Martin 1995). It has been estimated that a single hippocampal astrocyte may contact 120,000 synapses of different cell types and circuits (Bushong et al. 2002).

Astrocytes are functionally coupled to other astrocytes and to oligodendrocytes by gap junctions, a feature more prominent among protoplasmic astrocytes in grey matter (Orthmann-Murphy et al. 2008). These gap junctions create a “glial syncytium” that has been shown to be important in coordinating neuronal activity as well as oligodendrocyte maintenance and myelination.

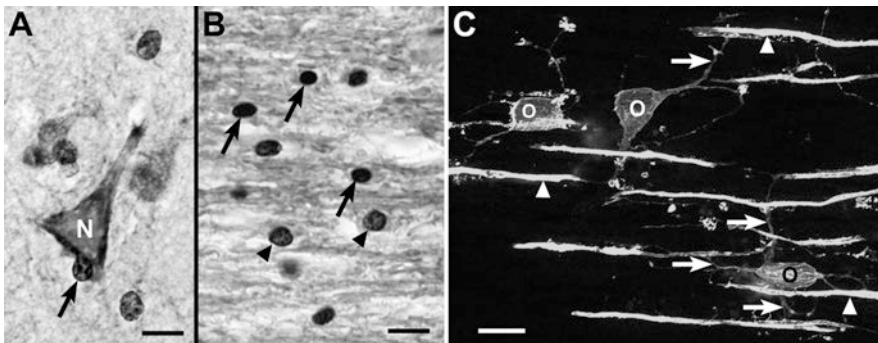
An intriguing function of astrocytes is their participation in the glymphatic circulation, a counterpart to peripheral lymphatic circulation. First described in 2012, the glymphatic circulation begins as cerebrospinal fluid (CSF) flows down the perivascular spaces surrounding penetrating cerebral blood vessels (Hablitz and Nedergaard 2021). After entering the brain parenchyma, the spaces become surrounded by astrocytic perivascular endfeet, forming a tunnel. In this location, astrocytes control brain CSF and interstitial fluid volume through aquaporin channels in their membranes (Salman et al. 2021) and provide clearance of soluble proteins and waste products to the cerebrospinal fluid (CSF). Interestingly, the glymphatic clearance is most active during periods of sleep.

In response to trauma, inflammation and disease, astrocytes undergo astrogliosis, marked by a morphological transformation whereby they become larger with thicker processes, and express higher levels of GFAP and vimentin (Liddelow and Barres 2017). Accompanied by only modest cell proliferation, reactive astrocytes do show altered expression of a number of genes. Astrogliosis is currently viewed as a multivariate reaction with various outcomes, reflecting astrocyte heterogeneity and the type of CNS challenge. A common feature of more severe astrogliosis is the formation of a glial scar, consisting of a shell of newly proliferated astrocytes, NG2 cells and microglia, surrounding a core of damaged tissue (Adams and Gallo 2018). The presences of the scar may inhibit axonal regeneration and myelination, but may also have the positive effect of preserving tissue integrity.

### 3.2 Oligodendrocytes

A second type of neuroglial cell is the oligodendrocyte or oligodendroglia (Butt 2005). Originally named based on their apparent minimal number of processes ('oligo' = few), oligodendrocytes are subdivided into two major groups based on their distribution and function: perineuronal or satellite oligodendrocytes and interfascicular or myelinating oligodendrocytes (Fig. 2.4). Perineuronal oligodendrocytes are located in grey matter areas, in close apposition to neuronal cell bodies and extend processes that contact the neuron (Fig. 2.4a). Perineuronal oligodendrocytes do not form myelin, rather they provide metabolic and functional support for the neuron, possibly by modulating glutamate and GABA metabolism (Bernstein et al. 2019).

Interfascicular oligodendrocytes produce the myelin sheath which surrounds and insulates axons with diameters greater than 0.2 microns. In the adult, they have small cell bodies with a dark, heterochromatic nucleus and minimal cytoplasm (Ross and Pawlina 2016). The cell bodies align in parallel groups among myelinated axons in white matter tracts (Fig. 2.4b, c). Myelin is an elaboration of the oligodendrocyte plasma membrane, with each oligodendrocyte capable of forming myelin on as many as 40 axons. Each oligodendrocyte extends many separate processes



**Fig. 2.4** Oligodendrocytes. The two main types of oligodendrocytes are distributed between grey matter and white matter areas. Panel (a) shows a perineuronal oligodendrocyte (arrow) in grey matter adjacent to a neuron (N). With this stain, only the nucleus is visible which is small and darkly stained. Panels (b) and (c) show interfascicular oligodendrocytes which are present in white matter and form the myelin sheath. Panel (b), shows a section of the corpus callosum where the small dark nuclei of oligodendrocytes (arrows) are visible among the myelinated axons. The slightly larger nuclei (arrowheads) are those of fibrous astrocytes. The cell processes of both types are not visible with this stain. Panel (c) shows oligodendrocytes in co-culture with dorsal root ganglion neurons. Here, the oligodendrocyte cell bodies (O) are visible along with their multiple extensions (arrows) which end as myelin internodes (arrowheads). Oligodendrocytes form multiple internodes, a feature demonstrated by the cell in the lower right. Panel (a): Cresyl violet, luxol fast blue stain; Panel (b): Hematoxylin and eosin stain; Panel (c): Myelin basic protein immunocytochemical staining; Scale bar in each panel = 10 microns; (Image in Panel (c) is courtesy of Dr. Babette Fuss, Dept. of Anatomy and Neurobiology, Virginia Commonwealth University)



that each spiral around the axon forming multiple concentric lamellae. Initially the lamellae contain intervening cytoplasm which is eventually extruded as the lamellae condense forming mature, compact myelin.

Myelination in humans begins before birth with the most rapid period occurring during the first 2 years of life. It continues throughout life, albeit at a slower rate. Myelination progresses in a stereotypic manner, generally in the caudal to rostral direction, with the frontotemporal subcortical regions myelinating last. Myelination is initiated by molecular signals such as neuregulin on the axonal membrane as well as the appearance of electrical activity in the axon (Pajevic et al. 2014). Once formed, the myelin sheath is dynamic, both in terms of macromolecule turnover and remodeling to adjust to circuit demands (Chang et al. 2016).

The myelin sheath consists of a series of segments, called internodes, that are spaced along the axon. Internodal length and thickness are proportional to the diameter of the axon. The gaps between the internodes expose regions of the axon called nodes of Ranvier that are rich in sodium channels. On either side of the node is the paranodal region, where the ends of the internode are anchored to the axonal membrane by tight junctions (Rasband and Peles 2021). These tight junctions are, in part, responsible for confining the sodium channels to the node. Beyond the paranode is the juxtaparanodal region of the axon which is rich in potassium channels. This segregation of the ion channels, between node and juxtaparanode, insures inward current at the nodes, via sodium channels (depolarization), and outward, rectifying current via potassium channels into the space between the internode and the axon membrane (Cohen et al. 2020).

The myelin sheath functions to increase the conduction velocity of the axon, by a process called saltatory conduction. Unlike unmyelinated axons, where conduction is continuous along the length of the axon, in myelinated axons, depolarization occurs only at nodes of Ranvier. Depending on the diameter of the axon, this node-to-node jumping of the action potential results in an increase in conduction velocity ranging from 70 to 120 m/s (faster than a Formula 1 race car!), or nearly a 50–200 fold increase over the 0.5–2.0 m/s speed of unmyelinated axons. (Suminaite et al. 2019). Based on the biophysical cable properties of axons, to attain the speed of a myelinated axon, the diameter of an unmyelinated axon would need to be of such dimensions, that it would result in an unacceptable brain size. As an example, to maintain conduction speeds of just 50 m/s solely by increasing the diameter of unmyelinated axons, would result in a spinal cord with a diameter of up to 1 meter! (Zalc 2016). By allowing faster conduction along thinner axons, myelin not only solves this space dilemma, it also saves energy in two ways (Knowles 2017; Perge et al. 2012). First, myelinated axons require fewer sodium channels and second, fewer energy-dependent depolarization-repolarization cycles are needed. From an evolutionary perspective, myelin was a key specialization in the success of vertebrates which are active animals that require rapid conduction speeds (Knowles 2017).

The myelin membrane is distinctively lipid-rich and also possesses a unique cohort of proteins (Aggarwal et al. 2011). Unlike most membranes where the proportion of lipid to protein is about 50:50, the ratio in human myelin is closer to 70:30. The myelin membrane also contains higher proportions of cholesterol and

glycolipids compared with other membranes. These lipids are critical for the formation and stability of myelin.

The protein composition of myelin is also simpler than most membranes. In CNS myelin, just two proteins, myelin basic protein (MBP) and proteolipid protein (PLP) constitute 60–80% of the total protein. Both function to stabilize and maintain the lamellar structure. These two proteins occupy different compartments within compact myelin (Aggarwal et al. 2011). PLP is inserted in the membrane whereas MBP is cytoplasmic, located adjacent to the inner face of the membrane where it plays a critical role in myelin compaction. Additional myelin proteins include the PLP-related protein, DM-20, cyclic nucleotide phosphohydrolase (CNPase), myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG). By electron microscopy, the compacted myelin membranes display a distinct periodicity with a prominent major dense line, representing the closely apposed cytoplasmic leaflets, and an intraperiod line, representing the less closely apposed outer leaflets of adjacent lamellae.

In the PNS, myelin is produced by Schwann cells. In contrast to oligodendrocytes however, each Schwann cell produces only a single myelin internode and rather than extending processes, the entire cell encircles the axon (Garbay et al. 2000). This simpler 1:1 stoichiometry is in part responsible for the better clinical recovery seen after peripheral demyelination. Fifty percent of the protein in PNS myelin is P<sub>0</sub> protein, which plays a similar role to that of PLP in CNS myelin. MBP is also present and, like in the CNS, it functions in myelin compaction. The periodicity pattern of PNS myelin is similar to that of CNS myelin.

Demyelination is the loss or destruction of myelin and is most often the result of inflammation. The most common example of CNS demyelination is multiple sclerosis (MS) with an onset between 20 and 40 years of age and is more prevalent in women (Murúa et al. 2022). MS is marked by a lymphocytic infiltration of the CNS with resultant destruction of myelin along with damage to the axon. A similar reaction in the PNS is found in Guillain-Barré syndrome.

### 3.3 *Microglia*

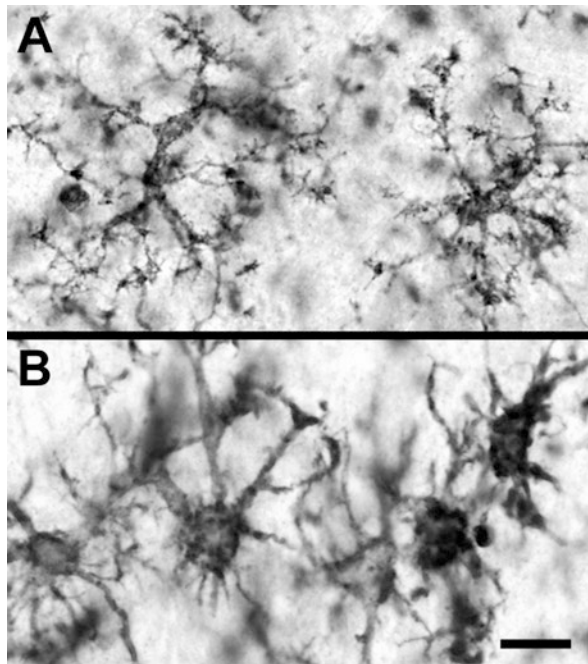
Microglia, or the “third element” as first described by Rio Hortega in 1919, are the third type of glial cell in the CNS. They are often referred to as the resident macrophages of the brain. Hemopoietic in origin, microglia arise early in embryological development from the yolk sac, the first blood forming structure in the vertebrate embryo. In humans, microglial precursors migrate from the yolk sac and can be detected in the brain and spinal cord as early as 4–6 weeks of gestation (Menassa and Gomez-Nicola 2018). Upon reaching the CNS, in response to local environmental factors, they undergo a wave of proliferation and differentiate into resident microglia. This early seeding of the CNS is the only source of microglia, with all subsequent generations being derived from these early progenitors. While microglia are related to peripheral macrophages, they have distinct transcriptomes,

epigenomes and proteomes (Prinz et al. 2019). These early microglia are highly phagocytic, functioning to refine neuronal circuits, including synapse elimination (pruning) and removal of supernumerary neurons by inducing apoptosis and phagocytosis (Prinz et al. 2019). Synaptic remodeling by microglia varies by brain region and continues well into adulthood, particularly in late-maturing regions like the prefrontal cortex.

In the adult, microglia constitute approximately 10% (7–15%) of all cells in the brain and are relatively evenly distributed in a tile-like pattern throughout the neuropil. They have small, round to rod-shaped cell bodies with multiple, highly ramified processes that extend over an area approximately ten times the diameter of the cell body (Fig. 2.5a). Ramified microglia were erroneously described as resting, until more recent observations through a cranial window into a living cortex revealed that microglial processes are rapidly extending and retracting, surveilling a large area of the surrounding tissue by contacting neurons, astrocytes, blood vessels and synapses (Davalos et al. 2005; Nimmerjahn et al. 2005). As a result of these observations, the term “surveying” rather than “resting” is the preferred description for these cells.

A key role for these surveying microglia is synaptic pruning and synaptic stripping, a process facilitated by complement factors C1q and C3 (Tenner et al. 2018). C1q and C3 are produced by neurons, astrocytes and microglia and are released into the extracellular space. They bind to presynaptic terminals, thereby marking them with an “eat me” signal for removal by microglia. In the process, the remaining

**Fig. 2.5** Microglia. Panel (a) shows two surveying microglial cells that demonstrate a ramified morphology. In this stage, microglia have small, round to rod-shaped cell bodies with multiple, highly branched processes that rapidly extend and retract, surveilling a large area of the surrounding tissue. Panel (b) shows activated microglial cells with larger, round cell bodies and fewer, thicker and shorter processes. Immunocytochemical staining for IBA1 antigen. Scale bar = 100 microns



synapses become strengthened. Dysregulation of this process is suggested to contribute to neurodegenerative diseases like Alzheimer's disease and neuropsychiatric conditions such as schizophrenia, depression and autism spectrum disorders.

In response to a variety of stimuli, including trauma, disease and viral or bacterial infection, the mostly sessile, surveying microglia can transform into activated forms which are motile (amoeboid) and possess larger, round cells bodies with fewer, thicker and shorter processes (Fig. 2.5b). They also display an accompanying change in the expression of stage-specific molecular markers (Prinz et al. 2019). After resolution of the challenge, activated microglia are capable of toggling back to the surveying (ramified) state but may retain some "memory" of the activated state, thus remaining primed to a later challenge.

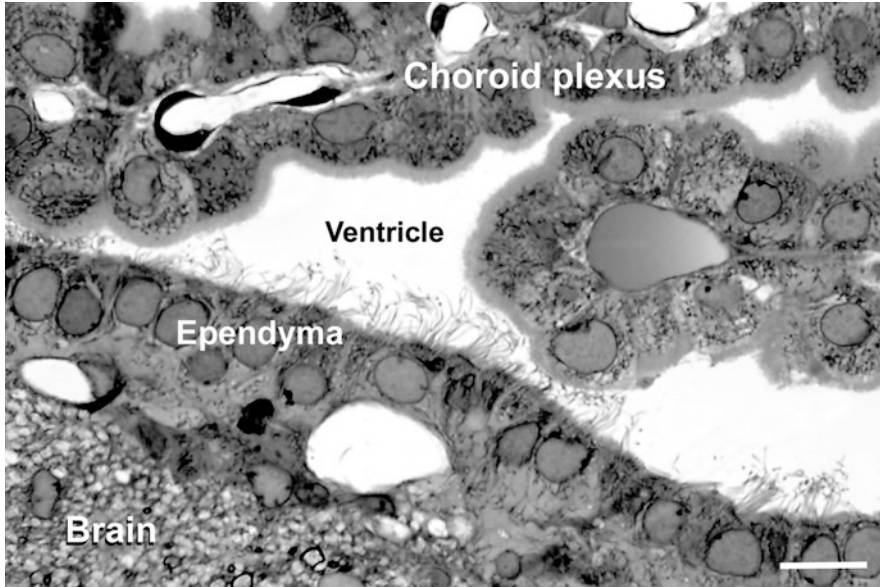
Initial attempts to classify the types of reactive microglia resulted in the nomenclature, M1 and M2, after the scheme proposed for peripheral macrophages (Mills et al. 2000). The M1 phenotype is considered pro-inflammatory (neurotoxic), releasing reactive oxygen species (ROS), nitrogen reactive species (NRS) and cytokines like tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ) or interleukin 12 (IL-12) (Tang and Le 2016). The M2 phenotype is considered anti-inflammatory and neuroprotective, releasing trophic factors, such as tumor growth factor- $\beta$  (TGF- $\beta$ ) and brain derived neurotrophic factor (BDNF). This binary M1/M2 classification scheme is being questioned as more sensitive techniques have revealed multiple phenotypes among reactive microglia with distinct gene expression profiles (Ransohoff 2016). Information based on sequencing data derived from microglia in defined pathological states indicates a great deal of phenotypic heterogeneity, suggesting that reactive microglia exist on a continuum (Masuda et al. 2019).

### 3.4 NG2 Cells (*Polydendrocytes*)

NG2 cells represent a fourth glial cell type, comprising 5–10% of all glial cells. They are distinguished by their expression of NG2 (nerve/glial antigen 2)/CSPG4 proteoglycan, a unique member of the chondroitin sulfate proteoglycan family that is also expressed by a subpopulation of macrophages and pericytes surrounding blood vessels in the CNS (Nishiyama et al. 2009). NG2 has a number of functions including cell proliferation, migration and adhesion (Schiffer et al. 2018).

First isolated from human brain in the 1992 (Armstrong et al. 1992), NG2 cells represent a heterogenous cell population of neuroectodermal origin. They are highly proliferative and evenly distributed in white and grey matter areas. NG2 cells are migratory and extend multiple, thin processes.

NG2 cells generate oligodendrocytes in both the adult and developing brain. They are contacted by both glutamatergic and GABAergic synapses throughout the brain and express receptors for both neurotransmitters, though the significance of this innervation is unclear (Trotter et al. 2010). While reports claim that NG2 cells can form neurons under certain culture conditions, it is not clear if this occurs in vivo. NG2 cells are also capable of forming protoplasmic astrocytes in grey



**Fig. 2.6** Ependyma. The ependyma lines the ventricles of the brain and central canal of the spinal cord. It consists of a single layer of cuboidal to columnar-shaped cells that are highly ciliated. The cilia help circulate the CSF in the ventricles. CSF is produced by the choroid plexus, a system of capillaries surfaced by a single layer of epithelial cells that possess microvilli forming a brush border. Toluidine blue stain. Scale bar = 25 microns

matter during development and following spinal cord injury. In all, under physiological conditions, NG2 cells are thought to maintain oligodendrocyte populations and influence synaptic plasticity. They also support remyelination and recovery from trauma by generating astrocytes (Dimou and Gallo 2015).

### 3.5 Ependymal Cells

Ependymal cells are unique supporting cells that line the ventricles of the brain and the central canal in the spinal cord (Jiménez et al. 2014). They form an epithelial lining, called the ependyma, consisting of a single layer of ciliated cuboidal to columnar-shaped cells (Fig. 2.6). Similar to other epithelial membranes, ependymal cells are tightly bound by junctional complexes. Developmentally, they are derived from radial glia.

Ependymal cells are morphologically and physiologically specialized for the passage of water and solutes in a bidirectional manner between the interstitial fluid and the CSF. The CSF is a colorless, watery fluid that fills the ventricles and surrounds the outside of the brain and spinal cord, both offering cushioning protection and serving as a carrier of nutrients and waste removal. The richly ciliated surface of the ependyma contributes to the flow of CSF within the ventricular system. The

majority of the CSF is generally believed to be produced by the choroid plexus, which consists of epithelial folds that are highly vascularized with fenestrated capillaries. The epithelium of the choroid plexus is continuous with ependyma, however the cells lack cilia and have an extensive brush border composed of microvilli. A choroid plexus is present in each of the brain ventricles.

## 4 Extracellular Matrix

The cellular elements of the CNS are surrounded by a narrow extracellular space that is not resolvable by light microscopy. Early results from electron microscopic studies led to the erroneous conclusion that no extracellular space was present in the CNS, since conventional tissue processing methods “obliterated” it. (Nicholson and Hrabetova 2017). With the use of refined methods of preparation and electrophysiological techniques, an extracellular space was confirmed (Lei et al. 2017). This space is tens of nanometers wide and is estimated to occupy up to 20% of the brain’s volume. It is filled with fluid, resembling CSF along with extracellular matrix (ECM) components consisting primarily of proteoglycans (chondroitin sulfate [CS] and heparin sulfate [HS]) and hyaluronic acid along with low levels of fibrous proteins. Components of the ECM are produced by both neurons and glia, with the majority of the hyaluronic acid coming from neurons. The ECM forms a highly hydrated net-like structure that aids in maintaining the structural integrity of the tissue as well as modulating learning, memory and synaptogenesis (Melrose et al. 2021). The ECM helps maintain neuronal network architecture and plays diverse roles in cell differentiation in early neural development. For example, repulsive CS cues and attractive HS cues serve as important guidance factors for growing axons in tract formation. The actions of growth factors can also be modulated through ECM interactions with surface integrins (Colognato et al. 2004).

A unique specialization of the extracellular matrix is the perineuronal net (PNN). The PNN, first described by Camillo Golgi in 1893, forms a honeycomb-like reticulum that appears at the end of the critical period after active synaptogenesis (Fawcett et al. 2019). The PNN in the CNS mostly surrounds inhibitory neuronal cell bodies and the proximal and middle portions of their dendrites. It is mostly composed of chondroitin sulfate proteoglycans (Melrose et al. 2021). It forms minute windows that surround and stabilize synaptic contacts while acting as a barrier for new synapses. PNNs have been proposed to function in learning and memory based on the timing of their appearance and experimental studies showing that removal of the nets by enzymatic digestion with chondroitinase ABC, restores a period of plasticity (Lensjø et al. 2017). A structure similar to the PNN also surrounds the node of Ranvier.

## 5 Concluding Remarks

Driven by increasingly robust experimental tools, the classical designation of cell types in the CNS is yielding to a much more complex picture of cellular diversity. Just as refinements in imaging led to a better understanding of cell structure, the ability to query the transcriptome of individual cells has illuminated the complexity even within specific cell types. The “neuron-centric” view of the nervous system is also giving way to a fuller understanding of how all the cell types function in concert to accomplish perception, integration, memory and the generation of behaviors.

**Conflict of Interest** The author declares no conflict of interest.

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# Chapter 3

## Synthesis, Processing, and Function of N-Glycans in N-Glycoproteins



**Erhard Bieberich**

**Abstract** Many membrane-resident and secreted proteins, including growth factors and their receptors are N-glycosylated. The initial N-glycan structure is synthesized in the endoplasmic reticulum (ER) as a branched structure on a lipid anchor (dolicholpyrophosphate) and then co-translationally, “en bloc” transferred and linked via N-acetylglucosamine to asparagine within a specific N-glycosylation acceptor sequence of the nascent recipient protein. In the ER and then the Golgi apparatus, the N-linked glycan structure is modified by hydrolytic removal of sugar residues (“trimming”) followed by re-glycosylation with additional sugar residues (“processing”) such as galactose, fucose or sialic acid to form complex N-glycoproteins. While the sequence of the reactions leading to biosynthesis, “en bloc” transfer and processing of N-glycans is well investigated, it is still not completely understood how N-glycans affect the biological fate and function of N-glycoproteins. This review will discuss the biology of N-glycoprotein synthesis, processing and function with specific reference to the physiology and pathophysiology of the immune and nervous system, as well as infectious diseases such as Covid-19.

**Keywords** Chaperones · Congenital disorders of glycosylation · ERAD · Glycolipids · N-glycoproteins · N-glycans · Processing · Trimming · Gangliosides · Glycosyltransferases

### Abbreviations

CDG	Congenital disorders of glycosylation
COG	Conserved oligomeric Golgi
Dol-PP	Dolicholpyrophosphate

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dNM	Deoxynojirimycin (dNJ)
EDEM	ER degradation enhancing mannosidase-like
ER	Endoplasmic reticulum
ERAD	ER-assisted degradation
ERES	ER-exit sites
Gal	Galactose
GalNAc	N-acetyl galactosamine
GD2	Ganglioside GD2
GD3	Ganglioside GD3
Glc	Glucose
GlcNAc	N-acetyl glucosamine
GM1	Ganglioside GM1
GM3	Ganglioside GM3
IgG	Immunoglobulin G
M6P	Mannose-6-phosphate
Man	Mannose
OST	Oligosaccharyl transferase

## 1 Introduction

Throughout the life cycle of neural cells undergoing a “metamorphosis” from neural stem cells to mature neurons, astrocytes, and oligodendrocytes (or Schwann cells in the peripheral nervous system), the differentiation and function of these cells relies upon their response to extra- and intracellular signaling cues. This response depends on the specificity and sensitivity of receptor proteins. It has become increasingly clear that the sensitivity of receptors is regulated by specific N-glycan residues that affect: (1) secretion, stability, and clearance of the receptor ligands; (2) surface expression, internalization, and recycling or turnover of the receptors; (3) adhesion of neurons and other cells via cell surface receptors and extracellular matrix proteins; and (4) signal induction and transduction by growth factor and neurotransmitter receptors and ion channels. In many of these cases, the N-glycan enhances (1) proper folding of ligand or receptor; (2) solubility or polarity of the ligand or receptor; and (3) binding to extracellular or intracellular factors that induce cell signaling pathways or mediate further processing of the N-glycoprotein. In particular, the latter has gained recent attention since specific N-glycans can regulate protein association in receptor/ligand complexes or sugar-specific binding proteins in the plasma membrane (e.g., galectins) that mediate endo- or exocytosis, transport or sorting, and recycling or turnover of the receptor (Dennis et al. 2009a, b; Lajoie et al. 2009; Lajoie and Nabi 2010; Boscher et al. 2011, 2012).

The latest example of N-glycan regulated receptor interaction is the binding of the Sars-Cov2 spike protein, the N-glycosylation of which critically regulates its binding with the ACE2 receptor and immunogenicity in the host (Li et al. 2020; Scudellari 2021; Zheng et al. 2021). In a broader sense, the immunological synapse, the interaction between an antigenic protein exposed at the surface of the

antigen-presenting cell (APC) and the T-cell eliciting the immune response toward the antigen, is probably one of the fastest developing research fields in which the function of N-glycans has been elucidated. Another field, in which research on N-glycan regulated receptor interaction is of growing importance is neural development, particularly with respect to glycosylation deficiency as the cause of congenital disorders, an area in which N-glycosylation of receptors and the interaction of receptors with glycolipids shares a common ground demonstrating the significance of glycans.

While these mechanisms are critical for the proportion of receptors expressed on the cell surface or the retrograde transport of signalosomes (protein complexes between ligand and receptor), it can also modulate the exocytotic transport and secretion of proteins with pathological effects such as amyloid or prion protein (McFarlane et al. 1999; Browning et al. 2011; Schedin-Weiss et al. 2014). It is not surprising that the particular structure of N-glycans and therefore, the sequence of enzymatic processing steps leading to their structure is the focus of intensive research, in particular for the identification of new drug targets. For more than three decades, specific inhibitors of glycosidases involved in N-glycoprotein processing have been tested for their application in antiviral and tumor therapy, including the Sars virus (Robina et al. 2004; Wrodnigg et al. 2008; Wohlfarth and Efferth 2009; Ritchie et al. 2010; Nash et al. 2011; Rajasekharan et al. 2021). On the other hand, mutations in proteins that mediate N-glycosylation and N-glycan processing can lead to severe diseases, including those of the nervous system (Freeze 2002; Jaeken 2010, 2011; Goreta et al. 2012). This is not limited to mutations in trimming or processing glycosidases, but encompasses proteins mediating the transport of N-glycoproteins for their processing in the ER or Golgi, as found in human congenital disorders of glycosylation (CDG) (Kelleher and Gilmore 2006; Reynders et al. 2011; Fung et al. 2012; Barone et al. 2014; Mohanty et al. 2020; Verheijen et al. 2020; Esmail and Manolson 2021). To define the function of N-glycans in normal physiology and disease one needs to know their precise structure and the enzymatic steps generating this structure. Currently, the rapid progress in high throughput mass spectrometric analysis has opened a growing field of comprehensive glycomics studies on N-glycans and other proteinogenic glycoconjugates such as O-glycans and proteoglycans (Moremen et al. 2012). Due to recent developments in imaging mass spectrometry, we are now able to investigate the spatial distribution of N-glycans at the resolution of individual cells (Blaschke et al. 2021, McDowell et al. 2021a, b). In addition to glycomics analysis, mutation analysis and the genome projects have provided us with the information and tools needed to study proteins involved in N-glycoprotein biosynthesis and processing. And yet, the dynamics of biochemistry on the cellular level requires knowledge beyond the statics of structure and sequence, an insight into the flux of biological reactions.

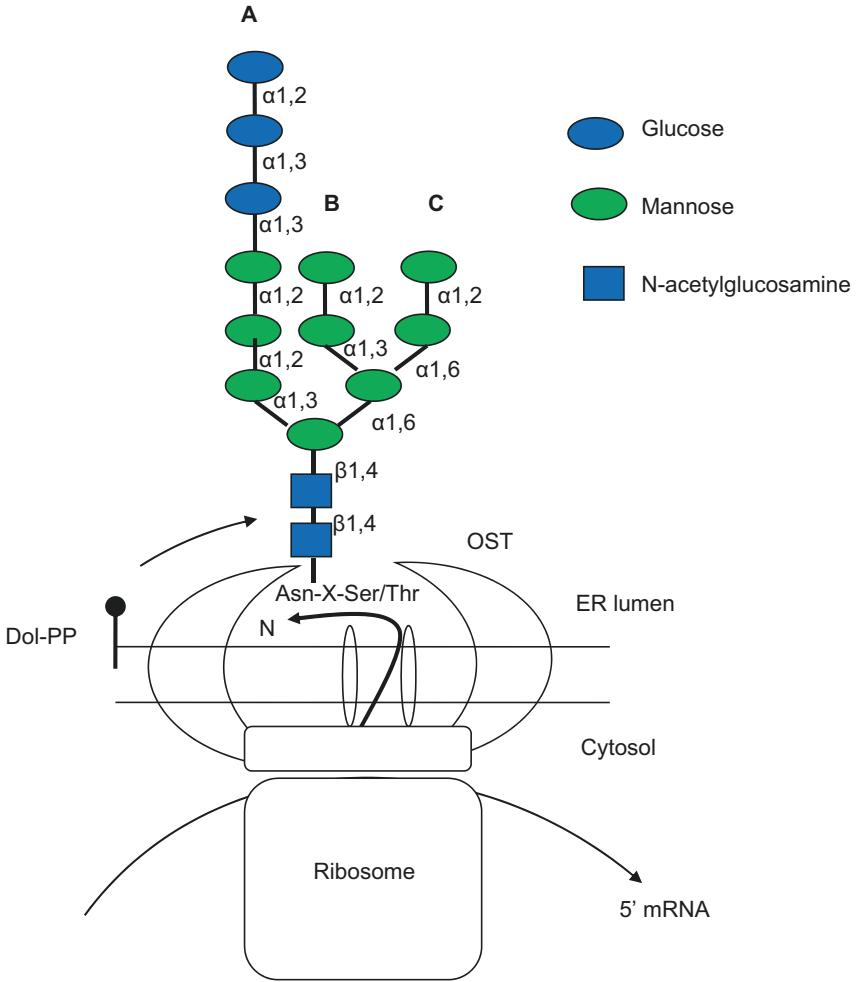
To understand this flux, one may envision the cell as a gigantic factory with sequential assembly lines for the generation and functional editing of N-glycans. This processing works like a flow chart with individual yes/no decision points for: (1) glycosylation leading to non-glycosylated or glycosylated proteins; (2) trimming and re-glycosylation leading to “high mannose” and “re-glycosylated” intermediates; and (3) further processing and re-glycosylation leading to “mannose-6-phosphate”, “hybrid”, or “complex” N-glycoproteins as end products.

As the result of this editing process, N-glycans act as specific addresses or tags that regulate the processing or function of their attached proteins by a simple rule: N-glycan-dependent association kinetics between enzymes, receptor proteins, and other factors keeping the N-glycoprotein in a particular compartment or moving it to the next. As a first step of understanding processing of N-glycans we will discuss the initial assembly of the N-glycan and its transfer to the protein.

## 2 N-glycans Are First Born on a Lipid and Then Transferred “En Bloc” onto the Nascent N-glycoprotein in the ER

N-glycans are oligosaccharides by their chemical nature: branched chains of sugar residues attached to each other by  $\alpha$ - and  $\beta$ -glycosidic linkages (Fig. 3.1). However, the N-glycan is not made on the protein, but pre-manufactured on an ER-resident lipid, the polyprenol dolichol pyrophosphate. Further, while after attachment to the protein, the N-glycan points to the lumen of the ER, the assembly of the initial 7 of the 14 sugar residues in the dolichol pyrophosphate (Dol-PP)-linked precursor oligosaccharide is accomplished at the cytosolic side. Hence, the N-glycosylation reaction relies on two critical steps: transport of the partial precursor oligosaccharide (Dol-PP-GlcNAc2Man5) across the ER membrane from the cytosolic to the luminal side and then after further glycosylation reactions, “en bloc” transfer of the mature precursor to the protein. In eukaryotes, the assembly of this precursor oligosaccharide is achieved by a set of ER-resident, transmembrane protein glycosyltransferases of the types (a), N-acyetylglucosaminyltransferases that attach two GlcNAc residues to Dol-PP in a  $\beta$ 1-N and then  $\beta$ 1-4 linkage, (b) mannosyltransferases catalyzing four different glycosidic linkages:  $\beta$ -1,4 (first mannose attached to second GlcNAc),  $\alpha$ -1,3 and  $\alpha$ -1,6 (mannose at the two branching points of the biantennary oligosaccharide), and  $\alpha$ -1,2 (mannose elongation of the middle (B) and two outer (A and C) branches, see Fig. 3.1) that attach a total of 9 mannose residues to Dol-PP-GlcNAc2 to form a branched Dol-PP-GlcNAc2Man9 structure, and c) glucosyltransferases that attach the terminal three glucose residues in two  $\alpha$ 1,3- and one terminal  $\alpha$ 1,2-glycosidic linkage onto the outer  $\alpha$ 1,3-mannosidic branch (A branch) of the precursor oligosaccharide (Fig. 3.1). These reactions depend on activated sugars that are provided on the cytosolic and luminal side of the ER in the form of UDP-GlcNAc, GDP-mannose (GDP-man), and UDP-glucose (UDP-glc).

Mutations in glycosyltransferases and the flippase transporting the Dol-PP-GlcNAc2Man5 partial precursor from the cytosol into the ER lumen lead to a spectrum of diseases known as congenital disorders of glycosylation type I (CDGs type I) (Leroy 2006; Mohorko et al. 2011). The symptoms of CDGs type I often involve the nervous system. For example, mutations of the RFT1 (Requiring Fifty Three 1, RFT1-CDG or CDG-In) yeast homolog of the mammalian flippase, cause sensorineural deafness (Leroy 2006; Jaeken 2010; Goreta et al. 2012). CDG-In is an extremely rare disease with only 10 known patients so far. Other CDGs type I



**Fig. 3.1** Co-translational, “en bloc” transfer of the  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  precursor oligosaccharide from dolichol pyrophosphate to asparagine, catalyzed by oligosaccharyl transferase in the ER lumen

resulting from mutations in precursor glycosyltransferases often show defects in multiple tissues that can lead to multiorgan failure as observed with glucosyltransferase II deficiency (CDG-Ih). It should be noted that, while CDGs type I can be very severe it is not clear which N-glycoprotein dysfunction due to hypoglycosylation accounts for a specific disease phenotype or symptom. The diagnosis of CDG type I is commonly based on testing for hypoglycosylation of transferrin in a patient’s plasma, although this aberrant glycosylation does not account for the entire spectrum of symptoms observed with this disease. As discussed later, the consequences of hypoglycosylation can be severe for a variety of proteins, the proper

folding of which relies on the intact N-glycan residue. Therefore, it may not be surprising that CDGs type I can lead to multiple tissue and organ failures.

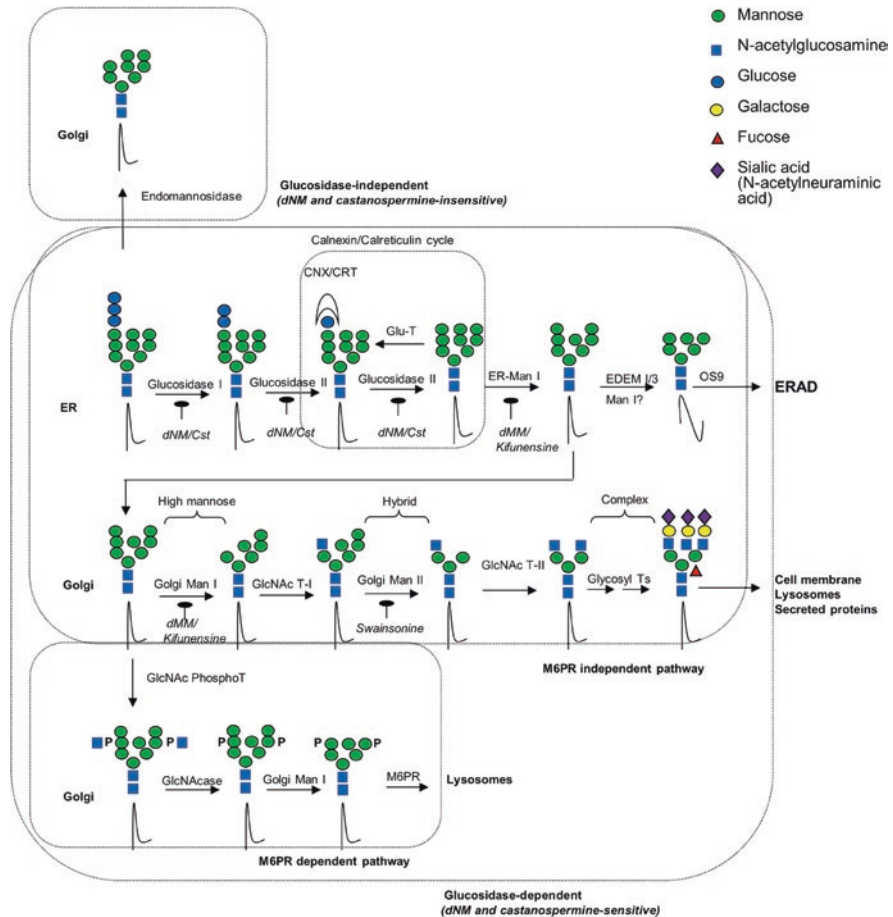
Following the assembly and the flipping reaction of the precursor oligosaccharide, co-translational “en bloc” transfer of the N-glycan from Dol-PP onto the nascent polypeptide is the next critical step in N-glycoprotein biosynthesis. This step is catalyzed by oligosaccharyl transferase (OST), a multimeric enzyme complex composed of 9 subunits in yeast and 8 (OST-A) or 9 (OST-B) subunits in higher eukaryotes (Pless and Lennarz 1977; Sharma et al. 1981; Kaplan et al. 1987; Kelleher et al. 1992; Bause et al. 1995; Hardt et al. 2000; Parodi 2000; Kelleher and Gilmore 2006; Ruiz-Canada et al. 2009; Roth et al. 2010; Moremen et al. 2012). In recent years, the precise function of these subunits has been clarified. N-glycosylation of the nascent polypeptide is predominantly mediated by OST-A, while OST-B is a proofreading enzyme that performs glycosylation of sites that were missed due to improper folding or disulfide linkage (Shrimal et al. 2015, 2019; Bai et al. 2018; Braunger et al. 2018; Shrimal and Gilmore 2019). OST manages association with the ribosome and signal recognition particle receptor for the nascent membrane glycoprotein, the recognition of the acceptor sequence Asn-X-Ser/Thr (X cannot be proline), and the catalytic transfer of the oligosaccharide from Dol-PP onto an asparagine (Fig. 3.1).

As with mutations in glycosyltransferases involved in the precursor oligosaccharide assembly, deficiencies of OST subunits lead to several CDGs of type I. The catalytic subunit of OST, Stt3 in yeast or Stt3A and B in mammals, is a conserved subunit that is already expressed in archaeobacteria. It has been speculated that because of the redundancy conferred by the two mammalian Stt3 homologs, CDGs resulting from errors in them have not yet been discovered (Kelleher and Gilmore 2006; Ruiz-Canada et al. 2009; Vleugels et al. 2009; Mohorko et al. 2011). However, mutations in other OST subunits have been associated with various CDGs of type Ix. For example, deficiencies of the ribophorin I subunit RPN2 have been found by screening for hypoglycosylation defects without impairment of precursor oligosaccharide assembly (Vleugels et al. 2009). At present, the pathology of these CDGs is unclear, but it is likely to involve nervous system defects. Once OST has transferred the precursor oligosaccharide onto the nascent polypeptide, a process starts that entails an important decision for the newly born N-glycoprotein: *fold or fail*. In particular, the glucose residues on the outer mannosidic ( $\alpha$ 1,3- or A) branch are instrumental in assisting the protein folding proofreading and refolding process, which will be discussed in the next section.

### 3 Trimming, Reglycosylation, and Remodeling: There Are Many Ways of N-glycoprotein Processing in the ER and Golgi

There are two pathways by which the N-linked Glc3Man9GlcNac2 oligosaccharides are processed (“trimmed”): the glucosidase-dependent and independent pathway (Fig. 3.2). The glucosidase-dependent pathway occurs in the ER and is mediated





**Fig. 3.2** N-glycoprotein processing: sequential removal (trimming) of glucose and mannose residues from the N-linked glycan in the ER and Golgi, followed by re-glycosylation  
 N-glycan processing generates signals for chaperone-assisted refolding, mannose-6-phosphate receptor-mediated transport of lysosomal enzymes, and other functions in protein trafficking, enzyme complex formation, and cell adhesion

by a sequential hydrolytic cleavage of the terminal glucose residues by glucosidases I and II (Fig. 3.2). This sequence is embedded into chaperone-assisted proofreading of protein folding: the calnexin/calreticulin cycle. The glucosidase-independent pathway is catalyzed by an endomannosidase which cleaves off a Glc1-3Man1 residue in the Golgi (Lubas and Spiro 1987; Hamilton et al. 2005; Alonzi et al. 2013). Although endomannosidase single-nucleotide polymorphism has been associated with panic and anxiety disorder, function and regulation of this enzyme are largely unclear (Jensen et al. 2014). Therefore, we will focus on the glucosidase-dependent pathway and its interaction with the calnexin/calreticulin cycle for protein folding.

To date, the calnexin/calreticulin cycle has been discussed as one of the most important processes underlying the function of N-glycosylation (Hammond and

Helenius 1993, 1994; Hammond et al. 1994; Ellgaard et al. 1999; Molinari and Helenius 2000; Deprez et al. 2005). It was first described by Ari Helenius in 1994 based on two important observations: (a) calnexin and calreticulin are chaperones in the ER that bind to monoglucosylated (GlcMan<sub>9</sub>GlcNAc<sub>2</sub>) N-glycoproteins and (b) the ER harbors a glucosyltransferase that re-attaches glucose to N-linked Man<sub>9</sub>GlcNAc<sub>2</sub> (Trombetta et al. 1989, 1991; Sousa et al. 1992; Trombetta and Parodi 1992; Hammond and Helenius 1993, 1994; Hammond et al. 1994). Trimming or processing of the terminal glucose residues is thus crucial for the chaperone function of calnexin and calreticulin. The function of a chaperone is to recognize and bind misfolded proteins and then mediate refolding until the proper conformation of the protein is accomplished.

The properly folded protein is then bound by another family of lectins, the ERGIC (ER-to Golgi intermediate compartment) proteins, which mediate exit from the ER at ER exit sites (ERES) (Benyair et al. 2015; Tannous et al. 2015; Shenkman and Lederkremer 2019). In recent years, it was found that the Calnexin/calreticulin cycle is assisted by several ER-resident proteins (e.g., CypB, Erp29, Erp57, thioreductases) that chaperone refolding or reduce disulfide bonds in the misfolded protein (Caramelo and Parodi 2015; Lamriben et al. 2016; Kozlov and Gehring 2020). If the misfolded protein cannot adopt its proper conformation it will be demannosylated by EDEM (ER degradation enhancing mannosidase-like) proteins and degraded in the ER by the ERAD (ER-assisted degradation) system, a rather complicated organized sequential process of “unfolded protein response” that first shuttles the misfolded protein into the cytosol, where it is ubiquitinated and then removed by proteosomal degradation (Banerjee et al. 2007). These final steps of EDEM-mediated demannosylation and ERAD-mediated translocation to the cytosol occurs in a specialized ER compartment, the ER quality control compartment or ERQC. Another ERAD-associated system transports the misfolded proteins to the Golgi and then the lysosome for proteolysis. Failure of ERAD can result in severe diseases due to the accumulation and aggregation of misfolded proteins, particularly in the nervous system. Ablation of the ERAD factor Der12 in Schwann cells leads to myelin defects in the peripheral nervous system (Volpi et al. 2016, 2019). Another prominent example is Parkinson’s disease that can result from ERAD malfunction involving the E3-ubiquitinase parkin (Ron et al. 2010). Mutations in glucocerebrosidase (GCCase), a lysosomal  $\beta$ -glucosidase deficient in Gaucher disease, can lead to the accumulation of aberrant GCCase attached to parkin, which is currently discussed as one of the causes of Parkinson’s disease (Ron et al. 2010). Since GCCase cleaves off glucose from glucosylceramide to generate ceramide, it is an example of the interdependence of N-glycoprotein and glycosphingolipid metabolism, which will be discussed later in this chapter. In addition to the aggregation and accumulation of aberrant proteins, ERAD malfunction may also lead to pre-mature degradation of proteins that are still in the process of refolding, which has been discussed as one of the causes of cystic fibrosis (Farinha and Amaral 2005). From these examples, it is evident that the N-glycan assisted calnexin/calreticulin cycle for protein folding and thus the function of ERAD critically relies on proper N-glycan processing.

To generate the monoglucosylated N-glycan, glucosidases I and II remove the first two glucose residues from the N-linked Glc3Man9GlcNAc2 oligosaccharide (Peyrieras et al. 1983; Kalz-Fuller et al. 1995; Parodi 2000; Volker et al. 2002). The resulting GlcMan9GlcNAc2 is bound to calreticulin or calnexin and the protein (partially) refolded, which is followed by removal of the innermost glucose residue by glucosidase II. After this, the N-glycan (Man9GlcNAc2) is transiently reglucosylated to GlcMan9GlcNAc2 by UDP-glucose:glycoprotein glucosyltransferase to prevent further N-glycan trimming of a glycoprotein that has not yet adopted its proper folding state. After glucosidase II has cleaved off the newly added glucose residue, the Man9GlcNAc2 oligosaccharide re-enters the reglucosylation-refolding-trimming cycle until the native conformation of the N-glycoprotein is accomplished. Once the protein is correctly folded, the chaperones do not bind to the protein anymore and the Man9GlcNAc2 oligosaccharide is processed by a series of mannosidases (Fig. 3.2).

The removal of mannose residues from Man9GlcNAc2 is initiated in the ER and intimately associated with the ERAD response to misfolded proteins (Tokunaga et al. 2000; Wang and White 2000; Ruddock and Molinari 2006; Banerjee et al. 2007; Alonzi et al. 2013). If correct folding cannot be achieved the N-glycoprotein is not reglucosylated and ER mannosidase I cleaves off the terminal alpha1,2-mannose residue of the middle (B) branch in the N-glycan generating a Man8GlcNAc2 oligosaccharide (Fig. 3.2). How exactly the ERAD machinery distinguishes between properly folded and misfolded Man8GlcNAc2 bearing glycoproteins is not yet fully understood. As discussed earlier, this process involves a series of reactions by chaperones and EDEMs in the ERGQ, which accelerate the ERAD response to misfolded N-glycoproteins instead of allowing further exit to the Golgi (Helenius and Aebi 2004; Ruddock and Molinari 2006; Hosokawa et al. 2010). It is expected that mutations in trimming enzymes, in particular glucosidase I, II, and ER mannosidase I would lead to another type of CDGs, called CDG type II since these mutations will affect the N-glycan structure after assembly and transfer to the protein.

Indeed, the first CDG type II known to be caused by trimming glycosidase deficiency is CDG type IIb, which results from mutations in ER glucosidase I (Volker et al. 2002). CDG type IIb shows multiorgan deficiencies leading to a variety of symptoms such as hepatomegaly, hypoventilation, feeding problems, seizures, and in one patient a fatal outcome at 74 days after birth. Interestingly, other CDG type II diseases are not directly associated with mutations of trimming enzymes (or have not yet been found), but with proteins of the intercisternal Golgi transport machinery such as the “conserved oligomeric Golgi” (COG) complex proteins. Since trimming glycosidases and N-glycan-associated glycosyltransferases are located in distinct Golgi compartments it is expected that mutations in these transport proteins would also lead to disorders of N-glycosylation (Kelleher and Gilmore 2006; Reynders et al. 2011; Fung et al. 2012). The COG proteins form a complex of 8 proteins critical for retrograde vesicle transport within the Golgi. It is known to regulate Golgi distribution of mannosidases and glycosyltransferases important for N- and O-glycoprotein processing (Moremen et al. 2012). Mutations in COG

proteins are known to cause CDGs of type II, many of those with presentation of nervous system disorders.

The majority of CDGs caused by COG proteins are associated with abnormal reglycosylation of processed N-glycans due to mislocalization of the respective glycosyltransferases. This process of reshaping the N-linked oligosaccharide by reglycosylation is initiated by 3 distinct Golgi-resident mannosidases and follows (at least) two different routes (Fig. 3.2). The transport of lysosomal hydrolases requires the attachment of a Mannose-6-phosphate tag (mannose-6-phosphate or M6P-dependent pathway), while other N-glycoproteins of the “hybrid” and “complex” type are further trimmed by removal of additional mannose residues. We will discuss the pathway of further trimming first.

Golgi mannosidase I is distinct from ER mannosidase I in that it cleaves off 3  $\alpha$ 1,2-residues from the Man8GlcNAc2 precursor to yield Man5GlcNAc2 (Dunphy et al. 1981; Cummings et al. 1983; Helenius and Aebi 2004; Moremen et al. 2012). This  $\alpha$ 1,2 exomannosidase is also different from Man9 mannosidase, which has been cloned and characterized by the author’s former group and cleaves 3 mannose residues from Man9GlcNAc2 to yield Man6GlcNAc2, which can then be a substrate of Golgi mannosidase I that removes one additional mannose residue (Schweden et al. 1986; Bause et al. 1993; Bieberich and Bause 1995; Bieberich et al. 1997). Regardless of how the Man5GlcNAc2 oligosaccharide is generated, further trimming proceeds after attachment of one GlcNAc residue to the outer mannose (A) branch by UDP-GlcNAc transferase I (Fig. 3.2). The resulting GlcNAcMan5GlcNAc2 oligosaccharide is then either elongated by the addition of sugar derivatives such as GalNAc or sialic acid (hybrid N-glycans), or it is subjected to removal of an additional 2 mannose residues by Golgi mannosidase II, which generates N-linked GlcNAcMan3GlcNAc2, the “core” glycan structure that is the initial building block for all complex N-glycoproteins (Arumugham and Tanzer 1983; Cummings et al. 1983; Moremen and Touster 1985; Moremen and Robbins 1991; Herscovics et al. 1994; Moremen 2002). Recently, Golgi mannosidase II has become the focus of research to find specific inhibitors of its action for use as a cancer treatment (Armstrong et al. 2020; Lee et al. 2021).

As with hybrid N-glycans, complex N-linked oligosaccharides are reglycosylated with additional sugar derivatives such as GalNAc, sialic acid, or fucose (Fig. 3.2). These complex N-glycans come in a large variety of highly branched structures, which can far exceed the initial biantennary (2 branching points) structure of high mannose oligosaccharides. Biochemically, high mannose N-glycans can be distinguished from hybrid or complex ones by the use of two endoglycosidases that either cleave off the complete N-glycan (glycopeptidase F) or hydrolyze the  $\beta$ -glycosidic linkage between the two GlcNAc residues (chitobiose) of high mannose N-glycoproteins (endoglycosidase H). These enzymes and other glycosidases were discovered early in the history of glycobiology, which in combination with metabolic labeling using radioactive sugars tremendously facilitated the structural analysis of N-glycans (Tarentino et al. 1974).

Several glycosylation deficiencies are known to result from mutations of enzymes in the M6P-dependent pathway or from aberrant reglycosylation of hybrid or complex

N-glycoproteins. In the M6P-dependent pathway for lysosomal enzymes, the Man8GlcNAc glycan is first endowed with 2 GlcNAc phosphate residues that are attached by a Golgi transferase to the subterminal mannose residues of the two outer mannose branches (Waheed et al. 1981; Lemansky et al. 1984; Chao et al. 1990; Lubke et al. 1999; Ghosh et al. 2003; Gary-Bobo et al. 2007; Coutinho et al. 2012). Next, the mannose-bound phosphate residues are uncovered by N-acetylglucosaminidase and the terminal mannose residues of the outer (A and C) branches are removed by Golgi mannosidase I (Fig. 3.2). The resulting P2Man6GlcNAc2 oligosaccharide is now recognized by two types of M6P receptors (cation-dependent and independent) in the trans Golgi, which bind to the N-glycoprotein and initiates their transport to the late endosome. While the late endosome matures to lysosomes, the pH value drops and the M6P receptor releases the lysosomal enzyme. The M6P receptors are then recycled to the trans Golgi for further transport of lysosomal enzymes. Deficiencies in the M6P-dependent pathway, in particular caused by mutations of GlcNAc phosphotransferase, the enzyme attaching the GlcNAc phosphate residues to the N-glycan, can lead to severe disorders of glycosylation. Well known examples are I-cell disease or mucopolipidosis type II, and pseudo Hurler polydystrophy or mucopolipidosis type III (Coutinho et al. 2012). These diseases are usually not classified as CDG type II but as oligosaccharidosis or mucopolipidosis-type lysosomal storage diseases because failure of transporting enzymes to lysosomes will lead to the accumulation and lysosomal storage of the enzyme substrates, in particular glycosaminoglycans. Mucopolipidosis type II and III lead to severe abnormalities in multiple organs (hepatomegaly, splenomegaly) and delay in cognitive and motor skills development. Recently, it has been found that a cation-independent M6P receptor is identical to insulin growth factor receptor II (IGFRII), which has led to the hypothesis that this multifunctional receptor plays critical roles in several nervous system diseases, including Alzheimer's disease (Wang et al. 2017).

As mentioned earlier, the majority of CDGs type II are caused by mutations in glycosyltransferases that generate the complex N-glycan, e.g., CDG II<sub>d</sub>, a deficiency of  $\beta$ 1,4 galactosyltransferase I, which leads to psychomotor delay and macrocephaly (Leroy 2006; Jaeken 2010). Also, as discussed earlier, many CDGs type II of complex N-glycoprotein processing are caused by aberrant COG proteins, e.g., CDG-II<sub>e</sub> or COG7 deficiency, which leads to hyposialylation of complex N-glycans. In contrast to lysosomal storage diseases related to aberrant M6P-dependent transport of lysosomal enzymes, the molecular cause of the deficiency or the malfunction of the N-glycoprotein in various CDGs are not clearly defined. The reason is twofold: (a) glycosylation defects affect not only one but a variety of complex N-glycoproteins; and (b) for many N-glycoproteins it is not well understood what the physiological function of the N-glycan is, which makes it difficult to understand the malfunction as well. In the next two sections, we will discuss some of these functions, particularly with respect to the significance of N-glycoproteins for brain development and physiology. However, before we move on to these sections, it is necessary to briefly discuss important tools that have helped to elucidate the sequence of N-glycoprotein processing and the function of N-glycoproteins: trimming enzyme-specific inhibitors.

#### **4 The Essential Toolbox of a Glycobiologist: A Brief History of the Discovery of N-glycoprotein Biosynthesis Inhibitors and Their Impact on Our Understanding of N-glycan Processing**

The discovery and development of N-glycosylation and processing inhibitors is intimately linked to the history and progress in N-glycoprotein research – and the professional careers of many leading glycobiologists. The first inhibitor of N-glycosylation, tunicamycin was found 50 years ago in an attempt to screen for antiviral drugs made by bacteria, in particular strains of *Streptomyces* (Takatsuki et al. 1971; Takatsuki and Tamura 1971a, b, c; Schwarz et al. 1976; Ericson et al. 1977; Leavitt et al. 1977; Hart and Lennarz 1978). This finding is not as surprising as it may seem today since the screening procedures at that time were often based on virus hemagglutination and *in vitro* proliferation assays, which were critically affected by the glycoprotein nature of serum proteins and the virus envelope, a well-known fact even decades ago (Hewitt 1937). Therefore, one of the tests routinely performed was a competition assay determining whether addition of sugars, in particular N-acetyl aminosugars would reverse the effect of the antiviral antibiotic, as seen with tunicamycin (Takatsuki and Tamura 1971a, b, c). Later, Alan Elbein discovered that tunicamycin inhibits the transfer of N-acetylglucosamine to dolichol phosphate, the first step in the synthesis of the lipid-linked oligosaccharide that serves as the precursor for all N-glycoproteins (Chambers and Elbein 1975; Ericson et al. 1977). It was also found early on that tunicamycin induces the degradation of viral glycoproteins and it was hypothesized that proteolysis was due to the lack of glycosylation, the first inkling of what would later be known as chaperone-assisted N-glycoprotein proofreading or the calreticulin/calnexin cycle in the ERAD response to unfolded proteins (Schwarz et al. 1976). Nowadays, tunicamycin is commonly used to induce the “unfolded protein response” or ER stress, unfortunately often without paying attention to its effect on N-glycoprotein biosynthesis. Because of its toxicity tunicamycin has never made its way into use as a virus therapy, although recent studies suggest that it may have antiviral effects against Hepatitis C and probably SARS-Cov-2 at subtoxic doses (Reszka et al. 2010).

Another antibiotic acting on N-glycoprotein biosynthesis and isolated from *Streptomyces*, the imino sugar deoxynojirimycin (dNM or dNJ) was also discovered more than 40 years ago. It was identified as a member of the validamycin family in a screening assay for antifungal agents that would inhibit the breakdown of trehalose, the storage disaccharide in insects and fungi (Nash et al. 2011). Deoxynojirimycin is an inhibitor of  $\alpha$ -glucosidases and was first designed for treatment of type II diabetes because of its ability to prevent the breakdown of amylose (starch) and mobilization and uptake of glucose in the intestine. Nowadays, acarbose, a natural tetrasaccharide containing a structural isomer of deoxynojirimycin is widely used as an oral medication for diabetes type II. Deoxynojirimycin itself has done more to shape the history of research on N-glycoprotein processing than it has as a therapeutic for diabetes.

At the time when acarbose and dNM were discovered, it was well known that neuraminidase-sensitive, glycoprotein-bound sialic acid is important in virus agglutination. Gilbert Ashwell and Anatol Morell reported for the first time a galactose-specific receptor for asialoglycoproteins (Van Den Hamer et al. 1970; Morell et al. 1971). Yet, besides knowing that many glycoprotein-linked oligosaccharides contained terminal sialic acid, units of galactose- $\beta$ 1,4-N-acetylglucosamide, and other di-,tri-, and tetrasaccharides, the actual (branched) structure of the N-linked glycan remained unclear until 1978, when Ellen Li and Stuart Kornfeld proposed a structure with branched mannose chains linked to asparagine via N-acetylglucosamine (Li and Kornfeld 1978). The 1970's and early 1980's became a well-spring of new discoveries in glycobiology. Willam Lennarz worked out the biosynthesis pathway of the dolichol-linked precursor oligosaccharide and Amando Parodi reported the "en bloc" transfer of this precursor onto the nascent glycoprotein (Parodi et al. 1972; Waechter et al. 1973; Lucas et al. 1975).

Deoxinojimycin and other inhibitors such as castanospermine played essential roles in this discovery since they inhibited not only broad spectrum  $\alpha$ -glucosidases, but also glucosidase I and II, which are the other components of the N-glycan-driven proofreading machinery. Similar to tunicamycin, dNM and castanospermine prevented correct folding and secretory exit of viral N-glycoproteins and induced ER-resident protein degradation instead. However, inhibitors of Golgi mannosidases such as swainsonine or deoxymannojimycin, did not. Based on this observation and the concurrent characterization of GlcMan9GlcNAc2-binding ER chaperones, Ari Helenius then proposed the calreticulin/calnexin cycle, which is probably the most prominent example for the general function of N-glycans (Hammond et al. 1994). Besides their value in understanding the unfolded protein response to N-glycoproteins, glucosidase I and II inhibitors were also instrumental in the discovery of the M6P-dependent pathway by Kurt von Figura et al. in 1984 (Lemansky et al. 1984).

I came into contact with dNM through my graduate student mentor Dr. Gunter Legler, who contributed much to our understanding of the catalytic mechanism of  $\alpha$ - and  $\beta$ -glucosidases. He and his mentee, who then became my first post-doctoral mentor, Dr. Ernst Bause, were among the first to use dNM and its derivatives to characterize and purify glycosidases, including those involved in trimming of N-glycoproteins (Hettkamp et al. 1982, 1984; Peyrieras et al. 1983; Schweden et al. 1986). At this time, the pre-human genome era, one could not just "blast search" for cDNA sequences, but actually had to purify a protein to homogeneity and then identify the amino acid sequence in order to synthesize oligonucleotide primers that could be used for RT-PCR to generate a probe for screening of a lambda gt11 library, and eventually, isolate the protein-specific cDNA; a long-forgotten and extremely tedious technique. Using alkylated dNM derivatives for affinity chromatography of trimming enzymes proved to be extremely helpful in this endeavor. Despite this progress, it took several years and thousands of plasmid minipreps to generate specific probes that then led to the first cloned cDNAs for glucosidase I and Man9 mannosidase (Bause et al. 1993; Bieberich and Bause 1995; Kalz-Fuller et al. 1995).

Currently, alkylated dNM derivatives such as N-butyl dNM (miglustat) are being tested as antibiotics for treatment of several viral diseases, including HIV, SARS-Cov, and SARS-Cov-2 (Ratner and Vander Heyden 1993; Robina et al. 2004; Rajasekharan et al. 2021). Grabowski's and Legler's groups have reported that alkylated dNM derivatives inhibit the two enzymes that regulate the metabolic conversion of ceramide and glucosylceramide into each other, lysosomal  $\beta$ -glucosidase (the "Gaucher enzyme") and glucosyltransferase, with longer alkyl chain length being more specific for  $\beta$ -glucosidase and shorter chain length for glucosyltransferase (Legler and Liedtke 1985; Osiecki-Newman et al. 1986, 1987; Legler and Bieberich 1988; Greenberg et al. 1990). Therefore, N-butyl dNM has been discussed as a possible treatment for several lysosomal storage diseases involving accumulation of sphingolipids, including Gaucher disease (glucosylceramide accumulation), Niemann-Pick disease (sphingomyelin accumulation), and Tay Sachs or Sandhoff disease (GM2 accumulation) (Platt et al. 1994; Chavany and Jendoubi 1998; Jeyakumar et al. 1999; Cox et al. 2000; Mistry 2000; Patterson et al. 2007; Baek et al. 2008; Abian et al. 2011; Nash et al. 2011; Venier and Igdoura 2012).

Due to the recent Covid-19 pandemic, inhibitors of N-glycosylation and processing for virus therapy have regained attention and several pharmacological approaches such as the castanospermine prodrug Celgosivir, Miglostat, and several novel trimming inhibitors based on modified iminosugars are currently tested against SARS-Cov-2 and other viruses (Rajasekharan et al. 2021). While the use of trimming enzyme inhibitors has not (yet) led to breakthroughs in virus therapy, dNM, castanospermine, and swainsonine have certainly been invaluable in elucidating the sequence of trimming enzymes and the function of N-glycans. One of these more recently discovered functions intertwines N-glycoprotein processing with glycolipid biosynthesis: the role of N-glycosylation in the subcellular localization and enzyme complex formation of glucosyltransferases in ganglioside biosynthesis.

## **5 Sweetening the Bond or Sugar-Coating Bad News: Recent Advances in the Role of N-glycans for Virus Infections and Immune Responses**

The observation that N-glycans critically contribute to and regulate interactions between host cells and pathogens as well as the immune response to them is almost as old as the hemagglutination (inhibition) assay (HA), a serological assay measuring the aggregation of red blood cells (typically from chicken) induced by binding of sialylated surface glycoproteins of the virus to sialic acid receptors on erythrocytes (Spackman and Sitaras 2020). As discussed in the previous section, the HA is also used to identify neutralizing antibodies or drugs interrupting N-glycosylation and therefore, the development of new antiviral drugs. In the 1940s, Influenza was one of the first viruses characterized and quantified by HA, demonstrating the importance of N-glycoproteins for binding of viruses to host cells (Pedersen 2014). In light of the



Covid-19 pandemic, research on the importance of viral N-glycoproteins for infection and antigenicity has rekindled attention to this old observation. The spike (S) protein of Sars-Cov-2 contains 22 N-glycosylation sites, which “sugar coat” the entire protein except the receptor binding site on the top (Li et al. 2020; Ramirez Hernandez et al. 2021; Scudellari 2021; Zheng et al. 2021). It is still not clear if the N-glycans are critical to shape the S-protein conformation for binding to the ACE receptor or whether they participate in the molecular interaction with it. However, it has been found that the heavy N-glycosylation of Sars-Cov-2 surface proteins is not only instrumental for binding, membrane fusion, and endocytosis of the virus, but also for its antigenicity. In fact, “glycan shielding” by N-glycosylation of virus envelope proteins is one of the main reasons for failure to develop effective vaccines against a variety of viruses, the most prominent examples being Ebola, Zika, and HIV (Watanabe et al. 2019). This viral (and bacterial) “molecular mimicry” of adopting the N-glycan landscape of the host plasma membrane is not only the prevalent cause of immune evasion, but it can also lead to autoimmune disease and is one of the reasons why RNA vaccines are critical for future vaccine design (Klasse et al. 2020; Negrini et al. 2020). It has been known for many years that the successful immune response (or vaccination) of the host against bacterial or virus proteins can trigger autoimmune diseases such as rheumatism (Esmail and Manolson 2021). More recently, it has become clear that the erroneous response of the host immune system against N-glycans on the pathogen surface is among the triggers for the autoimmune response. Hence, it will be critical to expand our arsenal of vaccines beyond inactivated (but still N-glycosylated) pathogen particles and proteins and tailor specifically vaccines toward protein sequences that are not glycosylated and yet instrumental for virus infection and propagation. The RNA vaccine against the receptor binding domain of Sars-Cov-2 is one of the most recent examples for this strategy.

So far, we have discussed the role of N-glycans in evading the host’s immune system and to facilitate virus infection. However, there is also the opposite function in that N-glycosylation helps trigger and sustain a specific immune response toward a pathogen. The oligosaccharide composition of cell surfaces, the glycocalyx, is mainly determined by three families of glycoconjugates: glycoproteins, glycolipids, and proteoglycans. One well known example of how these families generate a glycocalyx unique for specific cell types is the composition of blood group antigens commonly known as “ABO” blood groups (correct would be AB0). Blood group antigens are based on a composition of terminal sugar residues (fucose, N-acylglucosamine, and galactose) that is shared between glycans from glycoproteins and glycolipids on the surface of red blood cells. It provides prominent examples of how glycans and their receptors, lectins, interact to ensure a specific cell type antigenicity and recognition by immune cells.

Immune responses are not only regulated by a family of glycans in the glycocalyx, but also by the glycans linked to specific proteins or lipids in the immune system. Any immune response is initiated by the interaction of antigen-presenting cells (APCs) and several lineages of T-cells. In this moment, the immune system decides between “self” and “foreign”, a critical decision that if erroneously executed will lead to fatal infection or autoimmune disease (Shahine 2018; Shahine et al. 2021).

In Natural Killer T-cells (NKTs), the molecular basis of this decision is the interaction of T-cell receptors (TCRs) with the N-glycoproteins of the CD1d family on APCs. CD1d is a class I major histocompatibility complex (MHC) protein that lifts membrane lipids into a hydrophobic pocket, which is then recognized by NKTs to either execute the immune response or not. Type I NKTs recognize  $\alpha$ -galactosylceramide bound to CD1d, which is an archetypical glycolipid that in contrast to  $\beta$ -galactosylceramide, does not occur naturally in our body or have any other physiological function. It is thought that “self-lipids”, particularly plasma membrane-resident phospholipids and glycolipids presented by the CD1 family of glycoproteins maintain homeostasis of T-cells, whereas their exchange with foreign lipids triggers their activation and immune response. Whoever has had an unpleasant encounter with Poison Ivy or Oak (*Toxicodendron*) knows how powerful the immune response to the plant lipid Urushiol can be when presented by CD1d to NKT cells (Shahine et al. 2021). Although the role of N-glycosylation of CD1d for its surface expression and potential to activate T-cells was found some time ago, it is still not clear how this may affect its presentation of glycolipids.

In contrast to N-glycans linked to CD1d or other MHC molecules, interest in the function of N-glycosylation of immunoglobulins has grown rapidly, particularly in the context of glycoengineering of monoclonal antibodies for immunotherapy, a research field also fueled by the Covid-19 pandemic (Wang et al. 2015; Zhou and Qiu 2019; Li et al. 2021). Further, aberrant N-glycosylation of immunoglobulins was found to be associated with a variety of allo- and autoimmune diseases, implying a critical role of N-glycans for the function of immunoglobulins. Most studies focus on the function of N-glycans in the interaction of the constant (Fc) region of IgGs, which regulates its interaction with Fc receptors on B-cells, NKTs, macrophages and other cell types initiating antibody-dependent cell-mediated cytotoxicity (ADCC). Particularly, endowment of these complex type N-glycans with terminal fucose residues was found to weaken the interaction with Fc receptors (Li et al. 2021). Hence, small inhibitors of glycoprotein processing such as castanospermine or swainsonine that prevent remodeling of complex N-glycans are used to “glycoengineer” monoclonal antibodies with improved activity. Novel approaches include the development of inhibitors for glycosyltransferases, which are the enzymes critical for the remodeling of the N-glycan structure and ultimately, its specific function. The next section will take a closer look at glycosyltransferases and their interdependence in the biosynthesis and function of glycoproteins and glycolipids.

## **6 Sweet Encounters of Glycoproteins and Glycolipids: N-glycans Affect the Subcellular Distribution and Complex Formation of Enzymes in Glycolipid Biosynthesis**

Glycosyltransferases not only transfer sugar residues onto protein linked-glycans, but also onto lipids, in particular sphingolipids. Glycosphingolipids are synthesized from ceramide, a sphingolipid consisting of sphingosine attached to various fatty

acids, by sequential glycosylation reactions catalyzed by a series of ER- or Golgi-resident glycosyltransferases (Sandhoff and Kolter 2003; Ngamukote et al. 2007; Gault et al. 2010; Yu et al. 2010; Maccioni et al. 2011a, b; Sonnino et al. 2018). As expected, the substrate specificity of these enzymes is different from that of glycosyltransferases in N-glycoprotein biosynthesis and processing. After attachment of glucose and then galactose to ceramide, which generates lactosylceramide, the most basic ganglioside, GM3, is synthesized by attachment of sialic acid. This reaction, catalyzed by GM3 synthase, is followed by enzymatic reactions that split ganglioside biosynthesis into three distinct pathways: a-, b-, and c-series gangliosides (Svennerholm 1956; Yu and Ledeen 1972; Ando and Yu 1977; Yu and Ando 1980; Yu 1984, 1994; Yu et al. 1988, 2004, 2012). If N-acetyl galactosamine is the next sugar residue added to GM3, a reaction catalyzed by GM2/GD2 synthase, ganglioside biosynthesis will exclusively follow the a-series pathway. However, if another sialic acid residue is added first, a reaction catalyzed by GD3 synthase, then ganglioside biosynthesis follows the b- or c-series pathway. Note that GD3 synthase can only act on GM3 (thereby generating GD3), while GM2/GD2 synthase can use GM3 (thereby generating GM2) or GD3 (thereby generating GD2) as the substrate. Therefore, the relative location of GD3 and GM2/GD2 synthase determines which pathway of ganglioside biosynthesis is taken. If GM2/GD2 synthase acts first, only a-series gangliosides are made, whereas a sequential reaction of first GD3 synthase and then GM2/GD2 synthase channels ganglioside biosynthesis towards the b-series pathway. Likewise, c-series gangliosides are made if GT3 synthase acts on GD3 before GM2/GD2 synthase does.

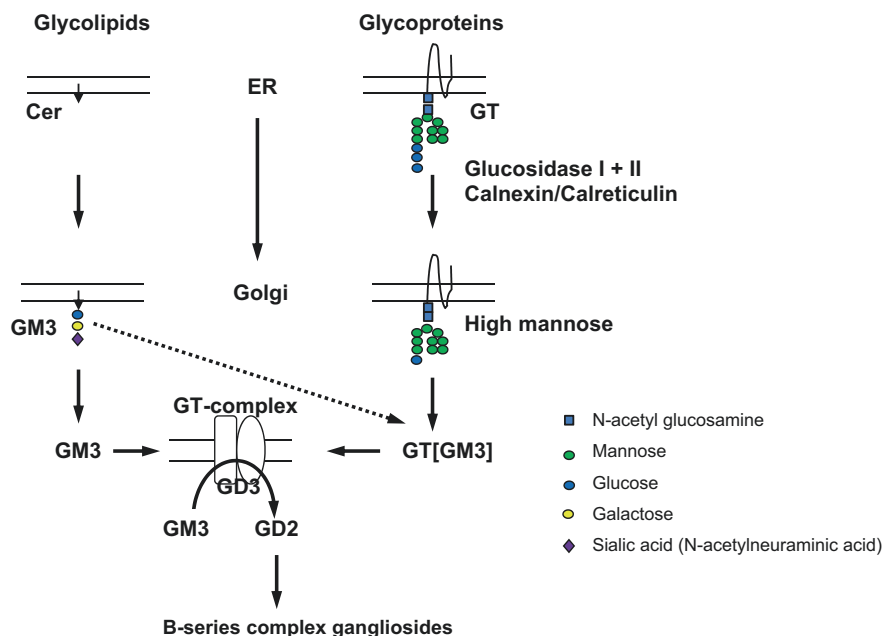
Regulation of biosynthetic pathways by the relative location of enzymes in a reaction sequence is not just of academic curiosity but may determine the composition of gangliosides in an organism, tissue, or cell. Robert K. Yu, whose laboratory I joined after my post-doctoral work on N-glycoproteins, found about 35 years ago that the ganglioside pathways undergo a rapid switch from a- to b-series during embryonic brain development (Yu et al. 1988), just at the time point when neuroprogenitor cells start to divide asymmetrically and many intermediate neurons are born (Yu 1994; Bieberich and Yu 1999; Yu et al. 2012). Bob Yu gave me the opportunity to pursue my own ideas about the regulation of ganglioside biosynthesis by the interdependence of glycosylation reactions in glycolipid and glycoprotein biology.

To understand the meaning of this interdependence one has to know that most glycosyltransferases are type II transmembrane proteins with 3–4 N-glycosylation sites. Hence, bearing in mind what we have discussed before – N-glycans are critical for protein folding and transport – N-glycoprotein processing may regulate the subcellular localization, and therefore relative location, of glycosyltransferases in ganglioside biosynthesis. I quickly realized that I was not the only one who pursued this idea. Hugo Maccioni's and our group published in 1998 that inhibition of trimming glucosidases I and II with dNM and castanospermine, but not inhibition of Golgi mannosidase I and II with deoxymannojirimycin and swainsonine prevented transport of GD3 synthase from the ER to the Golgi (Martina et al. 1998; Bieberich et al. 2000). There was some discrepancy between both studies with respect to the effect of glucosidase inhibition on enzyme activity. While in Maccioni's study,

dNM and castanospermine were found to preserve activity of GD3 synthase, castanospermine increased its proteolytic turnover in our study. Since it was known from the Helenius model that N-glycans are required to achieve chaperone-assisted protein folding via the calreticulin/calnexin cycle, both groups concluded that N-glycosylation was necessary to attain and maintain catalytic activity, while glucose trimming was required for the ER-to- Golgi exit of the enzyme (Martina et al. 1998; Bieberich et al. 2000).

In the following years, Maccioni's group made great strides toward understanding the regulation of glycosyltransferase transport and enzyme complex formation, and its significance for glycolipid biosynthesis (Maccioni et al. 1999, 2002, 2011a, b; Martina et al. 2000; Giraud et al. 2001; Giraud and Maccioni 2003; Maccioni 2007; Ferrari et al. 2012; Spessott et al. 2012). However, it still remained unclear how this would switch a- to b-series gangliosides and whether N-glycans are actually critical for this pathway switch. I was working in Bob Yu's group on this problem and discovered that GD3 synthase forms a disulfide bridge-mediated homodimer that turns into a heterodimer with GM2/GD2 synthase (Bieberich et al. 1998). Interestingly, binding to GM3 as well as inhibition of trimming by glucosidases I and II retained the GD3 synthase homodimer in the ER, suggesting that the enzyme-substrate complex may participate in protein folding or transport (Fig. 3.3). The observation of enzyme (–substrate) complexes in ganglioside metabolism was in line with Saul Roseman's original idea that the biosynthesis of specific gangliosides is best achieved by forming multi-enzyme complexes of glycosyltransferases (“cooperative sequential specificity” model) (Roseman 1970). Moreover, using matrix analysis of networks borrowed from electrical engineering (“multi-enzyme kinetic analysis”), we calculated that by forming the complex between GD3 synthase and GM2/GD2 synthase, ganglioside biosynthesis would be efficiently channeled into the b-series pathway, an enzymatic switch that provided an explanation for the rapid change in the composition of gangliosides during embryonic brain development (Bieberich et al. 1998; Bieberich and Yu 1999).

In addition to the GD3 synthase homodimer and GD3 synthase-GM2/GD2 synthase heterodimer complex identified by Bob Yu's group, Hugo Maccioni's group discovered that enzyme complexes were also formed between LacCer-, GM3-, and GD3-synthase, and GM2/GD2- and GM1/GD1a synthase (Martina et al. 2000; Giraud et al. 2001; Giraud and Maccioni 2003; Maccioni et al. 2011a, b; Ferrari et al. 2012; Spessott et al. 2012). Common to two of the glycosyltransferase complexes described so far is that the stability and/or subcellular localization of at least two of their subunits, GD3 synthase and GM1/GD1a synthase, are critically dependent on N-glycosylation and trimming by glucosidases I and II. Moreover, inhibition of this trimming (by castanospermine) dramatically changes the ganglioside composition by preventing synthesis of higher sialylated, complex b-series gangliosides such as GT1b, the most prominent ganglioside after the “a-to-b series switch” in mouse (and human) embryonic brain at a time point of intense neural progenitor (and intermediate neuron) proliferation and migration (Bieberich et al. 1998). It should be noted, that Bob Yu's group not only was the first to discover this



**Fig. 3.3** Regulation of ganglioside biosynthesis pathways by N-glycan-dependent glycosyltransferase distribution and complex formation. Inhibition of trimming by glucosidases I and II increases proteolytic turnover of GD3 synthase and prevents enzyme complex formation with GM2/GD2 synthase in the Golgi, suggesting that N-glycoprotein processing of glycosyltransferases is critical for ganglioside metabolism. The GD3 synthase-GM2/GD2 synthase complex is hypothesized to promote b-series complex ganglioside biosynthesis. Moreover, our group has proposed that binding of GD3 synthase to GM3 may facilitate enzyme complex formation (“lipid co-chaperone” hypothesis)

ganglioside pathway switch, but also demonstrated that the simplest b-series ganglioside, GD3, is a robust cell surface marker for mouse (and human) neural progenitor cells (Yanagisawa et al. 2004; Ngamukote et al. 2007; Nakatani et al. 2010). Yet, it remains to be determined, which functional role the pathway switch plays for brain development and how it is integrated with the regulation for N-glycan processing of glycosyltransferases in ganglioside metabolism.

## 7 Conclusions and Epilogue: The Tale of the Tail That Wags the Dog

At the end of this chapter on N-glycosylation and N-glycoprotein processing, one may miss a discussion of the important functions that cell surface N-glycans play in cell-to-cell recognition and adhesion, in particular in the brain. For example,

galectins, cell surface lectins that bind to N-acetylgalactosamine in N-linked glycans, have been found to regulate growth factor receptor endocytosis/recycling, which may contribute to glioma metastasis (Le Mercier et al. 2010). Another non-discussed example for lectin-like cell surface binding is the interaction of myelin-associated glycoprotein (MAG) with specific gangliosides, which has been suggested to be critical for myelination of axons (Yang et al. 1996). Loss of b-series complex gangliosides as well as abnormal N-glycoprotein processing of MAG leads to severe nervous system symptoms such as Wallerian degeneration and demyelination (Konat et al. 1987; Sheikh et al. 1999). The tail (protein or lipid-linked glycan) wags the dog (neuron or glia), so to speak.

One may also criticize that other types of protein glycosylation such as O-glycosylation or protein-associated glycans in general, such as proteoglycans are not mentioned (see (Moremen et al. 2012) for a comprehensive review on these). The reason for this is twofold: for one, these glycoconjugates are reviewed in other Chaps. 4 and 5 of this book. However, more importantly, I wanted to focus on biological processes that are dynamically regulated by the morphing and reshaping of protein-linked N-glycans. As we have seen, the glycosylation and trimming machinery is intimately connected with proofreading and editing of N-glycoproteins. In this regard, it should be noted that the stability, subcellular distribution, and complex formation of glycosyltransferases in ganglioside biosynthesis is the first example of enzymes in a metabolic pathway that may be regulated by N-glycoprotein processing. Moreover, as we have discussed, the function of N-glycoprotein processing is by far not completely understood and may involve substrates such as the ganglioside GM3 as “lipid co-chaperones”. And finally, the interdependence between protein-linked N-glycan processing and glycolipid metabolism, a theme that brought me as a researcher trained in glycoprotein biology to pursue studies on glycolipids, holds promise to make future discoveries in uncharted territories with impact on systems biology; a fascinating area of research that tears down the boundaries between over-specialized disciplines in biology and goes back to a more classical, Humboldtian view on life as emerging from the self-organized biology of interacting metabolic systems, such as glycoprotein processing and glycolipid biosynthesis. Therefore, this chapter is not only meant to give an account of what is known about N-glycosylation (certainly not in an exhaustive manner), but also to ignite interest in young scientists to pursue this area of research in their careers.

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# Chapter 4

## Synthesis of O-Linked Glycoconjugates in the Nervous System



Jin-Ichi Inokuchi, Shinji Go, and Yoshio Hirabayashi

**Abstract** Glycoproteins carrying O-linked N-acetylgalactosamine, N-acetylglucosamine, mannose, fucose, glucose, and xylose are found in the nervous system. Lipids are glycosylated by distinct glycosylation enzymes as well. Membrane lipid, ceramide, is modified by the addition of either glucose or galactose to form glycosphingolipid, galactosylceramide, or glucosylceramide. Recent careful analyses by MS have identified glucosylated lipids of cholesterol and phosphatidic acid. These O-linked carbohydrate residues are found primarily on the outer surface of the plasma membrane or in the extracellular space. Their expression is cell or tissue specific and developmentally regulated. Due to their structural diversity, they play important roles in a variety of biological processes such as membrane transport, metabolic stress responses, cell–cell interactions and so on. Discoveries of human diseases associated with glycosylation enzyme deficits have proved modification of lipids and proteins with carbohydrates play critical roles in human health and disease in the nervous systems.

**Keywords** Ceramide · Glycosphingolipid · Glucosylceramide · Ganglioside · Heparan sulfate · Chondroitin sulfate · Keratan sulfate · Mucin · Glycosyltransferase · Sulfation

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## Abbreviations

CGT	UDP-Gal:ceramide galactosyltransferase
CST	Cerebroside sulfotransferase
GalCer	Galactosylceramide or cerebroside
GalNAc-T	UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase
GCS	UDP-Glc:ceramide glucosyltransferase, UGCG or GlcT-1
GlcCer	Glucosylceramide
GlcChol	Cholesterylglucoside
GSL	Glycosphingolipid
LacCer	Lactosylceramide
LARGE	The like-acetylglucosaminyltransferase
NCAM	Neural cell adhesion molecule
POFUT1	Protein O-fucosyltransferase 1
POMGNT1	Protein O-linked mannose N-acetylglucosaminyltransferase 1 (beta 1,2-)

## 1 Introduction

O-linked glycans are found in proteins and lipids, both of which can carry a great variety of glycan chains. Most typical protein/peptide O-glycans in nonneural tissues possess an  $\alpha$ GalNAc residue linked to a serine or threonine hydroxyl group to which other sugars can be added. These protein/peptide O-glycans are classified as mucin-type glycans and they also exist in brain. Glycoproteins carrying O-linked mannose, fucose, glucose, and xylose are also present in the nervous system. Lipids can be glycosylated as well. Ceramide, a membrane sphingolipid, can be modified by the addition of either glucose or galactose through a  $\beta$ -glycosidic linkage to form the glycosphingolipid identified as a cerebroside (ceramide-O-glc/gal). Recent analyses have identified glycosylated lipids of cholesterol and phosphatidic acid. These O-linked carbohydrate residues are found primarily on the outer surface of the plasma membrane or in the extracellular space. Their expression is cell or tissue specific and developmentally regulated. Due to their structural diversity, they play important roles in a variety of biological processes such as membrane transport and cell–cell interaction, and host-parasite (pathogen) interaction. Recent studies demonstrate that glial cells contain glycogen as reservoir of glucose and glucosamine, supporting neuronal activities. The glycosylation reactions occur primarily in the Golgi apparatus and/or in ER membranes. Protein O- $\beta$ -GlcNAc modification can also occur in the cytoplasm. This review describes the basic structures of O-glycans and the synthetic enzymes involved with focus on the initial step of glycosylation.

## 2 Biosynthesis of O-Linked Proteins

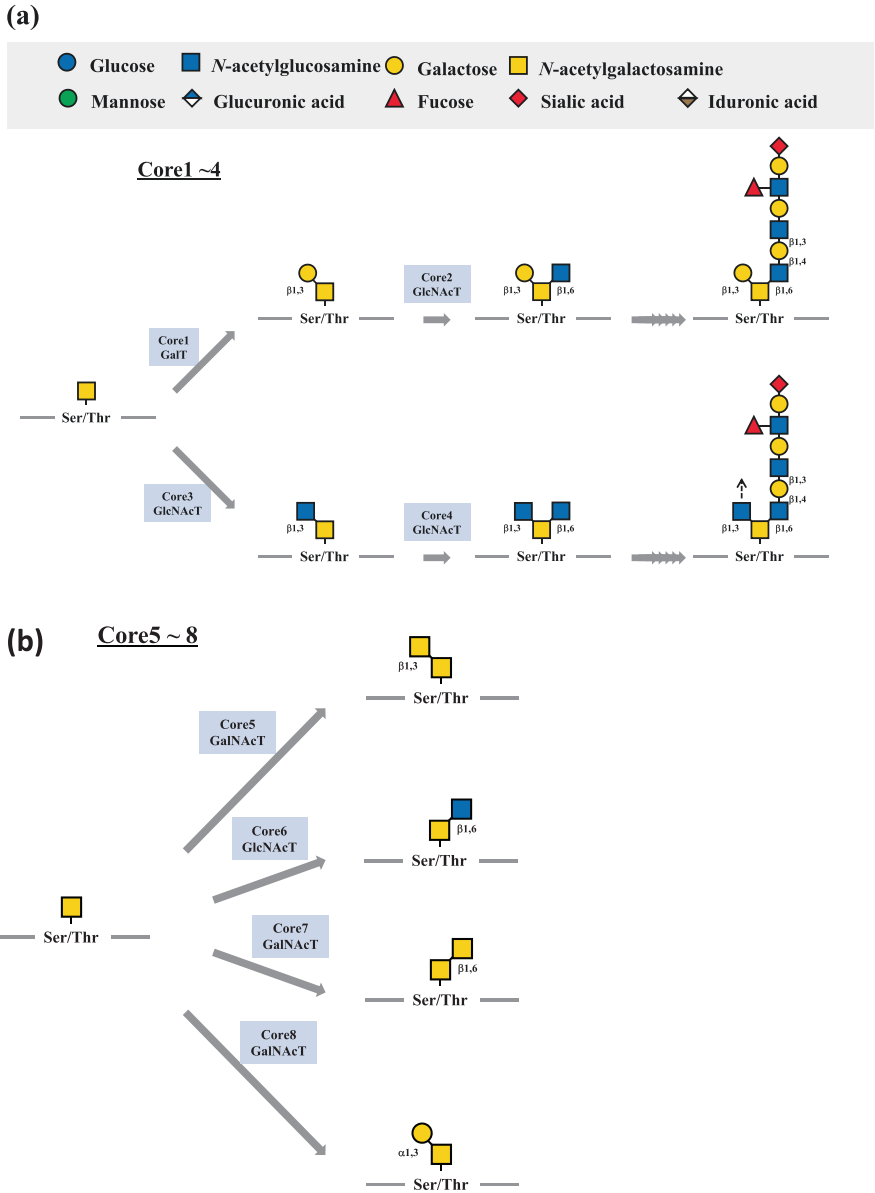
Cell surface glycan chains in the nervous system have critical roles during differentiation, development, regeneration, synaptic plasticity, and aging (Kleene and Schachner 2004). Their tremendous structural diversity contributes to cell–cell communication, including neuron–glia and cell–matrix interactions. There are at least six different O-glycan-protein linkages that have been identified in the mammalian nervous system (Fig. 4.1a, b).

### 2.1 O-GalNAcylation

O-GalNAc modification of proteins occurs most frequently and is abundant in mucin-type glycoproteins. The glycan chains are very heterogeneous, and eight different core structures are known (Fig. 4.1a, b). However, little is understood about their detailed structure, distribution, and function in the nervous system. To understand precisely how and where O-GalNAc is attached to each protein, a novel method of O-GalNAc glycoproteome was developed using lectin enrichment of glycans coupled to LC-MS/MS (Steenfot et al. 2013; King et al. 2017). This type of approach is absolutely necessary for advancing our knowledge of O-GalNAc glycans in the brain.

UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase (GalNAc-T) is the key Golgi enzyme catalyzing the initial step in the biosynthesis of O-GalNAc glycans. Among the 20 or 21 GalNAc-T genes (*GALNT*) that have been identified (Bennett et al. 2012), only a few genes, such as *GalNAc-T9*, *T-13*, and *T-17* (also called *T-16*), have been isolated and found in brain tissues. The latter gene is also known as WBSR17 and is associated with Williams–Beuren syndrome (WBS) (Nakayama et al. 2012). The effects of mouse T-17 knockout on brain function are interesting, but have not yet been reported. Syndecan-3 (*SCD3*) is suggested to carry this type of glycan (Zhang et al. 2003) and *SCD3* plays an important role in inflammation-associated diseases (Arokiasamy et al. 2019).

Neural cell adhesion molecule (NCAM) and neuropilin-2 contain O-glycans, which are polysialylated with  $\alpha$ 2–8 sialosyl residues. Although a detailed functional study has not yet been carried out, modification of O-GalNAc chains is thought to be involved with important physiological functions in the central nervous system. For more detailed information, see Bagdonaite et al. (2021).



**Fig. 4.1** (a) Synthesis of O-GalNAc glycan (Cores 1–4). Sugar species are depicted using the symbols given in “Essentials of Glycobiology” (Varki et al. 2009). (b) Synthesis of O-GalNAc glycans (Cores 5–8)

## 2.2 O-Mannosylation

Krusius and co-workers first reported that more than half of the carbohydrate–peptide linkages in brain proteoglycans are of the mannosyl-O-serine/threonine type and contain keratan sulfate (Krusius et al. 1986). The same group identified the sialylated tetrasaccharide NeuAc $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Man-Ser/Thr and its related glycans (Krusius et al. 1986).

O-Mannosylated glycans, isolated from peripheral nerve tissues, have slight structural differences compared to sialylated tetrasaccharides present in those of the central nervous system (Endo 1999; Nickolls and Bonnemann 2018). This glycan has the following structure: NeuAc $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-2Man-Ser/Thr. The linkage between the GlcNAc and the Man residue is 3-substituted in the case of the brain tetrasaccharide. The O-glycan in peripheral nerve tissue is a major sialylated O-glycan in  $\alpha$ -dystroglycan. The O-glycans isolated from  $\alpha$ -dystroglycan possess an evolutionarily conserved structure, being observed in animals from *Drosophila* to mammals. They have been found in only a limited number of proteins in the brain, such as  $\alpha$ -dystroglycan, chondroitin sulfate, and PTPRZ1/RPTP $\beta$  (Krusius et al. 1986; Nakamura et al. 2010). Because O-mannosylated glycans in  $\alpha$ -dystroglycan have essential biological roles as well as pathobiological roles in muscle and the nervous system, their structures have been extensively studied (Fig. 4.2) (Endo 1999).

O-Mannosylation is catalyzed by O-mannosyltransferase, which is encoded by the *POMT1* and *POMT2* genes. POMT1 and POMT2 complex formation is essential for POMT activity. Unlike other peptide O-glycosyltransferases, POMT is a member of the dolichyl-phosphate-mannose-dependent mannosyltransferase

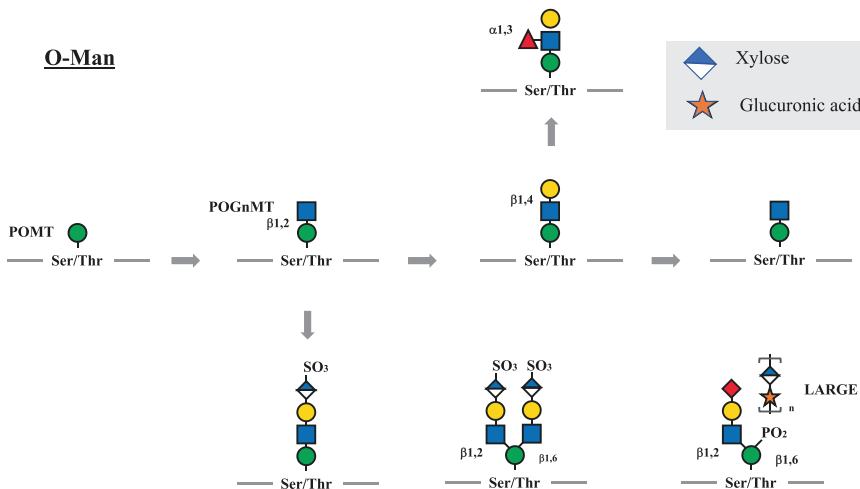


Fig. 4.2 Synthesis of O-Man glycans

located in ER membranes. Mutations in either *POMT1* or *POMT2* cause Walker-Warburg syndrome, a congenital muscular dystrophy with abnormal neuronal migration (van Reeuwijk et al. 2005).

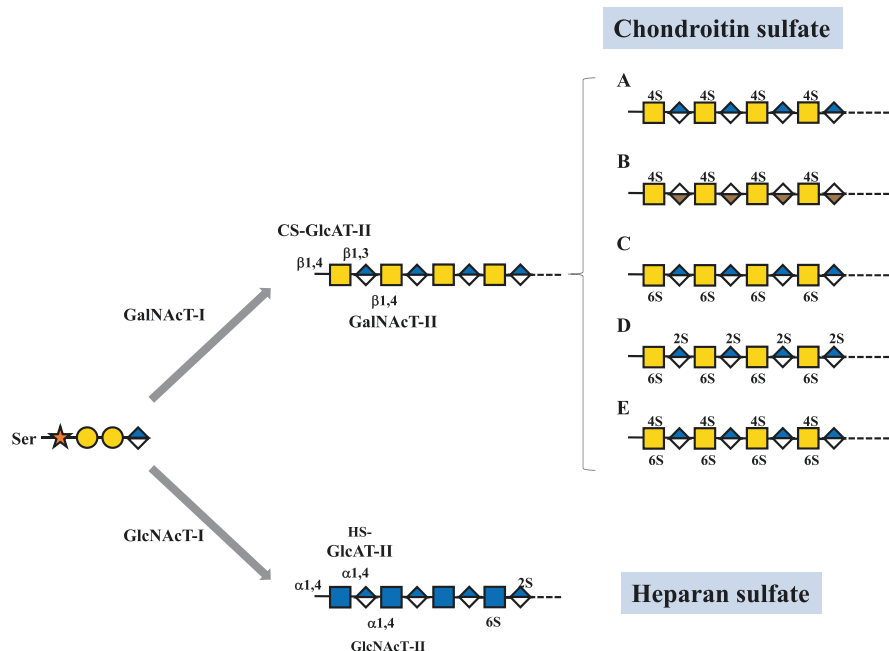
Mannose residues are further glycosylated by protein O-linked mannose N-acetylglucosaminyltransferase 1 (POMGnT1) to form disaccharides that are further elongated by glycosyltransferases to make three major forms: non-branched, branched, and LARGE-dependent glycan chains (Fig. 4.2). *LARGE* encodes a bifunctional glycosyltransferase that has both xylosyltransferase and glucuronyltransferase activities. It is of interest to note that LARGE-dependent glycan structures have a phosphate residue at the 6-position of the core mannose. Recent studies indicated that SGK196 is a typical kinase that phosphorylates the 6-hydroxy position of mannose and is required for dystroglycan receptor function (Yoshida-Moriguchi et al. 2013).

### 2.3 O-GlcNAcylation

O-GlcNAc is covalently attached to serine or threonine residues of intracellular proteins through a  $\beta$ -glycosidic linkage. This modification is evolutionarily conserved and is found in nuclear and cytoplasmic proteins, such as nuclear pore proteins, chromatin histone proteins, transcriptional factors, and P53 (Wells et al. 2001). The GlcNAc residue is not modified by further glycosylation. Importantly, the modification site can also be phosphorylated by a serine/threonine kinase. O-GlcNAc modification is suggested to be involved in the homeostatic mechanism of energy metabolism, since UDP-GlcNAc—the donor for O-GlcNAc transferase (OGT)—is metabolically derived from all metabolites, including glucose, nucleotides, fatty acids, and nitrogen (Ruan et al. 2013). Further detailed information on the physiological significance of O-GlcNAc modification is described in Chap 9 by Lagerlof and Hart.

### 2.4 O-Xylosylation

O-Xylosylated proteins are present in proteoglycans including chondroitin sulfate/dermatan sulfate and heparan sulfate (Fig. 4.3), important components for brain development and physiology. The most characteristic feature of these two groups is that they have highly complicated, heterogeneous glycan chains. These proteoglycans are found as constituents of the extracellular matrix and function as cell adhesion molecules in the brain. Proteoglycans interact with extracellular or cell surface molecules expressed in neighboring cells. Both chondroitin sulfate/dermatan sulfate and heparan sulfate have common basic tetrasaccharide structures: GlcUA $\beta$ 1-3Gal $\beta$ 1-3Gal $\beta$ 1-4Xyl( $\pm$ 2-O-phosphate)  $\beta$ -Ser (Fig. 4.3). Xylosyltransferase



**Fig. 4.3** Synthesis of both heparan sulfate and chondroitin sulfate

encoded by the *XYLT1* gene catalyzes the transfer of xylose from UDP-xylose to *serine* residues within the transferase recognition sequences of protein substrates. The enzyme is localized in the early *cis*-Golgi apparatus (Sarrazin et al. 2011), and its activity is upregulated during myofibroblast differentiation in skin fibrosis (Faust et al. 2013).

### Heparan Sulfate

The basic tetrasaccharide is further modified by an  $\alpha 4$ GlcNAc-transferase, termed Ext13. Then, an enzyme complex composed of Ext1 and Ext2 alternately adds GlcA and GlcNAc to the nascent chain (Fig. 4.3). *Ext* genes are evolutionarily conserved and are characterized as tumor suppressors. The oligosaccharide chains simultaneously undergo a series of processing reactions, including N-deacetylation and sulfation, to yield mature glycans (for details, see (Ruan et al. 2013)). Variation in the number and length of the chains produces enormous chemical diversity. Brain heparan sulfate glycan structures are typically found in neuropilin-1, syndecan-3, and glypican-1. Recent studies on proteoglycan syntheses and core proteins in genetically engineered animals have demonstrated that neural proteoglycans play critical roles not only in brain development and neuronal network formation but also in neuronal regeneration in injured nervous tissues, in formation and deposition of A $\beta$ -amyloid peptide in Alzheimer brains, and in autism (Irie et al. 2012).

### Chondroitin Sulfate

In the case of chondroitin sulfate, the core tetrasaccharide is first modified by the addition of GalNAc catalyzed by a  $\beta$ 4GalNAc-transferase and then by the addition of GlcA catalyzed by a  $\beta$ 4GlcA-transferase. The combination of the two glycosyltransferases is responsible for forming the repeating disaccharide unit (GlcA-GalNAc). Sulfation on the disaccharide unit generates the diverse structures of chondroitin sulfate proteoglycans (Fig. 4.3; structures A to E) (Silbert and Sugumaran 2002). The chondroitin-sulfated proteoglycans, NG2 and neurocan, are synthesized by both neurons and glia. Accumulating evidence has shown that chondroitin sulfate proteoglycans play key roles in neural development, axon guidance cues, neural plasticity, and neural repair after injury (Kwok et al. 2012).

## 2.5 O-Fucosylation

O-Fucosylation is a conserved posttranslational modification of proteins that is catalyzed by two glycosyltransferases, protein O-fucosyltransferase 1 (POFUT1) and protein O-fucosyltransferase 2 (POFUT2). Molecular genetic studies have proven that POFUT1 is essential for normal development in both flies and mice. Analysis of *POFUT1* null embryos demonstrated that it is implicated in the Notch signaling pathway (Okajima and Irvine 2002).

## 2.6 O-Glucosylation

$\beta$ -linked O-Glc proteins are found in the extracellular EGF repeats of proteins such as CRB2, F7, F9 and NOTCH2, and this glucosylation is catalyzed by POGLUT1. It can only glycosylate serine residues in the CXSXPC motif, and it possesses dual donor substrate specificity, that is the transfer of glucose and xylose from UDP-glucose and UDP-xylose, respectively (Urata et al. 2020). Importantly POGLUT1 is localized mainly in the luminal ER compartment and recognizes the folding state of the substrate proteins.

O- $\alpha$ Glc is present in glycogenin (GYG1 and 2). Transfer of glucose to glycogenin is catalyzed by the glycogen initiator synthases (UDP-glucose-glycogen  $\alpha$ -glucosyl-transferases; *GYS1*, *GYS2*), which enable the glucose residue to act as an acceptor for subsequent glucosylation. The glucose molecules are attached through a hydroxyl group on a specific tyrosine side chain of the glycogenin. Elongation of the chain is accomplished by formation of an  $\alpha$ 1,4-glycosidic linkage that is catalyzed by glycogen synthase. Studies have shown that the brain contains glycogen (Brown and Ransom 2007). Brain glycogen functions as an energy source when the ambient glucose concentration is unable to meet immediate energy demands (Brown and Ransom 2007). In addition to glucose, glucosamine is rich in glial glycogen and glucosamine is an essential amino-sugar for N-linked glycoprotein synthesis (Sun

et al. 2021). A summary of the vertebrate brain glycans and enzymes catalyzing their initial glycosylation is provided in Table 4.1.

### 3 Biosynthesis of O-Linked Lipids

There are three major classes of membrane lipids: cholesterol, glycerolipids, and sphingolipids. All of these lipids can be modified by the addition of sugars (see Table 4.2). Among the three, sphingolipids are the most abundant glycolipids in the brain. They have been extensively studied in efforts to elucidate their biological functions in the nervous system.

#### 3.1 Glucosylceramide and Glycosphingolipids

Glycosphingolipids (GSLs) are a large and heterogeneous family of sphingolipids that form complex patterns on eukaryotic cell surfaces. Their diverse structures result from various combinations of their long-chain (sphingoid) bases, amide-linked fatty acids, and hundreds of head group variants. The subcellular biosynthetic machinery of GSLs is summarized in Fig. 4.4. Most GSLs are generated from glucosylceramide (GlcCer), which is formed when glucose is attached to the primary alcohol group (C1-OH) of a ceramide molecule of the endoplasmic reticulum (ER)/Golgi compartment. This glucosylation is catalyzed by UDP-Glc:ceramide glucosyltransferase, UGCG/GlcT-1/GCS (Ichikawa and Hirabayashi 1998).

**Table 4.1** Molecular diversity of vertebrate brain O-glycans and initial glycosylation enzymes

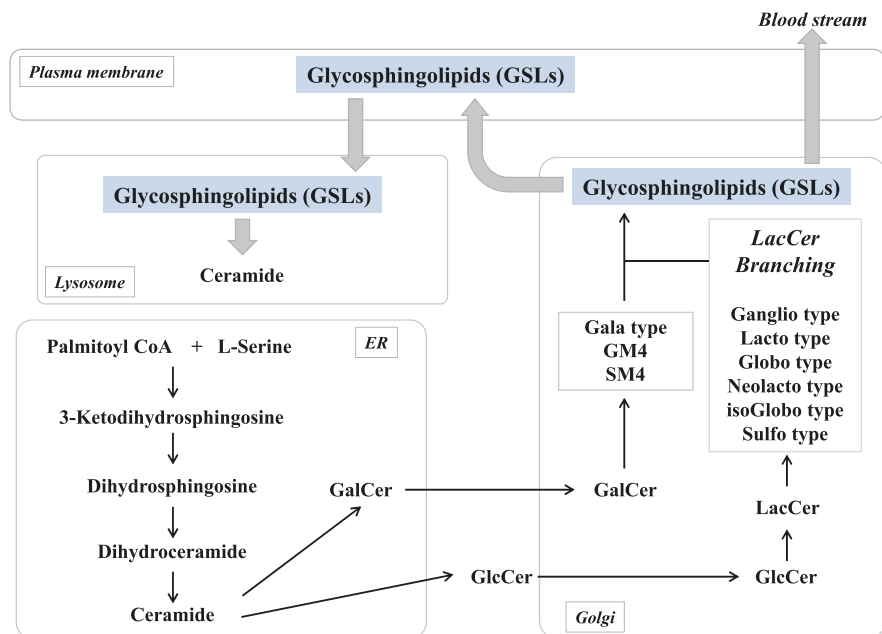
O-glycosylation	Acceptor protein/ lipid	Responsible gene	Major class of glycan or protein
$\alpha$ -GalNAc	Thr/Ser	GALNT17	Mucin type. Plasma membrane
$\beta$ -GlcNAc	Thr/Ser	OG1	Cytoplasmic, nuclear protein
$\alpha$ -Man	Thr/Ser	POMT1.2	$\alpha$ -Dystroglycan
$\alpha$ -Fuc	Thr/Ser	POFUT1,2	Notch receptor (EGF repeats and TSRs)
$\alpha$ -Glc	Thy	GYS1.2	Glycogen
$\beta$ -Xylose	Ser	XYLT1	Heparan sulfate, Chondroitin sulfate, Dermatan sulfate
	Ceramide	UGCG	XylCer
$\beta$ -Glc(Xylose)	Ser	POGLUT1–3	Notch receptor (EGF repeats & TSRs)
	Ceramide	UGCG	GlcCer
$\beta$ -Gal	Ceramide	UGT8	GalCer
	Alkyl-acylcramide	UGT8	Seminolipid (SM4)
	Cholesterol	GBA	Cholesterylgalactoside

*EGF* epidermal growth factor-like, *TSR* thrombospondin type 1 repeat



**Table 4.2** Lipids modified with carbohydrate. Enzyme promiscuity generates new glycolipids

	Localization	Substrate	Product	Function	Associated disease
UGCG	Golgi/ER	Ceramide	GlcCer	Precursor lipid for GSLs, Ceramide reduction, MDR	Genetic disorder (Ichthyosis) <sup>c</sup>
			XylCer <sup>a</sup>	Unknown	
GBA1	Lysosome	GlcCer	Ceramide	Degradation of GlcCer/XylCer	Gaucher disease
		XylCer	XylChol	Unknown	
GBA2	Golgi/ER	GlcCer	CholGlc, Ceramide	Removal of excess Chol/GlcCer	Spastic paraplegia 46 <sup>d</sup>
				Mitigate ER stress <sup>b</sup>	

<sup>a</sup>Boer et al. (2021)<sup>b</sup>Sorli et al. (2013)<sup>c</sup>Monies et al. (2018)<sup>d</sup>Martin et al. (2013)PA phosphatidic acid, *GlcCer* glucosylceramide, *mdr* Multiple drug resistance**Fig. 4.4** The subcellular biosynthetic machinery of GSLs

Glycan chains of GSLs are mainly synthesized in the lumen of the Golgi apparatus. Especially notable is that GlcCer is biosynthesized on the cytoplasmic leaflet of the Golgi membrane. To execute further elongation, GlcCer has to be translocated from the cytosolic leaflet to the inner leaflet of the Golgi membrane by a specific translocase, which has yet to be identified. This topological orientation of the

catalytic sites of glycosyltransferases is supported by their membrane protein type III structures. GlcCer synthase is a type III membrane protein with an N-terminal signal-anchor sequence, whereas other glycosyltransferases are type II membrane proteins with an N-terminal membrane-spanning domain and catalytic domains in the C-terminal region.

GlcCer is then modified by a  $\beta$ 1-4galactosyltransferase to generate lactosylceramide (LacCer). LacCer plays a pivotal role as a precursor for the synthesis of complex GSLs. The common LacCer structure is then elongated by different glycosyltransferases, to form the six classes of GSLs bearing the core structures known as gala-, globo- (Gb), isoglobo- (iGb), ganglio (Gg), lacto (Lc), and neolacto- (nLc) (Fig. 4.5). Detailed maps of the biosynthetic pathways for each class of GSL in mammals are depicted schematically in Figs. 4.5, 4.6, 4.7, 4.8, 4.9, and 4.10. Gangliosides are typical membrane GSLs and are found in both neurons and glia. Some gangliosides are markers for particular sets of neuronal cells. For example,  $\alpha$ -series gangliosides such as GQ1b $\alpha$  are specific to cholinergic neurons.

Every tissue or cell has a unique set of glycosyltransferases, which can be regulated via tissue- or cell-specific transcriptional control and by posttranslational modifications. Additionally, the possibility of an epigenetic control mechanism for glycosyltransferases has been demonstrated, which could result from environmental factors (Tsai and Yu 2014). Many genetically modified animal models, including tissue-specific (conditional) glycosyltransferase knockout mice, have been generated. These models have contributed greatly to our understanding of the evolutionary, physiological, and pathological significance of GSL synthesis. In particular, studies

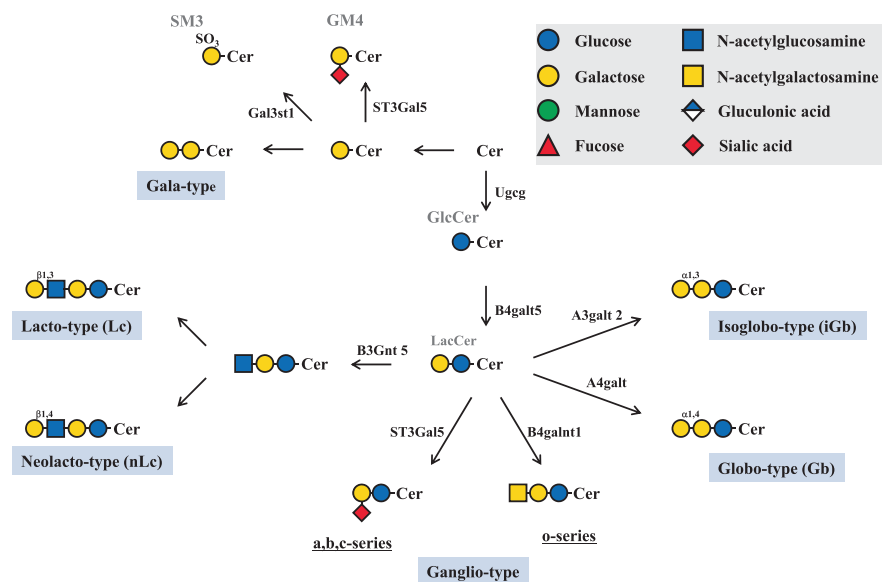


Fig. 4.5 Overview of biosynthetic pathway of GSLs

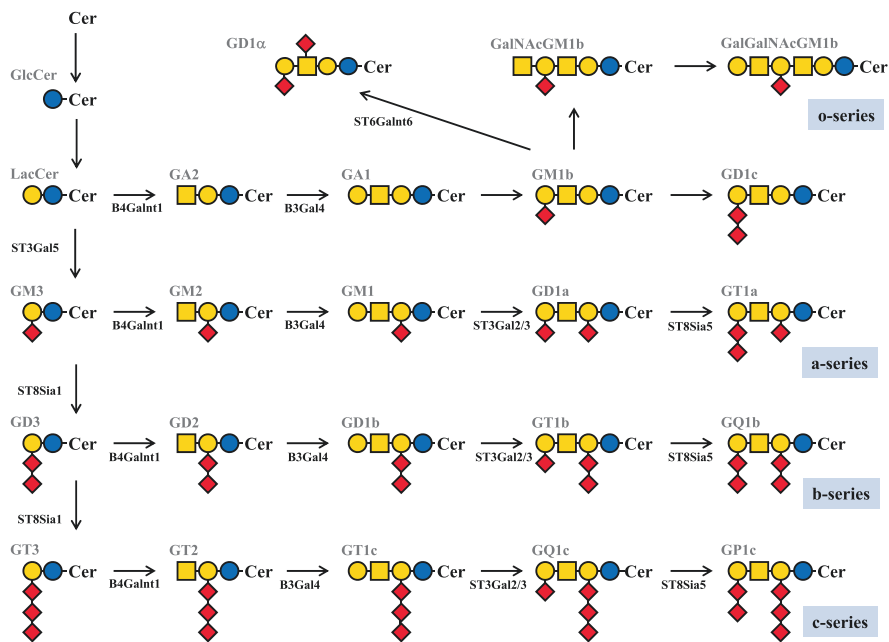


Fig. 4.6 Ganglio-type GSLs (1)

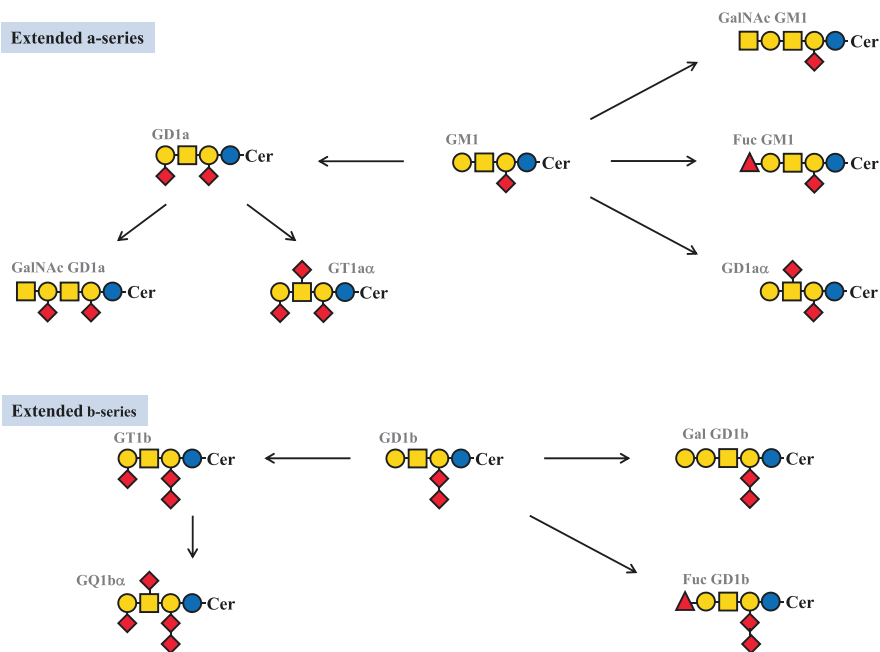


Fig. 4.7 Ganglio-type GSLs (2)

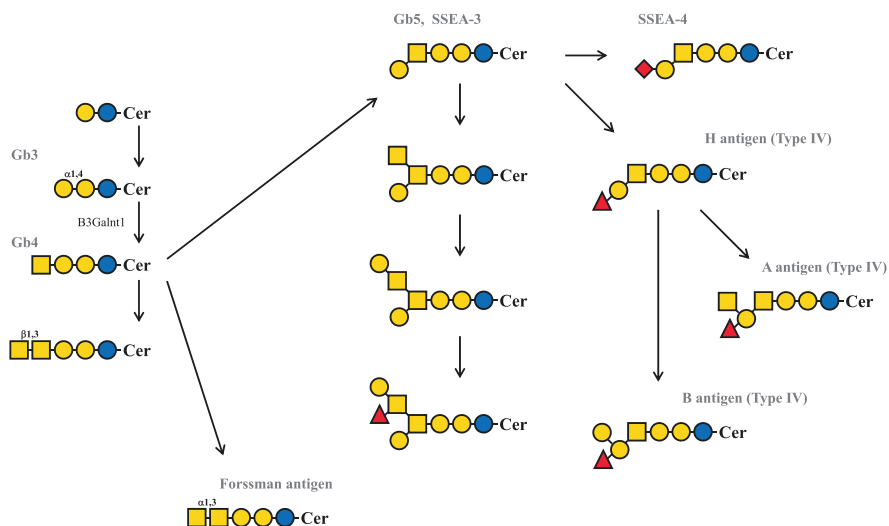


Fig. 4.8 Globo-type GSLs

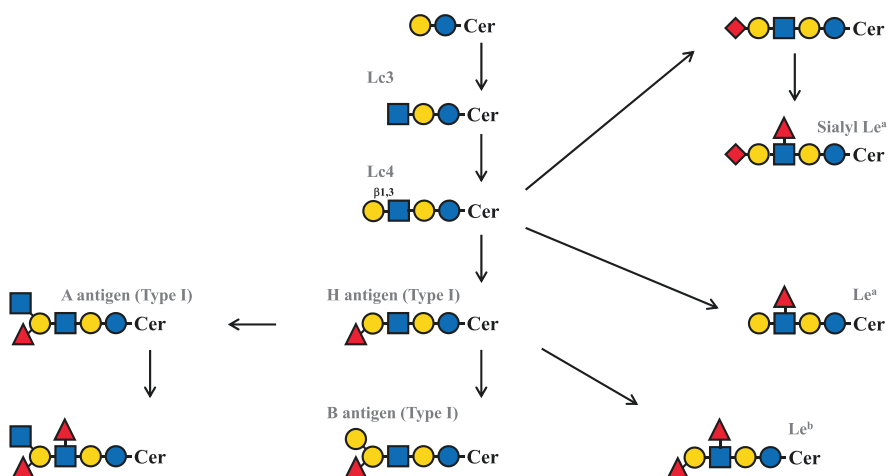


Fig. 4.9 Lacto-type GSLs

of mice in which the GlcCer synthase gene (*Ugcg*) was knocked out in specific tissues indicated that GSLs have tissue-specific biological functions (Ishibashi et al. 2013; Jennemann and Grone 2013). Importantly, GlcCer itself is thought to play an important role in the homeostatic regulation of energy metabolism (Fig. 4.11) (Ishibashi et al. 2013).

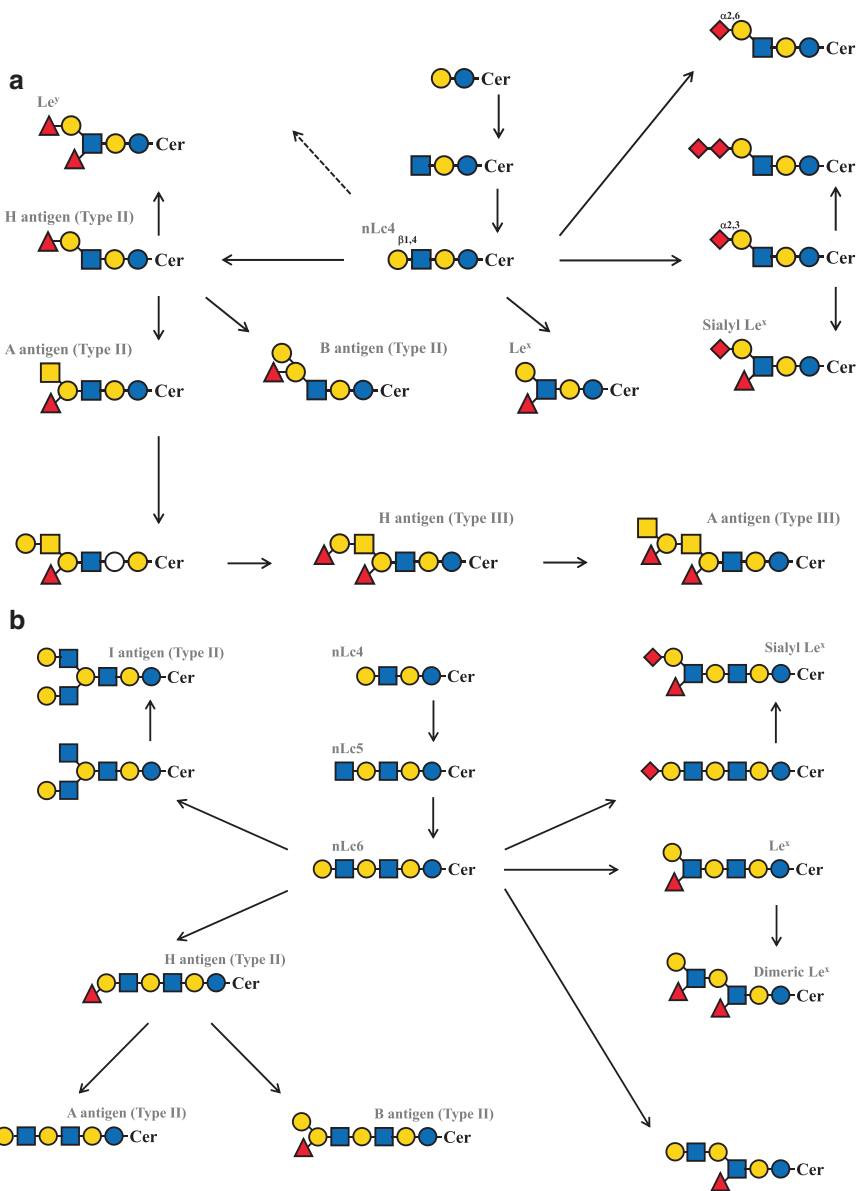
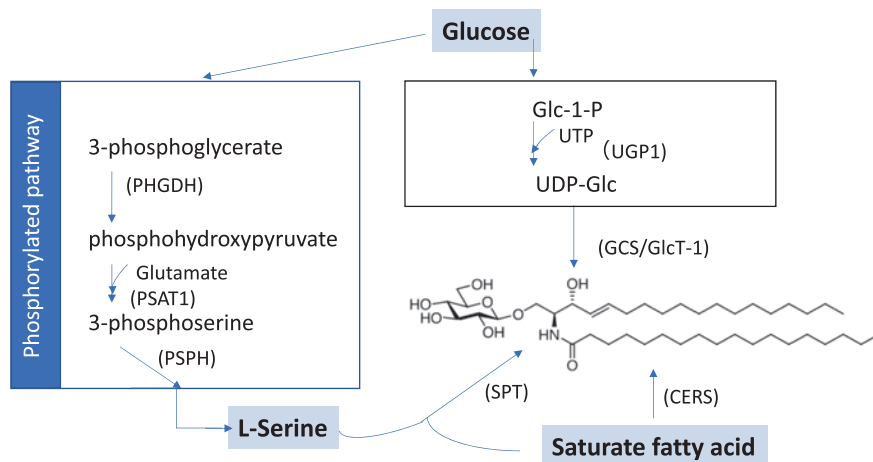


Fig. 4.10 (a, b) Neolacto-type GSLs



**Fig. 4.11** Glucosylated lipid synthesis links to cellular energy metabolism. Animal model studies demonstrate that sphingoglycolipid synthesis regulates the accumulation and release of stored lipids such as triacylglycerides in adipose tissue. This regulatory role of glucosylceramide and its downstream GSLs on energy homeostasis is not surprising, since the basic building blocks of glucosylceramide synthesis, namely, UDP-glucose, palmitoyl-CoA, and serine (derived from glucose), are directly related to energy metabolism. Lipid droplets are also present in glial cells in the central nervous systems

### 3.2 Galactosylceramide and Glycosphingolipids

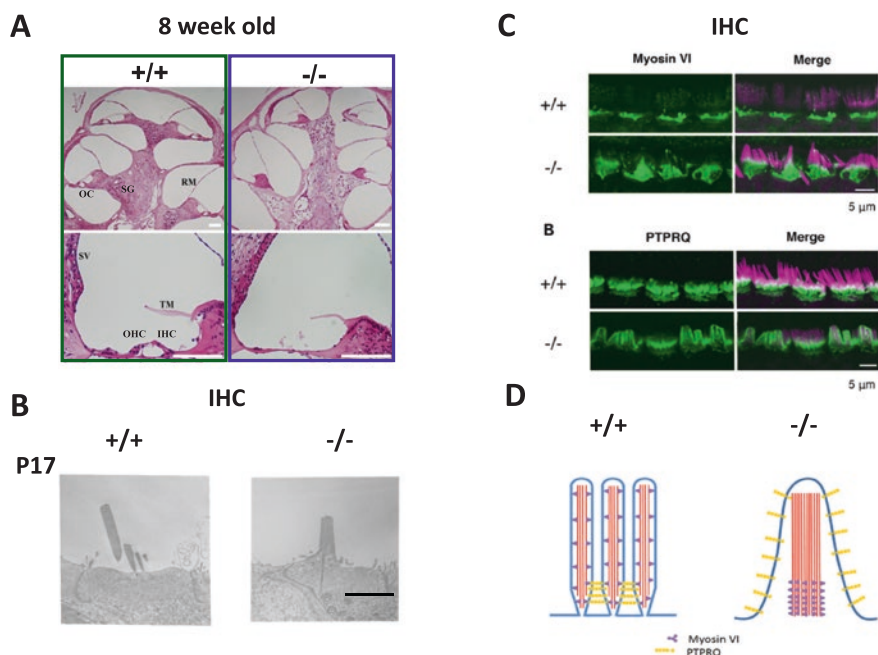
GalCer is characteristically abundant in the myelin membranes of oligodendrocytes. GalCer formation occurs on the luminal side of ER membranes in a reaction catalyzed by UDP-Gal:ceramide galactosyltransferase (CGT). GalCer is translocated from the inner leaflet of the ER to the lumen of the Golgi apparatus prior to synthesis of NeuAc $\alpha$ 2-3GalCer (GM4) and sulfated GalCer (SM4), both of which are also abundant in myelin membranes. The sialylation and sulfation of GalCer to form GM4 and sulfatide are catalyzed by GM3 synthase (ST3Gal5) and sulfotransferase (CST), respectively. These glycolipids play an essential role in myelin functions.

### 3.3 GlcCer and GM3 Synthase Deficiency in Human and Mouse

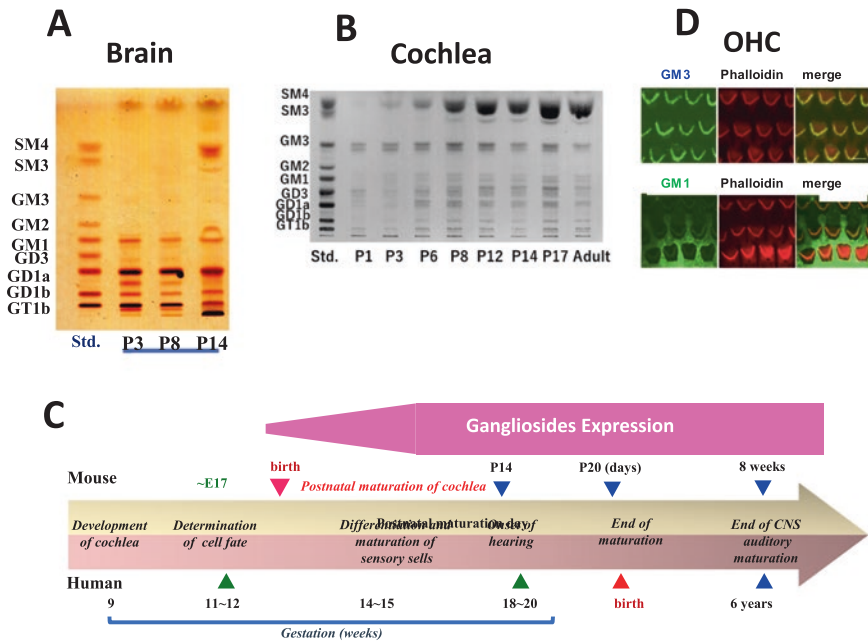
Genetic deficits of lipid glycosylation and its down-stream glycolipid synthesis clinically cause severe phenotypes. In mouse, knockout of *Ugcg* proved that GlcCer synthesis is essential for embryo survival. However it is not clear why the glycolipid synthesis is required for early embryo development (Hirabayashi 2012). In humans, UGCG deficit causes ichthyosis demonstrating the critical role of GlcCer formation for corneal ceramide generation (Monies et al. 2018).

GM3 synthase (GM3S, ST3GAL5) is the first biosynthetic enzyme of  $\alpha$ - and  $\beta$ -series gangliosides. Patients with GM3S deficiency suffer severe neurological disability (Simpson et al. 2004). Inokuchi's group found that GM3S null mice exhibit deafness which accompanied the selective degeneration of the organ of Corti (Yoshikawa et al. 2009). After this finding, hearing difficulties of patients of GM3S deficiency was reported (Fragaki et al. 2013), but the "hearing loss" was not described. Yoshikawa et al., examined carefully auditory function of the patients with homozygous ST3GAL5 c.694C>T mutation (Yoshikawa et al. 2015) and found that the patients have the absence of middle ear muscle reflexes, distortion product otoacoustic emissions and cochlear microphonics, as well as abnormal auditory brainstem responses and cortical auditory-evoked potentials.

The same research group examined the mechanism of hearing loss using GM3S<sup>-/-</sup> mice in more detail. GM3S<sup>-/-</sup> mice showed severe hearing loss at the time of normal hearing onset accompanied by degeneration of cochlear hair cells of the organ of Corti (Fig. 4.12a). Stereocilia of inner hair cells (IHCs) were fused by P17



**Fig. 4.12** Selective degeneration of the organ of Corti of GM3S null mice. Histology of cochlea. Tissue sections of cochlea in 8-week-old GM3S<sup>+/+</sup> and GM3S<sup>-/-</sup> mice were prepared and stained with hematoxylin and eosin (a). The organ of Corti, with OHCs, IHCs, RM, TM, and SV, appears to be normal in GM3S<sup>-/-</sup> mice. In contrast, the organ of Corti is completely missing in the cochlea of GM3S<sup>-/-</sup> mice. Scale bars: 100  $\mu$ m. (b) TEM images of the IHC stereocilia in GM3S<sup>+/+</sup> and GM3S<sup>-/-</sup> mice at P17. Scale bar: 2  $\mu$ m. (c) IHCs of GM3S<sup>+/+</sup> and GM3S<sup>-/-</sup> mice stained for myosin VI and PTPRQ (green) and F-actin (phalloidin, magenta). (d) Schematic images for dislocalization of PTPRQ and myosin VI. OHCs outer hair cells, IHCs inner hair cells, RM Reissner's membrane, TM tectorial membrane, and SV stria vascularis



**Fig. 4.13** Marked increase of gangliosides during postnatal maturation period in the organ of Corti of wild type mice. HPTLC analysis of gangliosides in brain (a) and cochlea (b) during postnatal periods. (c) Summary of stage-specific expression of gangliosides during maturation periods. Comparative development of the cochlea of both humans and mice are also shown. (d) Distinct expression of GM1 and GM3 in the organ of Corti in wild type mice. Scale bar: 5  $\mu$ m

(Fig. 4.12b), and protein tyrosine phosphatase receptor Q (PTPRQ), normally linked to myosin VI at the tapered base of stereocilia, was maldistributed along the cell membrane (Fig. 4.12c, d). Stereocilia of outer hair cells (OHCs) showed signs of degeneration as early as postnatal Day 3 (P3); thereafter, blebs devoid of actin or tubulin appeared at the region of vestigial kinocilia, suggesting impaired vesicular trafficking. GM3 plays an essential role in the postnatal maturation and function of the organ of Corti (Yoshikawa et al. 2015). GM3-dependent membrane microdomains might be essential for the proper organization and maintenance of stereocilia in mammalian auditory hair cells.

Healthy mice begin to recognize sound by postnatal Day 12 (P12), designated the ‘onset of hearing’ (Fig. 4.13c). Marked increase of gangliosides is notable in the cochlea during early postnatal maturation (Fig. 4.13b). GM3 is the dominant cochlear GSL at P1. After P3, there is marked increase of GM3 as well as complex gangliosides (GM1, GD1a, GD3, GD1b, GT1b) and sulfatides (SM3 and SM4), suggesting these molecules contribute to hearing onset. After auditory maturation, GM3 and GM1 show a distinctive distribution among cellular elements of the organ of Corti (Fig. 4.13d). Of note, expression profiles and amounts of gangliosides in brain during early postnatal periods was similar, already achieving the adult profile



at P3 (Fig. 4.13a) but those in cochlea reached the adult expression profile at P8 (Fig. 4.13b), confirming distinct and different regulation of ganglioside expression between brain and cochlea during early postnatal development.

### 3.4 Other O-Linked Lipids – New Glucose-Related Lipids

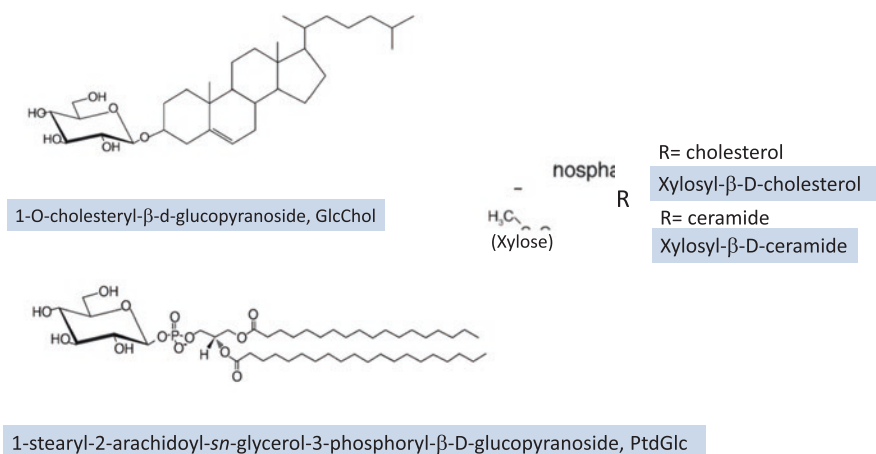
Recent studies show that cholesterol and phosphatidic acid are also modified with glucose (Fig. 4.14) to form cholesterylglucoside and phosphatidylglucoside, respectively.

#### Cholesterylglucoside

Glucosylated cholesterol (1-O-cholesteryl- $\beta$ -D-glucopyranoside, GlcChol) was found in mammalian brains. Mammalian GlcChol synthesis is not dependent on UDP-Glc but glucosylceramide (GlcCer). GlcCer is a glucose donor for GlcChol synthesis. UGCG-deficient GM-95 cells are incapable of synthesizing GlcChol without the exogenous addition of GlcCer. Later, it was found that glucocerebrosidase GBA transfers glucose from GlcCer to Cholesterol by retained transglucosylation reaction (Akiyama et al. 2013). The physiological function of brain GlcChol is still unknown. In addition to GlcChol, xylose is also transferred to cholesterol through  $\beta$ -configuration by the action of GBA1 (Boer et al. 2021). Xylose is derived from Xylosylceramide that is synthesized by CGS/GlcT-1. XylChol is reduced in spleen from patients with GBA1-deficient Gaucher disease.

#### Phosphatidylglucoside

PtdGlc was first isolated from fetal rodent brains and purified to homogeneity in 2006 (Nagatsuka et al. 2006). Its complete structure is 1-stearyl-2-arachidoyl-*sn*-glycerol-3-phosphoryl- $\beta$ -D-glucopyranoside (Fig. 4.14). Acetylated PtdGlc (1-ste



**Fig. 4.14** New glucosylated lipids in the nervous system tissues

aryl-2-arachidoyl-*sn*-glycerol-3-phosphoryl  $\beta$ -D-(6-O-acetyl)glucopyranoside) also exists in fetal rat brain. Very interestingly and importantly, PtdGlc isolated from fetal rat brain has only one fatty acid combination: its *sn*-1 and *sn*-2 chains are exclusively stearic acid (C18:0) and arachidic acid (C20:0), respectively. A single molecular species rarely occurs in natural phospholipids. It is not fully understood how this glycolipid is biosynthesized in cells. Glycosylation is dependent on UDP-glucose synthesized by a  $\beta$ -glucosyltransferase in the ER. The glycolipid is localized in lipid rafts and is involved in astroglial differentiation (Ishibashi et al. 2013). PtdGlc is degraded by phospholipase A2 to form a water-soluble Lyso-PtdGlc. The lyso-glycolipid functions as an endogenous ligand for the atypical cannabinoid receptor GPR55 (Guy et al. 2015). Lyso-PtdGlc/GPR55 signaling axis plays an important role in axonal guidance of pain-sensing neurons and neutrophil migration (Li et al. 2021). Since the glucose head group is critical for GPR55 activation, it is essential to identify the gene involved in the lipid modification with glucose. This issue is currently under investigation.

## 4 Conclusions

Brain contains a large number of carbohydrate chains with different core structures on both proteins and lipids. To understand their physiological and pathophysiological functions, it is crucial to identify and characterize the glycosyltransferases involved in the initial step in the synthesis of each type of glycan. While many of the glycosyltransferases have been identified, endogenous structures of O-GalNAc chains and the corresponding GalNAc-Ts have not been completely identified as yet.

Many questions remain to be addressed including how glycosyltransferase expression is regulated during development and how their glycosylation activities are related to neural activities such as memory and learning, formation, and maintenance. Of particular importance, proteins and lipids in the CNS are heavily involved in the pathological processes in the aging-associated diseases such as Parkinson disease, dementia and Alzheimer diseases.

Recent developments in MS-technology have resulted in the identification of cell-type-specific and disease-associated glycan chains present in mammalian cell surface membranes. Examples of this can be seen in the identification of an O-mannose modified with phosphate in  $\alpha$ -dystroglycan and of new lipids modified with glucose in the nervous system. Since glucose is a conserved essential compound for life, it is anticipated that all glucosylated lipids will have conserved, basic biological functions. The sialic acid containing GlcCer-derived lipid, GM3, is dominantly expressed in insulin responsive organs such as human skeletal muscle, liver and adipose tissue, and brain. Studies of these tissues showed that GM3 was enriched in membrane microdomains (lipid rafts) involved in regulation of energy metabolism. In order to understand the precise roles of GlcCer- and/or GM3-dependent lipid rafts, generation of tissue-specific knockout mice for each synthase is essential since metabolic homeostasis is maintained by communication between tissues.

Although not discussed in detail in this chapter, characterization of the structural diversity of the sphingoid base and the N-acyl chain of ceramide is essential for understanding the dynamics of GSLs in living cell membranes as well as their interactions with lipid raft-associated proteins. “Sphingolipidomics” should enable investigators to determine the precise structure of sugar chains as well as ceramide components of GSLs. This will aid in elucidation of the functional supra-biomolecular complex consisting of GSLs (gangliosides) and functional proteins in lipid microdomains. Current advancement in MS will accelerate the elucidation of the physiological roles of each glycolipid. However, the information of glycans and fatty acids obtained by MS does not reveal the exact molecular structure, e.g., the position of double bonds and branching structure of fatty acid chains, so interpretation of MS data requires great care. Further technological development of MS is needed.

**Conflict of Interest** The authors declare that they have no conflict of interest.

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# Chapter 5

## Chemistry and Function of Glycosaminoglycans in the Nervous System



Nancy B. Schwartz and Miriam S. Domowicz

**Abstract** Proteoglycans, and especially their GAG components, participate in numerous biologically significant interactions with growth factors, chemokines, morphogens, guidance molecules, survival factors, and other extracellular and cell-surface components. These interactions are often critical to the basic developmental processes of cellular proliferation and differentiation, as well as to both the onset of disease sequelae and prevention of disease progression. In many tissues, proteoglycans and especially their glycosaminoglycan (GAG) components are mediators of these processes. The GAG family is characterized by covalently linked repeating disaccharides forming long unbranched polysaccharide chains. Thus far in higher eukaryotes, the family consists of chondroitin sulfate (CS), heparin/heparan sulfate (HS), dermatan sulfate (DS), keratan sulfate (KS) and hyaluronan (HA). All GAG chains (except HA) are characteristically modified by varying amounts of esterified sulfate. One or more GAG chains are usually found in nature bound to polypeptide backbones in the form of proteoglycans; HA is the exception. In the nervous system, GAG/proteoglycan-mediated interactions participate in proliferation and synaptogenesis, neural plasticity, and regeneration. This review focuses on the structure, chemistry and function of GAGs in nervous system development, disease, function and injury response.

**Keywords** Proteoglycan · Glycosaminoglycan · Chondroitin sulfate · Heparan sulfate · Dermatan sulfate · Glycosyltransferase · Sulfotransferase · Brain injury response · Axon guidance molecule · Growth factor interaction · Stem cell niche · Inflammation · Tumorigenesis

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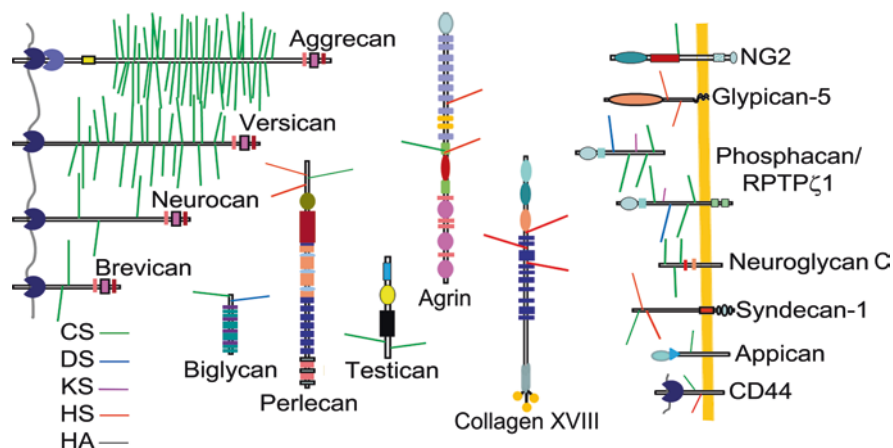
Advances in Neurobiology 29, [https://doi.org/10.1007/978-3-031-12390-0\\_5](https://doi.org/10.1007/978-3-031-12390-0_5)

## Abbreviations

APP	$\beta$ -amyloid precursor protein
CS	Chondroitin sulfate
DS	Dermatan sulfate
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
Gal	Galactose
GalNAc	N-acetylgalactosamine
GB	Glioblastoma
GlcA	Glucuronic acid
GlcN	Glucosamine
GlcNAc	N-acetyl glucosamine
HA	Hyaluronan
HS	Heparan sulfate
IdoA	Iduronic acid
LAR	Leukocyte common antigen-related phosphatase
NDST	N-deacetylase/N-sulfotransferases
NSC	Neural stem cell
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PNN	Perineuronal net
VZ	Ventricular zone

## 1 Introduction

Glycosaminoglycan (GAG) chains are predominantly found in nature covalently attached to multidomain core proteins, together denoted as proteoglycans (Schwartz 2000). These macromolecules are chemically complex and structurally diverse due to variation in: (i) primary sequence, modular arrangements and repetition of core protein domains; (ii) abundance, distribution and composition of the GAG chains; and (iii) position and distribution of GAG modifications including sulfation, phosphorylation and epimerization. From their early discovery, proteoglycans were characterized based on their GAG constituents, of which there are six distinct classes: heparan sulfate (HS), heparin, chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and hyaluronan (HA); only HA is not synthesized covalently bound to protein. Increasingly, this nomenclature of proteoglycans is complicated by the identification of molecules having more than one type of GAG chain attached to the same core protein. Both proteoglycan core proteins and their GAG constituents contribute significantly to the structural and functional roles proteoglycans play in numerous biological interactions critical to development and disease progression. In particular, the brain contains significant amounts and types of proteoglycans (and HA) that change with stage of development, state of cellular



**Fig. 5.1** Representation of the extracellular and membrane-bound proteoglycan families in the central nervous system. Potential GAG modifications are color-coded according to the type. Yellow bar signifies the plasma membrane. All the members of the lectican family of CSPGs are represented, while only representative members of the syndecan, glypican and small leucine-rich repeat PG (biglycan) families are shown. Many of the PGs have alternative splice forms

differentiation, migration patterns, regional specificity, and pathological processes. In this review, we focus on the chemistry and function of GAGs as components of proteoglycans in the nervous system. Some of the major types of proteoglycans in brain are represented in Fig. 5.1.

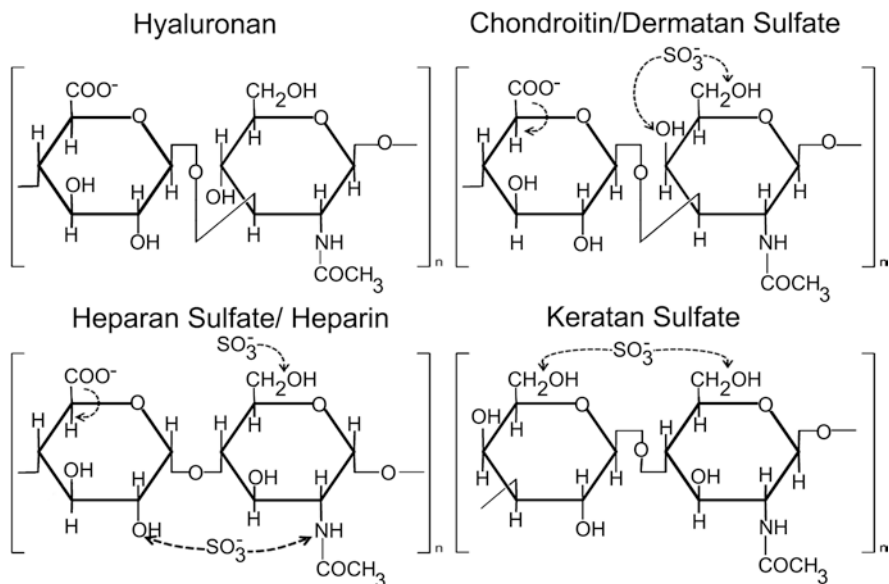
## 2 Glycosaminoglycan Structure and Chemistry

Although six classes of GAGs exist, certain features are common across classes: (i) the long, unbranched heteropolysaccharide chains consist largely of repeating disaccharide units composed of an hexosamine and uronic acid is characteristic of each type of GAG; (ii) the most common substituents are sulfate groups, linked either by ester bonds to certain monosaccharides or by amide bonds to the amino group of the hexosamine; the exception again is HA which is not modified. The abundance of sulfate groups on the GAG chains and the carboxyl groups of the uronic acids contribute to the high net negative charges on GAG chains. Recent findings that correlate structural and chemical properties of the various GAG families with biological activity are summarized in this section.

### 2.1 Chondroitin Sulfate (CS)

Throughout nature, CS is the most abundant type of GAG.





**Fig. 5.2** Chemical structure of GAGs. The repeating disaccharide backbone structure of the six classes of GAGs are shown. Arrows indicate possible modifications

### 2.1.1 Structure and Chemistry

CS chains are characterized by a repeating disaccharide of N-acetylgalactosamine (GalNAc) linked by a  $\beta$ 1,3-glycosidic bond to a glucuronic acid (GlcA), which is then linked via a  $\beta$ 1,4 bond to another disaccharide unit (Fig. 5.2). The disaccharide units may be sulfated on the C-4 (designated CS-A) or C-6 (CS-C) position of GalNAc. A functionally important disulfated CS disaccharide has been identified that is sulfated on both the 4 and 6 position of GalNAc (CS-E), is expressed in brain and may modulate neurite out-growth (Mikami et al. 2009). CS chains typically contain between 30–50 disaccharide units (15–25,000 Da) and are linked covalently to the various protein cores of different chondroitin sulfate proteoglycans (CSPG) via a specific tetrasaccharide linkage region, which consists of xylose-galactose-galactose-glucuronic acid, and is O-glycosyl linked to serines within the various core proteins, i.e., GlcA $\beta$ 1-3Gal $\beta$ 1-3Gal $\beta$ 1-4Xyl $\beta$ 1-O-Ser (Schwartz 2009). The number, length, distribution and degree of sulfation of the CS chains are highly variable among the various CSPGs.

### 2.1.2 Synthesis and Modification of CS Chains

In general, all GAG chains are synthesized by the sequential action of glycosyl-transferases which transfer a monosaccharide from a nucleotide-linked derivative to an appropriate acceptor, either the non-reducing end of another sugar or a

polypeptide, rather than en bloc as in glycoprotein or bacterial polysaccharide synthesis. This mechanism imposes strict substrate specificity for donor, acceptor and linkage type. For CS chains, formation of the unique tetrasaccharide linkage region is the first step, sequentially catalyzed by xylosyltransferase,  $\beta$ 1–4 galactosyltransferase,  $\beta$ 1–3 galactosyltransferase, and  $\beta$ 1–3 glucuronosyltransferase activities, which exist in multiple isoforms for each activity (Schwartz 2000). Polymerization then proceeds via alternating addition of GlcA and GalNAc catalyzed by repeated glucuronosyltransferase and N-acetylgalactosyltransferase activities, respectively, to form the characteristic CS disaccharide units (Schwartz 2010). To date, six homologous glycosyltransferases responsible for synthesis of the repeating disaccharide regions have been cloned. A comprehensive summary of the CS biosynthetic enzymes is provided in the review by Mikami and Kitagawa (2013).

Sulfation of GalNAc on the C-4 or C-6 positions occurs concomitantly with, or shortly after, chain polymerization, and is catalyzed by two types of sulfotransferases, 4-O-sulfotransferases forming CS-A and 6-O-sulfotransferases forming CS-C. Multiple isoforms have been cloned and each has been characterized. They may be functionally redundant except for one of the 4–O sulfotransferases (C4ST-1) that plays a distinct regulatory role in CS synthesis (Izumikawa et al. 2011). To form the disulfated CS-E species, a GalNAc 4-sulfate 6-O sulfotransferase catalyzes the transfer of sulfate to the C-6 position of a pre-existing 4-O-sulfated GalNAc residue (Ohtake et al. 2001). An uronyl 2-O-sulfotransferase catalyzes 2-O sulfation of a GlcA residue in CS, resulting in formation of a second disulfated disaccharide designated CS-D. For sulfation of all GAGs, the sulfate donor is 3'-phosphoadenosine 5'-phosphosulfate (PAPS), which is formed from ATP and sulfate in two steps catalyzed by the bifunctional PAPS synthetase (Li et al. 1995; Lyle et al. 1994; Schwartz 2005, 2010). The PAPS synthetases constitute a gene family, with two members expressed in a tissue- and developmental- specific manner (Kurima et al. 1998; Li et al. 1995; Schwartz 2005). While key steps governing biomolecular sulfation have been assumed to be only at the level of the multiple sulfotransferases, it is possible that regulatory mechanisms occur at other steps along the overall sulfation pathway (Schwartz 2002, 2005; Schwartz et al. 1998).

Lastly, the tetrasaccharide linkage region can be modified by a phosphate group on the C-2 position of xylose and by sulfate groups on the C-6 position of the first galactose and the C-4 and/or C-6 positions of the second galactose, (Sugahara and Kitagawa 2000; Uyama et al. 2007). These modifications have been suggested to be involved in the processing of the growing linkage region, although the biological significance of these modifications are still not clear (Koike et al. 2009; Tone et al. 2008). More recently, the linkage region has been shown to undergo further modification, including sialylation of the galactose residues and fucosylation of the xylose residue (Gomez Toledo et al. 2015; Wen et al. 2014). Again, the impact of these modifications on the ultimate GAG structure or subsequent polymerization remains unknown (Persson et al. 2019). Formation of the tetrasaccharide linkage region starts in the endoplasmic reticulum with addition of xylose, continues with addition of the two galactoses in the cis/medial

Golgi and addition of the first GlcA in the medial/trans Golgi (Kearns et al. 1993; Vertel et al. 1993). Most ensuing sulfation/polymerization reactions occur in the trans Golgi (Silbert and Sugumaran 2002).

### 2.1.3 CS Proteoglycans (CSPGs) in Brain

The nervous system is rich in proteoglycans, especially CSPGs (Schwartz and Domowicz 2018) (Fig. 5.1), which function in neural cell growth and plasticity (Yamaguchi 2000) and are upregulated in scar formation thereby inhibiting axonal regeneration after injury (Bradbury and Carter 2011; Fawcett 2020; Fawcett and Asher 1999; Galtrey and Fawcett 2007) (see Sect. 3). Two types of CSPGs are found in the brain: (i) secreted extracellular CSPGs called lecticans that bind to HA and link proteins including aggrecan, neurocan, versican, and brevican; and (ii) membrane-bound CSPGs such as neuroglycan C and PTPR $\zeta$ . The transcript encoding the latter CSPG also can be alternatively spliced to generate an extracellular variant, phosphacan (Sakurai et al. 1996; Snyder et al. 1996). The expression of these CSPGs is developmentally and spatially regulated; moreover, the CS composition changes during development: CS-C is expressed in developing brain, while CS-A is more highly expressed in adult brain (Sakurai et al. 1996; Snyder et al. 1996). CSPGs (aggrecan, phosphacan/PTPRZ1 and versican) are important in synapse stabilization and maturation, mainly as components of perineuronal nets (PNN) which are specialized matrices that surround neurons and their dendrites in the CNS (Galtrey et al. 2008; Wang and Fawcett 2012). Recently, neuroglycan C (CSPG5) has been found associated with the peripheral margin of PNNs and typically as part of GABAergic and glutaminergic perisynaptic matrix assemblies (Pinter et al. 2020). In genome-wide association studies, certain neurocan alleles, which is another proteoglycan component of PNNs, have been associated with psychiatric disorders such as bipolar disorder, schizophrenia and ADHD (Kathuria et al. 2020; Muhleisen et al. 2012; Schimmelmann et al. 2013; Schultz et al. 2014; Wang et al. 2018). Functionally, CSPGs in PNNs participate in: ocular dominance, fear memory resilience and enhancement of long-term recognition memory (Fawcett et al. 2019; Mencio et al. 2021; Schwartz and Domowicz 2018). Other CSPGs are associated with the gliogenesis process; aggrecan is expressed in radial glia during the switch from neuronal to glial precursor production (Domowicz et al. 2008; Schwartz and Domowicz 2018). These changes may reflect the observed interactions of CSPGs with various growth factors, chemokines, and guidance molecules during brain development. Various members of the fibroblast growth factor (FGFs) family bind highly sulfated CS/DS chains and guidance molecules such as slit2, netrin1, and ephrin A1 and A5. Semaphorin 3A, 5A, and 5B bind CS in a sulfate-dependent manner (Maeda 2010) (see Sect. 3.1 for further discussion of functions of CSPGs in the nervous system).

## 2.2 *Dermatan Sulfate (DS)*

DS is a stereoisomer of CS, differing from CS in that some of its GlcA residues are converted to iduronic acid (IdoA) by DS epimerases, although the glycosidic linkages have the same positions and configurations as in CS (Fig. 5.2). Two DS epimerases have been identified, and one (DS-epi2) is expressed in developing brain and appears to regulate the proportion of IdoA-containing units in cerebellar DS during postnatal development (Akatsu et al. 2011; Thelin et al. 2013). The iduronated disaccharides are sulfated at the C-4 position of GalNAc by a DS 4-O sulfotransferase. IdoA may also be sulfated at the C-2 position by the same uronyl 2-O sulfotransferase that sulfates GlcA in CS, yielding another type of di-sulfated disaccharide (IdoA (2-SO<sub>4</sub>)-GalNAc (4-SO<sub>4</sub>)). Interestingly, rare over-sulfated DS disaccharide units have been implicated as critical elements in neuritogenesis (Hikino et al. 2003; Tovar et al. 2005; Volpi 2010). In addition to acting as a neuritogenic molecule, DS has been reported to function in modulating axonal growth, and in inhibiting or stimulating regeneration in injured CNS. These various functions are attributed to the structural diversity of DS chains (de Sousa et al. 2020; Li et al. 2013; Mizumoto et al. 2015; Rost et al. 2016).

## 2.3 *Heparin*

Heparin and HS (see Sect. 2.4) differs from other GAGs by containing  $\alpha$ -glycosidic linkages and having glucosamine (GlcN) as the hexosamine joined with either GlcA or IdoA to form the disaccharide repeat. Because of their highly varied modifications, heparin and HS are considered the most complex GAGs. In heparin, almost all GlcN residues contain sulfamide linkages, and some GlcN residues are N-acetylated. Heparin may be N-sulfated or O-sulfated on C-6 of GlcN, and O-sulfated on C-3 of the hexosamine and C-2 of GlcA, with the average disaccharide unit having 2.5–2.7 sulfate groups. Heparin functions mainly as an anticoagulant and lipid-clearing agent (Cui et al. 2012; Hughes 2012; Oyagi and Hara 2012), although it does bind strongly to numerous brain-specific chemokines and growth factors (McCanney et al. 2019), and is actively being investigated as a potential therapeutic treatment for aneurysmal subarachnoid hemorrhage and cerebral edema (Khattar et al. 2020; Li et al. 2020).

## 2.4 *Heparan Sulfate (HS)*

As a proteoglycan, HS may be extracellular or in most cell types an integral membrane component.

### 2.4.1 Structure and Chemistry

HS contains disaccharide repeat units similar to those of heparin, i.e., GlcA/IdoA  $\beta$ 1–4GlcN, but has more N-acetyl groups and fewer N-sulfate and O-sulfate groups in general (Fig. 5.2). However, structural heterogeneity arises due to: (i) variability in epimerization at the C-5 of uronic acid; (ii) having either acetyl or sulfo groups on the N-position of GlcN; (iii) and sulfation at C-2 of the uronic acid or at C-6 and C-3 of GlcN. In addition to this microheterogeneity, an HS chain can be differentially modified only in certain regions, leading to domain-specific patterns along the GAG chain, many of which may be biologically important (Kreuger and Kjellen 2012; Shriver et al. 2012).

### 2.4.2 Synthesis and Modification of HS Chains

The structural diversity of HS is biosynthetically generated in a non-template-dependent manner by specific glyco- and sulfo-transferases and sugar-modifying enzymes acting in a highly sequential pattern. Modification of a growing HS chain, consisting of the same tetrasaccharide linkage region found in CS, and repeating disaccharide units of GlcU and GlcNAc, is initiated by N-deacetylation of GlcNAc residues and replacement of the acetyl groups with sulfate groups on the resulting GlcN residues by N-deacetylase/N-sulfotransferases (NDST). Some of the GlcA residues are epimerized to IdoA by HS C-5- epimerase, followed by O-sulfation at either the C-2 of uronic acid by HS 2-O sulfotransferase or on the C-6 of GlcN by HS 6-O sulfotransferase. The final modification step is O-sulfation of the C-3 of GlcN by HS 3-O sulfotransferases. Each subsequent reaction is dependent to some extent on the preceding modification, i.e., the product of one reaction is the substrate for the next. While reactions often do not proceed to completion, yielding chains with variable modifications in different regions, the ultimate structure is not entirely random and seems to be regulated during development in a tissue-specific manner (Maeda et al. 2011). Although HS contains the same modifications found in heparin, the modifications are not as complete leading to greater structural heterogeneity; an average sulfate content of only 1 sulfate group per disaccharide is common in HS chains. Interestingly, due to the presence of IdoA in their linear sequences, heparin and HS exhibit conformational flexibility, allowing better binding to some proteins (Shriver et al. 2012).

### 2.4.3 Heparan Sulfate Proteoglycans (HSPGs) in Brain

HS-containing proteoglycans are usually associated with cell surfaces, either as integral membrane proteins such as syndecans, glycosylphosphatidylinositol (GPI)-anchored proteins like glypican, or secreted extracellular HSPGs like perlecan (Fig. 5.1). Each HSPG type has multiple family members that may be differentially

expressed temporally or spatially. The HS GAG constituents of these HSPGs are the preferred binding partners for multiple target proteins due to their specific sulfated and modified motifs within the HS chains. The structure/chemistry-dependent binding of HS has been demonstrated for several biologically critical interactions: e.g., FGF family members fall into 5 distinct groups based on specific HS structures required for efficient binding (Ashikari-Hada et al. 2004); axon guidance molecules like slit2 prefer HS with 6-O- and N-sulfate, netrin1 prefers 2-O-, 6-O- and N-sulfate, and semaphorin 5B, ephrin A1 and ephrin A5 prefer 2-O- and N-sulfate modifications (Shipp and Hsieh-Wilson 2007). However, attempts to correlate sulfation with function have proven difficult. In more recent *in vitro* studies, chemically de-sulfated oligosaccharides exhibited differential binding to Slit and Robo and affected retinal growth cone collapse, with no consistency between binding and biological activity (Ahmed et al. 2016). Furthermore, eliminating 2-O-sulfation by deletion of *Hs2st* results in complementary neuronal migration and cranial axon defects, while elimination of 6-O-sulfation by deletion of *Hs6st1* and 2 causes defective nerve axon extension (Tillo et al. 2016). Clearly, HS sulfation contributes to neuronal development and axonal growth, but how it relates to signaling pathways needs further investigation (Yu et al. 2018). Also notable is that many HSPGs are hybrids containing CS and HS chains (Fig. 5.1) (Noborn et al. 2016), providing potential opportunities for each type of GAG to interact with binding partners.

## 2.5 Keratan Sulfate (KS)

KS is composed predominantly of a repeating disaccharide in which Gal is linked  $\beta$ 1–4 to N-acetyl glucosamine (GlcNAc) and differs from the other GAGs by containing no uronic acid but may contain other monosaccharides such as mannose, fucose, sialic acid, or GalNAc (Fig. 5.2) as well. It is the only GAG that may contain these sugars in branched positions. Sulfate content is variable, with sulfation on C-6 of either or both of the Gal and hexosamine residues. KS also exists in two types that differ in their linkage to protein cores, carbohydrate content and tissue distribution. KS-I is linked to protein by a GlcNAc asparaginyl bond, typical of glycoproteins, and KS-II is linked through GalNAc to serine or threonine and is often found on the same core proteins as CS. KS expression, (as well as CS) is upregulated in glial scar formation in response to injury, shown both by detection of KS with a KS-specific monoclonal antibody and in a mouse model deficient in N-acetylglucosamine 6-O-sulfotransferase-1 (Zhang et al. 2006a, b). Using surface plasmon resonance binding and proteomics approaches, corneal KS was shown to interact with a large number of nerve regulatory proteins (Conrad et al. 2010); the interactive capability of KS with neuro-regulatory ligands suggest KS may also play significant roles in neural processes (Fu et al. 2009; Scranton et al. 1993; Takeda-Uchimura et al. 2015).

## 2.6 Hyaluronan (HA)

HA is a copolymer of GlcNAC and GlcA (Fig. 5.2) and differs from other GAGs in several respects: (i) it contains no modifications (e.g., no sulfation); (ii) it is not covalently linked to a core protein (e.g., not a proteoglycan component); and (iii) it is produced by bacteria as well as by eukaryotic cells. Although often considered to be the least complex GAG structurally and chemically, HA chains can reach molecular masses of  $10^5$ – $10^7$  Da, which contribute to its roles as a lubricant and shock absorber in many tissues. In brain it helps to structure the extracellular milieu and is especially abundant in developing brain (Frischknecht and Gundelfinger 2012; Preston and Sherman 2011). In particular, HA is a prominent component, along with HA-binding CSPGs (aggrecan, versican, neurocan, and brevican), of PNNs, which are insoluble macromolecular aggregates of ECM molecules surrounding synaptic contacts on soma and proximal dendrites of certain neurons (Fawcett et al. 2019). The formation of PNNs occurs late in postnatal development and are important to the acquisition and storage of memories (Banerjee et al. 2017), suggesting that PNNs drive the transition from more plastic, juvenile neural circuits to more stable adult circuits. Furthermore, knockout models of the three HA polymerizing enzymes develop epileptic seizures, likely due to reduction in brain extracellular space, with Has3 mutants showing the most extreme phenotype (Arranz et al. 2014). More recent studies indicate that the solubilities of HA and aggrecan increase during aging, accompanied by degradation and loss of HA (Sugitani et al. 2021), although the consequences of these age-related changes are not clear.

## 3 Function of GAGs in the Brain

GAGs, as components of proteoglycans, have been implicated in an extensive list of brain-development, aging, and disease processes. The structural differences between GAG chain types often dictate distinct binding partners, thus associating them with unique functionalities. As well, the types of core proteins to which GAGs are covalently bound determine distinct subcellular localizations, association with unique receptors and participation in intracellular-signaling cascades. Furthermore, GAG localization can be controlled by transcriptional regulation of core protein expression, availability of biosynthetic enzymes, intracellular degradation, and extracellular modification by proteases, sulfatases, and deacetylases. All these aspects have added to the complexity of studying these molecules and continue to challenge our understanding of GAG function. This section covers recent advances in understanding the functional properties of GAG chains with respect to brain development, function, and disease.

### 3.1 *GAG Interactions and Binding Partners*

As mentioned, HS chains interact specifically and in a structure-dependent manner, with growth factors (FGFs, midkine, pleiotrophin), axon guidance molecules (slit2, netrin1, semaphorin 5B, ephrins), chemokines (chemokine ligand 2) and morphogens (Hedgehogs, Wnts) (Bornemann et al. 2008; Haerry et al. 1997; Lin 2004; Mizumoto et al. 2013a; Shipp and Hsieh-Wilson 2007; Sweeney et al. 2006; Zou et al. 2003; Schwartz and Domowicz 2022). Consequently, they play critical roles in cell proliferation, survival, differentiation, and migration during brain development. One of the best-described systems is the tripartite molecular complex of FGF2, FGFR1 and the HS chain of a proteoglycan. This interaction is required to induce receptor dimerization and ensuing intracellular signaling. The functional dependency on particular sulfation and acetylation patterns has been highlighted by studies using carbohydrate microarrays (Shipp and Hsieh-Wilson 2007) which found that, even for modifications resulting in equal charge distribution, binding is dependent on the specific position of the charge. Thus, the expression of specific sulfotransferases in a tissue or cell type may dictate the type of gradient a particular HS-binding protein can form.

CS chains also have been implicated in interactions with many of the same molecules that interact with HS and heparin (Deepa et al. 2002; Djerbal et al. 2017; Kawashima et al. 2002; Kuschert et al. 1999; Nandini et al. 2005), but often do so with reduced affinity. In general, higher binding affinities are found with CS-E polysaccharides. For example, netrin1, slit2, and semaphorin 5B bind to CS-E with higher affinity than to heparin and HS, while they bind weakly to CS-A, CS-B, CS-C, and CS-D (Shipp and Hsieh-Wilson 2007). In contrast, heparin-binding proteins such as midkine and pleiotrophin bind strongly to sulfated CS-E with affinities similar to that of heparin (Deepa et al. 2002; Maeda et al. 2006), and with lower affinity to CS-A, CS-C, and CS-D (Mizumoto et al. 2013a). Usually, HSPGs are on the cell surface while CSPGs for the most part are secreted; in general, HSPGs are found in lower concentrations than CSPGs. Together, all of these differing properties can lead to complex biological consequences.

Structural influence also has been observed in the axon-guidance action of semaphorin 5A in the diencephalon, where HS exerts an attractive signal and CS a repulsive one (Kantor et al. 2004; Perez et al. 2021). Furthermore, functional complexity may be based on variants of one PG class, as occurs in neural stem niches during brain development, where significant changes in types and quantity of HSPGs occur during neurogenesis (Hagihara et al. 2000; Litwack et al. 1998). Likewise, later in development CSPG compositional changes also occur during the critical switch from neurogenesis to gliogenesis (Domowicz et al. 2008; Ishii and Maeda 2008; Schwartz and Domowicz 2018; Shimazaki et al. 2005). Presumably these GAG composition changes could differentially regulate the availability and interaction of growth factors, signaling molecules and other extracellular matrix partners during development.



## 3.2 *GAG Functions During Brain Development*

The above-mentioned interactions are critical to the function of GAGs in all aspects of neural development, neurological abnormalities (disease) and response to injury. Some specific examples of the role GAGs play in the nervous system are described now.

### 3.2.1 **Neural System Development and Lineage Specification**

During brain development, expression of different proteoglycan types closely parallels the timing of their required functions as binding partners for signaling molecules or as permissive substrates for emerging cell types. For example, HSPGs regulate signaling by members of the FGF and WNT families, that function in controlling proliferation and self-renewal of neural precursors and differentiation of neuronal and glial subtypes (Bernfield et al. 1999; Ornitz 2000); (reviewed in Schwartz and Domowicz (2018)). HSPGs function in the potentiation of the WNT pathway by interacting with R-spondins, known to be important in morphogenesis (Dubey et al. 2020). Similarly, changes in expression patterns and concentrations of CSPGs occur during the course of rodent brain maturation as required for proliferation, differentiation, and neurogenesis of neural stem progenitor cells (Domowicz et al. 2008; Schwartz and Domowicz 2018; Sirko et al. 2007). As mentioned, interactions of proteoglycans with signaling molecules depends on the protein core, GAG chain type and length, and degree and position of sulfate substitutions; all of which vary depending on stage of development and physiological state, in order to provide a system for selective activation or inhibition of various signaling pathways during brain development. Elucidation of GAG function in these interactions has often come from studies of naturally occurring or knockout animal models for the individual proteoglycan core proteins, which exhibit a range of phenotypes (Brakebusch et al. 2002; Rauch et al. 2005), or for GAG-synthesizing enzymes, which suggest that GAGs are essential for early embryogenesis (reviewed in Izumikawa and Kitagawa (2010), Maeda et al. (2011), Schwartz and Domowicz (2018)). Because of embryonic lethality due to elimination of some enzymes, studying GAG function during brain development has also required use of conditional knockout mice. As well, the use of GAG-degrading enzymes is a common strategy for assessing the functions of GAG chains under various experimental conditions (Maeda 2010). For instance, disrupting CSPGs via addition of chondroitinase ABC in the developing mouse neocortex, results in reduction of neural progenitor proliferation and generation of neurons, while the number of astrocytes increased, suggesting a switch in fate from neuronal to glial lineage (Long and Huttner 2019; Sirko et al. 2007, 2010a) (see Sect. 3.2.4).

### 3.2.2 Brain Patterning

Developmental processes like neuronal migration, axon pathfinding and synaptogenesis are fundamental to the organization and expansion of the nervous system. While neurons and axons have to travel considerable distances to reach their final positions, the extracellular matrix (ECM) that they travel through presents both physical barriers and contains guidance molecules that aid migration. Interestingly, there are important feedback mechanisms between the migrating cell/axon and the ECM that aid movement toward the final destinations (Reig et al. 2014; Tessier-Lavigne and Goodman 1996). The molecularly diverse proteoglycans and their GAG chains, as components of the ECM, contribute extensively to these aspects of neuronal development. For example, based on the important interactions of HS in FGF-signaling paradigms, it is not surprising that brain patterning defects are associated with HS-biosynthetic-enzyme mutations. Nestin-Cre-Ext1 conditional-knockout mice (Inatani et al. 2003) lack a cerebellum due to defects in establishing the midbrain/hindbrain boundary, which is known to be controlled by FGF8 and WNT signaling, rather than to specification defects (reviewed in Yamaguchi et al. (2010)). Another patterning defect in this model is agenesis of the olfactory bulb; however, the molecular basis for this phenotype is less clear, although both FGF8 hypomorphic mutants (Meyers et al. 1998) and FGFR1-null mice (Hebert et al. 2003) lack olfactory bulb formation. Patterning defects with variable penetrance have also been observed in HS-Ndst1<sup>-/-</sup> mutants, leading to lack of olfactory bulbs, absence of the hippocampal and anterior commissures, and microcephaly (Grobe et al. 2005). KAL-1/anosmin-1, a neural cell adhesion molecule mutated in Kallman syndrome (Bulow et al. 2002) has been shown to interact with HSPGs and its receptor, FGFR (Diaz-Balzac et al. 2014). Loss-of-function mutations in FGFR, which are required for formation of the olfactory bulb (Hebert et al. 2003), as well as mutations in HS biosynthetic genes and in different HSPG core proteins (glypican, perlecan, and syndecan) have also been shown to be needed for KAL-1 function (Witte and Bradke 2005). Together, these results support a three-dimensional model of HSPG and KAL-1/anosmin-1 mediating intercellular communication through complex/cooperative interactions (Diaz-Balzac et al. 2014). Less is known about the role of CSPGs in olfactory patterning. In a recent study of ECM expression in the olfactory bulb, PNNs, which are composed mainly of CSPGs were rarely present and exhibited thin or diffuse appearances (Hunyadi et al. 2020). This finding may explain the life-long plasticity of the olfactory system (Imai 2014) since PNNs reduce neural plasticity in adulthood by acting as a scaffold for molecules that inhibit synapse formation, thereby limiting receptor mobility at synapses (Wang and Fawcett 2012).

### 3.2.3 Neurite Outgrowth, Axonal Pathfinding and Migration

Several lines of evidence have strongly supported the participation of GAGs in axon guidance and pathfinding in both the peripheral and central nervous systems. With respect to HS participation in these important functions, axonal-guidance defects have been described in many of the HS-synthesizing enzyme mutants. Most notably, Nestin-cre-EXT1 null embryos show severe axon guidance defects in three major commissural fiber tracts of the forebrain (corpus callosum, hippocampal commissure, and anterior commissure) (Inatani et al. 2003). Even though these defects, as well as defects in guidance through the optic chiasm, are similar to those of slit1 and 2 double mutants, it is possible that some of these defects are due to defective FGF signaling (Yamaguchi et al. 2010). Studies in a Wnt1-Cre-mediated ablation of Ext1, in which HS was eliminated only in the dorsal part of the spinal cord, found commissural axon-path-finding defects, demonstrating that commissural neurons need to express HS for their axons to respond to netrin1 signals (Matsumoto et al. 2007). Brain phenotypes of Ndst1-/- mice include axon guidance defects similar to those of the Nestin-cre-EXT1 null embryos, implying that sulfation of HS chains is essential for their axonal-guidance function (Grobe et al. 2005). The subtle phenotypes in axon-guidance observed in 2-O-sulfotransferase- (Hs2st), 6-O-sulfotransferase- (Hs6st1), and C5-epimerase-knockout mice further support this notion (Li et al. 2003; McLaughlin et al. 2003; Pratt et al. 2006). Removal of HS sulfatases 1 and 2 also affects neurite outgrowth and cell survival in cerebellar granular neurons, likely by interfering with the FGF2, GDNF and NGF pathways (Kalus et al. 2015). The proteoglycan perlecan has also been identified as a requirement for sema-1a-PlexA-mediated repulsive guidance of motor axons in *Drosophila* (Cho et al. 2012). Agrin, a HS/CS proteoglycan, modulates the ability of FGF2 to stimulate neurite outgrowth (Kim et al. 2003) and this effect could be partially due to the composition of the GAG side chains (Bixby et al. 2002).

Strong evidence has accumulated for the influence of CS chains on dendritic and axonal growth in culture, based on de-glycosylation experiments with chondroitinase and tissue culture studies in artificial matrices. In culture, CS and purified CSPG have been shown to inhibit neuritogenesis and axonal growth by a number of neuronal types on different substrates (Bandtlow and Zimmermann 2000; Bao et al. 2005; Schmalfeldt et al. 2000; Ughrin et al. 2003), but there have also been reports of CS and DS promoting neurite outgrowth (Fernaund-Espinosa et al. 1994; Hikino et al. 2003; Lafont et al. 1992). In primary hippocampal neuronal cultures, CS is localized to focal contacts between neurons, and its removal by chondroitinase treatment or by knockdown of sulfotransferases involved in CS-E synthesis was followed by destabilization of focal contacts and induced formation of multiple axons (Nishimura et al. 2010). Thus, in culture, the effects of CS chains and their core proteins on neurogenesis strongly depends on the type of neuron, plating substrate and developmental stage. Similar evidence for their function in axonal guidance *in vivo* is limited. Although, CSPGs have been shown to participate in axonal pathfinding *in vivo* in different areas of the brain and at different developmental stages. For example, trigeminal-ganglion neurons show abnormal axon growth into

mesenchymal regions after treatment with chondroitinase, and this effect is mediated by semaphorin 5A (Kantor et al. 2004). CS also modulates retinal axonal growth towards the optic nerve (Brittis et al. 1992) and across the chiasm (Chung et al. 2000a, b; Ichijo and Kawabata 2001), and lastly, CS prevents the axons from crossing the midline when they reach the optic tectum (Carulli et al. 2005; Hoffman-Kim et al. 1998). Surprisingly, no biosynthetic knockout model of a single CSPG has exhibited major axonal-guidance problems, perhaps highlighting the functional redundancy of individual CSPGs (Rauch and Kappler 2006); as an example, no defects in axonal path-finding were found in a mouse knockout of chondroitin-6-sulfotransferase (Uchimura et al. 2002). Nevertheless, a zebrafish model of morpholino-knockdown of chondroitin-4-sulfate exhibited aberrant projections from spinal motor axons, implying a function in axonal guidance (Mizumoto et al. 2009). More recent *in vitro* studies demonstrated that CSPGs formed an inhibitory zone around mouse cerebellar granular neurons, that when removed enzymatically reversed the inhibitory effects of CSPGs on neurite outgrowth and expansion of the growth core area (Jin et al. 2018). However, how CSPGs modulate signaling pathways, other ECM components and guidance molecules in axonal pathfinding still remains incompletely understood (reviewed in Masu (2016) and Mutalik and Gupton (2021)).

### 3.2.4 Differentiation and Stem-Cell Niche

During development the brain is formed in a complex sequence of events from a simple neuroepithelium that lines the cerebral ventricles and spinal canal. Differentiation is characterized by a progressive wave of neurogenesis from radial glial progenitors, and later by gliogenesis which predominantly depletes the radial glial progenitors from the brain ventricular zone (VZ), leaving only two adult neurogenic niches (Rowitch and Kriegstein 2010). Complex changes in proteoglycans and associated GAG-chains have been described in areas where neural stem cells reside during brain development, suggesting that proteoglycans could regulate neural stem cell survival, proliferation, and /or differentiation (Akita et al. 2008; Sirko et al. 2010a). Even though we are still in the early stages of understanding how proteoglycans influence neural stem cell niches, several lines of evidence *in vivo* and in culture indicate interesting connections in this area.

As example, the HS profile and context of stem cells undergo modification as they progress through various stages of differentiation (Kraushaar et al. 2013), concomitant with increased expression of HS biosynthetic and modification enzymes (Forsberg et al. 2012; Wang et al. 2017). For instance, as cells switch from a proliferative state to lineage differentiation, increased 6-O-sulfation is accompanied by changes in FGF signaling from a requirement for FGF2 to FGF1 (Brickman et al. 1998). The presence of 6-O-sulfation sites is regulated by HS6ST and sulfatases 1 and 2, and is necessary for promoting interactions with HS and several heparin binding growth factors (Kalus et al. 2015). Different isoforms, which serve as recognition elements for growth factors and other signaling molecules often change

over time. Members of the glypican family of HSPGs have also been found to be expressed in a developmentally regulated manner in the VZ during neurogenesis. In rodents, expression of glypican-4, a marker of stem and progenitor cells, is down-regulated after neuronal commitment (Hagihara et al. 2000), while glypican-1 continues to be expressed in postmitotic neurons (Litwack et al. 1998). Glypicans-2 and -5 are only expressed in committed neurons and not in their precursors (Saunders et al. 1997). Interestingly, knockout of glypican-1 is characterized by a reduction in brain size, accompanied by impairment in FGF signaling and premature differentiation of post-mitotic neurons (Jen et al. 2009), which supports a key role for glypican-1 in neural stem cell proliferation and neuronal differentiation (Oikari et al. 2016). The HSPG perlecan is a component of the basal lamina of the neuroepithelium during development, and null mutants also display microcephaly with variable penetrance due to reduction of early mitotic precursors and impaired cell-cycle progression (Giros et al. 2007). Perlecan also contributes to differentiation of the astrocyte and oligodendrocyte lineages (Nakamura et al. 2015; Winkler et al. 2002). Other HSPGs, like syndecan-1 and -4, also are localized in the VZ where neural precursors reside (Ford-Perriss et al. 2003). Knock-down of syndecan-1 *in vivo* by *in utero* electroporation reduces neural stem cell proliferation and induces premature neuronal differentiation, possibly acting through the Wnt signaling pathway (Wang et al. 2012). Furthermore, in models of altered HSPG biosynthesis like the Nestin-cre-EXT1 null embryos and *Ndst1*<sup>-/-</sup> mutants, thinning of the frontal cortex was also observed (Grobe et al. 2005; Inatani et al. 2003). As these few examples show, identification of specific HSPGs and HS sulfation patterns have helped distinguish between neuronal and glial lineage specification from the stem cell state (Yu et al. 2017).

CSPGs have also been linked to neural stem/progenitor cell proliferation, survival and differentiation in culture (Cortes et al. 2022). Several CSPGs are enriched in the VZ of the telencephalon during neurogenesis, and their expression is maintained in neurosphere cultures derived from VZ cells (Ida et al. 2006). Degradation of CS with chondroitinase in neural stem cell (NSC) cultures results in reduced cell proliferation and impaired neurosphere formation (Sirko et al. 2007), while addition of exogenous proteoglycans to these cultures increases survival of the precursors (Tham et al. 2010), and alters their differentiation potential resulting in increased numbers of astrocytic precursors and decreases in neurogenesis (Sirko et al. 2007). Intrauterine injection of chondroitinase into the lateral ventricle during mid-neurogenesis provided results consistent with the *in culture* observations (Sirko et al. 2007), indicating a function for CSPG in precursor self-renewal, proliferation, and differentiation during the neuron-glial differentiation switch. Also, studies in neurosphere cultures established that CS mediates proliferation and maintenance through FGF-2, and that CS restrains maturation and gliogenesis through an EGF-dependent pathway (Sirko et al. 2010b). CSPG regulation of radial glial cell differentiation and maturation was also linked to the integrin signaling pathways (Gu et al. 2009). Some members of the lectican family are more strongly associated with the gliogenesis process. Brevican in rodents and aggrecan in birds starts to be expressed in radial glial cells of the VZ during the *switch* from *neuronal* to *glial*

precursor production (Domowicz et al. 2008; Jaworski et al. 1995); in particular, aggrecan expression is developmentally regulated and observed only in glial precursors and not in mature cells (Domowicz et al. 2008). Furthermore, in mutants in which expression of aggrecan is knocked out, increased differentiation to the astrocytic pathway is observed *in vivo* and *in culture*; addition of purified aggrecan into the culture system rescues this phenotype (Domowicz et al. 2008).

Interestingly, there is increasing evidence that HA, the main binding partner of lecticans, is also an important component of many stem cell niches (Preston and Sherman 2011), and in particular, HA in brain may play a role in neural stem cell precursor proliferation and differentiation during development and possibly in adult brain-stem-cell niches as well (Preston and Sherman 2011). HA degradation induces proliferation of quiescent astrocytes in adult spinal cord (Struve et al. 2005), indicating that HA might act to slow proliferation. On the other hand, an HA-rich matrix has been hypothesized to inhibit oligodendrocyte differentiation (Back et al. 2005; Srivastava et al. 2020). More recently, in a human neocortical explant culture model in which the folding of the cortical plate could be induced, endogenous levels of HA, the HA receptor RHAMM, and its downstream ERK signaling partners within the tissue were all observed to increase (Long et al. 2018). Depleting HA both blocked and reversed the ECM-induced folding suggesting that HA is a key component of the folding mechanism, and that modulating the ECM greatly affects the morphology of the developing neocortex (Long and Huttner 2019).

### 3.2.5 Synaptic Plasticity

As introduced earlier in Sect. 2.1.3, GAGs are important components of the perineuronal nets (PNNs), the specialized extracellular matrices surrounding many neurons and their dendrites in the CNS. PNNs are established as the final, mature synaptic circuitry is stabilized and have been linked to regulating brain plasticity during synapse stabilization and maturation. Neurocan, versican, and phosphacan/PTPR $\zeta$  are components of some PNNs, while aggrecan and link protein expression is upregulated postnatally during PNN formation, suggesting they also play a role in PNN formation (Galtrey et al. 2008; Wang and Fawcett 2012). In addition to being localized to PNNs, proteoglycans have been implicated in multiple synaptic-associated functions (Rowlands et al. 2018); a few of which are now summarized.

The mammalian visual cortex has the ability to undergo plasticity changes controlled by visual experiences during a critical period of postnatal development. Monocular deprivation causes an ocular dominance shift toward the non-deprived eye after enzymatic degradation of CS chains, indicating that GAGs are inhibitory for experience-dependent plasticity and their removal reactivates ocular plasticity, further suggesting GAGs play an essential role in the age-dependent decrease in ocular dominance plasticity (Pizzorusso et al. 2002, 2006). Furthermore, enhancement of long-term recognition memory has been observed after *in vivo* treatment with chondroitinase, and these effects are lost over time as PNNs are rebuilt (Carulli et al. 2010; Romberg et al. 2013). Similar memory effects also have been described

in link protein (*Hapln1*) knockout mice, which retain juvenile levels of ocular dominance plasticity (Pizzorusso et al. 2006), suggesting that regulation of memory and experience-driven synaptic plasticity may involve lectican-rich matrices (Carulli et al. 2010; Romberg et al. 2013). More recently, evidence has been forthcoming that PNNs and CS-GAG chains, the main effectors of PNNs, play important roles in memory acquisition and retention, as shown by chondroitinase ABC digestion and transgenic deletion of PNN components (Carulli et al. 2020; Romberg et al. 2013; Rowlands et al. 2018; Slaker et al. 2015). In animal models of Alzheimer's disease with pathology-associated memory loss, chondroitinase digestion or CS-GAG blocking antibodies also alleviate the memory loss (Vegh et al. 2014; Yang et al. 2015, 2017). Other examples of synaptic plasticity implicating action by the proteoglycan components of PNNs have been described in the rodent barrel cortex after sensory deprivation (McRae et al. 2007), in hippocampal slices after inducing spine remodeling (Orlando et al. 2012), by interfering with induction of long-term potentiation (Bukalo et al. 2001), in neuronal hippocampal cultures by regulating AMPA receptor motility (Frischknecht et al. 2009), in the lateral vestibular nucleus of the rat after unilateral labyrinthectomy (Deak et al. 2012) and in neostriatum during the emergence of behavior (Lee et al. 2008). The proteoglycan-interacting axonal-guidance molecule, semaphorin 3 (Vo et al. 2013), and the CSPG receptors (Ye and Miao 2013), LAR (leukocyte common antigen-related phosphatase) (Fisher et al. 2011) and Nogo-receptor (Dickendesher et al. 2012), have also been localized to PNNs.

The mechanisms through which CSPGs might regulate synaptic plasticity have been elusive, and include suggestions that PNN components could inhibit axonal growth and sprouting (Crespo et al. 2007; Fitch and Silver 1997; Grumet et al. 1996; Oohira et al. 1991; Wang and Fawcett 2012). A recent study which focused on the sulfation properties of CSPGs in PNNs involved in neuroplasticity required for memory in aging, suggests a potential mechanism (Yang et al. 2021). It is well established that the sulfation composition of CS changes with age, with a significant decline in CS-6 with age (Deepa et al. 2006; Foscarin et al. 2017). The CS-6 decrease and ensuing increase in CS-4/CS-6 ratio leads to a more inhibitory PNN matrix and diminished plasticity in aged brains (Foscarin et al. 2017). Transgenic deletion of chondroitin-6-sulfotransferase (ChSt3) reduced levels of CS-6 in young animals, simulating the age-related memory loss. Concomitantly, over expression of ChSt3 restored CS-6 levels in aged animals and restored cortical long-term potentiation and rescued memory deficits (Foscarin et al. 2017). Further support comes from the area of axonal injury and regeneration, where evidence of inhibition of axonal growth by proteoglycans continues to be accumulated (See next section).

Association of HSPGs with late synaptic maturation has been increasingly documented, in particular for syndecan-2 and -3 (Ethell and Yamaguchi 1999; Reizes et al. 2001), and as mentioned earlier, HSPGs are important to the synapse-formation process (Condomitti and de Wit 2018; Irie and Yamaguchi 2004; Johnson et al. 2006; Raulo et al. 2005). Mice with a conditional EXT1-knockout targeted to post-natal neurons developed autistic-like socio-communicative deficits with attenuation of excitatory synaptic transmission in glutaminergic amygdala pyramidal neurons

(Irie et al. 2012), strongly linking HS to synaptic plasticity. More recently, new experimental methodologies, like cell-specific transcriptome analysis (SCTA), proteomics, and interactome approaches have begun to identify roles for HSPGs in synaptic specificity. As example, SCTA of different neuron types in *Drosophila* olfactory bulb and GABAergic populations in mouse cortex revealed cell-type specific expression patterns of HSPGs and HS-modifying enzymes (Li et al. 2017a; Paul et al. 2017; Tasic et al. 2016). Other studies have elucidated region and cell-type specific binding partners, e.g. glypican-4 regulates excitatory synapse formation through a trans-synaptic interaction with the postsynaptic protein LRRTM8 (de Wit et al. 2013; Siddiqui et al. 2013). Thus far, HSPGs appear to be important regulators of synaptic specificity by interacting with binding partners that are restrictively expressed in discrete cell types to exert differential effects on synaptic function (Condomitti and de Wit 2018).

### 3.3 *GAG Function in CNS-Associated Disorders and Injury*

Just as the formation, development, and establishment of essential functions in the brain involve complex and reciprocal interplay between multiple cell types, signaling pathways and the extracellular environment, in brain injury, genetic disorders and malignancies similar interacting paradigms involving proteoglycans pertain. This section covers recent advances in understanding the role of GAGs in brain dysfunction.

#### 3.3.1 *Injury Response*

Upon CNS injury, glial scar formation is crucial in the healing process needed to seal the blood-brain barrier breach and reestablish homeostasis. However, a cascade of secondary pathological events occurs including: up-regulation of inflammatory cytokines and chemokines, activation of astrocytes and microglia, synthesis and release of CSPGs (largely by reactive astrocytes), and formation of a glial scar (Donnelly and Popovich 2008; Gesteira et al. 2016). The regeneration failure is due to formation of a barrier of reactive astrocytes and inhibitory CSPGs, which prevent growth cone advancement (Bradbury and Carter 2011; Fawcett 2020; Fawcett and Asher 1999; Galtrey and Fawcett 2007). As mentioned, the efficacy of removing GAGs with chondroitinase treatment in improving axonal growth and/or functional recovery has also been demonstrated in numerous adult-injury models (Bradbury and Carter 2011; Bradbury et al. 2002; Cafferty et al. 2007; Carter et al. 2011; Fawcett 2009; Garcia-Alias et al. 2011; Moon et al. 2001; Tom et al. 2009; Zhao et al. 2013; Zuo et al. 2002), thus, providing strong evidence for the role of CSPGs (reviewed in Bradbury and Burnside (2019), Fawcett (2020), Schwartz and Domowicz (2018)). Even though functional connections below the injury location have been confirmed in some cases (Bradbury et al. 2002), activation of alternative



sprouting patterns cannot be eliminated as a mechanism of repair (Bradbury and Burnside 2019; Bradbury and Carter 2011; Burnside and Bradbury 2014; Massey et al. 2006), nor can neuroprotective effects due to release of growth factors from the digested areas (Carter et al. 2011; Dudas and Semeniken 2012). Expression patterns of different CSPGs following brain or spinal cord injury have been assessed in several injury models and indicate that various GAG chains and CSPGs possess inhibiting properties (Burnside and Bradbury 2014). The type of CS sulfation that mediates these effects is still not totally clear, but *C6st1* knockout in mice has a positive influence on axonal regeneration in the CNS (Lin et al. 2011), while down-regulation of CS-E by siRNA targeting *GalNAC4S6ST* induced inhibition of neuronal attachment and neurite extensions in culture (Karumbaiah et al. 2011). This dichotomy could explain the lack of functional recovery often observed after chondroitinase treatment (Tom et al. 2009). However, other possibilities have been suggested such as an inhibitory effect on axonal growth by the remaining core protein. Thus, activation of extracellular matrix proteases could represent a potential therapeutic approach (Cua et al. 2013); alternatively, levels of proteoglycan core proteins could be regulated by specific transcription factors in the glia scar (Iseki et al. 2012). Toward this end, conditional ablation of Sox9, a transcription factor known to regulate matrix synthesis, reduced levels of CSPG biosynthetic enzymes and increased hind-limb function and locomotor recovery after spinal cord injury (McKillop et al. 2013). However, proteoglycans like lubricin (PRG4), known to inhibit activation of CD44, have recently been shown to reduce neuroinflammation and protect the blood-brain barrier after cortical impact traumatic brain injury (Bennett et al. 2021), highlighting a novel area for therapeutic approaches. This area of research has also revealed a number of CSPG receptors that may regulate CSPG inhibition of axonal growth through the glia scar. PTPR $\zeta$  binds to GAG chains, and RPTP $\sigma$  knockout mice show enhanced axonal growth after spinal cord injury (Shen et al. 2009). Similarly, LAR binds CSPGs with high affinity, and treatment with LAR-targeting peptides after thoracic spinal cord transection injuries in mice also promotes axonal growth (Fisher et al. 2011). Other types of brain injury models involving CSPGs have been reported; e.g. triple-null mutants for Nogo-receptors 1, 2, and 3 which bind CSPGs, exhibit enhanced axonal regeneration after retro-orbital optic nerve crush injury (Dickendeshner et al. 2012), formation of a CSPG-rich glial scar in stroke models (Gherardini et al. 2015), in traumatic brain injury (Galindo et al. 2017), and in perinatal and embryonic injury models that also show up-regulation of Sox9 (Domowicz et al. 2011, 2018). These receptors and signaling molecules may also represent therapeutic targets for novel traumatic-injury treatments.

Fewer examples exist for potential roles of HSPGs in regulation of axonal growth after injury, although increases in HS and *Hs2st* mRNA levels in glial scars after injury have been reported (Properzi et al. 2008). Glypican-1 is expressed by reactive astrocytes after brain injury in concert with its binding partner Slit2, which is known to be a negative regulator of axonal growth (Hagino et al. 2003a, b; Lau and Margolis 2010). Testican-1, a heparan/chondroitin sulfate hybrid proteoglycan recently implicated in regulating WNT signaling (Kopke et al. 2020), is also expressed around necrotic areas in similar experimental paradigms (Iseki et al. 2011). Glypican

infusion into the brain infarct cavity after cerebral ischemia produced the same level of improvement in functional recovery after injury as chondroitinase treatment (Hill et al. 2012), indicating that HS can also exert a positive influence on axonal regeneration. All if these examples suggest therapies involving matrix manipulations are potential strategies to be considered.

### 3.3.2 Neurological Disorders

A number of genetic diseases result from mutations of GAG biosynthetic and degrading enzymes, and some exhibit neural phenotypes. In addition, GAG changes have been described in several neuro-pathological conditions such as the mucopolysaccharidoses (MPSs), Alzheimer's disease, schizophrenia, Parkinson's disease and epilepsy; how these GAG changes affect the disease pathology is currently an area of active investigation.

MPSs are genetic metabolic diseases caused by mutations in genes encoding GAG-degrading (hydrolyzing) lysosomal enzymes; thus, accumulation of GAGs is the biochemical hallmark of these disorders. Some MPSs have no neurological involvement, while others are characterized by intellectual disabilities and/or a variety of CNS deficits such as hydrocephalus, spasticity, seizures, cerebral infarction, ataxia, sleep apnea, optic atrophy, hearing impairment, and hyperactivity/ aggressive behavior. Although there are MPSs associated with failure to degrade CSPGs, the most severe neurological symptoms are linked with accumulation of HS in neural cells (Wegrzyn et al. 2010). The MPSs with associated neurological impact illustrate that normal brain function requires dynamic turnover of GAGs and appropriate homeostasis of proteoglycans. Extensive examination of this family of disorders can be found in the following reviews (Banecka-Majkutewicz et al. (2012), Fecarotta et al. (2020), Lehman et al. (2011), McBride and Flanigan (2021), Pierzynowska et al. (2020)).

As mentioned earlier there are multiple genetic diseases associated with defects in GAG biosynthetic enzymes (reviewed by Mizumoto et al. (2013b) and Mizumoto and Yamada (2021)) which manifest neurological pathologies. Two missense mutations in chondroitin  $\beta$ 1,4-N-acetylgalactosaminyltransferase-1 (*CSGALNACT1*) have been found in patients with different types of motor and sensory neuropathies (Saigoh et al. 2011); in both cases, recombinant enzymes bearing the mutated amino acids showed total loss of enzymatic activity. In two large cohorts of European ancestry, genetic association was found between autism and the heparan sulfate 3-O sulfotransferase 5 (*HS3ST5*) gene (Wang et al. 2009). Chondroitin synthase-1 (*CHSY1*) mutations were reported in patients with Temtamy pre-axial brachydactyly who exhibit skeletal defects, sensorineural hearing loss and varied degrees of learning disabilities (Izumikawa et al. 2013; Li et al. 2010; Tian et al. 2010). Evidence of disrupted Gdf5 (Li et al. 2010) and Notch (Tian et al. 2010) signaling was found in two animal models created with *CHSY1* knockdowns. In familial and sporadic cases of the connective tissue diseases, Ehlers-Danlos syndrome and Adducted thumb-clubfoot syndrome, patients harbor mutations in dermatan

4-O-sulfotransferase, and exhibit mild cranial ventricular enlargement and psychomotor retardation as pathological manifestations (Dundar et al. 2009; Malfait et al. 2010; Miyake et al. 2010).

A genome-wide association study carried out on subjects with recurrent early-onset major depressive disorder revealed a chromosome 18q22.1 site as having the strongest evidence for association (Shi et al. 2011). This site encompasses the promoter regulatory area for the DS epimerase-2 gene, which has also been linked to other bipolar disorders (Goossens et al. 2003). In light of the findings indicating that DS epimerase-2 is the predominant epimerase expressed in post-natal developing mouse brain (Akatsu et al. 2011), and that over-sulfated dermatan structures could actively promote neurite growth, these studies make phosphacan (Hikino et al. 2003), the major DS proteoglycan in PNNs (Deepa et al. 2006), an attractive target for future studies in the schizophrenia field (Pantazopoulos et al. 2015). Interestingly, over-expression in mice of PTPR $\zeta$ 1, of which phosphacan is an alternative splicing form, produces schizophrenia-like behavior (Takahashi et al. 2011), and removal of PNNs with chondroitinase produced deficits in dopamine system function and enhanced response to psychostimulants, as is observed in schizophrenia patients (Shah and Lodge 2013). A representative list of human neurological phenotypes associated with mutations in proteoglycan core proteins and GAGs biosynthetic enzymes are summarized in Table 5.1.

A number of neurodegenerative disorders exhibit up-regulation and abnormal GAG distribution, including Alzheimer's and Parkinson's diseases (van Horsen et al. 2003). The accumulation of A $\beta$  peptide in Alzheimer's disease is produced by sequential cleavage of  $\beta$ -amyloid precursor protein (APP) by the  $\beta$ -site APP cleaving enzyme, BACE1, and  $\gamma$ -secretase. A $\beta$  peptide in senile plaques and neurofibrillary tangles co-localizes with HS- and CSPGs (reviewed in Ariga et al. (2010), Cui et al. (2013)). Sulfated GAGs regulate aggregation and/or stabilization of A $\beta$  amyloid in a structure-dependent manner (Cui et al. 2012), and heparin treatment reduces A $\beta$  production by disrupting BACE-1 processing of APP. As well, treatment with low-molecular-weight heparin reduces plaques and A $\beta$  accumulation in a mouse model of Alzheimer's disease (Beckman et al. 2006; Klaver et al. 2010; Leveugle et al. 1997; Parkin et al. 2007). Thus, the design of GAG derivatives which act specifically to inhibit BACE-1 cleavage of APP and efficiently cross the blood-brain barrier is being actively pursued (Mycroft-West et al. 2020, 2021). In Alzheimer disease, formation of neurofibrillary tangles is also mediated by HSPGs via mechanisms that are increasingly being better understood (Lehri-Boufala et al. 2015). Similar to tauopathies, HS has been implicated in the uptake and seeding of  $\alpha$ -synuclein fibrils as well as propagation to neighboring cells (Ghosh et al. 2017; Holmes et al. 2013). Furthermore, in cellular models of Parkinson disease, sulfated GAGs have been shown to regulate cathepsin D activity (Lehri-Boufala et al. 2015). In other neurodegenerative diseases the contribution of GAGs to pathology are linked to the neuroinflammatory process as discussed below.

**Table 5.1** Representative table of human congenital neural disorders associated with mutations in proteoglycan biosynthetic enzymes and core proteins genes

Gene	Protein	Disorder	MIM	Clinical features
GPC4	Glypican 4	Keipert syndrome	300037 312870	Craniofacial and digit abnormalities, variable learning disabilities and sensory neuronal deafness.
AGRN	Agrin	Myasthenic syndrome, congenital 8	103320 615120	Pre- and post-synaptic defects at neuromuscular junctions, muscular weakness.
COL18A1	Collagen 18a1	Knobloch syndrome type1	120328 267750	Vitreoretinal degeneration, macular abnormality, severe myopia and occipital encephalocele.
XYLT2	Xylosyltransferase 2	Spondyloocular syndrome	605822 608125	Osteoporosis, sensorineural hearing loss, cataracts, and mild learning disability.
B4GALT7	Galactosyltransferase 1	Ehlers-Danlos syndrome spondylodysplastic type 1; Ehlers-Danlos syndrome progeroid type 1	130070 604327	Short stature, joint hypermobility, craniofacial dysmorphism, verbal delay, mild mental retardation.
B3GALT6	Galactosyltransferase 2	Ehlers-Danlos syndrome spondylodysplastic type 2; Ehlers-Danlos syndrome progeroid type2	615349 615291	Short stature, joint laxity and dislocation, facial dysmorphism bone fragility, spondyloepimetaphyseal dysplasia, verbal and intellectual disability.
CHSY1	Chondroitin sulfate synthase 1	Temtamy preaxial brachydactyly syndrome	605282 608183	Short stature, symmetric preaxial brachydactyly and hyperphalangism of digits, sensorineural hearing loss, delay motor and mental development and growth retardation.
DSEL	Dermatan sulfate epimerase 2	Bipolar disorder; depressive disorder	611125	Alternating episodes of depression and mania or hypomania
CHST14	Dermatan-4-sulfotransferase 1	Ehlers-Danlos syndrome Musculo-contractual type1; Ehlers-Danlos syndrome typeVIB; Adducted thumb-clubfoot syndrome	601776 608429	Craniofacial dysmorphism, multiple contractures, progressive joint laxity, contracture of thumbs and feet, psychomotor developmental delay

(continued)

**Table 5.1** (continued)

Gene	Protein	Disorder	MIM	Clinical features
EXT2	HS-polymerase-2	Seizures-scoliosis-macrocephaly syndrome	616682	Moderate intellectual disability, seizure disorder, hypotonia, scoliosis, macrocephaly, coarse facies, bilateral cryptorchidism in males
EXT2 plus multiple gene deletion		Potocki-Schaffer syndrome	601224	Multiple exostoses, bilateral parietal foramina, intellectual disability, hearing loss, craniofacial dysmorphism, cardiovascular, ocular, and genitourinary tract abnormalities.
EXTL3	Exostosin like 3	Immunoskeletal dysplasia with neurodevelopmental abnormalities; Neuro-immunoskeletal dysplasia	617425 605744	Immunodeficiency with complete absence of T cells, intellectual disability, short stature, limb shortening, dysmorphic facial features and skeletal abnormalities.
NDST1	N-deacetylase/N--sulfotransferase	Intellectual disability autosomal recessive 46	616116 600853	Impairment in motor and cognitive function, muscular hypotonia, epilepsy, postnatal growth deficiency, seizures, cranial nerve dysfunction, ataxia developmental delay, distinctive facial features.
HS2ST1	Heparan sulfate 2-O-sulfotransferase 1	Neurofacioskeletal syndrome with or without renal agenesis	604844 619194	Facial dysmorphism with coarse face, developmental delay and/or intellectual disability, corpus callosum agenesis or hypoplasia, Brachydactyly of hand and feet and uni- or bilateral renal agenesis
UGDH	UDP- Glc dehydrogenase	Developmental and epileptic encephalopathy 84	603370 618792	Intractable epileptic seizures and developmental delay
SLC35A3	UDP- GlcNAc transporter	Arthrogryposis, intellectual disability and seizures	605632 615553	Arthrogryposis, knee and hip dislocations, anomalous vertebrae, hypotonia, autism, epilepsy, seizures and mild to moderate intellectual disability.

### 3.3.3 Inflammation

Following brain injury, cascades of inflammatory events are triggered including, activation of local (microglia and astrocyte) and infiltrated (leukocyte) cells, leading to production of cytokines and proteases that cause profound changes in the ECM (Stephenson and Yong 2018). As previously mentioned, all the major cell types in the brain are able to up-regulate production of CSPGs not only after traumatic injury but also during the evolution of neurodegenerative disease like multiple sclerosis, Parkinson and Alzheimer disease (Heindryckx and Li 2018). Besides the initial benefits of helping to limit the damage areas, upregulation of these proteoglycans may contribute to long-term detrimental roles not only by restricting axonal growth (see above) but also in their ability to enhance inflammatory cascades. In particular, CSPGs signal through TLR2/6 to induce TNF $\alpha$  secretion or TLR4 and its downstream pathway MyD88/NF $\kappa$ B (Kim et al. 2009; Zhang et al. 2015). GAGs, particularly highly sulfated CS chains, bind pro-inflammatory and regulatory cytokines as well as CXC and CC chemokines (Garnier et al. 2003; Hirose et al. 2001; Hoogewerf et al. 1997; Kuschert et al. 1999; Petersen et al. 1998), thus, allowing them to accumulate at the injury site and probably protect cytokines and chemokines from degradation (Stephenson and Yong 2018). As well, CS chains interact with CD44 and activate resident microglia to increase IGF-1 and metalloprotease (MMP) expression (Rolls et al. 2008). CSPGs form complexes with pro-MMPs and enhance their activation (Iida et al. 2007; Malla et al. 2013; Ra et al. 2009; Winberg et al. 2003) and CS chains enhance the activity of the ADAMTS family of proteases by binding TIMP3, a known physiological MMP inhibitor (Troeberg et al. 2014). Activation of MMP pathways is being increasingly implicated in the chronic neuroinflammatory process developed during the progression of neurodegeneration (Cabral-Pacheco et al. 2020; Domowicz et al. 2019, 2021; Mohamedi et al. 2020). Ultimately, CSPGs can regulate their own degradation to either facilitate repair (Rolls et al. 2008) or prolong inflammation, since the degradative fragments can act as damage-associated molecular patterns (DAMPs) (Adair-Kirk and Senior 2008; Gaudet and Popovich 2014). A novel intracellular function for sulfated GAGs has been described in the activation of the cGAMP sensor STING, a pathway well characterized as part of the innate immune response (Zhang et al. 2020). Fang et al. (2021) demonstrated that sulfated GAGs are necessary and sufficient to drive the polymerization and activation of STING in the Golgi apparatus, with heparin and HS displaying the highest activity. This pathway has been shown to be active in microglia (Jiang et al. 2021; Jin et al. 2021), and thus highlights a new area of investigation for regulation of neuroinflammation by GAGs.

### 3.3.4 Tumorigenesis

Proteoglycans have been implicated in several aspects of tumor biology, including migration, adhesion, cell proliferation, inflammation and angiogenesis (Fuster and Esko 2005; Iozzo and Sanderson 2011; Iozzo and Schaefer 2010, 2015; Phillips

2012; Theocharis et al. 2010). In brain cancers, studies suggest that proteoglycans regulate oncogenic pathways in tumor cells and promote critical tumor micro-environment interactions (Cecchi et al. 2012; Mellai et al. 2020; Sugiarto et al. 2011; Wade et al. 2013). However, the proteoglycans involved depend upon the cell type that becomes malignant and the molecular heterogeneity of the tumor (Wade et al. 2013). Glioblastoma (GB) is the most malignant and common primary tumor in adult brain (Louis et al. 2021). Abnormal activation of receptor tyrosine kinase (RTK) signaling pathways and invasive growth into adjacent non-neoplastic tissue are common characteristics of almost all GBs (Snuderl et al. 2011). Unfortunately, despite the importance of RTK signaling in GB, there has been little clinical success in targeting RTKs, which can be affected by alterations in receptor expression or by ligand availability (Georgescu 2021; Qin et al. 2021; Stommel et al. 2007). Alterations in proteoglycan and HA biosynthesis and expression of modifying enzymes have been implicated in increased tumorigenesis, angiogenesis and invasiveness in a variety of cancers, and brain tumors are no exception (Mellai et al. 2020; Park et al. 2008; Wade et al. 2013). For example, NG2 (CSPG4)-expressing oligodendrocyte precursors, have been implicated in the development of gliomas upon Ras activation and p53 depletion (Kondo and Raff 2000; Shoshan et al. 1999; Sugiarto et al. 2011). NG2 also has been found to be overexpressed in oligodendrogliomas and astrocytomas (Bouvier et al. 2003; Chekenya and Pilkington 2002; Ligon et al. 2004; Shoshan et al. 1999) and is involved in the EGFR-PI3K-AKT pathway by enhancing the activation of the EGFR tyrosine kinase domain and the proliferative capacity of GB cells (Al-Mayhany et al. 2019). Furthermore, NG2 levels correlate with malignancy grade and increased resistance to radiotherapy (Svendsen et al. 2011; Tsidulko et al. 2017). Glypican 3 (GPC3) has been found to be overexpressed in gliomas where it drives tumorigenesis and hyperexcitability (Yu et al. 2020). Versican forms are upregulated in glioma vasculature and promote local invasion via the glioma-associated microglia Toll-receptor 2 pathway (Hu et al. 2015; Miquel-Serra et al. 2006). Lastly, decorin expression levels have been negatively correlated with survival rate of GB multiforme patients, while overall CS content was positively associated with the proliferative activity of tumors (Tsidulko et al. 2020).

Another major type of brain tumor, neuroblastoma, which originates from the sympathoadrenal lineage of the neural crest and is the most common tumor in early development (Louis and Shohet 2015) is shown to be regulated by HSPGs. Interestingly, Glypican 2 is upregulated in neuroblastoma tissue and its high expression correlates with poor prognosis (Li et al. 2017b); soluble forms of HS are able to suppress proliferation in neuroblastoma, probably through the FGF2 signaling pathway (Knelson et al. 2013, 2014). Thus, HS mimetics have become attractive GB therapeutic candidates for various tumor subtypes (Dredge et al. 2011; Hossain et al. 2010; Johnstone et al. 2010; Liu et al. 2009; Zhou et al. 2011). With respect to CSPGs, neurocan has been found to be highly expressed in neuroblastoma which induces an undifferentiated state that stimulates cell division, thereby promoting tumor malignancy by providing a growth advantage (Su et al. 2017). Taken together these findings underscore the importance of proteoglycans, not only as biomarkers

for tumorigenesis, but also as therapeutic targets (Yan and Wang 2020). On the other hand, recent work highlights that changes in brain proteoglycan composition, induced by chemotherapy and radiation treatments, may generate microenvironments supportive of relapsing development (Politko et al. 2021; Tsidulko et al. 2018, 2021; Vitale et al. 2019). Thus, the potential adverse effects of ECM-targeted therapy in brain must be considered in order to avoid tumor relapse.

## 4 Concluding Remarks

This brief synopsis of the structure-function relationship of GAGs and GAG-containing proteoglycans highlights the importance of these cell-surface and extracellular constituents in numerous biologically significant interactions critical to the basic developmental processes of proliferation, differentiation, patterning, axonal pathfinding, synapsis formation etc. underlying the formation of a functional nervous system. GAGs are also implicated in regeneration, injury response and disease pathology of the brain. These functional revelations have only been able to be made due to the elucidation of the complex chemistry and structure of these multifaceted molecules. With the advent of the -omics era and the development of new methodologies such as human organoid cultures, glyco-proteomics, glyco-sequencing and automated glycan synthesis, additional progress should be forthcoming on deciphering GAG functions, which may then lead to new therapeutics to modulate GAG interactions vital to both development and disease.

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# Chapter 6

## Structural Analysis of Oligosaccharides and Glycoconjugates Using NMR



Yoshiki Yamaguchi, Takumi Yamaguchi, and Koichi Kato

**Abstract** Carbohydrate chains play critical roles in cellular recognition and subsequent signal transduction in the nervous system. Furthermore, gangliosides are targets for various amyloidogenic proteins associated with neurodegenerative disorders. To better understand the molecular mechanisms underlying these biological phenomena, atomic views are essential to delineate dynamic biomolecular interactions. Nuclear magnetic resonance (NMR) spectroscopy provides powerful tools for studying structures, dynamics, and interactions of biomolecules at the atomic level. This chapter describes the basics of solution NMR techniques and their applications to the analysis of 3D structures and interactions of glycoconjugates in the nervous system.

**Keywords** Nuclear magnetic resonance · Structure · Dynamics · Interaction · Oligosaccharide · Glycolipid

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## Abbreviations

A $\beta$	Amyloid $\beta$
DIS	Deuterium-induced isotope shift
FID	Free induction decay
HSQC	Heteronuclear single-quantum coherence
MD	Molecular dynamics
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NOESY	NOE spectroscopy
PCS	Pseudocontact shift
PRE	Paramagnetic relaxation enhancement
REMD	Replica exchange MD
RF	Radio frequency
STD	Saturation transfer difference
TRNOE	Transferred NOE
TROSY	Transverse relaxation optimized spectroscopy

## 1 Introduction

In the nervous system, cellular processes, including division, development, migration, and morphological changes, are dynamically controlled through molecular recognition events on cell surfaces. In these bio-organization processes, carbohydrate chains that modify proteins and lipids play critical roles in recognition and adhesion during cell–cell communication. To better understand the molecular mechanisms underlying these neurophysiological functions, atomic views are desirable to describe dynamic interactions of biomolecules such as glycoconjugates. In addition, recent evidence has demonstrated that gangliosides on neuronal cell membranes are targets for various amyloidogenic proteins that are associated with neurodegenerative disorders, e.g.,  $\alpha$ -synuclein in Parkinson's disease, amyloid  $\beta$  (A $\beta$ ) in Alzheimer's disease, and prion proteins in Creutzfeldt–Jakob disease (Taylor and Hooper 2006; Ariga et al. 2008; Fantini and Yahi 2010; Matsuzaki et al. 2010, 2018; Magistretti et al. 2019; Alessenko and Albi 2020; Yahi et al. 2021). Atomic descriptions of these pathological processes provide a basis for designing novel therapeutic molecules.

Nuclear magnetic resonance (NMR) spectroscopy is one of the most widely used techniques for atomic visualizations of biomolecules. The unique feature of this method is its ability to determine atomic coordinates of biomacromolecules in solution and those embedded in membranes and to characterize their dynamic motion at the atomic level. NMR spectroscopy also serves as a powerful tool for detailed analyses of functional intermolecular interactions and is now routinely used by a wide range of researchers, including glycobiologists. This chapter provides the basics of biomolecular NMR spectroscopy needed for applying this useful technique to address neuroglycobiological issues.

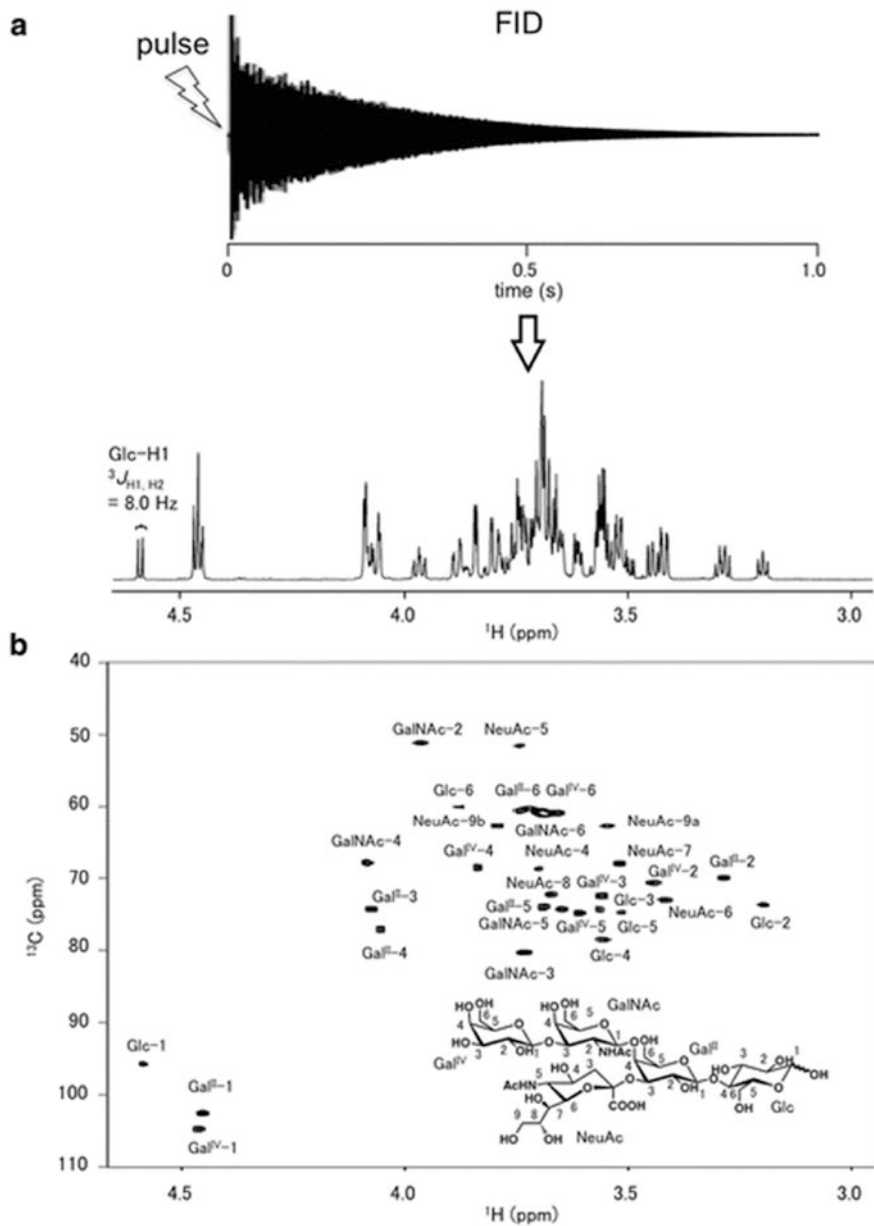
## 2 Basic NMR Phenomena

NMR is a physical phenomenon that reflects quantum mechanical magnetic properties of atomic nuclei in orientation with a strong magnetic field (Abragam 1961; Levitt 2008). Although all isotopes containing odd numbers of protons and/or neutrons have intrinsic magnetic moments and are therefore NMR active, the most commonly studied nuclei are  $^1\text{H}$  and  $^{13}\text{C}$ , which have spin quantum numbers of  $1/2$  and therefore exhibit high-resolution NMR spectra. These nuclei have two spin states with energy differences that depend on intrinsic magnetic moments and a given magnetic field. In comparison with  $^1\text{H}$ -NMR,  $^{13}\text{C}$ -NMR measurements suffer from low sensitivity because of the lower natural abundance of this isotope (1.1%) and its smaller magnetic moment. Hence, NMR samples are often enriched with  $^{13}\text{C}$  using metabolic labeling and chemical synthesis (Yamaguchi and Kato 2007b; Ohki and Kainosho 2008; Kato et al. 2010; Zhang et al. 2013; Yamaguchi et al. 2017; Kato et al. 2018a, b; Ikeya et al. 2018). Other nuclei with spin quantum number of  $1/2$ , such as  $^{15}\text{N}$ ,  $^{19}\text{F}$ ,  $^{31}\text{P}$ , and  $^{77}\text{Se}$ , can be used for analysis of structure, dynamics and interaction of biomolecules including oligosaccharides and glycoconjugates (Gorenstein 1994; Pomin 2013; Uzawa et al. 2018; Suzuki et al. 2019; Martínez et al. 2020).

Alignment of the nuclear magnetic moment in the magnetic field is perturbed by an electromagnetic field with a resonant radio frequency (RF) pulse that corresponds with the energy difference. After applying the RF pulse, nonequilibrium magnetization, as the sum of all the individual nuclear magnetic moments in the sample, precesses around the magnetic field with the resonant frequency, thereby producing corresponding voltage oscillations in the detection coil. The duration of the oscillating signal is limited and decays exponentially. This time-domain NMR signal, known as free induction decay (FID), is Fourier transformed to produce a frequency-domain spectrum. Figure 6.1a shows FID and Fourier-transformed  $^1\text{H}$ -NMR spectrum of the pentasaccharide of ganglioside GM1 dissolved in  $\text{D}_2\text{O}$ . The real part of the complex spectrum is typically displayed as the NMR spectrum, showing the absorptive Lorentzian line shape.

## 3 Chemical Shifts as Structural Probes

Individual protons in molecules are generally surrounded by differing electronic environments, which shield each proton against the magnetic field with various modes. As a result, resonant frequencies differ among protons depending on chemical environments, even under the same magnetic field. Hence, a  $^1\text{H}$ -NMR spectrum exhibits a number of peaks at different positions, and these chemical shifts are each assigned to distinct protons in the molecule. The same is true for the other NMR-active nuclei.



**Fig. 6.1** NMR spectral examples of the GM1 pentasaccharide. (a) A free induction decay (FID) and Fourier-transformed  $^1\text{H}$ -NMR spectrum and (b)  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectrum of the GM1 pentasaccharide. In (a), peak splitting (8 Hz) is observed for Glc-H1 signal at 4.6 ppm (see text)

In practice, chemical shift ( $\delta$ ) is measured in parts per million (ppm) relative to a reference resonance signal from a standard compound:

$$\delta = (\nu - \nu_{\text{ref}}) / \nu_{\text{ref}} \times 10^6$$

In the equation above,  $\nu$  and  $\nu_{\text{ref}}$  represent the resonance frequencies of sample and reference signals, respectively.

Chemical shifts offer unique conformational probes for biomolecules. For example, secondary structures of proteins can be determined by inspecting chemical shifts of backbone  $^1\text{H}$  and  $^{13}\text{C}$  atoms (Wishart and Sykes 1994; Cornilescu et al. 1999). In carbohydrate NMR spectroscopy,  $^1\text{H}$ -NMR chemical shifts have traditionally been used as *structural reporters* for identifying chemical structures of oligosaccharides (Vliegthart 1980, 2021).  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of carbohydrates can also be used to examine the effect of bisecting GlcNAc modification on conformation of *N*-glycans (Hanashima et al. 2018).

In carbohydrate NMR spectra, signals are typically in very close proximity of one another (Fig. 6.1a). To resolve chemical shift degeneracy of glycans, applications of higher magnetic fields are certainly advantageous (Kato et al. 2008). Signal overlapping in multiantenna glycans is also resolved by use of paramagnetic probes (Canales et al. 2013; Usachev et al. 2017).

## 4 Through “BOND” and Through “SPACE” Interactions

Chemical shift values are influenced by surrounding chemical environments. In contrast, scalar coupling (or *J*-coupling) splits NMR peaks and reflects indirect interactions between NMR-active nuclei that are mediated by the electrons participating in chemical bonds between nuclei. Scalar coupling constants (*J*) are defined by magnitudes of peak splitting and are independent of molecular orientations with respect to the magnetic field but depend on molecular geometry. The vicinal scalar coupling constant  $^3J$ , which pertains to atoms separated by three covalent bonds, is typically employed for conformational analyses of biomolecules. This constant is related to the dihedral angle  $\theta$  as described by the Karplus equation as follows:

$$^3J(\theta) = A \cos 2\theta + B \cos \theta + C$$

In this equation, A, B, and C are coupling coefficients, and  $\theta$  is the dihedral angle. This relationship has been applied to determinations of sugar-ring stereochemistry and characterization of glycosidic linkage conformations of oligosaccharides (Hadad et al. 2017; Hanashima et al. 2018; Hamagami et al. 2020). In Fig. 6.1a, the anomeric proton (H1) of the  $\beta$ -glucose residue at the reducing terminus of the GM1



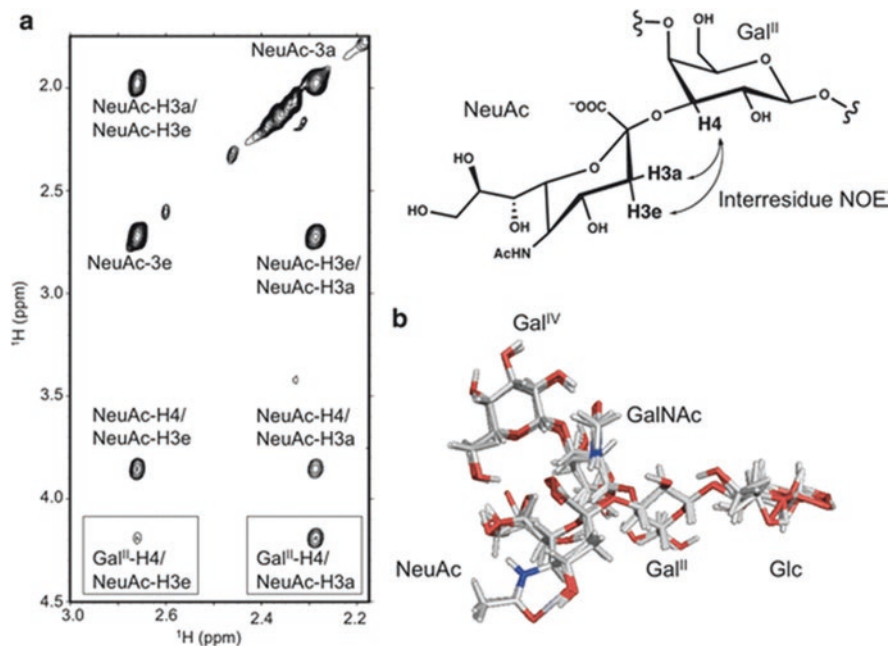
pentasaccharide exhibits peak splittings that originate from vicinal scalar coupling between H1 and H2 protons ( ${}^3J_{\text{H1, H2}} = 8.0$  Hz).

In many two- and multidimensional NMR experiments, migration of magnetization among correlated nuclei is a crucial process. Magnetization transfer that proceeds from one spin to another is mostly achieved through scalar coupling. Due to increased numbers and line widths of resonances, 2D homonuclear  ${}^1\text{H}$ -NMR methods are ineffective for biomolecules with molecular masses  $>10$  kDa. In addition, larger line widths result in decreased sensitivity for  ${}^1\text{H}$  correlation experiments that rely on small ( $<10$  Hz) homonuclear  ${}^3J$  scalar coupling for coherence transfer. The efficiency of magnetization transfer in heteronuclear NMR spectroscopy is improved by employing relatively large one-bond scalar coupling interactions of  ${}^1\text{H}$  with  ${}^{15}\text{N}$  or  ${}^{13}\text{C}$ . To measure  ${}^1\text{H}$ - ${}^{13}\text{C}$  heteronuclear single-quantum coherence (HSQC) spectra (Bodenhausen and Ruben 1980) (Fig. 6.1b), one-bond scalar coupling  ${}^1J_{\text{C-H}}$  (approximately 145 Hz) is used to transfer magnetization from  ${}^1\text{H}$  to  ${}^{13}\text{C}$  and vice versa.

In addition to the through-bond scalar coupling interaction, through-space dipolar interactions are utilized to transfer magnetization between spins. Due to the nuclear Overhauser effect (NOE), perturbation of populations of stationary states within a spin system causes time-dependent changes in the intensity of dipolar-coupled resonance signals, which follows polarization transfers between spin populations via dipolar cross-relaxation. The efficiency of the NOE depends on the distance between interacting spins. Thus, through-space rather than through-bond magnetization transfer generates cross-peaks according to the NOE in NMR spectra.  ${}^1\text{H}$ - ${}^1\text{H}$  NOE spectroscopy (NOESY) provides a measure of interproton distances of up to 5 Å, enabling identification of atomic coordinates of biomacromolecules such as proteins (Wüthrich 1986). The intensity of the NOE ( $I$ ) is related to the distance ( $r$ ) between proton pairs, as in

$$I = f(\tau_c) \times r^{-6},$$

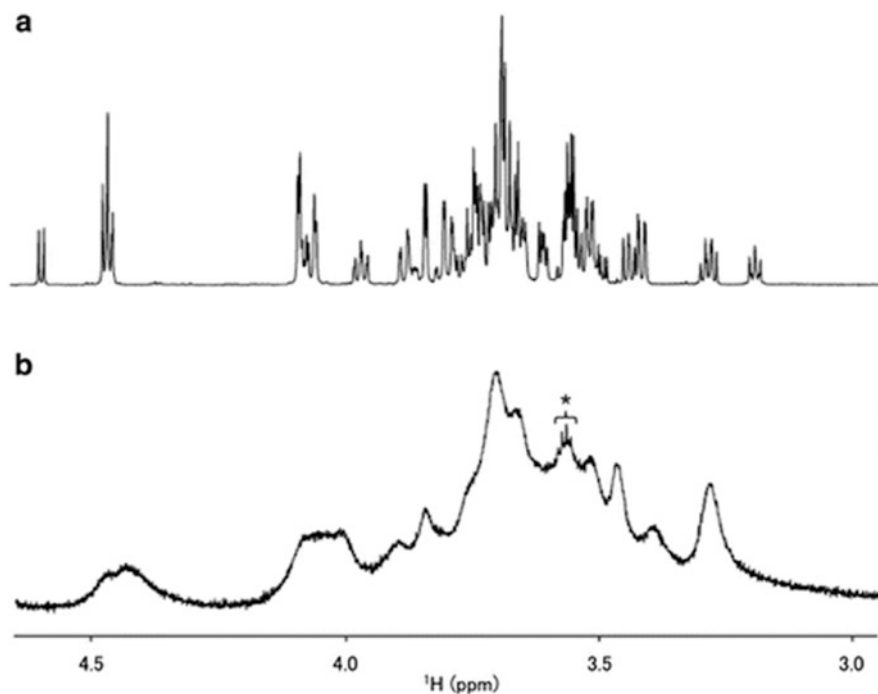
where  $f(\tau_c)$  is a function of the rotational correlation time  $\tau_c$  of the molecule. Figure 6.2a shows a part of the  ${}^1\text{H}$ - ${}^1\text{H}$  NOESY spectrum of micellar lyso-GM1. In addition to intraresidue NOEs, interresidue NOEs are observed between spatially proximal pairs of protons, as exemplified by the NeuAc H3 (axial)-Gal<sup>II</sup> H4 proton pair. Interresidual NOE observations are used to identify glycosidic linkages of unknown compounds and estimate dihedral angles along glycosidic bonds (Homans et al. 1987; Cumming and Carver 1987; Voisin et al. 2005). Combined NMR analyses based on through-bond and through-space interactions enable sequence-specific resonance assignments of oligosaccharide NMR signals and subsequently provide 3D structural information (Acquotti et al. 1990; Brocca et al. 2000; Prestegard et al. 1982; Yagi-Utsumi et al. 2010; Yu et al. 1986; Yagi-Utsumi and Kato 2015) (Fig. 6.2b).



**Fig. 6.2** Atomic distance information provided through NOEs. (a) A part of  $^1\text{H}$ – $^1\text{H}$  NOESY spectrum of the micellar lysoGM1 and (b) the lowest-penalty 3D models of the GM1 pentasaccharide calculated from interresidue NOE data. (Reprinted from (Yagi-Utsumi et al. 2010) with permission from Elsevier)

## 5 Relaxation and Molecular Motion

In principle, NMR experiments begin from the equilibrium state, in which all populations of energy levels of the system are described by the Boltzmann distribution. Although multiple pulses and multidimensional NMR techniques permit generation of nonequilibrium states, the equilibrium state is eventually restored. As in other spectroscopic techniques, recovery from the nonequilibrium state to equilibrium is called relaxation. Relaxation in NMR involves recovery of the nuclear spin magnetization component with an orientation that is parallel to the static magnetic field (called spin–lattice relaxation) and/or loss of phase coherence of individual nuclear spins (called spin–spin relaxation). Time constants of these two processes are termed  $T_1$  and  $T_2$ , respectively. In solution NMR spectroscopy, relaxation is governed by the dynamic properties of molecules, including overall molecular tumbling and internal motions. For example,  $T_2$  determines natural line widths of resonances detected during the acquisition period. In the comparison of  $^1\text{H}$ -NMR spectra for micellar ganglioside GM1 and the free oligosaccharide derived from it shown in Fig. 6.3, molecules with slower tumbling rates exhibit broader signal line widths, which are inversely proportional to  $T_2$ . Thus,  $T_2$  is a critical factor for detecting NMR peaks with higher signal-to-noise ratios. A sophisticated pulse sequence



**Fig. 6.3** One-dimensional  $^1\text{H}$ -NMR spectra of (a) liberated GM1 pentasaccharide (1.0 kDa) and (b) GM1 micelle with an approximate molecular mass of 140 kDa. Peaks originating from a low-molecular-weight contaminant are indicated by an asterisk

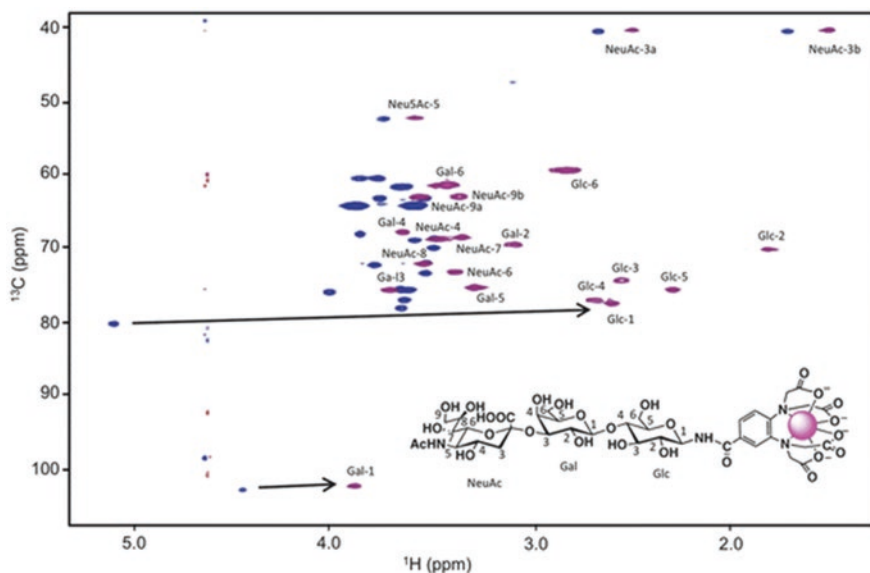
(transverse relaxation optimized spectroscopy (TROSY)) has been developed for NMR analyses of larger macromolecular complexes, in combination with optimal (generally higher) magnetic fields and sample deuteration for suppressing the magnetic dipole–dipole interactions caused by protons that are strong sources of relaxation (Pervushin et al. 1997).

## 6 Paramagnetic Effects as Sources of Long-Distance Information

Unpaired electrons can dramatically perturb NMR spectra, due to stronger dipole–dipole interactions with nuclei that have much larger magnetic moments than atomic nuclei. For example, through-space interactions between a paramagnetic center and neighboring protons cause increased relaxation rates with  $r^{-6}$  dependence on paramagnetic spin–proton distances (Solomon 1955). Such paramagnetic relaxation enhancement (PRE) offers long-distance information. Paramagnetic probes such as nitroxide radicals are used to characterize oligosaccharide conformations and

lectin–carbohydrate interactions (Johnson et al. 1999; Jain et al. 2001; Yamaguchi et al. 2013a; Kato and Yamaguchi 2015).

Chemical shifts can also be modulated in the presence of paramagnetic lanthanide ions (such as  $\text{Er}^{3+}$  and  $\text{Tm}^{3+}$ ). This perturbation is known as pseudocontact shift (PCS), which occurs when the magnetic susceptibility of metal ion is anisotropic. PCS is exploited to determine geometrical arrangements of individual nuclei in relation to the position of the metal ion with  $r^{-3}$  dependence (McConnell and Robertson 1958; Kurland and McGarvey 1970). Therefore, the atomic long-distance information for determining biomacromolecular conformations can be obtained by observing PCS following introduction of lanthanide probes into specific target molecule sites. Several NMR studies have used PCS to restrain protein and oligosaccharide conformations (Bertini et al. 2005; Otting 2010; Zhang et al. 2013). PCS of the GM3 trisaccharide (NeuAc $\alpha$ 2-3Gal $\beta$ 1-4Glc), the GM2 tetrasaccharide (GalNAc $\beta$ 1-4(NeuAc $\alpha$ 2-3) Gal $\beta$ 1-4Glc), and the GM1 pentasaccharide (Gal $\beta$ 1-3GalNAc $\beta$ 1-4(NeuAc $\alpha$ 2-3) Gal $\beta$ 1-4Glc) were analyzed by attaching a lanthanide-chelating tag to reducing ends (Yamamoto et al. 2012; Zhang et al. 2012, 2015; Yagi-Utsumi and Kato 2015). Using two-dimensional  $^1\text{H}$ – $^{13}\text{C}$  HSQC spectra, PCS values were measured as differences between  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts and those of diamagnetic compounds (Fig. 6.4). These analyses provide conformational information related to oligosaccharides. However, in general, oligosaccharide conformations dynamically fluctuate in solution. Therefore, observed PCS should be interpreted as averages of the dynamic conformational ensemble (*vide infra*).



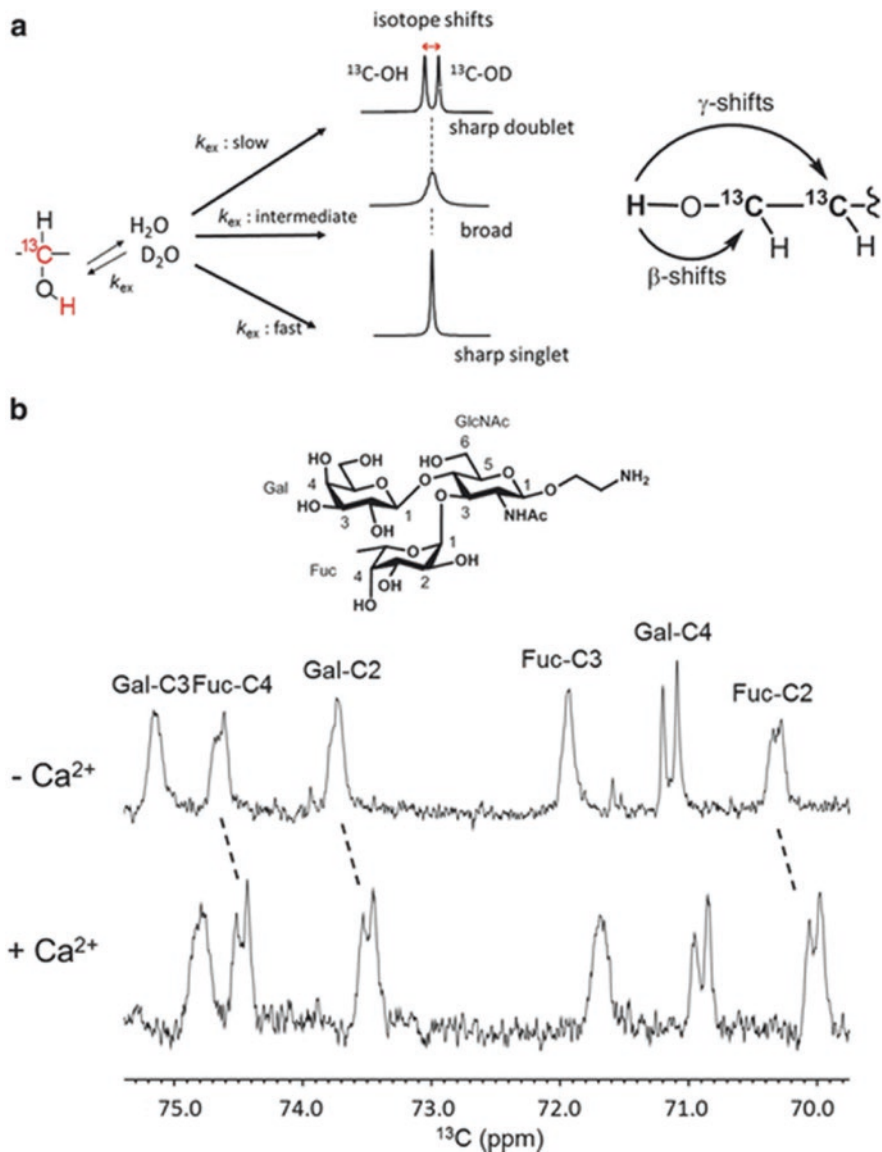
**Fig. 6.4** Comparison of  $^1\text{H}$ – $^{13}\text{C}$  HSQC spectra of the GM3 trisaccharide with lanthanide-chelating tag complexed with paramagnetic  $\text{Tm}^{3+}$  (red) and diamagnetic  $\text{La}^{3+}$  (blue). (Reprinted from (Yamamoto et al. 2012) with permission from The Royal Society of Chemistry)

## 7 Chemical Exchange: Dynamic Aspects in NMR

NMR spectroscopy provides unique information on the *exchange* of nuclei between different environments due to conformational transitions and/or intermolecular interactions (Lian and Roberts 1993). Suppose that a given nucleus exchanges with rate constant  $k$  between two sites with resonance frequencies that differ by  $\Delta\omega$  (Fig. 6.5a). If  $k$  is slow in terms of the frequency of chemical shift differences ( $k \ll \Delta\omega$ ), then two distinct signals corresponding to the nuclei of two sites are observed. In contrast, if the exchange rate is fast ( $k \gg \Delta\omega$ ), then a single resonance is observed, which reflects the population-weighted average chemical shift. Because conformational transitions of free oligosaccharides occur in a nanosecond time range (Yamamoto et al. 2012), the observed PCS shown in Fig. 6.4 are analyzed in the fast exchange regime. If the exchange rate is of the order of the chemical shift difference between two sites, the lines become considerably broad and coalesce at  $k \sim \Delta\omega$ . This is known as the intermediate exchange regime or coalescence, where  $k$  and  $\Delta\omega$  as well as populations of individual states can be estimated using sophisticated relaxation dispersion experiments (Loria et al. 1999; Mittermaier and Kay 2006; Sugase et al. 2007).

Biomolecules possess exchangeable protons, such as those in hydroxyl and amide groups. Proton exchange rates of these groups provide useful probes for characterizing conformational fluctuations and interactions of biomolecules such as oligosaccharides, because slower exchange rates indicate protecting factors such as hydrogen-bonding interactions at corresponding sites (Englander and Mayne 1992; Englander et al. 2007). Exchanges between protein amide protons and water occur in the slow exchange regime. The rates of these processes can be measured in several ways depending on the rate of exchange. When the exchange rate is comparable to or faster than the spin–lattice relaxation rate (typically,  $k_{\text{ex}} > 0.1 \text{ s}^{-1}$ ), the rate constant is most easily determined in saturation transfer experiments (Forsén and Hoffman 1963, 1964). In these experiments, saturation is performed by selectively irradiating the frequency of the water signal, and the exchange of amide protons with saturated water protons is quantitatively assessed according to NMR signal intensities (Spera et al. 1991). For slower rates ( $k_{\text{ex}} < 0.01 \text{ s}^{-1}$ ), exchanges are measured by observing progressive changes of NMR spectra, which exhibit time-dependent attenuation of peak intensities after rapidly transferring proteins from  $\text{H}_2\text{O}$  into  $\text{D}_2\text{O}$  (Jeng et al. 1990; Paterson et al. 1990).

Deuterium exchanges of rapidly exchanging protons, such as hydroxyl protons of oligosaccharides, cannot be quantitatively characterized using conventional  $\text{H}_2\text{O}/\text{D}_2\text{O}$  exchange monitoring. To overcome this difficulty in aqueous solution, NMR strategy has been developed using deuterium-induced isotope shifts (DIS; Hanashima et al. 2011; Battistel et al. 2017). DIS was utilized for detailed characterization of  $\text{Ca}^{2+}$ -dependent homophilic interactions of Lewis X trisaccharides ( $\text{Fuc}\alpha 1\text{-3}(\text{Gal}\beta 1\text{-4})\text{GlcNAc}$ ), which have been implicated as having a role in mediating compaction of the mouse embryo at the morula stage (Fenderson et al. 1984; Eggens et al. 1989).  $^{13}\text{C}$ -NMR of this trisaccharide was measured in a 50:50 mixture



**Fig. 6.5**  $^{13}\text{C}$ -NMR isotope shifts for analyzing proton exchange rates of sugar hydroxyl groups. (a) Schematic of NMR experiment using isotope shifts. All hydroxyl protons on glycans rapidly exchange to deuterons in  $\text{H}_2\text{O}/\text{D}_2\text{O} = 50:50$  solution. The  $^{13}\text{C}$ -NMR signal of the geminal carbons at the H/D exchanging hydroxyl protons shows characteristic signal shape, which is dependent on the H/D exchanging rate. Especially under significantly slow exchange conditions, a set of sharp doublet is provided due to isotope shifts ( $\beta$ -shifts). The chemical shifts difference is  $\sim 0.15$  ppm.  $k_{\text{ex}}$ ; exchanging rates of protons. (b) Parts of  $^{13}\text{C}$ -NMR spectra of 40 mM Lewis X trisaccharide without 1.0 M  $\text{CaCl}_2$  (upper) and with 1.0 M  $\text{CaCl}_2$  (lower) at  $5^\circ\text{C}$ . (Adapted with modifications from (Hanashima et al. 2011) with permission from The Royal Society of Chemistry)

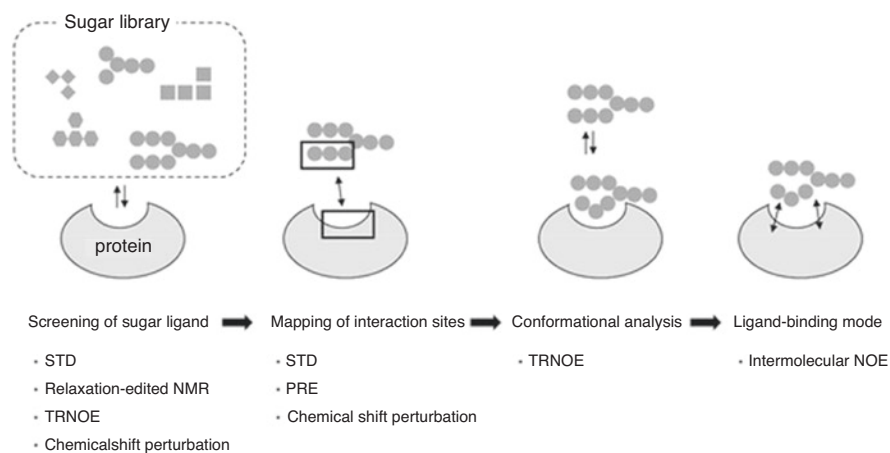
of H<sub>2</sub>O/D<sub>2</sub>O (Fig. 6.5). DISs were observed to be dependent on Ca<sup>2+</sup> concentrations. Sample conditions of 1.0 M Ca<sup>2+</sup> provided doublets originating from Fuc-C2, Fuc-C4, and Gal-C2. In contrast, under Ca<sup>2+</sup>-free conditions, no doublets originated from those carbon atoms, indicating that proton exchange became significantly slower upon Ca<sup>2+</sup> coordination. DIS was also used in the characterization of chain length-dependent secondary structure formation of short β-glucan chains (Hanashima et al. 2014).

## 8 NMR Tools for Intermolecular Interaction Analysis

### 8.1 Oligosaccharide–Protein Interactions

Analysis of sugar–protein interactions is an important step for elucidating structure–function relationships of glycans and designing drugs that target carbohydrate recognition systems (Kamiya et al. 2011). NMR spectroscopy provides invaluable tools for this purpose because it allows detection of weak sugar–protein interactions ( $K_d \sim$  mM), identification of glycotopes that are recognized by proteins, characterization of protein-bound oligosaccharide conformations, and determination of the modes of atomic interaction between oligosaccharides and proteins in solution (Fig. 6.6) (Yamaguchi and Kato 2007a; Ardá and Jiménez-Barbero 2018; Gimeno et al. 2020).

Saturation transfer difference (STD) NMR is now frequently used to analyze protein–ligand interactions (Mayer and Meyer 1999, 2001). One of the great advantages of this method is that it does not require expensive time-consuming stable-isotope labeling of either proteins or ligands. STD-NMR requires the alternate



**Fig. 6.6** A strategy for analyzing carbohydrate–protein interactions. (Adapted with modifications from (Yamaguchi and Kato 2007a) with permission from Yodoshia Co., Ltd.)

collection of an on-resonance spectrum for saturation of protein protons and an off-resonance spectrum, for reference. Upon irradiation of the protein with a saturation pulse, the saturation effect immediately spreads from irradiated points over the entire protein–ligand complex (Fig. 6.7). If ligand exchange between free and bound states is fast in terms of the time scale of spin–lattice relaxation, the saturation effect is readily transferred to the free fraction of the ligand, particularly to ligand protons located at the interaction interface. An example of STD-NMR is shown in Fig. 6.7, in which the interaction between trisialic acid and a specific monoclonal antibody is analyzed (Hanashima et al. 2013). The nonreducing terminus residue (c) and the central residue (b) showed higher relative values of STD amplification factor compared with the reducing terminal residue (a). The protons at C4, C6, and C7 on residues b and c also had higher values than those at C3, C5, and C8, indicating that this antibody preferentially binds to the  $\alpha$ -face of pyranose rings at residues b and c.

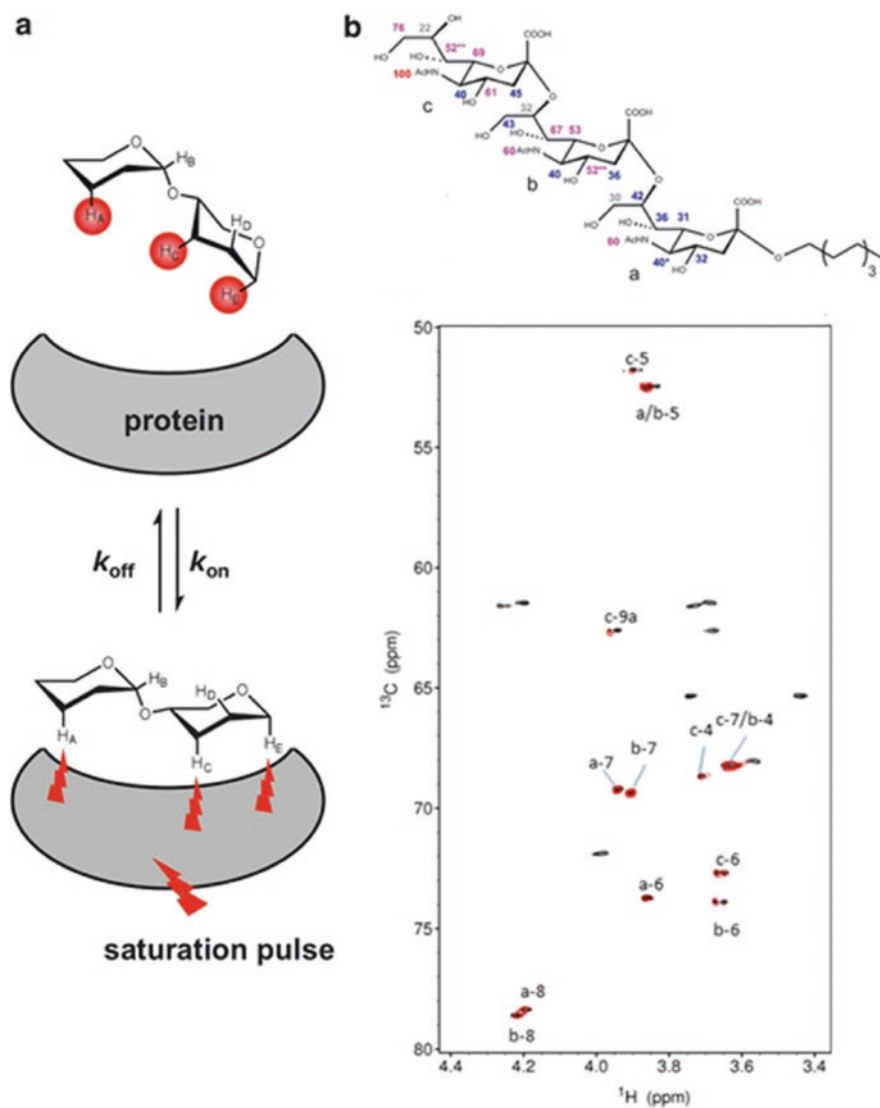
Atomic contacts can be identified by observing intermolecular NOE correlations between proteins and cognate ligands. Conformations of protein-bound ligands can also be determined by analyzing intramolecular NOE connectivities within ligands. The sign of the NOE signal depends on  $\tau_c$  of the molecular tumbling motion and becomes opposite when a fast-tumbling low-molecular-weight ligand binds to a slow-tumbling protein. Under conditions of excess ligand, if the ligand undergoes chemical exchange between the free and bound state more rapidly than longitudinal relaxation, intramolecular NOE connectivities reflecting the protein-bound state can be observed even for peaks exhibiting chemical shifts of free ligand (Clare and Gronenborn 1982, 1983; Glaudemans et al. 1990; Ni and Scheraga 1994). This type of NOE is referred to as transferred NOE (TRNOE). Figure 6.8 displays TRNOE data that characterize conformations of a trimannosyl ligand bound to the carbohydrate recognition domain of VIP36, an animal lectin involved in vesicular transport of glycoproteins between the endoplasmic reticulum and the Golgi (Yamaguchi and Kato 2008). TRNOE can also determine atomic contacts between proteins and carbohydrate ligands (Satoh et al. 2010).

## 8.2 Protein Binding to Glycolipid Clusters

In cell membranes, glycolipids such as gangliosides form clusters and play important roles in various biomolecular recognition events (Hakomori 2004). Because of their dynamic properties, crystallographic structural analyses of these glycolipid clusters are difficult. In contrast, NMR techniques provide detailed structural analyses of such dynamic clusters of glycolipids and their specific interactions with proteins.

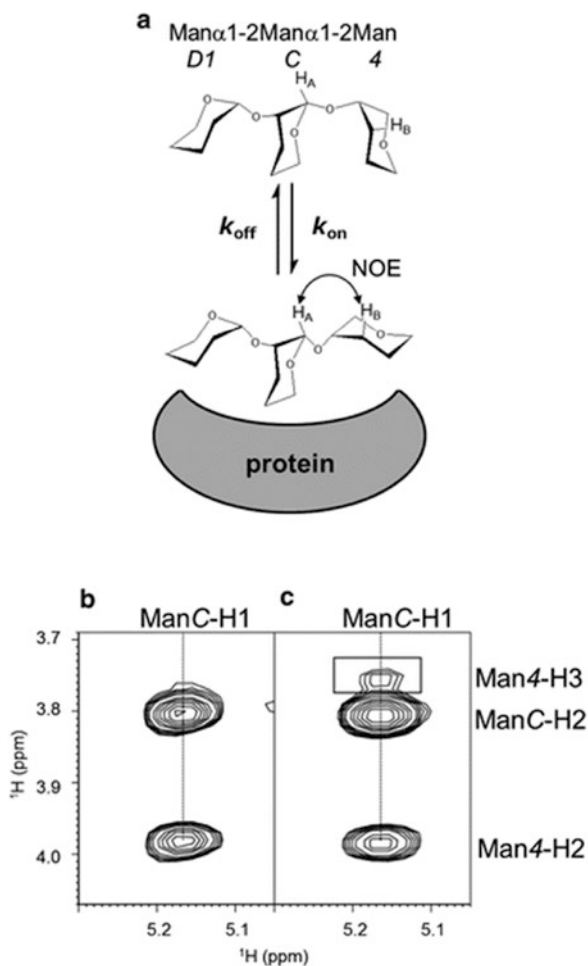
A $\beta$  has been reported to interact with GM1 gangliosides in Alzheimer's disease patients, thereby undergoing conformational transitions that result in pathogenic assemblies (Matsuzaki et al. 2010). To determine the interaction modes of A $\beta$  with ganglioside clusters, NMR experiments were conducted using deuterated  $^{15}\text{N}$ -labeled A $\beta$ (1–40) and aqueous gangliosidic micelles (Utsumi et al. 2009). Analyses of





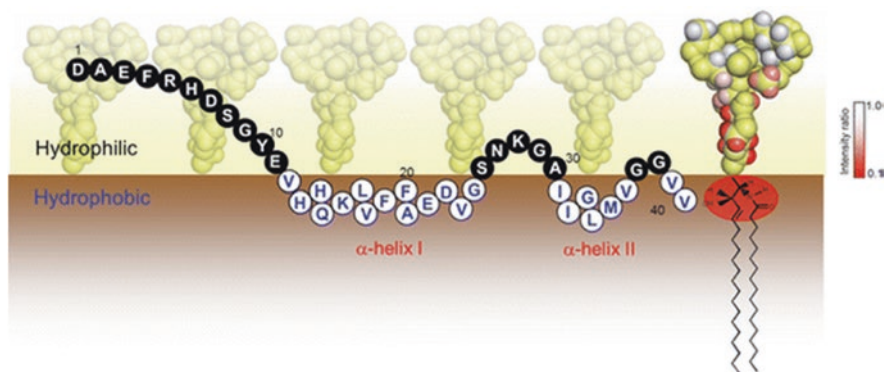
**Fig. 6.7** STD experiment. (a) Schematic of saturation transfer used for characterizing a carbohydrate–protein interaction; in this experiment, saturation transfer from protein to ligand is observed, thereby identifying the protons involved in the interaction, i.e.,  $H_A$ ,  $H_C$ , and  $H_E$ ; (b) Overlay of 2D  $^1H$ – $^{13}C$  STD-HSQC spectra of octyl-(NeuAc)<sub>3</sub> (50 equiv) with 20  $\mu$ M anti-oligo/polysialic acid IgM antibody 12E3 (red) and 2D  $^1H$ – $^{13}C$  HSQC spectrum (black) in PBS with 99 % D<sub>2</sub>O; Protein signal at 7 ppm was irradiated for saturation. (Adapted with modifications from (Hanashima et al. 2013) with permission from Elsevier)

**Fig. 6.8** TRNOE experiment. (a) Schematic of TRNOE used for characterizing carbohydrate–protein interactions; in this experiment, a spatially proximal proton pair,  $H_A$  and  $H_B$ , in the protein-bound state provides an NOE peak corresponding to chemical shifts of the unbound state under conditions of excess ligand. A part of NOESY spectrum of  $\text{Man}\alpha 1\text{-3Man}\alpha 1\text{-3Man}$  in (b) the absence and (c) the presence of the carbohydrate recognition domain of VIP36. An interglycosidic NOE between  $\text{ManC-H1}$  and  $\text{Man4-H3}$  (boxed) is observed only in the presence of lectin, indicating that these protons are spatially in close proximity of each other in the lectin-bound state of the trimannose. (Reprinted from (Yamaguchi and Kato 2008) with permission from Springer)



backbone chemical shift data of  $A\beta(1\text{--}40)$  indicated that this peptide forms discontinuous  $\alpha$ -helices upon binding to GM1 micelles. The saturation transfer data demonstrate that  $A\beta(1\text{--}40)$  lies on the hydrophobic/hydrophilic interface of the ganglioside clusters, exhibiting an up-and-down topological mode in which the two  $\alpha$ -helices (His14-Val24 and Ile31-Val36) and the C-terminal dipeptide are in contact with the hydrophobic interior (Fig. 6.9).

The  $A\beta$ -glycolipid interaction was also characterized by PRE (Yagi-Utsumi et al. 2010). The  $A\beta(1\text{--}40)$  peptide with an extra C-terminal cysteine residue was recombinantly produced and conjugated through a disulfide bond with a spin-labeled probe. On addition of the spin-labeled  $A\beta$  peptide to the solution containing micellar lyso-GM1, the  $^1\text{H}\text{-}^{13}\text{C}$  HSQC peaks originating from Glc and Gal<sup>II</sup> and those originating from the head group of the lyso-GM1 lipid moiety exhibited significant attenuation of intensity due to PRE line broadening (Fig. 6.9). These results



**Fig. 6.9** A topological model of A $\beta$ (1–40) bound to a ganglioside cluster, as deduced from NMR experiments; the regions of A $\beta$ (1–40) buried inside the hydrophobic interior of lyso-GM1 micelles and those exposed to hydrophilic environments were identified using saturation transfer experiments. Moreover, the PRE effect was used to identify atomic groups of lyso-GM1 that are proximal to the spin-labeled A $\beta$  peptide. The observed PRE effects are mapped on the 3D model of the carbohydrate moiety of lyso-GM1 with a color gradient from red to white. (Adapted with modifications from (Utsumi et al. 2009; Yagi-Utsumi et al. 2010) with permission from Springer and Elsevier, respectively)

indicate that the sugar–lipid interface was primarily perturbed upon interactions of A $\beta$  with the micelles.

Ganglioside micelle assemblies vary in size and curvature, depending on the size of the carbohydrate moiety. Such morphological variability can be a determining factor for ganglioside–protein interactions. Hence, for structural characterization of biomolecular interactions of glycolipid clusters, it is crucial to design appropriate membrane models that are suitable for sophisticated high-resolution spectral measurements. Small bicelles, in which a series of gangliosides were successfully embedded, have been developed as mimics of ganglioside-containing membranes for detailed NMR studies (Yamaguchi et al. 2013b). Using these standardized bicelles, chemical shift perturbation and relaxation data clearly indicated the ganglioside-specific involvement of N-terminal regions of  $\alpha$ -synuclein in membrane interactions (Yamaguchi et al. 2013b).

## 9 Liaisons Between NMR and Computation

As mentioned above, NMR data for flexible oligosaccharides, including chemical shifts,  $J$ , NOE, and PCS, should be interpreted as population-weighted averages of dynamic conformational ensembles rather than as one or two conformational states. Therefore, quantitative interpretations of NMR data are supported by theoretical calculations such as molecular dynamics (MD) simulations (Fadda and Woods 2010; Woods and Tessier 2010; Woods 2018; Re et al. 2020). Although motional

properties of systems can be obtained from suitable conditions under Newton's law, an inherent problem of this approach is its heavy dependence on simulation protocols, including initial structures, computational times, and force fields. It is therefore important to validate simulations by comparing with experimental observations. For example, the PCS-assisted NMR method has been successfully used to validate oligosaccharide conformational spaces sampled by MD simulations (Zhang et al. 2015). Replica exchange MD simulations (REMD) can enhance sampling using a parallel tempering technique (Sugita and Okamoto 1999). This method overcomes the multiple-minima problem by exchanging noninteracting replicas of the system at several temperatures. REMD simulations were applied to *N*-glycan and were consistent with both experimental NMR data (Re et al. 2011; Nishima et al. 2012; Kato et al. 2018a, b) and with the collisional cross sections determined using ion mobility spectrometry (Yamaguchi et al. 2012; Re et al. 2018). Thus, the combination of NMR spectroscopy and theoretical approaches promises atomic descriptions of dynamic conformations and interactions of glycoconjugates of neurophysiological and neuropathological interest (Kato et al. 2022).

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*Compliance with Ethics Requirements* The authors declare that they have no conflict of interest and that they have used no human subjects in work cited that was done in their laboratory.

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# Chapter 7

## Glycans and Carbohydrate-Binding/ Transforming Proteins in Axon Physiology



José Abad-Rodríguez, María Elvira Brocca, and Alonso Miguel Higuero

IN MEMORIAM Professor Hans-Joachim Gabius

**Abstract** The mature nervous system relies on the polarized morphology of neurons for a directed flow of information. These highly polarized cells use their somatodendritic domain to receive and integrate input signals while the axon is responsible for the propagation and transmission of the output signal. However, the axon must perform different functions throughout development before being fully functional for the transmission of information in the form of electrical signals. During the development of the nervous system, axons perform environmental sensing functions, which allow them to navigate through other regions until a final target is reached. Some axons must also establish a regulated contact with other cells before reaching maturity, such as with myelinating glial cells in the case of myelinated axons. Mature axons must then acquire the structural and functional characteristics that allow them to perform their role as part of the information processing and transmitting unit that is the neuron. Finally, in the event of an injury to the nervous system, damaged axons must try to reacquire some of their immature characteristics in a regeneration attempt, which is mostly successful in the PNS but fails in the CNS. Throughout all these steps, glycans perform functions of the outermost importance. Glycans expressed by the axon, as well as by their surrounding environment and contacting cells, encode key information, which is fine-tuned by glycan modifying enzymes and decoded by glycan binding proteins so that the development, guidance, myelination, and electrical transmission functions can be reliably performed. In this chapter, we will provide illustrative examples of how glycans and their binding/transforming proteins code and decode instructive information necessary for fundamental processes in axon physiology.

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## Abbreviations

AIS	Axon initial segment
CS	Chondroitin sulphate
DRG	Dorsal root ganglia
ECM	Extracellular matrix
ESC	Embryonic stem cells
GAG	Glycosaminoglycan
HA	Hyaluronan
HS	Heparan sulphate
iPSC	Induced pluripotent stem cells
KS	Keratan sulphate
NSC	Neural stem cells
OB	Olfactory bulb
OLG	Oligodendrocyte
OPC	Oligodendrocyte precursor
PG	Proteoglycan
PSA	Polysialic acid
RGC	Retinal ganglion cell
RMS	Rostral migratory stream

## 1 Introduction

Glycans play crucial roles in the nervous system by their influence in neuronal development, plasticity, differentiation or signal transduction, intervening also in the onset and evolution of neurodegenerative pathologies. However, only recent technological advances in bio-physicochemical and imaging analyses are permitting researchers to unveil and define the precise functions of specific glycan structures associated with glycoproteins and glycolipids during neuronal differentiation. In this chapter we will present and discuss the state-of-the-art and future perspectives of carbohydrate-related processes involved in neuron physiology, focusing on axon determination, development, structure and function.

## 2 Axon Determination/Early Neuronal Differentiation

Neuronal precursors go through several differentiation stages before turning into neurons, and it is becoming clear that this progression is precisely regulated by glycosylation and glycan interactions.

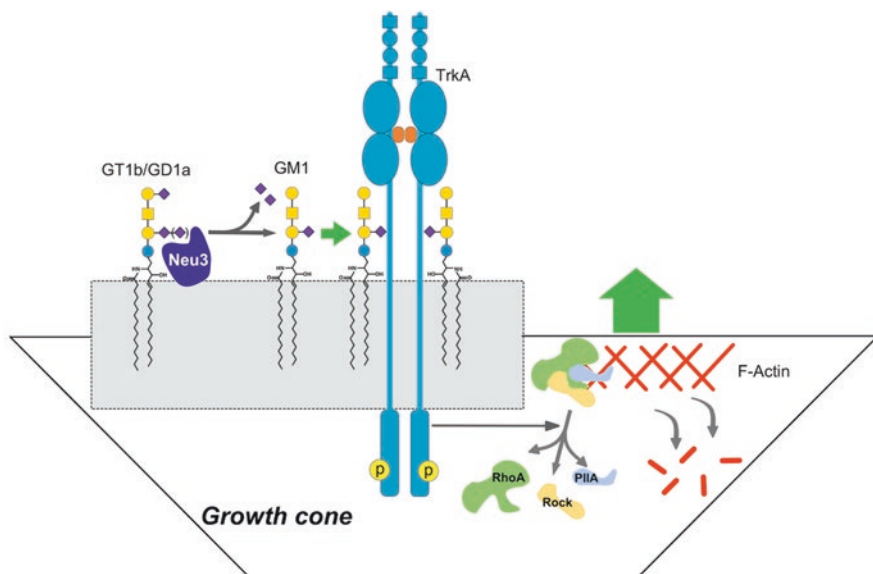
An instructive example of this is bisecting GlcNAc, a branch structure in N-glycans, most highly expressed in the nervous system where, besides other physiological and pathological functions (Kizuka and Taniguchi 2018), it plays a central role in neuronal differentiation. The expression of bisect-type N-glycans is up-regulated in human iPSC-derived neurons (Tateno et al. 2011), as shown by lectin microarray analyses. These kind of glycan was found to be up-regulated in both iPSCs and ESCs-derived murine neurons analysed by glycoblotting-based cellular glycomics (Terashima et al. 2014). These quantitative analyses revealed that bisecting GlcNAc glycans range from 1% to 2% of total glycans in iPSCs and ESCs, increasing up to 4% in derived NSCs, and reaching values between 11% and 12% in differentiated neurons, similar to those of cultured primary neurons, and very different to those of differentiated astrocytes shown to have between 1% and 4% of bisecting GlcNAc glycans (Terashima et al. 2014). However, these studies did not link glycan structures with their related glycoproteins, impeding detailed structural/functional pairing. New methods have been developed to address this drawback.

Using human-induced pluripotent stem cells (iPSCs) and iPSC-derived neuronal cells as a model of neuronal differentiation, and applying sequential LC/MS/MS analyses of protein extracts upon tryptic digestion, glycopeptide enrichment, and peptide deglycosylation, an altered production of a series of N-glycoproteins and their glycoforms has been shown during the differentiation into iPSC-derived dopaminergic neurons. In particular, there is an increase of N-glycoproteins bearing a bisected biantennary glycan structure with five N-acetylglucosamines, three mannoses, and a fucose. These dopaminergic and site-selective modifications are independent of the level of protein synthesis. These modifications target proteins functionally involved in neural cell adhesion (L1CAM, NCAMs, etc.) and in axon guidance/semaphorin-plexin signalling (PLXNs, SEMAs, etc.) (Kimura et al. 2021). These strategies that integrate glycoproteome analyses and resultant glycoprotein profiles with their evolving glycoforms, provide detailed information on the role of (N-)glycosylation in neuronal differentiation and could be the basis to a more precise understanding of neurodegenerative diseases and neural regeneration.

Neuron function requires different processes occurring in separate parts of the cell from the very early stages of differentiation (Dotti et al. 1988). Starting from the symmetric neuroblast stage, such a functional polarization is preceded by a precise molecular segregation that provides a single early neurite with axonal features. Underlining the relevance of glycans in early neuronal polarization, the plasma membrane ganglioside neuraminidase Neu3 (also known as PMGS) is located in a single neurite of non-polarized neurons (stages 1–2) before their morphological

polarization (stage 3), becoming a polarity landmark at the pre-axon plasma membrane.

The low filamentous actin (F-actin) content in the terminal segment of a neurite has been considered an early marker for the formation of fast-advancing growth cones occurring in axons (Bradke and Dotti 1999). In fact, Neu3 localizes at the neurite with lowest F-actin content specifying its axonal fate, and its local enzymatic inhibition triggers the polymerisation of actin filaments blocking axon development (Da Silva et al. 2005). Although the mechanism for the early polarized segregation of Neu3 is still unknown, its enzymatic activity locally removes neuraminic acid moieties from gangliosides GD1a or GT1b to produce GM1 bearing a pentasaccharide with a single sialic acid (Kopitz 2017; Ledeen and Wu 2015; Schengrund 2015). GM1 accumulation spatially-restricts neurotrophin receptor TrkA by direct interaction and stimulates signalling (Mutoh et al. 1995; Rabin and Mocchetti 1995) for phosphoinositide 3-kinase (PI3K)-induced inactivation of RhoA small-GTPase pathway proceeding through the disassembly of the complex RhoA/Rock (RhoA-associated kinase) from the brain actin-binding protein profilin-IIA (PIIA). This provokes a localized subcortical F-actin depolymerisation allowing microtubule protrusion and axon elongation (Da Silva et al. 2005; Da Silva et al. 2003) (Fig. 7.1). During subsequent stages of neuronal differentiation, Neu3



**Fig. 7.1** Neu3/GM1-induced axon outgrowth. Membrane neuraminidase Neu3 locally removes neuraminic acid moieties (purple diamonds) from gangliosides GD1a or GT1b to produce GM1, which spatially-gathers neurotrophin receptor TrkA by direct interaction, stimulating signalling for the inactivation of RhoA small-GTPase pathway, and disassembling the RhoA/Rock complex from the brain actin-binding protein profilin-IIA (PIIA). The subsequent localized subcortical F-actin (red long rods) depolymerisation allows microtubule to protrude supporting axon outgrowth (indicated by the green big arrow)

remains enriched in the axon membrane and its local enzymatic activity is required for normal axonal growth (Da Silva et al. 2005; Rodriguez et al. 2001).

Besides local F-actin regulation, GM1 also influences axon development by facilitating the trafficking of glycosylated neurotrophin receptors along the axon, and by its interaction with integrins and galectins such as Gal-1.

TrkA displays up to eleven putative N-glycosylation sites and only N-glycosylated TrkA forms bearing fully mature N-glycans can be targeted to the axon membrane (Watson et al. 1999). GM1 specifically binds to the mature form of TrkA, gathering it to transport vesicle membrane rafts for an efficient anterograde transport to the axon plasma membrane (Abad-Rodriguez and Díez-Revuelta 2015; Mutoh et al. 1995; Rabin and Mocchetti 1995). Cells lacking GM1, or expressing deficiently glycosylated TrkA, fail to target the receptor to the membrane precluding any neurotrophic effect (Mutoh et al. 2000; Mutoh et al. 2002). GM1 also interacts with glycosylated  $\alpha 5 \beta 1$ -integrin (Wu et al. 2007) and both molecules are stabilized in the complex by the specific cross-linking with homobivalent dimers of Gal-1. This membrane process stimulates axon growth by triggering the signalling cascade of focal adhesion kinase (FAK)/phospholipase C $\gamma$ /phosphoinositide-3 kinase (PI3K), and the opening of TRPC5 channels leading to a transient raise of intracellular Ca<sup>2+</sup> (Wu et al. 2016).

In all, variations in the glycosylated heads of gangliosides can induce local changes in the membrane, alone or in combination with glycan-crosslinking proteins such as galectins, gathering distinct molecular machineries and stimulating diverse signalling cascades that control axon outgrowth.

### 3 Axon Guidance

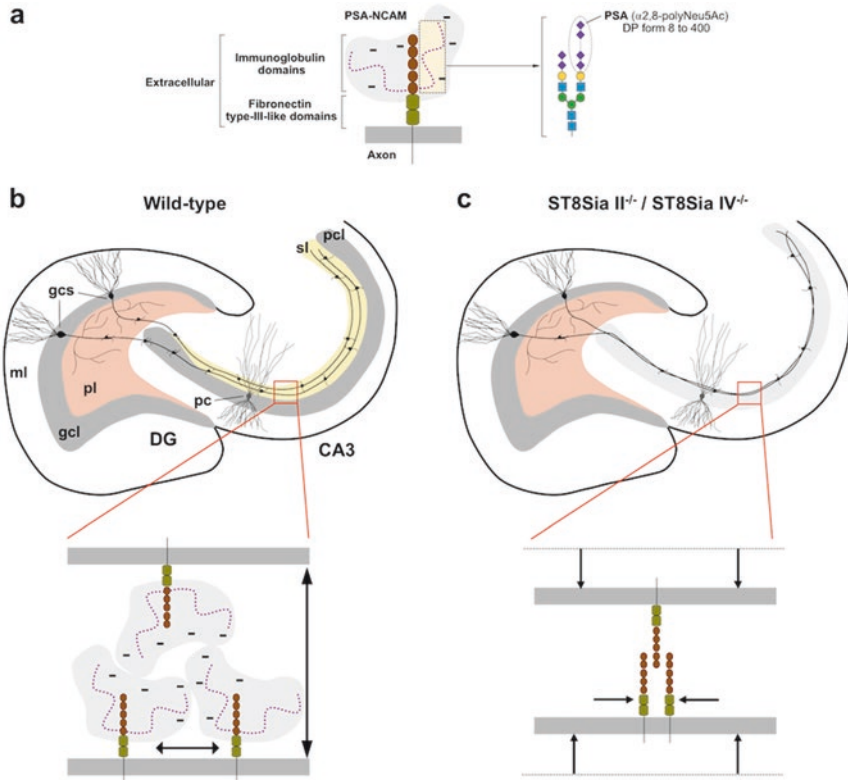
The correct anatomical and functional development of the nervous system requires the precise navigation of growing axons towards their specific targets. Axon guidance is based on different sets of tissue attractive/repulsive molecular cues whose disposition changes as the nervous system develops, and that are detected and interpreted by sensing systems at the axon membrane. Among others, classic axonal cues (netrins, semaphorins, slits, and ephrins) [4,5], neurotrophins (NGF, BDNF, NT3) and morphogens (sonic hedgehog, Wnt, BMP7) have been implicated in axonal guidance and their molecular mechanisms have been thoroughly studied (Seiradake et al. 2016; Ye et al. 2019). Playing a central role in these mechanisms, glycosylation confers a superior level of complexity to axon guidance pathways, allowing the refinement of their regulation for an optimized biological function. Within the next sections we will discuss the role of glycosylation of two major molecular groups involved in axon guidance, namely cell adhesion molecules and glycosaminoglycans. We will also explain the role of endogenous galectins and their interactions with glycosylated guidance cues in the navigation of olfactory axons in the adult olfactory system, as a paradigmatic case of physiological neuron regeneration within the central nervous system.

### 3.1 Axon Guidance by Glycosylated Cell Adhesion Molecules (CAMs)

Neuronal cell adhesion molecule (NCAM) presents a characteristic glycosylation with terminal polysialic acid (PSA), a polymer of  $\alpha$ 2–8 linked neuraminic acid. These polymers are formed by ST8Sia II and ST8Sia IV polysialyltransferases acting on hybrid, bi- to tetra-antennary complex glycans bearing, preferably, a terminal  $\alpha$ 2–6-linked neuraminic acid (Bhide and Colley 2016). Supporting the role of PSA-NCAM in guidance, impaired N-glycosylation or polysialylation by enzymatic ablation produces defective development of the mouse olfactory bulb and failed neuronal migration (Hildebrandt et al. 2009; Weinhold et al. 2005), projection errors in the chicken optic fiber layer due to the blockade of axon exit from the retina, and increased fasciculation of chicken spinal ganglia (Monnier et al. 2001). Similarly, impaired PSA synthesis affects granular cell projections (mossy fibers) along the hippocampal *stratum lucidum*. Their axons become misrouted due to over-adhesion to the pyramidal cell layer eliminating the lamination of the supra- and infra-pyramidal mossy fiber bundles (Fig. 7.2) (Koyama and Ikegaya 2018; Weinhold et al. 2005). There is a structural reason that explains these effects. In fact, PSA in NCAM confers numerous negative charges on its structure creating a strong electrostatic repulsion and increasing intercellular space that hampers adhesion (Rutishauser and Landmesser 1996). In all, PSA regulates NCAM-mediated neuronal migration and axonal fasciculation and guidance, becoming essential for the development of the nervous system.

Another member of the immunoglobulin cell adhesion molecule family (IgCAM) that is polysialylated is the synaptic cell adhesion molecule SynCAM. It functions as a guidance cue in the central and peripheral nervous system (Frei and Stoeckli 2017) by the expression of its PSA-SynCAM form in the NG2 glial subpopulation that integrates in different murine neuronal networks (Galuska et al. 2010).

Besides the regulation of axon growth and guidance by PSA-induced electrostatic hindrance, other adhesion molecules act through heterophilic interactions with glycosylated molecules on the membranes of adjacent cells. Such is the case of L1CAM that binds in *trans* to the highly glycosylated mucin glycoprotein CD24 carrying  $\alpha$ 2–3 linked sialic acid and Lewis<sup>x</sup> terminals on its structure. Interestingly, L1CAM/CD24 interaction can exert axon outgrowth stimulation in mouse cerebellar neurons or inhibition in DRG neurons (Kleene 2001). Neuronal L1 interacts in *trans* with  $\alpha$ 2–3 linked sialic acid in glial CD24 through a siglec homologous sequence, while CD24 Lewis<sup>x</sup> terminals interact with TAG1 and contactin in cerebellar neurons to assemble a neurite-growth stimulating complex. In contrast, TAG1 and contactin in DRG neurons, interacting in *trans* with CD24 Lewis<sup>x</sup> terminals, form complexes by *cis* interactions on neuronal surfaces with Caspr1 and Caspr2, respectively, triggering outgrowth inhibitory signalling (Lieberoth et al. 2009). Noteworthy, contactin binding to Caspr1 is also regulated by contactin glycosylation state. This will be discussed below within the Sect. 4.



**Fig. 7.2** Anomalous lamination of hippocampal mossy fibers in PSA-deficient mice. **(a)** PSA chains are synthesized on 5th and 6th N-glycosylation sites in immunoglobulin (Ig) domain 5 of NCAM, and are formed by repeated  $\alpha$ 2–8 N-acetylneuraminic acid units (degree of polymerization –DP– from 8 to 400 units). **(b)** Schematic design of wild type mouse dentate gyrus (DG) and hippocampus CA3 zone illustrating the mossy fibers, axons of granule cells (gcs) that project along the *stratum lucidum* (sl) in parallel to the pyramidal cell layer (pcl). These axons send collaterals to contact pyramidal cells (pc), constituting a fully laminated structure (ml, molecular layer; pl, polymorphic layer; gcl, granular cell layer). This laminated structure is partially maintained by PSA through the repulsive field provided by its bulky, polyanionic properties (**b**, bottom panel). Such an electrostatic repulsion regulates cell-cell interspace distance, avoiding the direct interaction of NCAM. **(c)** In PSA-deficient mice lacking the sialyl-transferases that synthesize PSA (ST8Sia II<sup>-/-</sup> /ST8Sia IV<sup>-/-</sup>) mossy fiber bundling and lamination is severely perturbed and axons become misrouted. The absence of PSA negatively charged “clouds” reduces the distance between axon membranes, and permit the direct interactions of NCAM with itself and other molecules in *cis* or *trans* (**c**, bottom panel) that provoke the over-adhesion among granular axons and to the pyramidal cell layer

Besides CAMs, other transmembrane glycoproteins such as endoglycan and dystroglycan play central roles in axon guidance. Endoglycan belongs to the CD34 family of sialomucins. It displays one membrane spanning peptide and heavily glycosylated extracellular domains. Similarly to PSA-bearing proteins, extracellular



domains of sialomucins display highly negative net charge due to multiple N- and O-linked glycosylations with sialylated terminals. This arrangement maintains a low adhesion level between the floor plate and the commissural axons, allowing them to exit the floor plate and navigate in the rostral direction (Baeriswyl et al. 2021). Dystroglycan is also a transmembrane protein, with a heavily glycosylated extracellular domain that is required for the precise patterning of different guidance proteins. Such is the case for slit, whose binding to glycosylated dystroglycan is required for its correct localization at the floor plate. As a matter of fact, genetic down regulation of dystroglycan or of its glycosylating enzymes produces misguidance of spinal commissural axons comparable to that in slit and slit receptor roundabout (robo) mutants. Other guidance-related molecules such as laminin, perlecan, etc. are also arranged in the basement membrane by scaffolding glycosylated dystroglycan, which is required, for instance, for the correct axonal sorting of contralateral and ipsilateral optical tracts in the optic chiasm by organizing guidance cues at the basement membrane (Clements et al. 2017; Lindenmaier et al. 2019; Wright et al. 2012). Underlining the relevance of glycosylated dystroglycan, though it is unknown whether guidance failures are involved, defective dystroglycan glycosylation produces a group of muscular dystrophies known as dystroglycanopathies, associated with abnormal development of the nervous system (Godfrey et al. 2011; Kanagawa 2021; Paprocka et al. 2021).

### 3.2 *Glycosaminoglycans in Axon Guidance*

Glycosaminoglycans (GAGs) are long linear polysaccharides built out of repeated disaccharides most usually containing GlcNAc or GalNAc, combined with uronic acid or galactose. There are several GAGs subfamilies, i.e. heparan sulfate (HS), chondroitin sulfate (CS), keratan sulfate (KS), that present sulfate groups at different positions, and the non-sulfated glycosaminoglycan hyaluronan (HA) (Higuero et al. 2017). Detailed information on GAG biosynthesis and structure is described in Chap. 5 by Schwartz and Domowicz. CS, HS, and KS covalently bound to proteins render the proteoglycans (PGs), CSPG, HSPGs, and KSPGs, respectively. HA is the only GAG that does not form PGs. GAGs are particularly enriched in the ECM where they modulate axonal directional outgrowth through the spatial patterning of many guidance cues. Consistently, deficient GAG biosynthesis induces failed nerve system formation and mistaken connectivity (Saied-Santiago and Bülow 2018).

HA is a key constituent of the nervous system ECM that surrounds neurons and glial cells. So far it has been found to function in axon guidance at the optic chiasm, where it colocalizes and binds CD44, a major local guidance cue. As a matter of fact, multiple guidance errors at the midline in the mouse optic chiasm are observed upon administration of exogenous HA or by genetic-driven impaired HA biosynthesis, demonstrating the regulation of axonal guidance through HA interaction with CD44 (Haupt and Huber 2008; Lin et al. 2007). Similar functions of HA have been suggested in other parts of the brain, such as the innervation of entorhinal fibers to

the hippocampus that is perturbed by hyaluronidase in vitro (Förster et al. 2001). Nevertheless, it is not known whether HA plays this role by modulating guidance cues, as it does in the optic chiasm.

HSPGs are major components of the ECM and are essential for axon guidance, as shown in basic studies using *Drosophila* (Inatani et al. 2003; Smart et al. 2011). Importantly, HSPGs organize the patterning of most of the relevant guidance cues during CNS development.

Syndecan is the major family of transmembrane HSPGs that regulates the localization of slit in the ECM, a repulsive cue located at the ventral midline that repels the growth of axons expressing slit receptor robo (Johnson et al. 2004b). Slit-mediated repulsion is also regulated by membrane-linked glypicans, whose extracellular domain bears multiple HS chains (De Pasquale and Pavone 2019). Perturbation of HS, either by enzymatic hydrolysis with heparinase III or by genetic down regulation of the *Ext1* gene (coding for the enzyme that polymerizes HS sugar chains), disrupts chemorepulsion dependent on slit-robo interaction (Piper et al. 2006) leading to severe defects in the development of the olfactory bulb and the cerebellum, and to major defects in the commissural tracts (Inatani et al. 2003). In contrast to the severe phenotypes of *Ext1* mutants, variations in the sulfation of HSPG (i.e. mutants for heparan sulfate sulfotransferases *hs2st* and *hs6st*) give rise to normal brain morphology with moderate and localized axon guidance issues at the optic chiasm and corpus callosum (Pratt et al. 2006), even though slit-mediated repulsion is reduced

Netrin-1 is another critical guidance molecule regulated by HSPGs. It shows chemoattraction or chemorepulsion depending on the axon projections (Boyer and Gupton 2018), and its deletion provokes embryonic lethality in mice by failed formation of the corpus callosum, and mistaken projections of spinal and hippocampal commissures (Yung et al. 2015). HS interacts with netrin-1 and genetic ablation of *Ext-1*, induces similar phenotypes to those of netrin-1 knockouts (Finci et al. 2014; Matsumoto et al. 2007). In addition, as shown in *C. elegans*, glypican modulates UNC6/netrin mediated axonal guidance by interacting with the netrin receptor UNC40/DCC (Blanchette et al. 2015). The fact that netrin and its receptors are evolutionarily conserved in mammals makes the regulation of netrin-1 by glypican feasible also in these organisms (Mutalik and Gupton 2021).

Like netrins, semaphorins are relevant cues in axonal guidance with both repulsive and attracting functions. Semaphorin 3A (*sema 3A*) is a secreted protein that binds to components of ECM (i.e. GAGs), regulating its patterning and interactions with its axonal co-receptors neuropilin and plexin for a correct directionality.

Similar to other guidance molecules mentioned above, down regulation of HS by genetic deletion of *Ext1* also affects axon guidance-related ephrins (Klein 2004). The growth cone collapse mediated by ephrin A (type-A ephrins are GPI-anchored proteins) is precluded in *Ext1* knockout mice, highlighting the role of HS in the regulation of ephrin A, though the underlying molecular mechanisms remain unknown (Irie et al. 2008). In the case of ephrin B (type-B ephrins are transmembrane proteins) the raise in specific N-glycosylation after crossing of the corpus callosum axons diminishes their response to semaphorins compared to pre-crossing

axons, which proves the capacity of HSPG to modulate the reaction to guidance cues in a precise manner (Mire et al. 2018).

CSPGs found at the ECM of the nervous system exert mainly repulsive functions. Their down regulation by chondroitinase enzymatic hydrolysis caused different axon pathfinding mistakes: (i) retinal projections at the midline of the optic chiasm grow axons in regions that normally do not allow their outgrowth (Chung et al. 2000); (ii) abnormal growth of the ventral motor nerve in the zebrafish embryo (Bernhardt and Schachner 2000), and (iii) axon navigation and targeting defects in RGC projections of *Xenopus* and chick embryos (Ichijo and Kawabata 2001; Walz et al. 1997). A recent *in vitro* study showed the direct inhibitory effects of CSPGs on the growth cones of mouse cerebellar granule neurons (Jin et al. 2018). Of note, semaphorin 5A is regulated and interacts with CSPGs and also with HSPGs. Interaction with the former confers sema5A a repulsive character, while interaction with the latter turns it into an axon attractive cue (Kantor et al. 2004), showing how different GAGs can re-define the signals for axonal pathfinding.

### ***3.3 Galectins and Their Interactions with Guidance Cues in the Adult Olfactory System***

Olfactory sensory neurons are periodically replaced with neuroblasts arisen from a stem cell niche in the olfactory mucosa. Those new neurons must project axons from the nasal neuroepithelium to the olfactory bulb (OB) in the brain, and they are guided by glycosylated cues interacting with endogenous galectins. Galectin-1 (Gal-1) notably contributes to this guidance, which is lost in Gal-1 knockout mice where axons fail to reach their targets at the OB (Puche et al. 1996). Non-covalent dimers of Gal-1 are proposed to associate ECM components such as laminin with unknown glycoconjugates on the axon membrane (likely GM1 or NAcLac-bearing glycoproteins) to guide the axon elongation, or to stimulate it by crosslinking different axons to generate fascicles (Tenne-Brown et al. 1998). Primary olfactory axons also express other galectins (Gal-3, -7, and -8), and the presence of different lactosamine-bearing guidance cues (i.e. NCAM), suggests the implication of these galectins in the axon guidance and targeting associated with the continuous renovation of the olfactory receptors (Storan et al. 2004).

Besides sensory olfactory neurons, in the subventricular zone (SVZ) neuroblasts derived from immature precursors migrate along the so-called “rostral migratory stream” (RMS) up to the OB, where they differentiate into granular and periglomerular interneurons (Luskin 1993). Along the RMS, ependymal cilia and astrocytes that form glial tubes to direct neuroblast migration (Peretto et al. 1997) express Gal-3. Gal-3-knockout mice display altered ependymal cilia and SVZ astrocytes with associated reduction of neuroblast migration and defective pathfinding. These effects correlate with the increased phosphorylation of epidermal growth factor receptor (EGFR) pointing to modulation of neuroblast migration either by direct

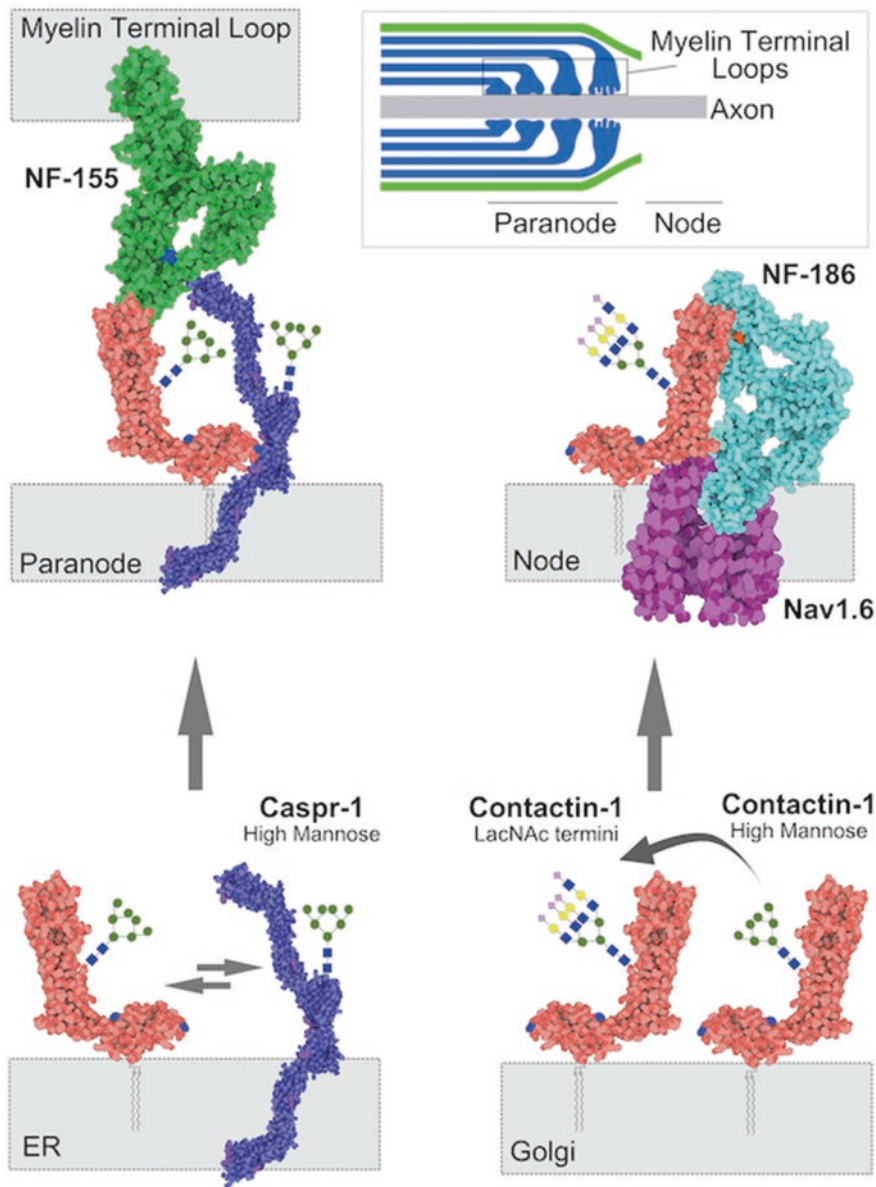
(Díez-Revuelta et al. 2010) or indirect (Comte et al. 2011) Gal-3 interactions with N-glycosylated EGFR.

## 4 Axon/Glia Interactions

Axon myelination is required for axon homeostasis and for the transmission of fast electrical impulses along nerves. Even though the mechanisms driving myelin formation are not completely understood, myelination is based on the interaction between axons and mature oligodendrocytes (OLGs), whose membrane-associated glycans are emerging as highly relevant actors. Immature neurons express and release Gal-4 that functions as a soluble regulator of oligodendrocyte progenitor (OPC) differentiation, keeping them in a proliferative non-myelinating stage through binding to unknown glycosylated receptors. As neurons undergo differentiation, Gal-4 expression is downregulated and OLGs advance towards a myelinating stage (Stancic et al. 2012), also secreting Gal-3 that, in an autocrine manner, reinforces the differentiation of OLGs and the integrity of myelin by binding to different glycosylated sites only present on the surface of the OLGs (Pasquini et al. 2011). Final lateral extension of myelin over the axonal surface gives rise to its final discontinuous display, with myelinated segments (internodes) and non-myelinated gaps (nodes), bordered by limiting interfaces (paranodes and juxtapanodes). Success in myelination depends on the glycosylation state of membrane molecules expressed by neurons and OLGs in the central nervous system (CNS), or Schwann cells in the peripheral nervous system (PNS), as discussed below.

Contactin1 and caspr1 bearing immature N-glycans (high mannose termini) in ER membranes associate in *cis* to leave the ER (Roth and Zuber 2017) using a raft-based trafficking mechanism targeted to the axon paranodes where they interact with NF-155 located at myelin terminal loop (Bonnon 2003; Faivre-Sarrailh et al. 2000; Gollan et al. 2003) (Fig. 7.3, left panels). In contrast, the maturation of contactin1 N-glycans by addition of N-acetyl-lactosamine (NAcLac) termini in the Golgi apparatus precludes the interaction with caspr1, and contactin1 is sorted to axon nodes, where it interacts with NF-186, gathering as well the Nav1.6 sodium channel involved in fast nerve impulse conduction (Fig. 7.3, right panels). In contrast, juxtapanodes involve the pairing in *cis* at the axonal membrane of caspr2 and contactin2 (also known as TAG-1) that subsequently interact in *trans* with contactin2 at the adjacent myelin sheet. This adhesion complex recruits and clusters Kv1-type potassium channels (Poliak and Peles 2003; Susuki et al. 2016; Traka et al. 2002). Based on the parallel but diverse N-glycosylation characteristics of contactin and caspr homologs (Hasler et al. 1993; Poliak et al. 1999), a likely glycan-based mechanism for the Caspr2/Contactin2 specific sorting to juxtapanode has been proposed though not proven so far.

In addition, the myelin-associated glycoprotein (MAG, Siglec-4), that binds gangliosides GD1a and GT1b with high affinity in vitro (Vinson 2001; Vyas et al. 2002;



**Fig. 7.3** Glycosylation role in the organization of nodes of Ranvier and paranodes. In the left panel it is shown how contactin1 and caspr1 bearing immature N-glycans (high mannose termini) in ER membranes associate *in cis* and target the axon paranodes where they interact with neurofascin-155 (NF-155) of the adjacent myelin terminal loop membrane. Alternatively, as shown in the right panels, contactin1 N-glycans can turn mature by addition of N-acetyl-lactosamine (NAcLac) termini within the Golgi apparatus. This blocks its interaction with caspr1, and targets contactin1 to the nodes of Ranvier, where it interacts with neurofascin-186 (NF-186) and recruits the Nav1.6 sodium channel required for fast nerve impulse conduction. Protein complex structures displayed in this figure are idealized for an intuitive comprehension of the processes. Protein 3D models have been obtained from [www.3dproteinimaging.com](http://www.3dproteinimaging.com)

Yang et al. 1996), is expressed in vivo at the membrane of myelinating OLGs and stabilizes the internode myelin by its interaction in *trans* with GD1a and GT1b in axonal membrane rafts (Posse De Chaves and Sipione 2010; Schengrund 2015; Schnaar 2010).

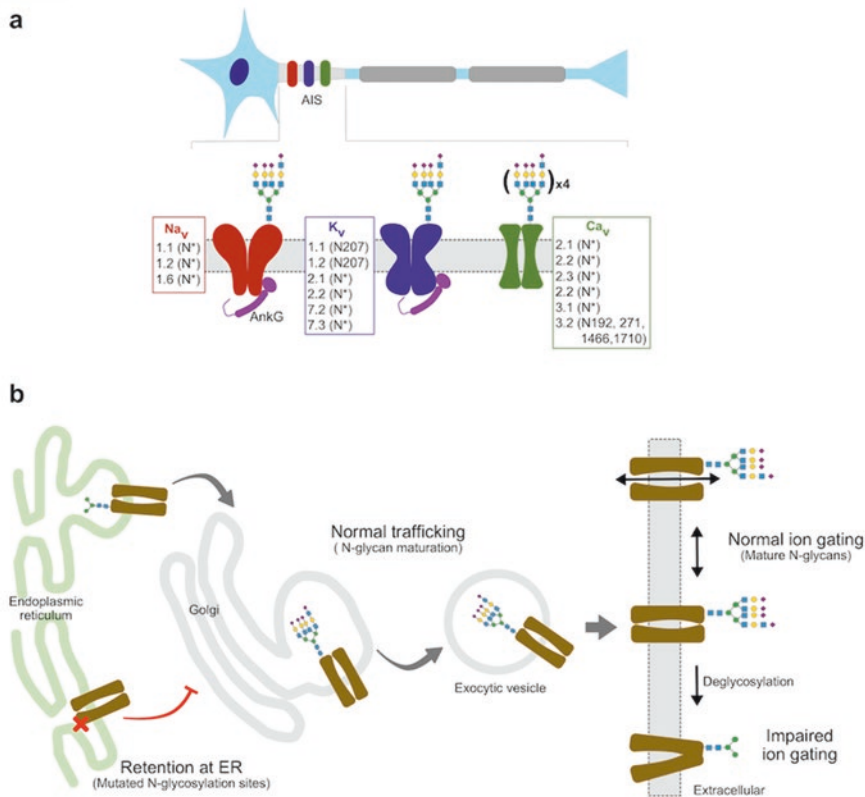
Recapitulating, lectins are involved in myelination at the level of OLG maturation and in the stabilization of myelinated internodes by MAG. This together with the strict glycosylation requirements for the correct sorting of main myelin molecules underscores the notion that glycan-related interactions are key to organize and regulate both non-myelinated and myelinated axon structures. Future research in this line promises significant insights into myelin-related physiopathology.

## 5 Nerve Impulse Generation/Transmission

In the nervous system, the directed flow of information relies on the polarized morphology of neurons, which typically consist of a cell body, multiple dendrites, and a single axon. A key structural and functional domain that integrates synaptic inputs received by the somatodendritic domain and in turn generates action potentials propagated by the axon is the axon initial segment (AIS) (Fig. 7.4a). As such, the AIS is a 10–60  $\mu\text{m}$  long domain strategically located between the somatodendritic compartment and the axon per se. It has unique structural features such as a dense network of membrane proteins characterized by a high concentration of glycosylated voltage-gated ion channels and cell adhesion molecules, and a specialized sub-membranous cytoskeleton enriched in ankyrin-G and spectrin  $\beta\text{IV}$  anchored to both actin filaments and microtubules (Leterrier 2016; Nelson and Jenkins 2017; Rasband 2010). Some of these features are also shared by the nodes of Ranvier in myelinated axons, which are responsible for propagating the action potentials generated at the AIS (Rasband and Peles 2021).

### 5.1 Voltage-Gated Ion Channels

Voltage-gated ion channels exert important neuronal functions such as the control of cell excitability, generation and propagation of action potentials, and modulation of synaptic transmission. Of particular importance for the correct functioning of the AIS and the nodes of Ranvier are several channels that regulate membrane permeability for sodium, potassium, and calcium ions. Generally, these ion channels and their auxiliary subunits are heavily glycosylated, with some subunits having up to 36% of their mass in the form of glycans (Messner and Catterall 1985; Roberts and Barchi 1987). The post-translational modification of these channels and/or their auxiliary subunits by N-glycan attachment can affect their function through different mechanisms (Fig. 7.4b). N-glycosylation can affect the folding and trafficking of ion channels to the cell surface. Once on the cell surface, N-glycosylation can



**Fig. 7.4** Role of glycosylation in voltage-gated ion channels of the Axon Initial Segment (AIS). **(a)** Schematic representation of ion channel expressed at the AIS. Channels of the three families (sodium, potassium and calcium) have been described in the AIS, all of them present putative N-glycosylation sites, although the presence of N-glycans have been only demonstrated in some of them, denoted as (N + number). Those sites with no current evidence of attached glycans are symbolised as (N\*). Updated information on channel glycosylation can be found at <https://www.glygen.org>. **(b)** The post-translational modification of voltage-gated ion channels (generic channel depicted in brown) and/or their auxiliary subunits by N-glycosylation can affect their function through different mechanisms. Lack of N-glycosylation can affect the channel folding as has been proven by directed mutagenesis of the different N-glycosylation sites. This usually induces the retention of non-glycosylated channels at the ER precluding their trafficking to the cell surface. In contrast, intact channels exit the ER and their N-glycans undergo full maturation before reaching the plasma membrane. Once on the cell surface, mature N-glycosylated channels can perform their normal ion gating function. Nevertheless, N-glycans hydrolysis by glycosidase enzyme activities (deglycosylation) at the membrane can also alter the channel distribution and stability (altered endocytosis and recycling) and impair their ion gating function, thus affecting the transmission of the nerve impulse

also alter the distribution and stability of these channels by modulating their endocytosis and recycling. Finally, N-glycosylation can also alter the biophysical characteristics of these channels thereby inhibiting or potentiating their function.

## 5.2 Voltage-Gated Sodium Channels

Voltage-gated sodium channels (VGSCs) are large integral membrane glycoproteins that regulate a fast and temporary sodium influx during the generation and propagation of an action potential. VGSCs are heterodimeric and heterotrimeric protein complexes composed of a large  $\alpha$  subunit and either one or two  $\beta$  subunits, all of which are heavily glycosylated. The  $\alpha$  subunit is the voltage-sensing pore-forming subunit, and as such is responsible for the influx of sodium ions, while the  $\beta$  subunits can modulate the gating kinetics (opening and closing), voltage-dependence, and surface expression of the channel (Hull and Isom 2018). In mammals, there are nine voltage-activated sodium channels (Nav1.1 – Nav1.9) encoded by a combination of nine  $\alpha$  subunit genes and four  $\beta$  subunit genes (Kruger and Isom 2016). In general, Nav1.1, Nav1.2, Nav1.3 and Nav1.6 are primarily expressed in the CNS while Nav1.7, Nav1.8 and Nav1.9 are expressed in the PNS (Catterall et al. 2005; Lai and Jan 2006; Vacher et al. 2008; Wang et al. 2017; Whitaker et al. 2000). Outside of the nervous system, Nav1.4 is primarily expressed in skeletal muscle and Nav1.5 in heart (Catterall et al. 2005). Early studies identified that VGSCs were concentrated at the axon initial segment and at nodes of Ranvier in myelinated axons (Catterall 1981; Ellisman and Levinson 1982; Waxman and Ritchie 1985; Wollner and Catterall 1986). In CNS neurons, Nav1.1, Nav1.2 and Nav1.6 are localized at the AIS, with Nav1.1 and Nav1.6 being also present at mature nodes of Ranvier (Caldwell et al. 2000; Duflocq et al. 2011; Hu et al. 2009; Lorincz and Nusser 2010; Lorincz and Nusser 2008; Ogiwara et al. 2007; Van Wart and Matthews 2006). As mentioned before, these channels are highly glycosylated and, in particular, highly sialylated. Early characterization of these channels demonstrated that glycan chains can account for up to 30% of their apparent molecular mass, with sialic acid residues representing almost 50% of the total carbohydrates (Elmer et al. 1985; Messner and Catterall 1985; Miller et al. 1983; Roberts and Barchi 1987). This rapidly prompted the question of the importance of these negatively charged residues on the activity of VGSCs. When purified channels in lipid bilayers were treated with neuraminidase to remove sialic acid residues, the result was a significant depolarizing shift in the average potential required for channel activation (Recio-Pinto et al. 1990). Additional experiments using sialylation-deficient cells or mutant forms of the sodium channels that could not be glycosylated led to similar results (Bennett et al. 1997). The removal of sialic acid residues had no effect on the structure or stability of the channels, confirming the idea that the presence of negatively charged extracellular residues causes the membrane around the channel to be slightly depolarized so that only a smaller change in membrane potential is required to induce channel gating (Cronin et al. 2005). Importantly, not all channels are uniformly glycosylated. For example, cardiac sodium channels are less glycosylated than those in muscle and brain (Cohen and Levitt 1993). In accordance with the role of sialylation in the regulation of sodium channel gating, the more heavily glycosylated Nav1.4 channel produced a large depolarizing shift following desialylation compared to the less glycosylated Nav1.5 channel which was unaffected. In



comparison to the muscle and cardiac channels, neuronal isoforms (Nav1.2 and Nav1.7) are moderately glycosylated and consequently showed moderate depolarizing shifts upon desialylation (Johnson et al. 2004a). Therefore, the effect of glycosylation on VGSC function is dependent not only on the specific isoform being expressed but also on the cell type in which it is expressed. Consequently, VGSC activity is regulated in a cell-specific manner by glycosylation. As mentioned before, VGSC  $\beta$  subunits are also extensively glycosylated (Yu et al. 2003; Zhou et al. 2012; Cortada et al. 2019). Although fewer studies have explored the role of glycosylation on their function, evidence indicates that  $\beta$  subunit glycosylation can regulate the trafficking of sodium channels to the cell surface. A recent study explored the functional significance of  $\beta 2$  subunit glycosylation on Nav1.5 channel trafficking (Cortada et al. 2019). The  $\beta 2$  subunit was glycosylated at three different sites (N42, N66 and N74). An unglycosylated triple mutant was mostly retained at the endoplasmic reticulum and was defective at promoting cell surface expression of the Nav1.5  $\alpha$  subunit.

### 5.3 Voltage-Gated Potassium Channels

Axonal voltage-gated potassium channels (VGKCs) are also involved in shaping the action potential and in controlling neuronal excitability. These types of channels are activated by membrane depolarization, which allows an outward flow of potassium ions that repolarizes the membrane and brings the action potential to an end. Following the action potential, these channels hyperpolarize the membrane and set the resting membrane potential of the cell. VGKCs are classified into 12 distinct subfamilies (Kv1 – Kv12) based on the similarity of their amino acid sequence (Gutman et al. 2005). Regarding their composition, VGKCs consist of homo- or heterotetramers of the pore-forming  $\alpha$  subunit (usually of the same family) and may also contain auxiliary  $\beta$  subunits that regulate the function and distribution of the channel. They are broadly expressed in a variety of tissues including the nervous system. In neurons, they have been localized throughout the cell although those located at the AIS are especially important for action potential generation and propagation. Kv1.1, Kv1.2, Kv2.1, Kv2.2, Kv7.2 and Kv7.3 channels are localized at the AIS in different type of neurons (Battfeld et al. 2014; Devaux et al. 2004; Goldberg et al. 2008; Jensen et al. 2017; Johnston et al. 2008; King et al. 2014; Klinger et al. 2011; Kole et al. 2007; Lorincz and Nusser 2008; Ogawa et al. 2008; Pan et al. 2006; Sanchez-Ponce et al. 2012; Sarmiere et al. 2008). Kv1.1, Kv1.2, Kv3.1b, Kv7.2 and Kv7.3 are also present at the node of Ranvier and axon terminal (Chung et al. 2006; De Wit et al. 2005; Devaux et al. 2003; Devaux et al. 2004; Kim and Rutherford 2016; Pan et al. 2006; Rasband et al. 1998; Sheng et al. 1993).

The importance of VGKCs was noticed in early studies with two *Drosophila melanogaster* excitability mutants with a leg shaking phenotype. The shaker and

hyperkinetic locus turned out to encode the  $\alpha$  and  $\beta$  subunits of potassium channels (Chouinard et al. 1995; Hotta and Benzer 1972; Ikeda and Kaplan 1970; Pongs et al. 1988; Salkoff and Wyman 1981). In mice, deletion of the Kv1.1 channel causes epilepsy (Smart et al. 1998). In humans, a variety of single base missense mutations in Kv1.1 channels have been associated with episodic ataxia (Adelman et al. 1995; Browne et al. 1994). Other type of VGKCs, Kv7.2 and Kv7.3, have an important role in the pathogenesis of neonatal epilepsies (Singh et al. 1998). Mutations in these channels cause neurons to become slightly depolarized and rhythmically fire multiple action potentials. These results highlight the importance of VGKCs in regulating cell excitability.

As found for most voltage-gated ion channels, VGKCs are also extensively glycosylated and this modification regulates the cell surface expression, stability, and gating properties of the channel (Fujita et al. 2006; Hall et al. 2015; Khanna et al. 2001; Lopez-Rodriguez and Holmgren 2018; Napp et al. 2005; Petrecca et al. 1999; Sutachan et al. 2005; Thayer et al. 2016; Vicente et al. 2018; Watanabe et al. 2003; Watanabe et al. 2015; Watanabe et al. 2004; Watanabe et al. 2007) (Fig. 7.4). Regarding the role of glycosylation on the function of VGKCs, the Shaker-related Kv1 subfamily is one of the most intensely studied. When Kv1.1 channels were expressed in CHO cell lines with reduced potential for glycosylation, the voltage-dependence of activation was shifted to more positive voltages and the activation kinetics were slower than for normally glycosylated channels. Sialidase treatment of glycosylated channels reproduced the effects observed on glycosylation deficient cells. Surface expression, however, was not affected by glycosylation (Thornhill et al. 1996). Site-directed mutagenesis of the Kv1.1 channel was used to study the effect of the complete lack of glycans on the functional properties of the channel. Kv1.1 has a single N-glycosylation site (N207) in its first extracellular loop. Expression of a N207Q mutant demonstrated that the lack of glycosylation, and in particular terminal sialylation, affected the steady-state activation and kinetic properties of the channel (Watanabe et al. 2003). Like Kv1.1, Kv1.2 channels also have a single N-glycosylation site (N207). However, the expression of Kv1.2 glycosylation mutants in hippocampal neurons revealed no apparent change in the activation and deactivation kinetics of the channel. Glycosylation at this site, on the other hand, was important for the cell surface expression and stability of the channel (Thayer et al. 2016).

Additionally, the Kv3.1 channel, which is present at the nodes of Ranvier, is also glycosylated at positions N220 and N229. Complex-type glycosylation at N229 increases trafficking of the channel to the cell surface. On the contrary, an unglycosylated mutant and a glycosylated form with high mannose-type glycans at position N220 were retained at the endoplasmic reticulum (Vicente et al. 2018). Moreover, replacement of the complex-type N-glycan with a hybrid type hindered the gating properties of Kv3.1 channels (Hall et al. 2015).

## 5.4 Voltage-Gated Calcium Channels

In excitable cells, voltage-gated calcium channels (VGCCs) elevate intracellular calcium concentrations after membrane depolarization. Particularly in neurons, these channels are the primary mediators of depolarization-induced calcium entry where they regulate the timing and generation of action potential bursts. VGCCs consist of a pore-forming  $\alpha 1$  subunit and two auxiliary subunits - a cytoplasmic  $\beta$  subunit and an extracellular  $\alpha 2\delta$  subunit. A great variety of channel subtypes can be co-assembled through the use of ten  $\alpha 1$  subunit genes, four  $\beta$  subunit genes, and four  $\alpha 2\delta$  subunit genes. VGCCs are classified in three channel subfamilies (Cav1, Cav2 and Cav3) according to their  $\alpha 1$  subunit, or into five classes (L-type: Cav1.1 - Cav1.4, P/Q-type: Cav2.1, N-type: Cav2.2, R-type: Cav2.3, and T-type: Cav3.1–3.3) based on their biophysical and pharmacological properties (Catterall 2011). There is a widespread expression of VGCCs throughout the nervous system. Cav1 channels are present in dendritic spines where calcium entry regulates long-term potentiation (Grover and Teyler 1990; Navakkode et al. 2022). Cav2 channels are present in presynaptic nerve terminals where calcium entry triggers neurotransmitter release (Mochida 2019). Cav3 channels are mainly present in the soma and dendrites of neurons, and they regulate cellular excitability and oscillatory behavior as well as neurotransmitter release (Cheong and Shin 2013; Perez-Reyes 2003; Turner and Zamponi 2014; Weiss et al. 2013; Weiss et al. 2012). Importantly, several VGCCs have been localized at the AIS including Cav2.1, Cav2.2, Cav2.3, and more recently, Cav3.1 and Cav3.2 (Bender and Trussell 2009; Lipkin et al. 2021). Experiments performed on different types of neurons revealed that the activation of these channels at the AIS is essential to the generation and timing of action potential bursts (Bender and Trussell 2009).

Like other voltage-gated ion channels, mature calcium channels are also glycosylated proteins, and their glycosylation regulates the cell surface channel density and their gating properties. *In silico* analysis of the amino acid sequence of the ten  $\alpha 1$  pore-forming subunits reveal canonical N-glycosylation sites (Lazniewska and Weiss 2017). Evidence pointing to the glycosylation of VGCCs have come from studies using lectin labelling to identify glycosylation sites, from biochemical analyses were the molecular mass of the channels change upon glycosidase treatment or N-glycosylation inhibition with tunicamycin, as well as from site-directed mutagenesis studies.

Results suggesting the potential glycosylation of T-type calcium channels came from experiments showing that the molecular mass of Cav3.1 and Cav3.3 channels were significantly different depending on the brain region and developmental time analyzed (Yunker et al. 2003). Furthermore, when the Cav3.3 channel was exogenously expressed in HEK293 cells and subjected to enzymatic deglycosylation with PNGase F, the differences in molecular weight were normalized, suggesting that the channel is an N-linked glycoprotein (Chen et al. 2007). A more detailed characterization of the glycosylation of T-type channels came from site-directed mutagenesis studies of the human Cav3.2 channel, which has four potential N-glycosylation sites

(N192, N271, N1466 and N1710). Disruption of these sites revealed that glycosylation at asparagine N192 and N1466 increases the steady-state expression and stabilizes the channel at the cell surface (Lazniewska et al. 2016; Weiss et al. 2013). Additionally, glycosylation at both N192 and N1466 sites is essential for regulation of the gating properties of the channels as they modulate the calcium permeability and/or opening probability of the channel (Ondacova et al. 2016).

In a similar manner, site-directed mutagenesis studies showed that N-glycosylation of L-type calcium channels is also critical for their correct function. When, either one, two or the four potential N-glycosylation sites found on Cav1.2 channel were mutated, the double (N124Q/N299Q) and quadruple mutants (N124Q/N299Q/N1359Q/N1410Q) showed a positive shift in voltage-dependent gating. Furthermore, the quadruple mutant showed a decreased cell surface expression with a concomitant decrease in current density (Park et al. 2015).

In addition to the pore-forming subunit, the  $\alpha 2\delta$  auxiliary subunit is also a glycosylated protein. It is highly N-glycosylated, with approximately 30 kDa of its mass in the form of glycans (Marais et al. 2001). The first studies concerning the functional significance of the glycosylation of this subunit revealed it has a role in stabilizing its interaction with the pore-forming subunit and for the functional increase in the channel's current amplitude (Gurnett et al. 1996). Site-directed mutagenesis studies identified positions N136 and N184 as functional N-glycosylation sites necessary for enhancement of calcium current amplitudes (Andrade et al. 2009; Sandoval et al. 2004). Glycosylation of the  $\alpha 2\delta$  subunit at position N633 was also identified as necessary for its surface expression and as essential for its role as a regulator of the channel's calcium current (Tétreault et al. 2016). Additional glycosylation sites (N348, N468 and N812) also contribute to the stability of this subunit (Tétreault et al. 2016).

## 5.5 O-Linked $\beta$ -N-Acetylglucosaminylation of AIS Proteins

O-GlcNAcylation is a posttranslational modification in which a single  $\beta$ -N-acetylglucosamine (O-GlcNAc) monosaccharide is attached to either serine or threonine residues of intracellular proteins. It is a unique type of intracellular glycosylation which contributes to numerous cellular functions and is dysregulated in a wide range of diseases (Chatham et al. 2021). Interestingly, O-GlcNAcylation is particularly abundant in the brain where the only two enzymes that add and remove O-GlcNAc moieties (O-GlcNAc transferase and O-GlcNAcase, respectively) are abundantly expressed (Lee et al. 2021; Liu et al. 2012; Wulff-Fuentes et al. 2021). Several studies have provided clues for the role of O-GlcNAcylation in the modulation of neuronal excitability and excitatory synaptic transmission.

Ankyrin-G is a scaffolding protein that functions as the master organizer of the AIS by clustering voltage-gated sodium and potassium channels, as well as cell adhesion molecules (e.g., neurofascin, NrCAM) (Jenkins and Bennett 2001; Pan et al. 2006; Zhou et al. 1998). It has long been known that ankyrin-G is glycosylated

at its serine-rich domain with O-linked GlcNAc residues (Zhang and Bennett 1996). Additionally, immunofluorescence experiments using an antibody that recognizes single O-linked GlcNAc residues revealed that O-GlcNAc modified proteins are particularly enriched at nodes of Ranvier (Zhang and Bennett 1996). However, progress towards identifying the functional significance of O-GlcNAcylation was hampered by the inability to identify the modified residues easily and precisely. It took some time until advances in affinity chromatography and mass spectrometry allowed robust enrichment methodologies and the dissociation of peptide backbone linkages without the elimination of O-GlcNAcylated moieties from O-GlcNAcylated peptides (Chalkley et al. 2009). Now, more than 5000 proteins comprise the human O-GlcNAcome including multiple voltage-gated sodium, potassium, and calcium channels (Wulff-Fuentes et al. 2021). Many of these are key AIS proteins (e.g., Kv7.3, Cav2.1, Cav2.2, Cav3.1 and ankyrin-G) with established roles in neuronal excitability (Chalkley et al. 2009; Ruan et al. 2014; Trinidad et al. 2012). In accordance with a relevant function for O-GlcNAcylation of AIS proteins in the regulation of action potential generation, an acute increase in O-GlcNAcylation strongly suppresses the intrinsic excitability of hippocampal CA1 neurons by cooperatively modulating three different voltage gated-cation channels: voltage-gated potassium channels, voltage-gated sodium channels and HCN channels (Hwang and Rhim 2019). Furthermore, in a recent study using a murine model of temporal lobe epilepsy as well as human epileptic hippocampal tissue, a global decrease of O-GlcNAcylation and OGT expression was found in the epileptic tissue compared to non-epileptic controls. Pharmacological inhibition of OGA enzyme, which elevated protein O-GlcNAcylation decreased both seizure duration and epileptic spike events.

In summary, the importance of protein glycosylation in the nervous system is evidenced by the fact that most congenital disorders of glycosylation, caused by mutations in genes of the glycosylation machinery, exhibit numerous neurological manifestations including epileptic seizures, mental and psychomotor retardation, ataxia, hyperreflexia, hypotonia as well as structural abnormalities (Baycin-Hizal et al. 2014). It is therefore conceivable that alterations in the glycosylation of target proteins, such as the AIS-localized glycoproteins discussed here (Fig. 7.4a), can contribute to the phenotypical manifestations observed in these diseases. In accordance with this, the activity of voltage-gated ion channels and mutations in the genes that code them have been associated with neurological disorders of neuronal excitability such as epilepsy and chronic pain (Ademuwagun et al. 2021; Goodwin and McMahon 2021). Many of these channels are localized at the AIS, and as such, the AIS has been suggested as a “common pathogenic node” in epilepsy (Wimmer et al. 2010).

## 6 Axon Regeneration

PNS axons upon injury can regenerate extensively, reconnecting with their targets and recovering their function. In contrast, the axons of CNS neurons mostly fail to regenerate, in part due to their intrinsically poor regenerating capacity even in permissive environments, and also to the formation of the glial scar at the injury site, where several inhibitory molecules contribute to CNS regeneration failure. Major components of the glial scar are CSPGs that exert a strong inhibitory effect on axon growth and regeneration (Laabs et al. 2005). In fact, injury producing damage to ECM leads to upregulation of growth inhibitory CSPG (Kim et al. 2018). Logically, CSPG has been targeted to stimulate regeneration, either directly by enzymatic hydrolysis by chondroitinase (Day et al. 2020; Mountney et al. 2013), or indirectly by manipulating the expression of specific interactors that reverse the CSPG-induced inhibition, such as the heparan-binding growth-associated molecule HB-GAM, also known as pleiotrophin, that injected after experimental spinal cord injury in mice enhances axon regeneration (Rauvala et al. 2017).

Importantly, the intrinsically low regenerative capacity of CNS axons can also be overcome by glycan-related mechanisms. As discussed in Sect. 2, early spatial confinement of neuraminidase Neu3 to a single neurite increases local GM1 providing the axon fate to that neurite, where a plethora of GM1-binding proteins such as Gal-1, TrkA, integrins, etc, signal and support subsequent axon outgrowth. Nevertheless, Neu3 activity decreases in CNS neurons as they mature and it is very low in fully differentiated neurons. Actually, elevating Neu3 activity by gene transfection induces a notable enhancement of regeneration of rat hippocampal and cortical axons upon in vitro axotomy (Dotti et al. 2001). In this line, a possible explanation to the different regenerative behaviour of injured CNS and PNS axons relies on the fact that the conversion of GD1a and GT1b to GM1 induced by Neu-3 occurs only in axotomized PNS but not CNS axons, as proven when comparing injured sensory and retinal axons of adult rats (Kappagantula et al. 2014). Attempts have been made to stimulate functional regeneration in experimental CNS injuries by raising local neuraminidase activity. Infusion of *Clostridium perfringens* sialidase enhances spinal axon outgrowth into implanted grafts of peripheral nerve in rats after brachial plexus injury (Yang et al. 2006). Similarly, sialidase from *Vibrio cholerae* infused to the site of a moderate thoracic spinal cord contusion induced significant improvements of the behavioural and anatomical outcomes (Mountney et al. 2013; Mountney et al. 2010). Unexpectedly, the combination of sialidase and chondroitinase did not show an additive reinforcement of injured axon regeneration when compared with that seen when each of the enzymes was used separately (Mountney et al. 2013).

## 7 New Perspective in CNS Regulation Through Glycan-Lectin Interactions in the Microbiota-Gut-Brain Axis

Over the last 20 years, the concept that the gut microbiota can influence the nervous system under physiological and/or pathological conditions has gained much attention as results of an exponentially growing number of studies are published in the scientific literature. A large proportion of this body of work is based on the association of a particular condition (e.g., behavior, disease, mood disorder, etc.) with changes in different populations of bacteria composing the gut microbiota in humans and animal models or with the complete lack of a gut microbiota using germ-free animals. Advances in high throughput technologies (metagenomics, metatranscriptomics, metaproteomics and metabolomics) have started to shed light on the mechanisms responsible for this long-range regulation, thus providing a causal relationship between an “altered” gut microbiota and nervous system dysfunctions (Liu et al. 2021; Wang et al. 2019). The most common mediators of this regulation include bacterially derived metabolites that can cross the blood-brain barrier, gut derived hormones, immune cell-derived cytokines, and a direct effect through the modulation of the vagus nerve (Cryan et al. 2019). Axon physiology is by no means an exception to this type of regulation. Two processes influenced by the gut microbiota are axon growth and axonal myelination (Hoban et al. 2016; Vuong et al. 2020). Microbiota-deficient mice have hypermyelinated axons in the prefrontal cortex, coincident with a marked upregulation of neural activity-induced genes as well as genes linked to myelination. Interestingly, these gene expression changes can be reverted by colonization of germ-free mice with a conventional microbiota (Hoban et al. 2016). Moreover, during the prenatal period, the maternal gut microbiota can regulate the growth of thalamocortical axons in the fetus (Vuong et al. 2020). Depletion of maternal microbiome resulted in decreased thalamocortical projections and reduced expression of genes related to axonogenesis. Gnotobiotic colonization with selected bacteria as well as maternal supplementation with selected microbial-derived metabolites prevented the axonal defects observed in the offspring.

Quite surprisingly, considerably less attention has been given to the role played by glycan-lectin interactions between hosts and microbes, and how those interactions impact the nervous system through the microbiota-gut-brain axis. A major component of the intestinal mucosa are epithelial-derived glycans. Host glycans are critical regulators of the gut microbiota colonization since they serve not only as a source of nutrients for bacterial metabolism but also as ligands for both commensal and pathogenic bacterial lectins (adhesins) (Koropatkin et al. 2012; Kudelka et al. 2020; Poole et al. 2018; Ringot-Destrez et al. 2017). In addition, host-derived lectins with bactericidal activity can shape the microbiota composition and establish intestinal homeostasis (Lehotzky et al. 2010; Miki et al. 2017; Vaishnavi et al. 2011). We therefore believe that more efforts aimed at comprehending the largely unexplored roles played by glycan-lectin interactions - not only within the nervous

system but also those that shape the host-microbe symbiosis - are necessary for a better understanding of the pathophysiology of the nervous system.

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# Chapter 8

## Neurological Consequences of Congenital Disorders of Glycosylation



Justyna Paprocka

**Abstract** The chapter is devoted to neurological aspects of congenital disorders of glycosylation (CDG). At the beginning, the various types of CDG with neurological presentation of symptoms are summarized. Then, the occurrence of various neurological constellation of abnormalities (for example: epilepsy, brain anomalies on neuroimaging, ataxia, stroke-like episodes, autistic features) in different CDG types are discussed followed by data on possible biomarkers and limited treatment options.

**Keywords** Congenital disorders of glycosylation · N-linked glycosylation · O-linked glycosylation · Neurological presentation · Glycosylphosphatidylinositol anchor synthesis · Glycosphingolipids · Biochemical markers · Brain abnormalities

### Abbreviations

ATP	Adenosine triphosphate
CCS	Cerebro-cutaneous syndrome
CD	Cluster of differentiation
CDG	Congenital disorders of glycosylation
CHIME	Mabry syndrome: colobomas, cardiac defects, ichthyosiform dermatosis, intellectual disability, conductive hearing loss, epilepsy
COG	Conserved oligomeric Golgi complex
DAF	Complement decay-accelerating factor

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EIEE	Early infantile epileptic encephalopathy
ESI-QTOF-MS	Flow injection-electrospray ionisation -quadrupole time- of -flight mass spectrometry
FLAER	Flow cytometry using proaerolysin variant
FS	Fryns syndrome (diaphragmatic defect, characteristic facial appearance, distal digital hypoplasia, pulmonary hypoplasia, at least one characteristic associated anomaly: polyhydramnios, cloudy corneas and/or microphthalmia, orofacial clefting, renal dysplasia/renal cortical cysts, malformations involving the brain, cardiovascular system, gastrointestinal system, genitalia)
GPIBD	Glycosylphosphatidylinositol biosynthesis defect
HPMRS	Hyperphosphatasia with mental retardation syndrome
ID/DD	Intellectual disability/developmental delay
LGMD	Limb-girdle muscular dystrophy
LLO	Primary oligosaccharide
MCAHS	Multiple congenital anomalies-hypotonia-seizures syndrome
MDDGA	Muscular dystrophy –dystroglycanopathy
MDDGA1	Muscular dystrophy- dystroglycanopathy (congenital with brain, eye anomalies), type A
MDDGB1	Muscular dystrophy- dystroglycanopathy (congenital with mental retardation), type B
MDDGC1	Muscular dystrophy- dystroglycanopathy (limb-girdle), type C
OST	Oligosaccharide transferase
TIEF	Transferrin isoelectric focusing
uPAR	Urokinase-type plasminogen activator receptor
VSD	Ventricular septal defect

## 1 Introduction

Disorders of glycosylation are caused by defects in the synthesis of glycan portion of proteins and lipids. It has been 42 years since a CDG was first described (Jaeken et al. 1980). This group of metabolic abnormalities with frequently seen multisystemic involvement is still rapidly growing. Looking back, never before have so many new entities within the same family of disorders been identified and described in such a short period of time. It is estimated that 2% of the human genome encodes proteins essential for glycosylation itself.

Around 150 types of CDG have been discovered. Most CDG have an autosomal recessive inheritance (Francisco et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018; Ondruskova et al. 2020; Lipiński and Tyłki-Szymańska 2021; Gardeitchik et al. 2018; Quelhas et al. 2021; Bogdańska et al. 2021; Wilson and Matthijs 2021) except for those that are

- Autosomal dominant: EXT1-CDG, EXT2-CDG, GANAB-CDG, POFUT1-CDG, POGLUT1-CDG, PRKCSH-CDG, SEC63-CDG

– or

- X-linked: ALG13-CDG, ATP6AP1-CDG, ATP6AP2-CDG, OGT-CDG, PIGA-CDG, SLC9A7-CDG, SLC35A2-CDG, SSR4-CDG and VMA21-CDG.

In recent years many abnormal glycosylation patterns were found in disease such as Alzheimer's disease (Regan et al. 2019), cardiovascular disorders (Gudej and Lauc 2018), diabetes (Rudman et al. 2019), chronic inflammatory diseases (Verhelst et al. 2020), immunodeficiencies (Ravell et al. 2020), and cancer (Gao et al. 2020). CDG multisystemic presentation is connected with the glycosylation process, which is the most common post-translational and co-translational modification in humans.

## 2 Classification

The most recent division of congenital disorders of glycosylation (Francisco et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018; Ondruskova et al. 2020; Lipiński and Tylki-Szymańska 2021; Gardeitchik et al. 2018; Quelhas et al. 2021; Bogdańska et al. 2021; Wilson and Matthijs 2021) covers

- Disorders of N-linked glycosylation
  - Disorders of N-acetylgalactosamine (GalNAc) glycosylation
  - Disorder of N-acetylglucosamine (GlcNAc) glycosylation
- Disorders of O-linked glycosylation
  - Disorders of O-mannosylation
  - Disorders of O-glycosylation
  - Disorders of O-xylosylation (glycosaminoglycan biosynthesis)
- Disorders of glycosylphosphatidylinositol anchor biosynthesis
- Disorders of glycolipid glycosylation
- Disorders of multiple glycosylation pathways
  - Disorders of Golgi pH and ion homeostasis
  - Disorders of vesicular trafficking

Approximately one fifth of all proteins in the existing databases and around half of human proteins are glycosylated. The glycosylation itself may contribute to protein folding, stability, transport to special subcellular destinations, modulate cell-cell interactions, signal transduction, trafficking and endocytosis.

Glycans are bound to a polypeptide chain by N- and O-glycosylation, among other processes. N-glycosylation is a multi-step process consisting of two main parts: the synthesis (referred to as basal glycosylation) and the processing of the primary N-glycan (terminal glycosylation). Primary glycosylation takes place in the cytoplasm and the endoplasmic reticulum (ER). As a result of this process, a primary oligosaccharide (LLO) is formed on the lipid carrier, dolichol phosphate (Dol-P), by addition of sugar residues, i.e. N-acetylglucosamine, mannose and glucose (Wilson and Matthijs 2021). The formed bi-antennary glycan with the structure of Man5 GlcNAc2 is transferred into the endoplasmic reticulum by the action of the

enzyme flipase. The oligosaccharide is elongated again in the ER space. The oligosaccharide Glc3Man9 GlcNAc2 is transferred from the lipid carrier to the nascent polypeptide chain by the enzymatic oligosaccharide transferase (OST) complex (Chang et al. 2018; Péanne et al. 2018; Wilson and Matthijs 2021). The oligosaccharide glycan binds to the asparagine of the protein molecule with an N-glycosidic bond. Defects associated with the synthesis of the primary oligosaccharide are referred to as type I glycosylation disorders.

The second part of glycosylation associated with LLO processing is also referred to as terminal glycosylation and begins within the ER. A quality control process of folding of the glycoprotein molecules is initiated to achieve the correct protein structure. The glycoprotein chain then travels via vesicular transport to the Golgi apparatus where the actual terminal glycosylation process takes place. Here, the glycan bound to the protein undergoes further enzymatic processing leading to the formation of Man5 GlcNAc2-Asn (Péanne et al. 2018; Wilson and Matthijs 2021). Glycans, which are to achieve a more complex structure, undergo further processing. N-acetylglucosamine residues are added, one for each arm of the glycan. The two antennae are then lengthened by the addition of galactose and sialic acid residues to form the structure Sia2Gal2GLcNAc2Man3GLcNAc2 (Wilson and Matthijs 2021). Fucosyltransferase eight attaches a fucose residue to some of the glycoproteins. This produces several types of glycans, whose structure is based on a common core consisting of two N-acetylglucosamine and three mannose residues. The glycoproteins are then transported from the Golgi apparatus to their target sites. Defects associated with processes occurring during terminal glycosylation are referred to as type II glycosylation disorders.

O-glycosylation is a less complicated process leading to the formation of an O-glycosidic bond. It takes place mainly within the Golgi apparatus. The hydroxyl group of threonine or serine (sometimes also proline) of the polypeptide chain attaches to N-acetylgalactosamine (GalNAc) (e.g. in mucin-type O-glycans) or mannose to form the primary O-glycan structure (Jaeken and Peanne 2017). This core can be modified by the addition of galactose, N-acetylglucosamine, sialic acid or fucose. There are three main types of O-glycoproteins:

I-mucin-type glycans,

II-glycans containing chains of glycosaminoglycans (PGs), formed by O-xylosylation, and

III-alphadystroglycans ( $\alpha$ DGs) containing O-mannosylated structures.

In patients with CDG, the fractions characterized by the absence of whole glycans are named type I or if their structure is abnormal due to the lack of terminal sugars and sialic acid as type II. Defects in which both the lack of whole glycans and abnormalities of their structure are diagnosed are referred to as CDG I/II. In the first type of congenital glycosylation disorders, the percentage of disialotransferrin and asialotransferrin increases. In type II, an increase (of different intensity) of asialo-, monosialo-, disialo- and trisialotransferrin fractions is observed. In type I/II, the percentage of asialo-, monosialo-, and disialotransferrin is elevated. Tetrasialotransferrin decreases in each type.

## 2.1 *N-Linked Glycosylation Defects*

The N-glycosylation process is more complex than that of O-glycosylation. The complete loss of N-glycosylation is lethal. Synthesis of N-linked glycan is possible due to around 700 glycosyltransferases and glycosidases (Schiff et al. 2017; Conroy et al. 2021; Mohanty et al. 2020). N-linked glycosylated proteins are essential for brain maturation, as well as differentiation and maturation of different organs and systems (Conroy et al. 2021; Mohanty et al. 2020). Table 8.1 presents N-glycosylation defects and associated neurological symptoms (Francisco et al. 2019; Jaeken and Peanne 2017; Ferreira et al. 2018). The main abnormalities observed during neurological examination include: cerebellar ataxia, central hypotonia, proximal muscle weakness (Francisco et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018; Ondruskova et al. 2020; Lipiński and Tyłki-Szymańska 2021; Gardeitchik et al. 2018; Quelhas et al. 2021; Bogdańska et al. 2021; Wilson and Matthijs 2021). Developmental delay/intellectual disability, peripheral neuropathy and stroke-like episodes (SLE), and epilepsy complete the clinical picture (Francisco et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018; Ondruskova et al. 2020; Lipiński and Tyłki-Szymańska 2021; Gardeitchik et al. 2018; Quelhas et al. 2021; Bogdańska et al. 2021; Wilson and Matthijs 2021).

PMM2-CDG, the most frequent disorder of N-glycosylation, may manifest with specific craniofacial dysmorphism: microcephaly, prominent forehead, flat nasal bridge, thin upper lip and large ears, abnormal fat distribution, and inverted nipples. Schiff et al. (2017) described the detailed percentage incidence of particular neurological symptoms associated with a PMM2-CDG disorder as follows:

- Developmental delay/intellectual disability (90–96%)
- Ataxia/cerebellar syndrome (96%)
- Cerebellar atrophy (95%)
- Hypotonia with frequent hyporeflexia (92%)
- Strabismus (84%)
- Epilepsy (11–12%), abnormal EEG findings (69%)
- Peripheral neuropathy (53%)
- Retinitis pigmentosa (22%)
- Nystagmus (9.5%)
- Stroke-like episodes (SLE): 89.3% – patients under 10 years of age, 68% at least one incident (provoking factors: fever, head injury).

SLE are characterized by disturbances of consciousness, focal neurological deficits (mono- or hemiparesis, dysphagia, transient visual loss), epileptic seizures, and in some patients features of severe encephalopathy (Schiff et al. 2017; Izquierdo-Serra et al. 2018; El-Hattab et al. 2015). The term SLE was created for mitochondrial disorders and pathognomonic for MELAS syndrome (mitochondrial encephalopathy, lactic acidosis, stroke-like episodes). By definition SLE do not follow corresponding vascular brain territories or vascular occlusion on MRI and usually involve temporal, parietal and occipital lobes, less frequently subcortical white matter

**Table 8.1** N-glycosylation defects with dominant neurological presentation (Francisco et al. 2019; Jaeken and Peanne 2017; Ferreira et al. 2018)

CDG, OMM	Gene	Inheritance	Affected enzyme/protein	Clinical picture <sup>a</sup>	Diagnostics
Interconversion of monosaccharides					
PMM2-CDG, 601785	<i>PMM2</i>	AR	Phosphomannomutase	DD/ID, hypotonia, cerebellar hypoplasia, cardiomyopathy, hepatopathy	TIEF, type I
N-glycan lipid-linked oligosaccharide (LLO) assembly					
DPAGT1-CDG, 191350	<i>DPAGT1</i>	AR	GlcNAc-1-P transferase	Congenital myasthenic syndrome	TIEF, type I
ALG13-CDG, 300776	<i>ALG13</i>	X-linked	UDP-GlcNAc transferase	EEIE36 in females, DD/ID	TIEF, type I
ALG14-CDG, 612866	<i>ALG14</i>	AR	UDP-GlcNAc transferase	Congenital myasthenic syndrome, DD	TIEF, type I
ALG1-CDG, 605907	<i>ALG1</i>	AR	$\beta$ 1–4 Man-transferase	DD, hypotonia, dysmorphism, cardiomyopathy	TIEF, type I
ALG2-CDG, 607905	<i>ALG2</i>	AR	$\alpha$ 1–3/6 Man-transferase	Congenital myasthenic syndrome, DD	TIEF, type I
ALG11-CDG, 613666	<i>ALG11</i>	AR	$\alpha$ 1–2 Man-transferase	DD/ID, epilepsy, hearing loss	TIEF, type I
RFT1-CDG, 611908	<i>RFT1</i>	AR	Man5GlcNAc2-PP-Dol flippase	DD/ID, epilepsy, hearing loss	TIEF, type I
ALG6-CDG, 604566	<i>ALG6</i>	AR	$\alpha$ 1–3 Glc-transferase	ID, hypotonia, ataxia, epilepsy, proximal muscle weakness, chronic diarrhoea	TIEF, type I
ALG8-CDG, 608103	<i>ALG8</i>	AR	$\alpha$ 1–3 Glc-transferase	ID, dysmorphism, chronic diarrhoea	TIEF, type I
Glycan transfer to nascent protein					
TUSC3-CDG, 601385	<i>TUSC3</i>	AR	OST subunit	Isolated ID	TIEF, type I
DDOST-CDG, 614507	<i>DDOST</i>	AR	OST subunit	DD, hypotonia, hepatopathy	TIEF, type I

(continued)



**Table 8.1** (continued)

CDG, OMIM	Gene	Inheritance	Affected enzyme/protein	Clinical picture <sup>a</sup>	Diagnostics
SST3A-CDG, 601134	<i>SST3A</i>	AR	OST subunit	DD/ID, hypotonia, epilepsy	TIEF, type I
SST3B-CDG, 608605	<i>SST3B</i>	AR	OST subunit	DD, microcephaly, hypotonia, epilepsy	TIEF, type I
SSR3-CDG, 606213	<i>SSR3</i>	AR	Translocon-associated protein, gamma subunit	Severe ID, hearing loss	TIEF, type I, only disialotransferrin
SSR4-CDG, 300090	<i>SSR4</i>	X-linked recessive	Translocon-associated protein, delta subunit	ID, hypotonia, microcephaly, strabismus, epilepsy, dysmorphism, gastrointestinal problems	TIEF, type I
N-glycan processing					
MAN1B1-CDG	<i>MAN1B1</i>	AR	$\alpha$ 1–2 mannosidase I	ID, dysmorphism, abdominal obesity	TIEF, type II
MGAT2-CDG	<i>MGAT2</i>	AR	$\beta$ 1–2 GlcNAc-transferase II	ID, dysmorphism, growth retardation, sometimes no speech	TIEF, type II
B4GALT1-CDG	<i>B4GALT1</i>	AR	$\beta$ 1–4 Gal-transferase	Macrocephaly (hydrocephalus), hypotonia, myopathy, dysmorphism, coagulation problems, myopia	TIEF, type II

<sup>a</sup>DD developmental delay, EEIE early infantile epileptic encephalopathy, ID intellectual disability

(Schiff et al. 2017; Izquierdo-Serra et al. 2018; El-Hattab et al. 2015). SLE typical for PMM2-CDG may resemble familial hemiplegic migraine (FHM) episodes related to *CACNA1A*, *ATPIA2*, *SCN1A* mutations (Izquierdo-Serra et al. 2018).

## 2.2 O-Linked Glycosylation Defects

Over the last two decades more than 30% of patients with congenital disorders of glycosylation have been identified with O-linked mutations (van Tol et al. 2019). O-glycosylation disorders show a high range of phenotypic variability. In contrast to N-glycosylation defects they lack the special biochemical markers/screening

tests. The patients with O-glycosylation disorders generally show the tissue-specific target and the types with neurological presentations are given in Table 8.2 (Francisco et al. 2019; Jaeken and Peanne 2017; Ferreira et al. 2018; van Tol et al. 2019).

Among O-glycosylation disorders are ones affecting O-fucosylation and O-galactosylation, that do not present with neurological problems.

### **2.3 Disorders of Glycosylphosphatidylinositol (GPI) Anchor Biosynthesis**

The GPI pathway consists of three steps: biosynthesis of the GPI anchor, attachment of protein to the GPI anchor catalyzed by transaminases complexes (PIG-K, hGAA1, PIG-S, PIG-T, and PIG-U) followed by remodelling of the GPI anchored proteins (Table 8.3) (Francisco et al. 2019; Jaeken and Peanne 2017; Ferreira et al. 2018; Wu et al. 2020; Knaus et al. 2018).

The spectrum of symptoms of errors in glycosylphosphatidylinositol anchor biosynthesis include encephalopathy, delayed motor development, developmental delay/intellectual disability (DD/ID), visual impairment, hypotonia, hypertonia/spasticity, dystonia, dyskinesia, seizures, cerebellar atrophy, nystagmus, strabismus, dysphagia, and dysarthria (Wu et al. 2020; Knaus et al. 2018). The phenotype caused by PIGA-CDG, PIGN-CDG, and PIGT-CDG is much more severe than those of the other GPIBDs.

Seizures described in GPI patients present with a dominance of focal onset and motor semiology. The two most frequent types of focal motor seizures are myoclonic and tonic (Paprocka et al. 2022). The most commonly used antiepileptic drugs are valproic acid, levetiracetam and topiramate (Wu et al. 2020; Knaus et al. 2018; Paprocka et al. 2022).

### **2.4 Disorders of Glycosphingolipid (GSL) Glycosylation**

The best known defects in the biosynthesis of gangliosides are those associated with a deficiency in activity of GM3 synthase encoded by the *ST3GALT5* gene and defects in activity of GM2/GD2/GA2 synthase encoded by the *B4GALNT1* gene (Table 8.4) (Francisco et al. 2019; Jaeken and Peanne 2017; Ferreira et al. 2018). In children with a deficiency of GM3 synthase activity seizures (tonic-clonic, myoclonic, polymorphic seizures) start within first year of life. Salt and pepper syndrome represents a severe intellectual disability, hyper- and hypo-pigmented skin maculae at various locations, dysmorphic facial features, scoliosis, choreoathetosis, spasticity and abnormalities on EKG (Wilson and Matthijs 2021; Reily et al. 2019). The neurological picture of B4GALNT1-CDG (four pediatric patients) includes hydrocephalus, Dandy-Walker malformation, hypotonia, and increased creatine kinase levels (Wilson and Matthijs 2021; Reily et al. 2019).

**Table 8.2** O-glycosylation defects with dominant neurological presentation (Francisco et al. 2019; Jaeken and Peanne 2017; Ferreira et al. 2018)

CDG, OMIM	Gene	Inheritance	Affected Enzyme/protein	Clinical picture <sup>a</sup>	Diagnostics
<b>O-GalNAc</b>					
GALNT2-CDG, 602274	<i>GALNT2</i> , 1q41	AR	Polypeptide GalNAc transferase	Developmental delay, epilepsy, chronic insomnia, white matter lesions	Genetics, ApoC-III IEF
<b>O-GlcNAc</b>					
OGT-CDG, 300255	<i>OGT</i> , Xq13.1	X-linked	O-GlcNAc transferase	X-linked mental retardation	Genetics
<b>O-glycosylation</b>					
POGLUT1-CDG, 615618	<i>POGLUT1</i> , 3q13.33	AR	O-glucosyl transferase	Limb-girdle muscle dystrophy	Genetics
<b>O-Man glycosylation</b>					
ISPD-CDG, 614631	<i>ISPD</i> , chromosome 7p21.2	AR	CDP-ribitol synthase	Walker-Warburg syndrome, muscle eye brain disease, limb-girdle muscle dystrophy, MDDGA7, MDDGC7	Genetics, II6/VIA4-Ion muscle biopsy
POMT1-CDG, 607423	<i>POMT1</i> , 9q34.13	AR	Protein O-mannosyltransferase	Walker-Warburg syndrome, muscle eye brain disease, limb-girdle muscle dystrophy, MDDGAI, MDDGB1, MDDGC1	Genetics, II6/VIA4-Ion muscle biopsy
POMT2-CDG, 607439	<i>POMT2</i> , 14q24.3	AR	Protein O-mannosyltransferase	Walker-Warburg syndrome, muscle eye brain disease, limb-girdle muscle dystrophy MDDGA2, MDDGB2, MDDGC2	Mannosyltransferase activity assay
POMK-CDG, 615247	<i>POMK</i> , 8p11.21	AR	Protein O-mannosyl kinase	Walker-Warburg syndrome, muscle eye brain disease, limb-girdle muscle dystrophy	Genetics, II6/VIA4-Ion muscle biopsy
POMGNT2-CDG, 614828	<i>POMGNT2</i> , 3p22.1	AR	O-mannose $\beta$ -1,4-GlcNAc transferase	Walker-Warburg syndrome	Genetics, II6/VIA4-Ion muscle biopsy
B3GALNT2-CDG, 610194	<i>B3GALNT2</i> , 1q42.3	AR	$\beta$ 1-3 GalNAc-transferase II	Walker-Warburg syndrome, muscle eye brain disease, intellectual disability	Genetics, II6/VIA4-Ion muscle biopsy

(continued)

Table 8.2 (continued)

CDG, OMIM	Gene	Inheritance	Affected Enzyme/protein	Clinical picture <sup>a</sup>	Diagnostics
FKTN-CDG, 607440	<i>FKTN</i> , 9q31.2	AR	Fukutin	Walker-Warburg syndrome, muscle eye brain disease, limb-girdle muscle dystrophy, Fukuyama congenital muscular dystrophy	Genetics, II6/VIA4-Ion muscle biopsy
FKRP-CDG, 606596	<i>FKRP</i> , 19q13.32	AR	Rbo5P-1 Rbo5P transferase	Walker-Warburg syndrome, muscle eye brain disease, limb-girdle muscle dystrophy	Genetics, II6/VIA4-Ion muscle biopsy
TMEM5-CDG, 605862	<i>TMEM5</i> , 12q14.2	AR	$\beta$ 1-4 xylosyltransferase	Walker-Warburg syndrome, muscle eye brain disease	Genetics, II6/VIA4-Ion muscle biopsy
B4GAT1-CDG, 605517	<i>B4GAT1</i> , 11q13.2	AR	$\beta$ -1,3 glucuronyltransferase I	Walker-Warburg syndrome	Genetics, II6/VIA4-Ion muscle biopsy
LARGE1-CDG, 603590	<i>LARGE1</i> , 22q12.3	AR	$\beta$ 1-3 GlcA-transferase/ $\alpha$ 1-3 Xyl-transferase	Walker-Warburg syndrome, muscle eye brain disease, limb-girdle muscle dystrophy	Genetics, II6/VIA4-Ion muscle biopsy
POMGNT1-CDG, 606822	<i>POMGNT1</i> , 1p34.1	AR	$\beta$ 1-2 GlcNAc-transferase	Walker-Warburg syndrome, muscle eye brain disease, limb-girdle muscle dystrophy	Genetics, II6/VIA4-Ion muscle biopsy
TMTC3-CDG, 617218	<i>TMTC3</i> , 12q21.32	AR	Putative O-mannosyl-transferase	Cobblestone lissencephaly, periventricular nodular heterotopia	Genetics

<sup>a</sup>*MDDGAI* muscular dystrophy-dystroglycanopathy (congenital with brain, eye anomalies), type A, *MDDGBI* muscular dystrophy-dystroglycanopathy (congenital with mental retardation), type B, *MDDGCI* muscular dystrophy-dystroglycanopathy (limb-girdle), type C

**Table 8.3** Disorders of glycosylphosphatidylinositol anchor biosynthesis and associated neurological symptoms (Francisco et al. 2019; Jaeken and Peanne 2017; Ferreira et al. 2018; Wu et al. 2020; Knaus et al. 2018)

CDG, OMIM	Gene	Inheritance	Affected enzyme/protein	Clinical picture <sup>a</sup>	Diagnostics, markers
PIGA-CDG, 311770	<i>PIGA</i>	X-linked	GlcNAc-transferase complex, catalytic subunit	MCAHS2, CCS, EIEE	CD14, CD24, CD48, CD 58, CD59, CD109
PIGC-CDG	<i>PIGC</i>	AR	GlcNAc-transferase complex	GPIBD16	CD16, CD55, FLAER
PIGQ-CDG	<i>PIGQ</i>	AR	GlcNAc-transferase complex	EIEE, GPIBD	–
PIGP-CDG	<i>PIGP</i>	AR	GlcNAc-transferase complex	EIEE55, GPIBD9	CD16, CD39, CD55, CD87, FLAER
PIGY-CDG	<i>PIGY</i>	AR	GlcNAc-transferase complex	HPMRS6	CD55, CD59
PIGL-CDG	<i>PIGL</i>	AR	GlcNAc-PI de-N-acetylase	HPMRS, CHIME	CD16, CD24, CD59, DAF, FLAER
PIGW-CDG	<i>PIGW</i>	AR	Inositol acyltransferase	HPMRS5	CD16, CD24, FLAER, DAF, uPAR
PIGM-CDG	<i>PIGM</i>	AR	Man-transferase 1	GPIBD1	CD24, CD59, FLAER
PIGV-CDG	<i>PIGV</i>	AR	Man-transferase 2	HPMRS1	CD16, FLAER
PIGN-CDG	<i>PIGN</i>	AR	EtNP-transferase 1	MCAHS1, FS, EIEE	CD16, CD24, CD59
PIGO-CDG	<i>PIGO</i>	AR	EtNP-transferase 3	HPMRS2	CD16, CD24, CD59, FLAER, uPAR
PIGG-CDG	<i>PIGG</i>	AR	EtNP-transferase 2	GPIBD15	CD16, CD24, CD48, CD55, CD59, DAF, FLAER
PIGT-CDG	<i>PIGT</i>	AR	GPI transamidase	MCAHS3	CD14, CD16, CD24, CD48, CD59
PIGS-CDG	<i>PIGS</i>	AR	Glycosylphosphatidylinositol class S deficiency,	GPIBD18	CD16, CD55, CD59, FLAER
PIGH-CDG	<i>PIGH</i>	AR	GPI mannosyltransferase 1	GPIBD17	CD16, CD55, CD59, DAF, uPAR, FLAER
GPAA1-CDG	<i>GPAA1</i>	AR	GPI anchor attachment protein 1	GPIBD15	CD16, FLAER
PGAP1-CDG	<i>PGAP1</i>	AR	Inositol deacylase	GPIBD9	Cd48, CD59, FAF, uPAR

(continued)

**Table 8.3** (continued)

CDG, OMIM	Gene	Inheritance	Affected enzyme/protein	Clinical picture <sup>a</sup>	Diagnostics, markers
PGAP3-CDG	<i>PGAP3</i>	AR	Phospholipase A2	HPMRS4	CD16, CD59, FLAER
PGAP2-CDG	<i>PGAP2</i>	AR	GPI-anchor remodeling protein	HPMRS3	CD14, CD24, CD59, CD157, DAF

<sup>a</sup>*CCS* cerebro-cutaneous syndrome, *CD* cluster of differentiation, *CHIME* Mabry syndrome: colobomas, cardiac defects, ichthyosiform dermatosis, intellectual disability, conductive hearing loss, epilepsy, *DAF* complement decay-accelerating factor, *EIEE* early infantile epileptic encephalopathy, *FLAER* flow cytometry using proaerolysin variant, *FS* Fryns syndrome (diaphragmatic defect, characteristic facial appearance, distal digital hypoplasia, pulmonary hypoplasia, and at least one characteristic associated anomaly: polyhydramnios, cloudy corneas and/or microphthalmia, orofacial clefting, renal dysplasia/renal cortical cysts, malformations involving the brain, cardiovascular system, gastrointestinal system, genitalia), *GPIBD* glycosylphosphatidylinositol biosynthesis defect, *HPMRS* hyperphosphatase with mental retardation syndrome, *MCAHS* multiple congenital anomalies-hypotonia-seizures syndrome, *uPAR* urokinase-type plasminogen activator receptor

**Table 8.4** Disorders of glycolipid glycosylation with dominant neurological presentation (Francisco et al. 2019; Jaeken and Peanne 2017; Ferreira et al. 2018)

CDG, OMIM	Gene	Inheritance	Affected enzyme/protein	Clinical picture	Diagnostics
ST3GAL5-CDG, 604402	<i>ST3GAL5</i>	AR	$\alpha$ 2–3 Sia-transferase	Amish infantile epilepsy syndrome (salt and pepper syndrome)	Genetics
B4GALNT1-CDG, 607091	<i>B4GALNT1</i>	AR	$\beta$ 1–4 GalNAc-transferase	Spastic paraplegia, transient cholestatic syndrome, hepatopathy, pulmonary symptoms, coagulation abnormalities	Genetics

## 2.5 Disorders of Multiple Glycosylation Pathways

As in other CDG subtypes a large majority of disorders of multiple glycosylation pathways have neurological involvement. Disorders of this group with neurological involvement are described in Table 8.5 (Francisco et al. 2019; Jaeken and Peanne 2017; Ferreira et al. 2018).

Proteins in the COG complex are involved in neurotransmitter release, receptor binding and uptake, affect the localization and functioning of copper transporters like ATP7A and SLC31A1, and play a role in the synthesis of glycosphingolipids. From the neurological perspective, among all of the COG-CDG the most severe types are mutations affecting COG7-CDG and COG6-CDG (D-Souza et al. 2020). The neurological presentations seen in COG-CDG are (Wilson and Matthijs 2021; D-Souza et al. 2020):

**Table 8.5** Disorders of multiple glycosylation pathways with dominant neurological presentation (Francisco et al. 2019; Jaeken and Peanme 2017; Ferreira et al. 2018)

CDG, OMIM	Gene	Inheritance	Affected enzyme/protein	Clinical picture	Diagnostics
Disorders of multiple pathways					
Monosaccharide synthesis					
GFPT1-CDG, 138292	<i>GFPT1</i>	AR	Glutamine:FoP amidotransferase	Congenital myasthenic syndrome	Genetics TIE normal
GN-CDG, 603824	<i>GN</i>	AR, AD	UDP-GlcNAc 2-epimerase/ ManNAc kinase	Myopathy	Genetics
NANS-CDG, 605202	<i>NANS</i>	AR	N-acetylneuraminic acid-9-phosphate synthase	DD/ID <sup>a</sup>	Genetics
Monosaccharide interconversion					
PGM1-CDG, 612934	<i>PGM1</i>	AR	Phosphoglucomutase	Myopathy, cardiomyopathy, Pierre-Robine sequence	Genetics
Dolichol biosynthesis					
NUS1-CDG, 610463	<i>NUS1</i>	AR	Cis-isoprenyl transferase subunit	ID, refractory epilepsy, hearing problems, macular atrophy	Genetics
SRD5A3-CDG, 611715	<i>SRD5A3</i>	AR	Polyprenol reductase	ID, ataxia, visual problems: cataract, visual loss, glaucoma, ichthyosis	Genetics
DOLK-CDG, 610746	<i>DOLK</i>	AR	Dolichol kinase	Multisystemic presentation (neurological and endocrinological), dilated cardiomyopathy, ichthyosis	Genetics
Dolichol-P-sugar biosynthesis and utilization					
DPM1-CDG, 603503	<i>DPM1</i>	AR	Dol-P-Man synthase	Cardiomyopathy, muscular dystrophy	Genetics
DPM2-CDG, 603564	<i>DPM2</i>	AR	Dol-P-Man synthase	Cardiomyopathy, muscular dystrophy	Genetics
DPM3-CDG, 605951	<i>DPM3</i>	AR	Dol-P-Man synthase	Cardiomyopathy, muscular dystrophy	Genetics
Nucleotide-sugar synthesis					

(continued)

Table 8.5 (continued)

	Gene	Inheritance	Affected enzyme/protein	Clinical picture	Diagnostics
CDG, OMIM					
CAD-CDG, 114010	<i>CAD</i>	AR	Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	EEIE50	Genetics
GMPPA-CDG, 615495	<i>GMPPA</i>	AR	GDP-mannose pyrophosphorylase $\alpha$ subunit	Alacrima, achalasia and mental retardation syndrome	Genetics
GMPPB-CDG, 615320	<i>GMPPB</i>	AR	GDP-mannose pyrophosphorylase $\beta$ subunit	MDDGA14, MDDGB14, MDDGC14	Genetics
Transporters					
SLC35A1-CDG, 605634	<i>SLC35A1</i>	AR	CMP-Sia transport	DD, epilepsy, ataxia, thrombocytopenia, bleedings, frequent infections	TIEF, type II, genetics
SLC35A2-CDG, 314375	<i>SLC35A2</i>	AR	UDP-Galactose transport	EIEE22, hipotonia, congenital brain abnormalities, dysmorfy	TIEF, type II, genetics
SLC35A3-CDG, 605632	<i>SLC35A3</i>	AR	UDP-GlcNAc transport	ID, epilepsy, arthrogyposis	TIEF, type II,
SLC39A8-CDG, 608732	<i>SLC39A8</i>	AR	Cation transporter	Epilepsy, skeletal dysplasia, rhizomyelic limb shortenings, short stature, low blood and high urine manganese and zinc concentration	TIEF, type II, genetics
Vesicular trafficking					
COG1-CDG, 606973	<i>COG1</i>	AR	Conserved oligomeric Golgi complex subunit 1	DD, hypotonia, microcephaly, cerebellar atrophy	TIEF, type II
COG2-CDG, 606974	<i>COG2</i>	AR	Conserved oligomeric Golgi complex subunit 2	DD, hypotonia, microcephaly, spasticity, low blood ceruloplasmin level, hepatopathy	TIEF, type II
COG4-CDG, 606976	<i>COG4</i>	AR	Conserved oligomeric Golgi complex subunit 4	DD, dysmorfy, axial hipotonia, peripheral hypertonia, recurrent infections	TIEF, type II
COG6-CDG, 606977	<i>COG6</i>	AR	Conserved oligomeric Golgi complex subunit 6	Refractory epilepsy, DD, microcephaly, hepatomegaly, cirrhosis, immunodeficiency	TIEF, type II
COG7-CDG, 606978	<i>COG7</i>	AR	Conserved oligomeric Golgi complex subunit 7	Microcephaly, VSD, hyperthermia, cutis laxa, dysmorfy	TIEF, II type
COG8-CDG, 606979	<i>COG8</i>	AR	Conserved oligomeric Golgi complex subunit 8	ID, hipotonia, cerebellar hypoplasia, dysmorfy	TIEF, II type



CDG, OMIM	Gene	Inheritance	Affected enzyme/protein	Clinical picture	Diagnostics
COP2-CDG 606990	<i>COPB2</i>	AR	COP-I subunit beta-2	Congenital microcephaly	Genetics
ARCNI-CDG	<i>ARCNI</i>	AD	COP-I subunit delta	Microcephaly, short stature, DD, micrognathia	Genetics
TRAPPC6B-CDG, 610397	<i>TRAPPC6B</i>	AR	Subunit of TRAPP tethering complex	Microcephaly, epilepsy, cerebral atrophy, ID	Genetics
TRAPPC9-CDG	<i>TRAPPC9</i>	AR	Subunit of TRAPP tethering complex	ID	Genetics
TRAPPC11- CDG	<i>TRAPPC11</i>	AR	Subunit of TRAPP tethering complex	LGMD	Genetics
TRAPPC12- CDG	<i>TRAPPC12</i>	AR	Subunit of TRAPP tethering complex	Muscular dystrophy, myopathy, ID, achalasia-Addison disease-alaetrimia	Genetics
Golgi homeostasis					
ATP6V1A-CDG, 607027	<i>ATP6V1A</i>	AR	Subunit of vacuolar ATPase	DD/ID, cutis laxa, cardiomyopathy, hipercholesterolemia	TIEF, II type
ATP6V1E1- CDG, 108746	<i>ATP6V1E1</i>	AR	Subunit of vacuolar ATPase	DD/ID, cutis laxa, cardiomyopathy, hipercholesterolemia	TIEF, II type
TMEM199- CDG, 616815	<i>TMEM199</i>	AR	Assembly factor for vacuolar ATPase	ID, liver diseases(cholestasis, cirrhosis, fibrosis), hipercholesterolemia, low ceruloplasmin level (similar to Wilson disease)	TIEF, II type
CCDC115- CDG, 613734	<i>CCDC115</i>	AR	Assembly factor for vacuolar ATPase	ID, liver diseases(cholestasis, cirrhosis, fibrosis), hipercholesterolemia, low ceruloplasmin level (similar to Wilson disease)	TIEF, II type
VMA21- CDG, 300913	<i>VMA21</i>	X-linked	Assembly factor for vacuolar ATPase	Myopathy, excessive autophagy	TIEF, II type
TMEM165- CDG, 614726	<i>TMEM165</i>	AR	Ca2+/H+ antiporter	ID, skeletal dysplasia, osteopenia, short stature, growth hormone deficiency	TIEF, II type, altered LAMP2

<sup>a</sup>ATP adenosine triphosphate, *ID/DD* intellectual disability/developmental delay, *EIEE* early infantile epileptic encephalopathy, *LAMP2* lysosomal associated membrane protein 2, *LGMD* limb-girdle muscular dystrophy, *MDDGA* muscular dystrophy –dystroglycanopathy, *TIEF* transferrin isoelectric focusing, *VSD* ventricular septal defect

- Developmental delay (COG1-CDG, COG4-CDG, COG5-CDG, COG6-CDG, COG8-CDG)
- Microcephaly (COG1-CDG, COG2-CDG, COG6-CDG, COG8-CDG)
- Seizures (COG2-CDG- tonic seizures, COG4-CDG, COG6-CDG- intractable focal seizures, COG8-CDG)
- Hipotonia (COG1-CDG, COG5-CDG, COG6-CDG, COG7-CDG)/hipertonia (COG2-CDG-spastic quadriplegia)
- Cerebellar (COG1-CDG, COG5-CDG)/cerebral atrophy (COG5-CDG, COG7-CDG) and
- Friedreich’s ataxia-like phenotype (COG5-CDG)

Major neurological presentation is also typically seen in people with errors in *subunit* 8 of the conserved *oligomeric Golgi complex* COG8-CDG. The clinical spectrum includes acute encephalopathy, hipotonia, cerebellar ataxia, oculomotor apraxia, chronic axonal neuropathy with dyssinergia oculocephalica, and pseudoptosis (D-Souza et al. 2020).

## 2.6 Recently Described CDG with Neurological Presentation

This section summarizes the newest CDG with usually complex phenotypes and follows the same system of categorization within each respective section (disorders of N-linked and O-linked glycosylation, disorders of glycosylphosphatidylinositol anchor biosynthesis GPIBDs)- see Tables 8.6, 8.7, 8.8, and 8.9 (Ondruskova et al. 2021; Wilson and Matthijs 2021; Ferreira et al. 2018).

## 3 Markers/Biochemical Abnormalities

First introduced in 1984, monitoring serum transferrin (Trf) levels by isoelectric focusing (TIEF) is considered to be the gold-standard method for CDG screening (Francisco et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018). In patients with CDG, fractions appear that are characterized by the absence of whole glycans (in CDG type I) or with glycans lacking terminal sugars and sialic acid (in CDG type II). Defects in which both the lack of whole glycans and abnormalities in their structure are diagnosed are referred to as CDGI/II. In the first type of congenital glycosylation disorders, the percentage of disialotransferrin and asialotransferrin increases. In type II, an increase (or different intensities) of asialo-, monosialo-, disialo- and trisialotransferrin fractions is observed. In type I/II, the percentage of asialo-, monosialo-, and disialotransferrin is elevated. Tetrasialotransferrin decreases in each type. In CDG, it shows typical patterns with increased disialo and asialo Trf in most CDG-I samples and of increased trisialo relative to monosialo Trf in most CDG-II samples (Ondruskova et al. 2021; Wilson

**Table 8.6** The newest disorders of N-linked glycosylation with neurological symptoms (Ondruskova et al. 2021; Wilson and Matthijs 2021; Ferreira et al. 2018)

CDG, OMIM	Gene	Inheritance	Affected enzyme/protein	Clinical picture	(TIEF)
FUT8-CDG, 618005	14q23.3	Autosomal recessive	alpha-1,6-fucosyltransferase	Multisystemic presentation: Intellectual disability, epilepsy, dysmorphism, skeletal abnormalities, IUGR, failure to thrive	Normal
MAGT1-CDG, #301031	Xq21.1	X-linked recessive	Magnesium transporter protein 1	Multisystemic presentation: Intellectual disability, immunodeficiency, liver disease	Type 1
MAN2B2-CDG	4p16.1	Autosomal recessive	Mannosidase alpha class 2B member 2	Multisystemic presentation: Severe coagulopathy, pancytopenia, stroke, immunodeficiency, chronic diarrhoea, failure to thrive, vasculitis, short stature	Normal
SSR3-CDG	3q25.31	Autosomal recessive	Signal sequence receptor subunit 3	Multisystemic presentation: Intellectual disability, dysmorphism, vomiting, diarrhoea, IUGR, failure to thrive, deafness, skeletal abnormalities, strabismus	Type 1

and Matthijs 2021). The altered glycosylation of Trf responsible for the variability of the isoform may be a pattern.

The early stages of glycosylation may not allow for quick and easy detection by isoelectric isofocusing and by the biomarker of some O-glycosylation disorders, apolipoprotein CIII level. It is also possible that proper glycosylation of specific proteins (i.e. transferrin or apolipoprotein CIII) does not exclude incorrect glycosylation of other proteins since glycosylation is not under direct genetic control. Table 8.10 emphasizes the pros and cons of selected CDG markers.

An altered glycosylation pattern of specific biomarkers may provide the key for identification of the glycosylation step altered and that, in turn, could allow identification of the specific mutation affecting the pathway. For O-xylosylation defects, a promising biomarker may be biconin (Bkn), a serum glycoprotein synthesized in the liver (B4GALT7-CDG, B3GALT6-CDG, B3GAT3-CDG, CHSY1-CDG). Simple structure of this proteoglycan is not subjected to important polymorphisms and the analysis of its level may be cost-effective and quick. In contrast, for defects in O-mannosylation, the study of alpha-dystroglycan found in muscle cells is used

**Table 8.7** The newest disorders of O-linked glycosylation with neurological symptoms (Ondruskova et al. 2021; Wilson and Matthijs 2021; Ferreira et al. 2018)

Disorders of O-xylosylation					
CDG, OMIM	Gene	Inheritance	Affected enzyme/protein	Clinical picture	TIEF
EXTL3-CDG, 617425	8p21.1	Autosomal recessive	Exostosin-likeglycosyltransferase 3	Intellectual disability, seizures, skeletal dysplasia, severe combined immunodeficiency disease (SCID)	Normal
Disorders of N-acetylgalactosamine (GalNAc) glycosylation					
GALNT2-CDG, 618885	1q42.13	Autosomal recessive	Polypeptide N-acetylgalactosamine transferase 2	Intellectual disability, epilepsy, autism, dysmorphism, insomnia	Variable
Disorders of N-acetylglucosamine (GlcNAc) glycosylation					
OGT-CDG, 300997	Xq13.1	Autosomal recessive	O-GlcNAc transferase subunit p110	Intellectual disability, dysmorphism, hypotonia, eye and hearing abnormalities, short stature, behavioral problems	NA

(Ondruskova et al. 2021; Wilson and Matthijs 2021). Serum apolipoprotein C-III (apoC-III, used for O-glycosylation disorders) IEF is abnormal in a very small number of serum samples from CDG patients and is usually performed after finding a type 2 serum TIEF to confirm a combined N- and O-glycosylation defect (Francisco et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018; Ondruskova et al. 2021; Lipiński and Tylki-Szymańska 2021; Gardeitchik et al. 2018; Quelhas et al. 2021; Bogdańska et al. 2021; Wilson and Matthijs 2021).

Hyperphosphatemia is a feature of GALNT3-CDG, while accumulation of serum hypogammaglobulinemia and urine tetrasaccharide (Glc-Glc-Glc-Man) are seen in MOGS-CDG (Francisco et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018; Ondruskova et al. 2021; Lipiński and Tylki-Szymańska 2021; Gardeitchik et al. 2018; Quelhas et al. 2021; Bogdańska et al. 2021; Wilson and Matthijs 2021).

For PIGA-CDG, PIGL-CDG, PIGN-CDG, PIGP-CDG, PIGQ-CDG, PIGW-CDG, PIGY-CDG and PGAP1-CDG, data about serum ALP elevation are less clear and additional patients' need to be studied [5,10]. Other biomarkers for identifying deficiencies in GPI biosynthesis are GPI itself and GPI-anchored proteins expressed at the surface of cells such as CD16, CD24, and CD55 (or DAF –decay accelerating factor), CD59. A recently described monoclonal antibody, T5-4E10mAb (recognizes unlinked GPIs that have a GalNAc side chain linked to the first mannose at the nonreducing terminus), may become another biomarker of GPI anchor defects (reference?).

**Table 8.8** The newest disorders of glycosylphosphatidylinositol anchor biosynthesis GPIBDs with neurological symptoms (Ondruskova et al. 2021; Wilson and Matthijs 2021; Ferreira et al. 2018)

CDG, OMIM	Gene	Inheritance	Affected enzyme/protein	Clinical picture	TIEF
GPAAI-CDG, 617810	8q24.3	Autosomal recessive	Phosphatidylinositol glycan anchor attachment protein 1	Intellectual disability, refractory epilepsy, dysmorphism, hypotonia, cerebellar syndrome, osteopenia	Normal
PIGB-CDG, 618580	15q21.3	Autosomal recessive	Phosphatidylinositol glycan anchor biosynthesis class B protein	Intellectual disability, refractory epilepsy, hypotonia, visual and hearing impairment, skeletal abnormalities (hands, feet), dysmorphism	Normal
PIGH-CDG, 618010	14q24.1	Autosomal recessive	Phosphatidylinositol glycan anchor biosynthesis class H protein	Intellectual disability, autism, epilepsy	Normal
PIGK-CDG, 618879	1p31.1	Autosomal recessive	Phosphatidylinositol glycan anchor biosynthesis class K protein	Intellectual disability, hypotonia, epilepsy, cerebellar atrophy, ataxia	Normal
PIGP-CDG, 617599	21q22.13	Autosomal recessive	Phosphatidylinositol glycan anchor biosynthesis class P protein	Intellectual disability, refractory epilepsy, cerebral atrophy, cortical visual impairment, dyskinesia, dysmorphism, feeding problems, recurrent infections of the respiratory system	Normal
PIGS-CDG, 618143	17q11.2	Autosomal recessive	Phosphatidylinositol glycan anchor biosynthesis class S protein	Intellectual disability, refractory epilepsy, hypotonia, cerebral/cerebellar atrophy, ataxia, visual impairment, dysmorphism,	Normal
PIGU-CDG, 618590	20q11.22	Autosomal recessive	Phosphatidylinositol glycan anchor biosynthesis class U protein	Intellectual disability, epilepsy, cerebral/cerebellar atrophy, strabismus, cortical blindness, dysmorphism, scoliosis	Normal

None of these biochemical CDG tests can screen for all CDG, and should not be used independently from each other but rather conjointly (done rarely) for getting deeper and more precise insight into biochemical mechanisms leading to the abnormal protein glycosylation and observed phenotype.

**Table 8.9** The newest disorders of multiple metabolic pathways with neurological symptoms (Ondruskova et al. 2021; Wilson and Matthijs 2021; Ferreira et al. 2018)

CDG, OMIM	Gene	Inheritance	Affected enzyme/protein	Clinical picture	TIEF
Disorder of monosaccharide synthesis and interconversion					
FCSK-CDG, 618324	16q22.1	Autosomal recessive	Fucose kinase	Intellectual disability, refractory epilepsy, hypotonia, congenital brain abnormalities, visual impairment, feeding difficulties, failure to thrive, recurrent infections of the respiratory tract	Normal
Disorders of Golgi pH and ion homeostasis					
ATP6AP2-CDG, 301045	Xp11.4	X-linked recessive	ATPase H(+) transporting lysosomal accessory protein 2	Intellectual disability, ataxia, cutis laxa, hepatosplenomegaly with cirrhosis and ascites, immunodeficiency, dysmorphism	Type 2
SLC9A7-CDG, 301024	Xp11.3	X-linked recessive	Solute carrier family 9 member 7	Intellectual disability, hipotonia, muscle weakness, hyperreflexia, dysmorphism	Normal
SLC10A7-CDG, 618363	4q31.22	Autosomal recessive	Solute carrier family 10 member 7	Intellectual disability, skeletal abnormalities: dysplasia, short stature, deformities, osteoporosis, visual and hearing impairment, IUGR, dysmorphism	Type 2
Disorders of vesicular trafficking					
GOSR2-CDG, 604027	17q21.32	Autosomal recessive	Golgi SNAP receptor complex member 2	Epilepsy, ataxia, myoclonia, nystagmus, progressive muscle weakness, areflexia, elevated CK, scoliosis	Normal

## 4 Congenital Brain Abnormalities

Congenital brain abnormalities most frequently observed in CDG are shown in Tables 8.11, 8.12, 8.13, and 8.14. (Schiller et al. 2020; van Dijk et al. 2018; Devisme et al. 2012; Chan et al. 2010; Barone et al. 2014; Feraco et al. 2012; Hedberg et al. 2014; Larsen et al. 2019). Taking into account that glycosylated proteins are crucial for brain development and functioning, the high frequency and diversity of brain abnormalities is understandable. Apart from cortical malformations, midline brain structure anomalies, brain volume anomalies, myelin disorders (ALG2-CDG-delayed, ALG9-CDG-delayed, ALG13-CDG, DDOST-CDG, DPM1-CDG-delayed, PGM3-CDG, PIGA-CDG, SLC35A2-CDG-delayed) and venous sinus thrombosis (PGM1-CDG, PMM2-CDG) have been described (Schiller et al. 2020; van Dijk et al. 2018; Devisme et al. 2012; Chan et al. 2010; Barone et al. 2014; Feraco et al. 2012; Hedberg et al. 2014; Larsen et al. 2019).

**Table 8.10** Biochemical markers and biochemical abnormalities frequently seen in CDG (Francisko et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018; Ondruskova et al. 2021, 2018; Bogdańska et al. 2021; Wilson and Matthijs 2021; Ruel et al. 2018)

Markers/ biochemical abnormalities	Pros	Cons
Transferrin IEF profile	Routine CDG screening for N-glycosylation defects (gold standard) Identification of type I and II abnormal transferrin IEF profile (IEF profile allows for identification 54 CDG subtypes: 29 with CDG I, 24 with CDG II, 1 with CDGI/II)	The normal IEF profile is seen in: DHDDS-CDG, FUT8-CDG, GANAB-CDG, GMPPA-CDG, GMPPB-CDG, GNE-CDG, MOGS-CDG, NANS-CDG, PGM3-CDG, PRKCSH-CDG, SEC23B-CDG, SLC35A1-CDG, SLC35A3-CDG, SLC35C1-CDG, TUSC3-CDG, also in case of O-glycosylation abnormalities. The so-called secondary glycosylation disorders: <ul style="list-style-type: none"> <li>• Fructosemia and galactosemia (the isoform pattern resembles that of type I CDG),</li> <li>• In alcohol abuse, with a pattern characteristic of type I.</li> </ul> In newborns, during the first weeks of life, a slight increase of cathodic fractions can be observed, mainly asialo-, monosialo- and sometimes disialotransferrin. The occurrence of a specific genetic variant of transferrin is the reason for the variability of the isoform pattern
Serum apoC-III IEF	Blood biomarker in core 1 mucin type O-glycosylation disorders (for example COG-CDG, ATP6VOA2-CDG, CCDC15-CDG, GALNT2-CDG)	Hypoglycosylation of apoC-III have been observed in glycogen storage disorders types I and III
Serum alkaline phosphatase (ALP)	Increased defects in the GPI anchor synthesis pathway: PIGL-CDG, PIGO-CDG, PIGV-CDG, PIGW-CDG, PIGY-CDG, PGAP2-CDG, PGAP3-CDG	Decreased in PIGT-CDG Normal serum ALP: PIGA, PIGN, PIGP, PIGQ, PIGW, PIGY and PGAP1 defects
Serum N-glycan profiling (ESI-QTOF-MS <sup>a</sup> )	Novel potentially characteristic polymannose changes in ALG3-CDG, STT3B-CDG, DDOST-CDG, SSR4-CDG	–

<sup>a</sup>ESI-QTOF-MS flow injection-electrospray ionization -quadrupole time- of -flight mass spectrometry

About 50% of patients with ATP6VOA2-CDG showed cortical malformations, especially pachygyria. Cerebellar hypoplasia is observed in ~83% of PMM2-CDG patients and in all SRD5A3-CDG patients. The midline malformations are frequent in PGM1-CDG (Schiller et al. 2020; van Dijk et al. 2018; Devisme et al. 2012; Chan et al. 2010; Barone et al. 2014; Feraco et al. 2012; Hedberg et al. 2014; Larsen et al. 2019).

**Table 8.11** Cortical malformations in CDG (Schiller et al. 2020; van Dijk et al. 2018; Devisme et al. 2012; Chan et al. 2010; Barone et al. 2014; Feraco et al. 2012; Hedberg et al. 2014; Larsen et al. 2019; Paprocka et al. 2021)

Cortical malformations			
Pachygyria	Polymicrogyria	Lissencephaly	Cortical dysplasia, heterotopia
ATP6V0A2 -CDG	B3GALNT2-CDG	B3GALNT2-CDG	ISPD-CDG
FKRP-CDG	POMGNT1-CDG	(cobblestone)	(heterotopia)
FKTN -CDG	POMT1-CDG	FKRP-CDG (cobblestone)	POMGNT1-CDG
LARGE -CDG	POMT2-CDG	FKTN-CDG	(dysplasia)
POMGNT1-CDG		ISPD-CDG (cobblestone)	POMT2-CDG
POMT1 -CDG		POMGNT1-CDG	(heterotopia)
POMT2-CDG		POMT1-CDG (cobblestone)	TMTC3-CDG
TMEM5 -CDG		POMT2-CDG (cobblestone)	
		TMEM5-CDG (cobblestone)	
		TMTC3-CDG (cobblestone)	

**Table 8.12** Midline brain structures anomalies in CDG (Schiller et al. 2020; van Dijk et al. 2018; Devisme et al. 2012; Chan et al. 2010; Barone et al. 2014; Feraco et al. 2012; Hedberg et al. 2014; Larsen et al. 2019; Paprocka et al. 2021)

Midline brain structures anomalies	
Corpus callosum (CC) anomalies	Pontocerebellar hypoplasia
ALG3 -CDG	B3GALNT2-CDG
ALG6-CDG	PMM2-CDG
COG2-CDG (thin CC)	
COG4-CDG (thin CC)	
FKRP-CDG	
FKTN-CDG	
ISPD-CDG (CC atrophy)	
MOGS-CDG (small CC)	
NANS-CDG/Spondyloepimetaphyseal dysplasia, Camera-Genevieve type	
PGAP1-CDG	
PIGA-CDG (thin CC)	
PIGG-CDG (thin CC)	
PIGP-CDG (thin CC)	
POMGNT1-CDG	
POMT1-CDG	
POMT2-CDG (CC aplasia)	
SLC35A2-CDG (thin CC)	
SSR4-CDG (thin CC)	
VPS13B-CDG Cohen syndrome	

## 5 Epilepsy

An interesting study performed by Silver et al. found an ~4.4% prevalence of CDG in children with epilepsy (Silver et al. 2021). They also found a significant correlation between abnormal liver enzymes, valproic acid therapy and abnormal TIEF results (Silver et al. 2021).



**Table 8.13** Brain volume anomalies in CDG (Schiller et al. 2020; van Dijk et al. 2018; Devisme et al. 2012; Chan et al. 2010; Barone et al. 2014; Feraco et al. 2012; Hedberg et al. 2014; Larsen et al. 2019; Paprocka et al. 2021)

Brain volume anomalies	
Brain atrophy	Ventriculomegaly and hydrocephalus
ALG1-CDG	ALG12 -CDG
ALG9-CDG	ALG13-CDG
B4GALNT1-CDG (cortical)	B3GALNT2-CDG (hydrocephalus)
B3GALTL-CDG	FKRP-CDG (both)
COG1-CDG (cortical)	FKTN -CDG (hydrocephalus)
COG2-CDG	ISPD-CDG (both)
COG4-CDG	NANS-CDG Camera-Genevieve type (hydrocephalus)
COG5 -CDG (cerebellum and cortex)	POMGNT1-CDG (both)
COG7-CDG	POMT2 -CDG (hydrocephalus)
COG8-CDG	
DPAGT1-CDG	
DPM1-CDG	
FKTN -CDG type A (cortical)	
MOGS-CDG	
NANS -CDG Camera-Genevieve type	
NUS1-CDG (cortical)	
PGAP2-CDG	
PIGG-CDG	
PIGN-CDG	
PIGT -CDG	
POMT2-CDG	
SLC35A2-CDG	
SLC35C1-CDG	
SLC39A8-CDG	
SRD5A3-CDG	
ST3GAL5-CDG (cortical)	
STT3A-CDG	
STT3B-CDG	
TMEM5-CDG	
TRAPPC11 -CDG	

The pathomechanism of epilepsy in CDG is complex due to diverse biochemical abnormalities. Hypotheses of epilepsy causes discussed by Freeze et al. (2015) include:

- A disturbed balance between excitatory and inhibitory neuronal activity (improper function of the voltage-gated ion-channels proteins within the cell membrane connected with a lack of N-glycans causing improper folding, shifted gating)
- Mutations in special transporters for example the X-linked UDP-Galactose transporter SLC35A2, in the UDP-GlcNAc transporter SLC35A3
- Decreased localized production of the protein-free GAG hyaluronan
- Deletions in glycoprotein neurexin1 (NRXN1)
- Congenital structural brain abnormalities most frequently connected with neuronal migration disorders (disturbed O-mannosylation of dystroglycans, different

**Table 8.14** Cerebellar atrophy and cerebellar hypoplasia in CDG (Schiller et al. 2020; van Dijk et al. 2018; Devisme et al. 2012; Chan et al. 2010; Barone et al. 2014; Feraco et al. 2012; Hedberg et al. 2014; Larsen et al. 2019; Paprocka et al. 2021)

Cerebellar atrophy	Cerebellar hypoplasia
ALG1-CDG	ALG1-CDG
ALG3-CDG	ALG3-CDG
ALG6-CDG	DPM2-CDG
ALG8-CDG	GMPPB-CDG
ALG9-CDG	ISPD-CDG
COG8-CDG	PGAP1-CDG
DPM1-CDG	PIGA-CDG
FKRP -CDG	PIGG-CDG
FKTN -CDG	PIGT -CDG
PIGN -CDG	PMM2-CDG
PMM2-CDG	POMGNT1-CDG
SLC35A2-CDG	POMT1 -CDG
SLC39A8-CDG	POMT2 -CDG
SRD5A3-CDG	VPS13B-CDG
STT3A-CDG	
STT3B-CDG	
TRAPPC11-CDG	

proteins like cadherin, abolishing polysialic acid, which presents on N-glycans in NCAM1).

Based on literature data defined and clearly characterized epilepsy connected with CDG may be divided into: epileptic spasms/West syndrome, early myoclonic encephalopathy of infancy, Ohtahara syndrome, epilepsy of infancy with migrating focal seizures.

**Epileptic spasms and West syndrome** (Fiumara et al. 2017; Pereira et al. 2017; Huo et al. 2020; Barone et al. 2012; Morava et al. 2016; de Koning et al. 1998; Kranz et al. 2007; Rind et al. 2010; Allen et al. 2013; Helander et al. 2013; Wu et al. 2003; Schenk et al. 2001; Edvardson et al. 2013a, b; Kodera et al. 2013; Aeby et al. 2016; Kato et al. 2014; Chiyonobu et al. 2014; Maydan et al. 2011; Simpson et al. 2004):

- ALG13- CDG, early infantile epileptic encephalopathy-36 (EIEE36),
- DOLK -CDG,
- DPAGT1-CDG,
- SLC35A2-CDG, early infantile epileptic encephalopathy-22 (EIEE22),
- ST3GAL3-CDG, early infantile epileptic encephalopathy-15 (EIEE15),
- ST3GAL5-CDG, salt and pepper developmental regression syndrome,
- ST3GAL5-CDG,
- PIGA-CDG,
- PIGW-CDG,
- PIGN-CDG,
- ALG1-CDG,
- ALG3-CDG,

- MPDU1-CDG,
- RFT1-CDG

**Early myoclonic encephalopathy of infancy (EMEI)** (Fiumara et al. 2017; Pereira et al. 2017; Huo et al. 2020; Barone et al. 2012; Morava et al. 2016; de Koning et al. 1998; Kranz et al. 2007; Rind et al. 2010; Allen et al. 2013; Helander et al. 2013; Wu et al. 2003; Schenk et al. 2001; Edvardson et al. 2013b; Kodera et al. 2013; Aeby et al. 2016; Kato et al. 2014; Chiyonobu et al. 2014; Maydan et al. 2011; Simpson et al. 2004):

- PIGA-CDG,
- ALG3-CDG,
- ALG6-CDG,
- DPM2-CDG,
- ALG1-CDG

**Ohtahara syndrome** (Aeby et al. 2016; Martin et al. 2014; Johnston et al. 2012):

- PIGA-CDG,
- PIGQ-CDG (EIEE77)

**Epilepsy of infancy with migrating focal seizures** (Barba et al. 2016)

- ALG3-CDG,
- ALG1-CDG,
- RT1-CDG

Difficult to control epileptic seizures are also seen in DPM1-CDG, ALG2-CDG, ALG8-CDG, ALG9-CDG, ALG11-CDG, ALG12-CDG, and RTF1-CDG [Bogdańska et al. 2021; Freeze et al. 2015; Fiumara et al. 2017; Pereira et al. 2017; Huo et al. 2020; Barone et al. 2012; Morava et al. 2016; de Koning et al. 1998; Kranz et al. 2007; Rind et al. 2010; Allen et al. 2013; Helander et al. 2013; Wu et al. 2003; Schenk et al. 2001; Edvardson et al. 2013a, b; Kodera et al. 2013; Aeby et al. 2016; Kato et al. 2014; Chiyonobu et al. 2014; Maydan et al. 2011; Simpson et al. 2004). Increased risk of hypoglycemia in CDG may limit treatment with a ketogenic diet (KD) although in many CGD subtypes eating a KD is very helpful. In GPI HPMRS vitamin B6 administration could improve the electroencephalography tracing and reduce the amount of epileptic seizures. Treatment of epileptic seizures in CDG is in accordance with the guidelines for antiepileptic therapy of special epileptic syndromes or of dominant semiology of seizures (Paprocka et al. 2021).

## 6 Developmental Delay/Intellectual Disability

Almost every CDG is connected with DD/ID. Although there are some individuals with a CDG that have normal intellectual abilities such as

- N-linked glycosylation defects: MPI-CDG,

- disorders of dolichol biosynthesis: MPDU1-CDG, DHDDS-CDG,
- O-linked glycosylation defects: EXT1-CDG, EXT2-CDG, FKTN-CDG, SLC35D1-CDG, FKRP-CDG, LFNG-CDG, CHST6-CDG, CHST14-CDG,
- multiple glycosylation defects: SLC35A1-CDG, ATP6VOA2-CDG, SEC23B-CDG, and
- GPI anchor disorders: PIGA-CDG, PIGM-CDG

(Francisco et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018; Ondruskova et al. 2014; Lipiński and Tylki-Szymańska 2021; Gardeitchik et al. 2018; Quelhas et al. 2021; Bogdańska et al. 2021; Wilson and Matthijs 2021; Paprocka et al. 2021; Ondruskova et al. 2014; Gadomski et al. 2017; Vals et al. 2017; de la Morena-Barrio et al. 2020)

## 7 Ataxia

Ataxia may be a clinical feature of ALG6-CDG, COG8-CDG, DPM1-CDG, PMM2-CDG, COG4-CDG, COG5-CDG, GMPPB-CDG, MPDU1-CDG, NANS-CDG, PGM3-CDG, SLC35A1-CDG, TRAPPC11-CDG (Barone et al. 2014; Paprocka et al. 2021).

According to some authors hypoglycosylation of the  $\alpha\delta$  subunit of the CaV2.1 channel, by the gain-of-function effect, may promote the voltage-dependent opening of the CaV2.1 channel, leading to ataxia (Barone et al. 2014; Paprocka et al. 2021). A similar mechanism is postulated to result in stroke-like episodes and in cerebellar syndrome in PMM2-CDG. Use of acetazolamide therapy reduced cerebellar symptoms, anxiety, stereotypic movements, improved verbal communications and coagulation parameters (Martínez-Monseny et al. 2019).

## 8 Neuromuscular Presentation

The neuromuscular junction is heavily glycosylated. Mutations in the N-glycosylation pathway in ALG2-CDG, ALG14-CDG, DPAGT1-CDG, GFPT1-CDG, GMPPB-CDG are known to result in myasthenic syndromes (Ferreira et al. 2018; Paprocka et al. 2021; Yand et al. 2013; Cossins et al. 2013). In some dystroglycanopathies myasthenic features are combined with the limb-girdle dystrophic pattern. In ALG14-CDG myasthenic and myopathic features are accompanied by cerebral frontoparietal atrophy, white matter loss, ventriculomegaly and refractory epilepsy.

Peripheral neuropathy is present in ~53% of PMM2-CDG patients (Schiff et al. 2017).

Clinical signs of muscle dystrophy with increased creatine kinase (CK) levels are reported in DPM1-CDG, DPM2-CDG, DPM3-CDG and less often and sometimes without CK elevation in POMT1-CDG, POMT2-CDG, POMGNT1-CDG,

FKRP-CDG, FKTN-CDG, LARGE-CDG, ISPD-CDG, GTDC2-CDG, B3GNT1-CDG, SGK196-CDG, GMPPB-CDG, TMEM5-CDG (Péanne et al. 2018; Wilson and Matthijs 2021; Paprocka et al. 2021).

## 9 Spasticity

Spasticity is a distinguishing feature of CDG due to rarity of its occurrence in this group of diseases. Spasticity helps to limit the causative problem causing the CDG due to the rarity of its occurrence in this group of diseases. The dominant abnormality is hypotonia: global or axial = central. Mutations in ST3GAL5-CDG (salt and paper syndrome) are associated with extreme spasticity coupled with severe intellectual disability, seizures, hyperkinetic movement disorders, and altered pigmentation (Bowser et al. 2019). Hereditary spastic paraplegia (HSP26) had been observed due to mutations in B4GALNT1-CDG. Spastic quadriplegia was reported in COG-CDG and peripheral hipertonia in PIGP-CDG (Trincherà et al. 2018).

## 10 Autistic Features

Autistic behavior is not a typical clinical symptom for CDG. To date, autistic features have only been observed in ALG6-CDG, DOLK-CDG, SLC35A3-CDG, GALNT2-CDG (Morava et al. 2016; Helander et al. 2013; Edvardson et al. 2013a, b; Paprocka et al. 2021; Dwyer and Esko 2016). Autistic disorders may also appear in CDG patients as a consequence of long-lasting epileptic encephalopathy.

## 11 Extrapiramidal Manifestation

Movement disorders in CDG patients are usually connected with hyperkinetic forms like chorea, athetosis, dystonia, stereotypies, tremor (Table 8.15) (Paprocka et al. 2021; Mostile et al. 2019; Boccuto et al. 2014; Lam et al. 2017; Ng and Freeze 2018; Lo Barco et al. 2021). Detailed observation and investigation may prove that their incidence in CDG patients is much higher.

## 12 Treatment Options

There is no causative treatment in congenital disorders of glycosylation. Treatment is usually symptomatic and preventive. Effective therapy can be used in: ALG2-CDG, ALG14-CDG, CAD-CDG, DPAGT1-CDG, GFPT1-CDG, GMPPB-CDG,

**Table 8.15** Extrapyrimalid manifestation in CDG (Paprocka et al. 2021; Mostile et al. 2019; Boccuto et al. 2014; Lam et al. 2017; Ng and Freeze 2018; Lo Barco et al. 2021)

Disorder, OMIM	Athetosis	Chorea	Dystonia	Streotypies	Tremor
PMM2-CDG	x	x	x	x	x
ALG3-CDG			x		
ALG6-CDG	x	x	x	x	x
ALG8-CDG		x			
ALG13-CDG		x			
MGAT2-CDG				x	
DPAGT1-CDG					x
DDOST-CDG					x
COG5-CDG		x		x	
MOGS-CDG			x		
SRD5A3-CDG				x	
TRAPPC11-CDG		x			x
PGAP1-CDG				x	
PIGN-CDG		x			x
PIGV-CDG	x		x		
ST3GAL5-CDG	x	x			x
B4GALNT1-CDG			x		

GNE-CDG, MPI-CDG, PGM1-CDG, PGM3-CDG, TMEM165-CDG, SLC39A8-CDG, SLC35A2-CDG, SLC35C1-CDG (Verheijen et al. 2020).

A number of CDG can be treated with simple sugars, In MDPI-CDG the deficit can be compensated for by the use of oral mannose supplementation. The effect of treatment is the normalization of transferrin isoforms and coagulation factors, and the disappearance of enteropathy (Altassan et al. 2019). Some patients require a high intake of mannose to fully compensate. However, such high doses can lead to hemolysis, jaundice, and seizure episodes. Mannose therapy does not prevent further liver damage and approximately 1/3 of patients develop liver cirrhosis. In CAD-CDG supplementation with oral uridine reduces the frequency of seizures. In PGM1-CDG, phosphoglucosmutase converts glucose 1-P to glucose 6-P and is a key enzyme in glycolysis, glycogenesis and glycogenolysis (Francisco et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018; Ondruskova et al. 2014; Lipiński and Tyłki-Szymańska 2021; Gardeitchik et al. 2018; Quelhas et al. 2021; Bogdańska et al. 2021; Wilson and Matthijs 2021; Verheijen et al. 2020). In PGM1-CDG oral supplementation with galactose improves glycosylation of transferrin and normalizes the isoform pattern, but not to reference values. Improvement in endocrine and other biochemical parameters, such as transaminases, coagulation factors, decrease in the frequency of hypoglycemia, and no episodes of rhabdomyolysis. In SLC35A2-CDG caused by the deficiency of the UDP-galactose transporter, galactose was observed to influence on reduction in the frequency of seizures. When the deficiency affects the manganese transporter SLC39A8-CDG, treatment with galactose improves glycosylation, and manganese supplementation reduces

the frequency of epileptic seizures. The use of galactose and  $Mn^{2+}$  in TMEM165-CDG improves glycosylation and blood coagulation parameters (Francisco et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018; Ondruskova et al. 2014; Lipiński and Tylki-Szymańska 2021; Gardeitchik et al. 2018; Quelhas et al. 2021; Bogdańska et al. 2021; Wilson and Matthijs 2021; Verheijen et al. 2020). In SLC35C1-CDG, oral fucose therapy reduces the respiratory tract infection rate.

A number of other methods have been used to treat specific or small numbers of CDG. In PGM3-CDG, stem cell transplantation corrects neutropenia and lymphopenia. In CDG occurring as congenital myasthenic syndrome (DPAGT1-CDG, ALG2-CDG, ALG14-CDG, GFPT1-CDG and GMPPB-CDG), cholinesterase inhibitors are used (Bogdańska et al. 2021; Freeze et al. 2015; Verheijen et al. 2020; Adam et al. 2018). In GNE-CDG, supplementation with sialic acid may stabilize muscle strength. There are no therapeutic suggestions for most of the CDG, and also for PMM2-CDG, the most common inherited glycosylation disorder. In PMM2-CDG patients actazolamide improved cerebellar symptoms, cognitive and social abilities and the coagulopathy. Attempts to supplement with oral or intravenous mannose did not bring any clinical benefits despite a marked biochemical improvement in *in vitro* studies on patient fibroblasts.

Organ transplantation is known to relieve selected symptoms: liver transplantation may be beneficial in MPI-CDG, CCDC115-CDG, heart transplantation in DOLK-CDG and hematopoietic stem cell transplantation in PGM3-CDG. Research is underway on the so-called pharmacological chaperones (PCs), which bind specifically to proteins, stabilizing them and preventing their degradation, and on over regulators of proteostasis acting as stabilizing molecules. Gene therapy is based on adeno-associated virus vectors (AAV) and nonviral transgene delivery (zinc-finger nucleases, TALENs, CRISPR/Cas9 technology) (Verheijen et al. 2020; Adam et al. 2018). Antisense gene therapy shows promise when used to treat fibroblasts from patients with PMM2-CDG or TMEM165-CDG (Verheijen et al. 2020; Adam et al. 2018).

### 13 Conclusions and New Perspectives

According to Francisco et al. (2019) about 50% of known CDG can not be detected by serum TIEF (Francisco et al. 2019; Marques-da-Silva 2019; Adam et al. 2018; Ng and Freeze 2018; Ferreira et al. 2018; Gilflix 2019). Glycosylation requires the action of glycosyltransferases, glycosidases and nucleotide-sugar transporters, localized in the endoplasmic reticulum and Golgi apparatus. Almost all CDG present with multisystemic, predominantly neurologic involvement during the first few years of life, but the constellation of symptoms may differ, as they do in mitochondrial cytopathies (Ng and Freeze 2018; Ferreira et al. 2018; Gilflix 2019). Selected CDG have special unique features, which may facilitate their diagnosis for example: midline malformations in PGM1-CDG, chronic diarrhoea in MPI-CDG, inverted nipples and abnormal fat distribution in PMM2-CDG, cataract/coloboma/optic disc

hypoplasia/nystagmus in SRD5A3-CDG, cutis laxa syndrome in ATP6V0A2-CDG and COG7-CDG (Francisco et al. 2019; Jaeken and Peanne 2017; Chang et al. 2018; Péanne et al. 2018; Ondruskova et al. 2014; Lipiński and Tylki-Szymańska 2021; Gardeitchik et al. 2018; Quelhas et al. 2021; Bogdańska et al. 2021; Wilson and Matthijs 2021; Foulquier and Legrand 2020; Freeze et al. 2015; Adam et al. 2018; Ng and Freeze 2018; Ferreira et al. 2018; Gilfix 2019).

The difficulty in identification of some CDG is caused by the lack of convenient and suitable biomarkers as is the case for GPI and GSL defects. Due to the huge diversity of symptoms the diagnosis is usually based on genetic analyses (next generation sequencing (NGS), whole exome sequencing (WES), or whole genome sequencing (WGS)). Collecting more data from biochemical, clinical and genetic studies may lead to development of easier diagnosis and more effective treatment of congenital disorders of glycosylation.

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# Chapter 9

## Brain O-GlcNAcylation: From Molecular Mechanisms to Clinical Phenotype



Batuhan Uygur and Olof Lagerlöf

**Abstract** O-GlcNAc is the attachment of  $\beta$ -N-acetylglucosamine to the hydroxyl group of serine and threonine in nuclear and cytoplasmic proteins. It is generally not further elongated but exists as a monosaccharide that can be rapidly added or removed. Thousands of proteins involved in gene transcription, protein translation and degradation as well as the regulation of signal transduction contain O-GlcNAc. Brain is one of the tissues where O-GlcNAc is the most highly expressed and deletion of neuronal O-GlcNAc leads to death early in development. O-GlcNAc is also important for normal adult brain function, where dynamic processes like learning and memory at least in part depend on the modification of specific proteins by O-GlcNAc. Conversely, too much or too little O-GlcNAc in the brain contributes to several disorders including obesity, intellectual disability and Alzheimer's disease. In this chapter, we describe the expression and regulation of O-GlcNAc in the nervous system.

**Keywords** O-linked N-acetylglucosamine · O-GlcNAc · Learning and memory · Neurodegeneration · Obesity · Food intake · Alzheimer's disease · Signaling · Nutrient sensing · Post-translational modifications

### Abbreviations

aCamKII	Alpha calcium/calmodulin-dependent protein kinase II
Agrp	Agouti-related peptide
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic
APP	Amyloid precursor protein
AP-2	Adaptor protein complex 2
CNS	Central nervous system
CREB	Cyclic AMP-response element binding protein
CTD	Carboxyl terminal domain

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CTRC	CREB-regulated transcription coactivator
Gtfs	Glycosyltransferases
HBP	Hexosamine biosynthesis pathway
ECD	Electron capture dissociation
eIF2	Eukaryotic initiation factor 2
ER	Endoplasmatic reticulum
ETD	Electron transfer dissociation
MAPK	Mitogen-activated kinase
MeCP2	Methyl CpG binding protein 2
mTOR	Mammalian target of rapamycin
NSF	N-ethylmaleimide-sensitive fusion protein
LTD	Long-term depression
LTP	Long-term potentiation
OGA	O-GlcNAcase
O-GlcNAc	O-linked N-acetylglucosamine
OGT	O-GlcNAc transferase
P	Phosphate
PET	Positron emission tomography
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PPF	Paired-pulse facilitation
PTM	Post-translational modification
PVN	Paraventricular nucleus of the hypothalamus
TPR	Tetratricopeptide
UDP	Uridine diphosphate

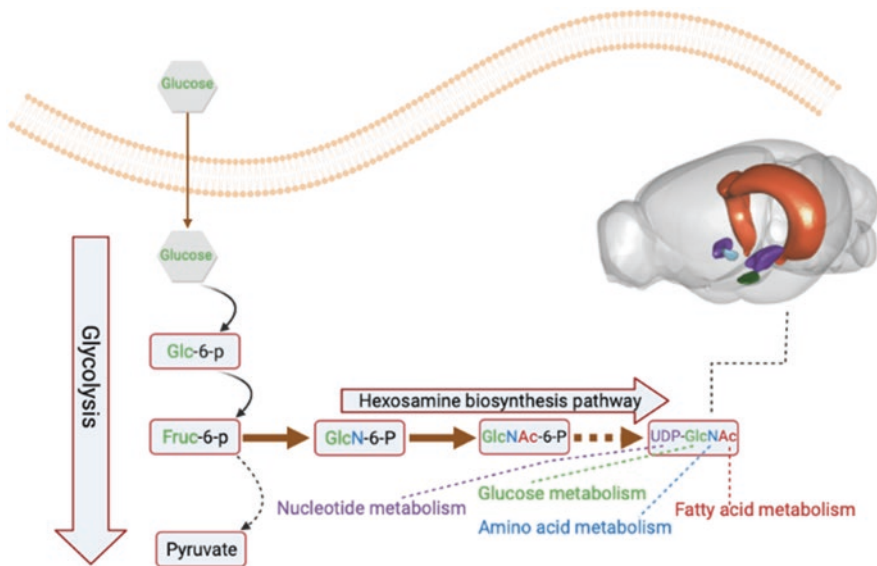
## 1 Introduction

O-GlcNAc is the attachment of  $\beta$ -N-acetylglucosamine to the hydroxyl group of serine and threonine in nuclear and cytoplasmic proteins (Torres and Hart 1984). It is generally not further elongated but exists as a monosaccharide that can be rapidly added or removed (Hart et al. 2011). Thousands of proteins involved in gene transcription, protein translation and degradation as well as the regulation of signal transduction contain O-GlcNAc (Trinidad et al. 2012; Alfaro et al. 2012). Brain is one of the tissues where O-GlcNAc is most highly expressed and deletion of neuronal O-GlcNAc leads to death early in development (Kreppel et al. 1997; O'Donnell et al. 2004). O-GlcNAc is also important for normal adult brain function, where dynamic processes like learning and memory at least in part depend on the modification of specific proteins by O-GlcNAc (Tallent et al. 2009; Rexach et al. 2012). Conversely, too much or too little O-GlcNAc on other proteins participates in neurodegenerative processes underlying diseases such as Alzheimer's and Parkinson's (Arnold et al. 1996; Liu et al. 2004; Yuzwa et al. 2012; Wang et al. 2010a, b; Marotta et al. 2012). Conversely, too much or too little O-GlcNAc in the brain contributes to several disorders including obesity, intellectual disability and Alzheimer's disease (Lagerlöf et al. 2016; Lagerlöf 2018; Pravata et al. 2020). In this chapter, we describe the expression and regulation of O-GlcNAc in the nervous system.

## 2 O-GlcNAc Is a Ubiquitous Monosaccharide That Cycles Onto and Off Serine and Threonine

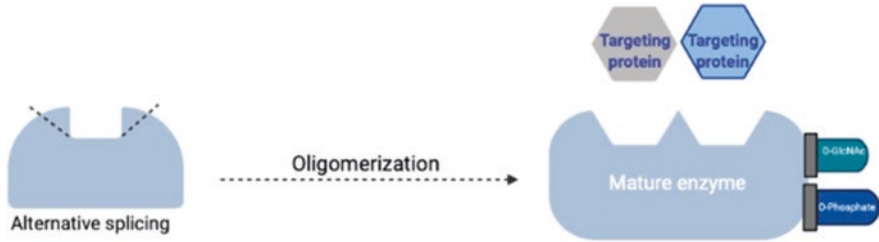
### 2.1 O-GlcNAc Is Not Elongated to Yield Complex Oligosaccharides

O-GlcNAc is the covalent modification of nuclear and cytoplasmic proteins by  $\beta$ -N-acetylglucosamine (Torres and Hart 1984). O-GlcNAc is formed as a derivative of glucose through the hexosamine biosynthesis pathway (HBP). In the HBP, the oxygen on the second carbon of fructose-6-phosphate is exchanged for nitrogen forming GlcN-6-P, prior to acetylation of the nitrogen to yield GlcNAc-6-P. This is then coupled to the high-energy molecule uridine diphosphate (UDP), UDP-GlcNAc (Fig. 9.1; Figs. 9.1, 9.2, and 9.3 were created with BioRender.com and the Allen Brain Explorer from the Allen Institute for Brain Science). Upon modification of proteins by O-GlcNAc, the GlcNAc is cleaved from the UDP and attached in  $\beta$ -position to the hydroxyl group of serine or threonine (O- $\beta$ -GlcNAc, O-GlcNAc).

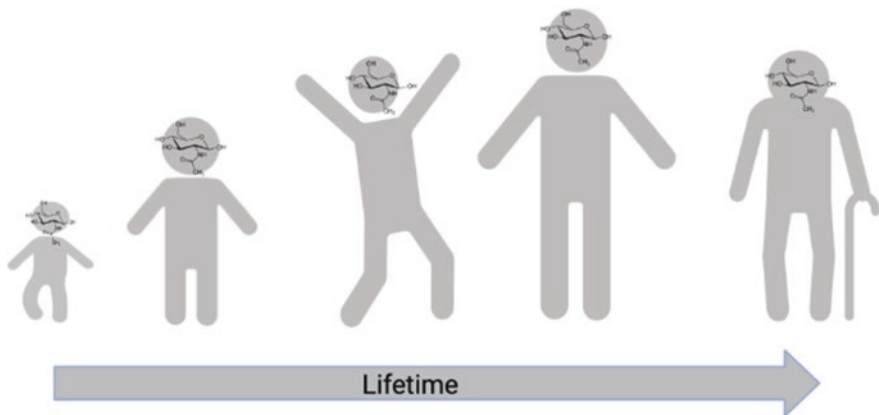


**Fig. 9.1** O-GlcNAc cycling depends on several metabolic pathways and is important for many brain functions. The donor substrate for O-GlcNAc, UDP-GlcNAc, is produced by the hexosamine biosynthesis pathway (HBP). Many metabolites feed into the HBP and thereby affect UDP-GlcNAc production. O-GlcNAc transferase (OGT) cleaves the UDP-GlcNAc and adds the GlcNAc to serine or threonine on proteins. The GlcNAc can then be removed from the protein by another enzyme, the O-GlcNAc hydrolase, OGA. The cycling on and off proteins has been shown to be important for many brain functions in diverse brain areas including the hippocampus, amygdala and hypothalamus





**Fig. 9.2** The regulation of the O-GlcNAc transferase, OGT, is complex. In cells, alternative splicing produces several isoforms of OGT. At least the full-length form of OGT, ncOGT, forms an oligomer that interacts with many other proteins. The interacting proteins direct OGT to its substrates at specific points in time and space. OGT can modify a large range of different proteins while showing some sequence specificity as to what amino acid becomes modified by O-GlcNAc. OGT activity can also be regulated by posttranslational modifications such as O-GlcNAc, O-phosphate and UDP-GlcNAc abundance



**Fig. 9.3** O-GlcNAc underlies normal brain development and function and contributes to the development of neurocognitive disease by regulating diverse cellular processes. (a) O-GlcNAc is expressed in the brain from early development until late in life. O-GlcNAc is essential for brain development and modulates many normal functions in the adult, e.g. learning and memory. Disturbed O-GlcNAc cycling may contribute to the pathology behind many neurodegenerative diseases. How O-GlcNAc impacts neuron function is exemplified in (b). (a) When the transcription factor CREB is modified by O-GlcNAc, it cannot bind the co-activator CRTC. This leads to the repression of many genes that are involved in synaptic plasticity. (b) Tau is normally modified by O-GlcNAc. In Alzheimer's disease, tau loses its O-GlcNAc and precipitates into cytotoxic aggregates. (c) It is believed that dynamic changes in synaptic function underlie learning and memory. At presynaptic terminals, O-GlcNAc may regulate synaptic plasticity by affecting the recycling of neurotransmitter-containing vesicles, possibly through affecting the function of the vesicle-binding protein synapsin I. In the postsynapse, the O-GlcNAc effect on learning and memory may be related to modulation of neurotransmitter receptor trafficking. (d) One mechanism by which O-GlcNAc impacts normal development is its regulation of axonal growth and branching

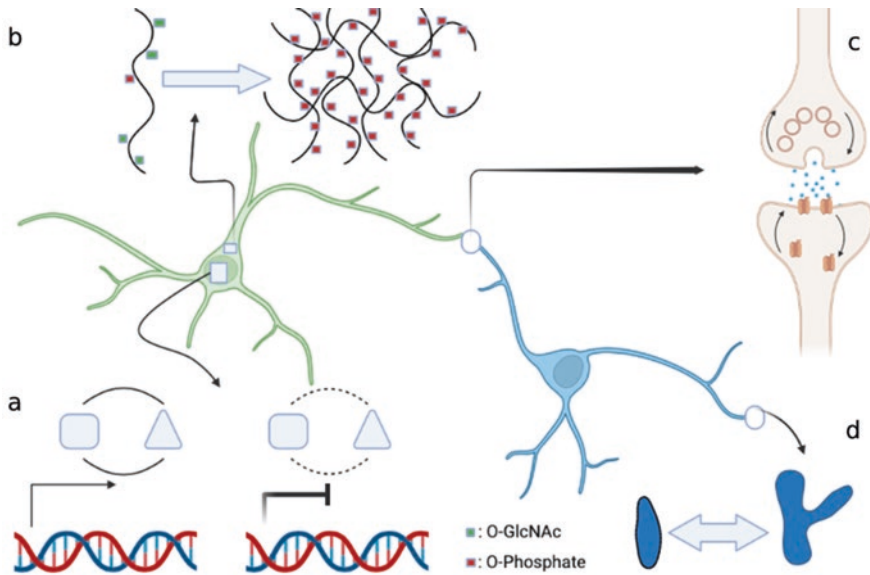


Fig. 9.3 (b)

The reaction is catalyzed by the O-GlcNAc transferase, OGT. The removal of GlcNAc is catalyzed by the O-GlcNAc hydrolase, O-GlcNAcase (OGA).

Unlike ‘classical’ O- and N-linked protein glycosylation the GlcNAc is generally not elongated but exists as a monosaccharide. In fact, when O-GlcNAc is artificially capped by galactose, its biological function is lost (Fang and Miller 2001). Although, O-GlcNAc is smaller than complex oligosaccharides, it is still much larger than many other protein modifications, such as protein methylation or protein phosphorylation (Hart et al. 2011).

## 2.2 O-GlcNAc Is Mostly Expressed on the Inside of Cells in Multicellular Organisms

O-GlcNAc is a highly conserved posttranslational modification. It has been found in evolutionary distinct clades like plantae, fungi and animalia (Kreppel et al. 1997; Webster et al. 2009). In multicellular organisms, all types of cells investigated so far contain O-GlcNAc (Hart et al. 2011). O-GlcNAc has also been identified in some unicellular organisms, e.g. giardia - the oldest eukaryote, and inside several types of virus (Banerjee et al. 2009; Benko et al. 1988; Caillet-Boudin et al. 1989). Nonetheless, most studies on unicellular organisms fail to report the presence of  $\beta$ -O-GlcNAc. Protozoans modify proteins by O-linked GlcNAc but primarily on extracellular proteins and in  $\alpha$ -linkage rather than in  $\beta$ -linkage. Yeasts appear to lack

O-GlcNAc entirely. Bacteria are also largely devoid of cytoplasmic O-GlcNAc even though some bacterial proteins have been shown to carry O-linked GlcNAc (Schirm et al. 2004; Fredriksen et al. 2012). Interestingly, the bacterium *Clostridium novyi* exploits O-GlcNAc by encoding an O-GlcNAc transferase that modifies small G-proteins in the infected cell (Selzer et al. 1996; Hart et al. 2011; Hart et al. 2007).

O-GlcNAc is expressed almost exclusively on the inside of cells (Torres and Hart 1984). Until the discovery of O-GlcNAc protein glycosylation was known to occur only on proteins exposed to the extracellular matrix, or in cellular organelles topographically similar to the outside of the cell such as the endoplasmic reticulum (ER) and the Golgi apparatus. In contrast, nearly all proteins that contain O-GlcNAc are expressed in the cytosolic or nuclear fraction of the cell. Proteins anchored to the cell membrane are modified with O-GlcNAc but usually only on parts stretching into the cytosol. This comes as no surprise as the O-GlcNAc transferase, OGT, is mainly nucleocytoplasmic rather than present in the Golgi or ER as other glycosyltransferases (there are at least two O-GlcNAc transferases with their active sites in the lumen of the ER, called, eOGTs, but these enzymes are distinct from the enzyme regulating nucleocytoplasmic O-GlcNAc. O-GlcNAc has been detected on extracellular domains of a handful of proteins, e.g. notch (see Sect. 3.2; Alfaro et al. 2012). Also the hexosaminidase removing O-GlcNAc, OGA, is cytosolic and active at neutral pH (see Sect. 3.3). By comparison, cellular glycosidases breaking down glycoconjugates retrieved from the cell surface are primarily found in the lysosome and prefer an acidic milieu.

Importantly, the concentration of O-GlcNAc is not uniform across the cell. Some parts, like the nuclear membrane, are heavily modified whereas other parts, like the mitochondria, contain O-GlcNAc but to a lesser degree. All major organelles and other cytosolic substructures, e.g. the proteasome and ribosome, express O-GlcNAc (Holt and Hart 1986; Zhang et al. 2003; Zeidan et al. 2010). The precise level varies over time and is finely tuned to meet the conditions of the cell (see Sects. 2.3, 4 and 5).

### 2.3 *O-GlcNAc Can Be Dynamically Attached and Removed*

Whether or not a protein is modified by O-GlcNAc varies substantially over time. On many proteins, including the heat-shock protein  $\alpha$ B-crystallin, the O-GlcNAc half-life is much shorter than the half-life of the peptide backbone (Roquemore et al. 1996). In fact, studies using selective inhibitors of the enzyme that removes O-GlcNAc from proteins, OGA, show that cycling rates are often on the order of minutes, making O-GlcNAc more akin to protein phosphorylation than 'classical' protein glycosylation. 'Classical' N- and O-linked glycosylation of proteins, glycosylation of proteins exposed to the extracellular matrix or within the secretory pathway, is, largely, stable once the mature glycan has been attached. There are examples of proteins, e.g. the nucleoporins that form pores through the nuclear membrane,

where the O-GlcNAc does not appear to turnover faster than the protein itself (Holt et al. 1987; Miller et al. 1999).

It has been proposed that O-GlcNAc cycling works like a light switch with only two modes of operation - either 'on' or 'off'. One argument in favor of this idea is the fact that there are only two enzymes that add and remove O-GlcNAc from proteins, OGT and OGA, respectively (see Sect. 3). Indeed, in some situations cells react by either elevating or suppressing global O-GlcNAc levels. For example, abundant nutrient supply can lead to a general increase in O-GlcNAc and scant supply to a general decrease (see Sects. 3.2 and 5.3). Likewise, cellular stress is associated with raised O-GlcNAc throughout the cell (Zachara et al. 2004). All the same, early studies showed that activation of lymphocytes causes O-GlcNAc levels in the cytosol to go up while they go down in the nucleus (Kearse and Hart 1991). A recently developed FRET (fluorescence resonance energy transfer) reporter that measures OGT activity in real-time demonstrated further that during serum stimulation of transformed cell lines, OGT was activated manifold in some parts of the cytosol whereas in nearby areas OGT activity remained at baseline (Carillo et al. 2011). Work on the regulation of signal transduction by O-GlcNAc describes the same picture; upon stimulation, in a single pathway there can be proteins that become better substrates for OGT but also proteins that become worse substrates for OGT (Whelan et al. 2010). Thus, despite there being only two enzymes that add and remove O-GlcNAc, changes in O-GlcNAc can occur 'locally' within the cell. For example, by forming dynamic multi-partner complexes OGT and OGA can be directed towards select targets among a broader range of available substrates (see Sects. 3.2, 3.3, and 4; Whelan et al. 2008; Cheung and Hart 2008; Housley et al. 2009).

The spatiotemporal regulation of O-GlcNAc cycling is complex and occurs on many levels. While nutrients and stress can cause global changes in O-GlcNAc, binding partners to OGT and OGA tune O-GlcNAc occupancy locally. Below we will discuss in detail how O-GlcNAc cycling is controlled in the nervous system and how the dynamic modification of proteins by O-GlcNAc helps the brain to develop and respond to challenges in the environment.

### **3 O-GlcNAc Is Added to Proteins by OGT and Removed by OGA**

#### ***3.1 Only Two Enzymes Regulate the Cycling of O-GlcNAc***

O-GlcNAc exists as a monosaccharide on nuclear and cytoplasmic proteins and can cycle rapidly and repeatedly over the lifetime of the polypeptide chain (see Sect. 2). In a single cell, including neurons, thousands of proteins carry O-GlcNAc (Trinidad et al. 2012; see Sect. 4.2). Change in O-GlcNAc can happen globally throughout the cell but also locally on individual proteins or sites within a protein (see Sect. 2.3).

In mammals, there are only two enzymes that add and remove O-GlcNAc. The O-GlcNAc transferase (OGT) adds O-GlcNAc to proteins (Haltiwanger et al. 1990; Kreppel et al. 1997). The O-GlcNAc glycosidase, O-GlcNAcase (OGA), removes O-GlcNAc from proteins (Dong and Hart 1994; Gao et al. 2001). As we will see in Sects. 4 and 5, loss or deregulation of O-GlcNAc cycling leads to severe developmental brain defects, impaired brain function in the adult and risk for many brain-related disorders, e.g. Alzheimer's disease. In this section we will discuss how OGT and OGA are both promiscuous in order to accept a broad range of targets while at the same time being specific enough to ensure that O-GlcNAc cycles on the correct site at the correct time and place.

### **3.2 *O-GlcNAc Transferase; a Highly Conserved Glycosyltransferase Present in the Nucleus & Cytosol***

In mammals, O-GlcNAc transferase (OGT) is encoded by a single gene. The gene is highly conserved and lies close to the centromeric region of the X chromosome (Xq13) (Shafi et al. 2000; Nolte and Muller 2002; Kreppel et al. 1997). It spans about 45 kb and its locus is linked to Parkinsonian dystonia, a neurodegenerative movement disorder (Nolte and Muller 2002; Muller et al. 1998). In most organs there are five major OGT transcripts ranging from 4.2 kilobase pairs (kb) to 9.5 kb. The transcripts undergo alternative splicing and two 4 kb transcripts may arise from an internal promoter. In brain the larger 9.5 kb and 6.4 kb transcripts, which include exons located 5' of the internal promoter, dominate (Hanover et al. 2003; Nolte and Muller 2002). Most studies so far argue that the total expression of *OGT* is stable during most conditions. However, little is known about the regulation of the OGT gene and at least in neuroblastoma cells OGT mRNA increases after depriving the cells of glucose (Cheung and Hart 2008). OGT transcription may also be stimulated by the metabolic hormone ghrelin (Ruan et al. 2014).

The protein encoded by *OGT* contains two major domains. The N-terminal half is comprised of several tetratricopeptide (TPR) repeats and the C-terminal half binds UDP-GlcNAc and harbors the glycosyltransferase activity. The TPR repeats form a flexible superhelix that can accommodate many protein-protein interactions (Lyer et al. 2003; Kreppel et al. 1997; Kreppel and Hart 1999; Jinek et al. 2004). *In vitro* experiments have shown that if the TPR domain is removed from OGT, the C-terminus alone can modify peptides with O-GlcNAc. In contrast, for protein substrates to be modified, the TPR domain is required (Kreppel and Hart 1999; Lyer and Hart 2003). It has been hypothesized based on computer simulations that the TPRs induce a conformational change in the substrate that enables the O-GlcNAc site to dock at the catalytic groove. (Trinidad et al. 2012; Lazarus et al. 2011). The C-terminus exhibits a more compact structure and resembles members of the GT-B superfamily of glycosyltransferases (Gtfs) but adopts some unique folds as well (Lazarus et al. 2011). While most OGT-interacting proteins are believed to bind the

TPR repeats the C-terminus includes regions that are necessary and sufficient for some interactions, e.g. to the mitogen-activated kinase (MAPK) p38 in neurons (Cheung and Hart 2008). The C-terminus also mediates translocation to the cell membrane upon insulin stimulation, probably via a cluster of lysines that pairs electrostatically with negatively charged phosphatidylinositol (3,4,5)-triphosphate (PIP3) (Yang et al. 2008).

Three major isoforms of OGT have been described (Hanover et al. 2003). All share the same catalytic domain but differ in their N-terminus. Nucleocytoplasmic OGT (ncOGT) is the full-length variant and includes 11–12 TPRs, depending on the species. Mitochondrial OGT (mOGT) starts with a mitochondrial targeting sequence, followed by a membrane-spanning region and continues with the last 9 TPRs found in ncOGT. The third, and shortest, isoform is soluble OGT (sOGT). It contains only 3 TPRs. According to most studies, only ncOGT is present in total brain lysate (Kreppel et al. 1997; Marz et al. 2006). However, sOGT may become upregulated in older animals and little is known about whether mOGT or sOGT is present in specific regions of the brain or in specific subcellular compartments (Liu et al. 2012). Apart from mOGT, which is anchored to the inner membrane of the mitochondria, almost all OGT activity is found in the nucleocytoplasm or as a non-integral membrane protein associated with the cytosolic face of cell membranes (Hanover et al. 2003; Haltiwanger et al. 1990).

*In vivo*, OGT oligomerizes into a dimer or trimer (Kreppel et al. 1997; Marz et al. 2006). OGT was first purified from rat liver and described as a heterotrimer consisting of two ncOGT and one 78 kDa unit. The 78 kDa unit is enriched in certain tissues and may correspond to sOGT, but may also be a proteolytic fragment of ncOGT (Haltiwanger et al. 1992; Kreppel et al. 1997). The major form of OGT expressed in brain is more likely a homodimer of ncOGT (Marz et al. 2006; Lagerlöf et al. 2017). Dimerization occurs over an evolutionary conserved hydrophobic region in the TPR domain (TPR 6). Dimerization is stable even in very high salt concentrations and probably not subject to posttranslational regulation (Kreppel and Hart 1999; Jinek et al. 2004).

OGT is exquisitely regulated adding O-GlcNAc only to particular sites at any given time and place. *In vitro*, OGT exhibits sequence specificity. If purified OGT is mixed with UDP-GlcNAc and a peptide that contains several possible O-GlcNAc sites, often only one or a few sites become significantly modified. Likewise, substrate peptides derived from different proteins are often modified with different efficiency (Kreppel and Hart 1999). Also UDP-GlcNAc levels influence peptide substrate specificity (Kreppel and Hart 1999; Shen et al. 2012). Nevertheless, there is no absolute substrate consensus sequence for OGT. Indeed, the catalytic site of OGT interacts primarily with the peptide backbone of the substrate and not particular side chains (Lazarus et al. 2011). *In vivo*, O-GlcNAc sites concentrate on disordered regions of proteins and close to proline and valine, so called PVS motifs (Alfaro et al. 2012; Trinidad et al. 2012; Hart et al. 2011). Presumably, the reason is that such regions can be made to fit OGT's catalytic groove more easily.

Primary sequence plays a role in determining the major O-GlcNAc sites on a given protein. However, in cells, whether a particular protein will be modified

depends also on OGT-binding proteins. OGT operates as a holoenzyme, where its interacting proteins direct OGT to its substrates (Whelan et al. 2008; Cheung and Hart 2008; Cheung et al. 2008; Housley et al. 2009; Marz et al. 2006; Yang et al. 2002). For example, in neurons, neurofilaments become O-GlcNAcylated only after activated p38 binds OGT (Cheung et al. 2008). OGT-interacting proteins can enhance OGT activity towards peptide substrates as well (Marz et al. 2006). Moreover, OGT is multiply phosphorylated and it modifies itself with O-GlcNAc (Kreppel et al. 1997; Song et al. 2008; Whelan et al. 2008). Phosphorylation of OGT can both activate OGT, e.g. CaMKIV during neuronal depolarization, and alter OGT's substrate specificity (Whelan et al. 2008; Song et al. 2008; Bullen et al. 2014). OGT is strongly inhibited by free UDP but, unlike most other glycosyltransferases that utilize a UDP-bound sugar as donor, OGT does not require divalent cations (Haltiwanger et al. 1990). Surprisingly, in addition to its glycotransferase activity OGT can also function as a protease (Capotosti et al. 2011). For a summary of the regulation of OGT, see Fig. 9.2.

OGT has emerged as a key cellular nutrient sensor. The donor for O-GlcNAc, UDP-GlcNAc, is produced by the hexosamine biosynthesis pathway, the HBP (see Sect. 2.1). UDP-GlcNAc is an abundant high-energy small molecule and ranges in concentrations within cells from 0.1 to 1 mM. Almost all metabolic pathways feed into the HBP and contribute to the production of UDP-GlcNAc, including fatty acids, nitrogen and 2–5% of all cellular glucose. A rich energy supply elevates UDP-GlcNAc, and, subsequently, protein O-GlcNAcylation whereas a scarce supply is associated with reduced levels of both UDP-GlcNAc and O-GlcNAc (Hart et al. 2011; Hart et al. 2007). Also in the brain fasting has been shown to decrease O-GlcNAc generally, a change reversed upon re-feeding (Liu et al. 2004; Li et al. 2006; Banerjee et al. 2016). Interestingly, depending on the substrate, the  $K_m$  for UDP-GlcNAc can vary more than 20-fold but most become better substrates in the presence of higher concentrations of UDP-GlcNAc (Shen et al. 2012; Kreppel and Hart 1999). Therefore, although metabolic flux is associated with global changes in O-GlcNAc, some proteins are more sensitive to nutrient supply than others. In Sect. 5.3 we will discuss how OGT as a nutrient sensor is involved in the pathology behind neurodegenerative disease.

### ***3.3 O-GlcNAcase; a Cytosolic O- $\beta$ -GlcNAc Hydrolase with Neutral pH Optima***

In accord with the O-GlcNAc transferase, the enzyme that removes O-GlcNAc from proteins, the O-GlcNAcase (OGA), is expressed from a single gene. The gene was first cloned as an antigen associated with meningioma and it is localized to the long arm of chromosome 10 (10q24), a locus tightly linked to late-onset Alzheimer's disease (Heckel et al. 1998; Bertram et al. 2000). In addition, alternatively spliced

OGA transcripts have been associated with sporadic cases of Alzheimer's disease (Twine et al. 2011).

OGA hydrolysis results in the displacement of GlcNAc from the protein (Dennis et al. 2006). Contrary to hexosaminidases, OGA has a neutral pH optima and is not inhibited by GalNAc. Nor does it remove GalNAc from proteins but is specific for  $\beta$ -linked O-GlcNAc (Dong and Hart 1994). OGA is also genetically and immunogenically distinct from other glycosidases (Gao et al. 2001).

OGA has been identified in all tissues so far investigated. It is expressed in the nucleus and cytoplasm of cells and the highest expression is found in brain (Gao et al. 2001). OGA is highly conserved but contains a middle, intrinsically unfolded region that exhibits more variability (Gao et al. 2001; Heckel et al. 1998; Butkinaree et al. 2008). Like OGT, OGA consists of two major domains. The glycosidase domain is C-terminal. The N-terminus contains a histone acetyl transferase (HAT) domain. While the HAT domain was reported to be functionally active (Toleman et al. 2004; Toleman et al. 2006), not all groups have been able to repeat this finding (Butkinaree et al. 2008). Caspase 3 cleaves OGA between the glycosidase domain and the HAT domain during apoptosis without any loss of O-GlcNAcase activity (Butkinaree et al. 2008).

Very little is known about the regulation of OGA. It is believed that OGA forms multi-partner complexes that direct OGA to its substrates, much like how OGT is regulated (Hart et al. 2011; Groves et al. 2017; see above). Recently, highly specific pharmacological inhibitors of OGA have been developed (Yuzwa et al. 2008; Yuzwa et al. 2012). In Sect. 5 we will learn more about the way in which OGA is important for the brain's ability to learn and form memories and serves as a drug target for neurodegenerative disorders.

## 4 O-GlcNAc Is Highly Expressed in the Nervous System

### 4.1 *O-GlcNAc Is Found Throughout the Brain*

Brain is one of the organs where O-GlcNAc is the most abundant. OGT and OGA are expressed in comparatively high levels in the brain (Kreppel et al. 1997; Lubas et al. 1997; Gao et al. 2001). Both enzymes are present across brain regions, including cortex, the cerebellum and subcortical nuclei such as the amygdala (Liu et al. 2012; Rexach et al. 2012). Within neurons, O-GlcNAc has been identified in all subcellular structures investigated, albeit to differing degrees. Biochemical fractionation was used to show that O-GlcNAc transferase activity was present in synapses (Cole and Hart 2001). Immunoelectron microscopy indicated that OGT and O-GlcNAc were present in both the post- and pre-synapse. In the presynapse, OGT is concentrated on synaptic vesicles storing neurotransmitter (Akimoto et al. 2003). Most research on O-GlcNAc in the brain has focused on O-GlcNAc's role in



neurons of the central nervous system. Less is known about its expression in neurons of the peripheral nervous system or in glia.

## 4.2 *Thousands of Neuronal Proteins Are Modified by O-GlcNAc*

One difficulty in studying the role of O-GlcNAc for brain function is its vast abundance. Technical breakthroughs have allowed identification of O-GlcNAc sites *en masse* by coupling selective enrichment of O-GlcNAc modified proteins with electron capture dissociation (ECD) or electron transfer dissociation (ETD) mass spectrometry (types of mass spectrometry that can fragment peptides without losing the GlcNAc) (Wang et al. 2010a, b; Trinidad et al. 2012). According to some estimates, about 40% of all neuronal proteins are modified by O-GlcNAc (Trinidad et al. 2012). Some proteins, like bassoon, which is important for neurotransmitter release, have more than a dozen O-GlcNAc sites. On other proteins, e.g. CaMKII, only a single site or a very few sites have been detected (Trinidad et al. 2012). As discussed in Sect. 3.2 O-GlcNAc sites concentrate on structurally disordered regions of proteins and there is some preference for a sequence context rich in proline and valine. For any particular site, O-GlcNAc is usually present in substoichiometric levels (Hart et al. 2007). The absolute occupancy is dynamic and depends on the activity of the neuron, possibly due to CaMK-dependent stimulation of OGT (Khidekel et al. 2007; Rexach et al. 2010; Song et al. 2008).

Proteins modified by O-GlcNAc belong to all functional classes. Many O-GlcNAc proteins are shared across cell types and underlie constitutive cellular functions such as transcription, translation and protein degradation. Nevertheless, the role played by O-GlcNAc on these factors is often neuron-specific. For example, in neurons, the common transcription factor cyclic AMP-response element binding protein (CREB) is modified by O-GlcNAc on serine 40 upon cell depolarization. Once modified, CREB is prevented from binding to its co-activator CRTC (CREB-regulated transcription coactivator). This inhibits CREB and leads to an inactivation of several transcription pathways involved in synaptic plasticity (see Sect. 5.2; Rexach et al. 2012). Other O-GlcNAc proteins are particular to neurons, including certain scaffolding proteins, signaling proteins and cytoskeletal proteins. Many of these are proteins found in the synapse. One interesting case is CaMKII- $\alpha$ . CaMKII- $\alpha$  is modified by O-GlcNAc in a small region known to contain one phosphorylation site that can activate the enzyme and another phosphorylation site that deactivates it (Trinidad et al. 2012). The regulation of CaMKII- $\alpha$  activity is crucial for synaptic events that underlie learning and memory (Lisman et al. 2002). Therefore, elucidation of how O-GlcNAc may fine tune CaMKII- $\alpha$  activity is important not only to our understanding of synapse biology but also higher-order brain function. Moreover, in the synapse, there is a significant overrepresentation of

kinases, enzymes that add O-phosphate to proteins, among the proteins that are modified by O-GlcNAc (Trinidad et al. 2012).

Several proteins that contribute to the development of neurocognitive disease carry O-GlcNAc. The transcription factor methyl CpG binding protein 2 (MeCP2) coordinates activity-dependent gene transcription and is modified by O-GlcNAc. Loss of MeCP2 causes Rett syndrome, a developmental disorder classically associated with repetitive and stereotypical hand movements and mental retardation. It has been shown that only the MeCP2 molecules that do not carry O-GlcNAc were activated in response to neuronal depolarization (Wang et al. 2010a, b; Rexach et al. 2012). Seemingly on MeCP2, O-GlcNAc serves as a checkpoint for turning on or off activity-dependent gene transcription. Other O-GlcNAc proteins that are intimately involved in neurocognitive disease include tau (Alzheimer's disease) and  $\alpha$ -synuclein (Parkinson's disease) (Arnold et al. 1996; Wang et al. 2010a, b).

### 4.3 O-GlcNAc Regulates Diverse Cellular Processes

Proteins modified by O-GlcNAc regulate diverse cellular processes such as gene transcription, protein translation and degradation and signal transduction. The function of the O-GlcNAc modification depends on the specific protein that is modified as well as the site on that protein that is modified (Hart et al. 2007; Hart et al. 2011). Here we will briefly discuss different mechanisms by which O-GlcNAc underlies normal cell function.

#### Gene Transcription

*RNA polymerase II.* O-GlcNAc may directly affect basal transcription by inactivating RNA polymerase II. RNA polymerase II in the so-called pre-initiation complex is heavily modified by O-GlcNAc in its carboxyl terminal domain (CTD). However, the RNA polymerase II in the so-called elongation complex is devoid of O-GlcNAc. Instead, upon transcription initiation the CTD of RNA polymerase II becomes phosphorylated. It has been suggested that the loss of O-GlcNAc is required before it can be phosphorylated and thereby activated (Kelly et al. 1993; Comer and Hart 1999; Ranuncolo et al. 2012).

*Histones.* Histones are modified by multiple posttranslational modifications, including O-GlcNAc (Sakabe et al. 2010). The combination of these modifications is hypothesized to form a 'code' that either facilitates or prevents the access of transcription factors to DNA. The O-GlcNAc on serine 112 enhances the (mono-) ubiquitination of histone 2B thereby activating gene transcription (Fujiki et al. 2011).

*Transcription factors.* O-GlcNAc takes advantage of several different mechanisms to regulate the activity of transcription factors toward gene transcription (Ozcan et al. 2010). O-GlcNAc activates NeuroD (important for neuronal differentiation) by promoting its shuttling from the cytosol to the nucleus and protects p53 by saving it from ubiquitin-mediated degradation (Andrali et al. 2007). O-GlcNAc

on serine 228 on Oct4 (regulating self-renewal of stem cells) does not affect its general activity but influences its promoter specificity (Jang et al. 2012).

### Protein Translation

O-GlcNAc plays several distinct roles during the translation of messenger RNA to polypeptide chains. OGT and OGA bind very tightly to the ribosome and overexpression of OGT was shown to facilitate ribosome assembly. Several ribosomal proteins are modified by O-GlcNAc, including the mammalian target of rapamycin (mTOR) pathway protein S6 (Zeidan et al. 2010). Also associated translational factors are modified by O-GlcNAc. The eukaryotic initiation factor 2 (eIF2) initiates translation by forming a complex with p67. It has been suggested that the O-GlcNAc on p67 is required for p67's ability to protect eIF2 from phosphorylation and thereby inactivation (Datta et al. 1989; Ray et al. 1992).

### Protein and Vesicle Trafficking

The subcellular distribution of multiple proteins has been shown to depend on O-GlcNAc (Sayat et al. 2008; Geng et al. 2012). For instance, E-cadherin is a cell junction protein that, among other things, is important for inhibitory synapse formation (Fiederling et al. 2011). If the O-GlcNAc on E-cadherin's cytosolic tail is not removed, E-cadherin becomes trapped in the endoplasmic reticulum (ER) and cannot be transported further to the Golgi apparatus (Geng et al. 2012). O-GlcNAc may also play a role in the transport of microvesicles within cells, e.g. vesicles that mediate the release of neurotransmitter. There is no direct evidence showing that O-GlcNAc regulates microvesicle trafficking *per se*. However, proteins such as adaptor protein complex 2 (AP-2) that mediates clathrin-dependent endocytosis and N-ethylmaleimide-sensitive fusion protein (NSF), which is an ATPase involved in vesicle fusion, are modified by O-GlcNAc (Clark et al. 2008). A recent report indicates that OGT is located in the presynaptic terminal, where synaptic vesicles release neurotransmitter, and is critical for GABA neuron function in *drosophila*. However, OGT-dependent glycosylation was dispensable for this effect (Giles et al. 2019).

### Protein Degradation

O-GlcNAc is intimately involved in the control of protein degradation. O-GlcNAc sites often fall in regions with high PEST-scores (the Pro-Glu-Ser-Thr sequence is associated with rapid degradation of proteins) and an increase in global O-GlcNAc leads to an increased ubiquitination of proteins whereas a decrease in global O-GlcNAc decreases protein ubiquitination (Hart et al. 2007; Guinez et al. 2008). In addition, O-GlcNAc can directly inhibit the proteasome by modifying the 26S and 19S proteasomes (Zhang et al. 2003).

### Signaling and the Crosstalk Between O-GlcNAc and O-Phosphate

An emerging theme in the regulation of signal transduction pathways is the crosstalk between O-GlcNAc and O-phosphate. Many O-phosphorylated proteins are also O-GlcNAc proteins (Trinidad et al. 2012). O-GlcNAc and O-phosphate can directly and reciprocally inhibit each other by sharing the same site, as in the case of threonine 58 in the trans-activation domain of the transcription factor c-myc

(Chou et al. 1995a, b). On other proteins the crosstalk is indirect and can be both negative and positive. Removal of O-GlcNAc from threonine 57/serine 58 on CaMKIV, for example, prevents the phosphorylation of threonine 200, the main activation site on CaMKIV. At the same time, loss of O-GlcNAc on serine 189 facilitates threonine 200 phosphorylation (Dias et al. 2009). In fact, on a global level, the occupancy of most dynamic phosphorylation sites is affected by acute inhibition of O-GlcNAc cycling (Wang et al. 2008). As a group, kinases, the enzymes that add O-phosphate to proteins, are more often modified by O-GlcNAc than other kinds of proteins (Dias et al. 2012; Trinidad et al. 2012). Furthermore, as we discussed in Sect. 3.2, phosphorylation of OGT can activate OGT and influence its substrate specificity.

## 5 O-GlcNAc Is Essential for Brain Function

In Sects. 2, 3, and 4 we learned that O-GlcNAc dynamically modifies a vast array of neuronal proteins involved in many cellular processes, such as gene transcription and signal transduction. In the following section we will discuss how the regulation of neuronal function by O-GlcNAc underlies normal brain function and contributes to brain-related disorders (summarized in Fig. 9.3).

### 5.1 *Early and Late Brain Development Depends Upon O-GlcNAc Cycling*

In vertebrates, O-GlcNAc is essential for normal development. In many different cell types, it has been shown that perturbations of O-GlcNAc cycling interrupt the cell cycle by preventing cytokinesis (Slawson et al. 2005; Slawson et al. 2008; Wang et al. 2010a, b). In the embryo, this leads to failure of stem cell proliferation and subsequent death at the single cell stage (Shafi et al. 2000). Studies taking advantage of partial depression of OGT expression, where cell proliferation is preserved, indicated that renewal of stem cell pluripotency and stem cell differentiation depend upon O-GlcNAc. In fact, two members of the core pluripotency network in embryonic stem cells, Oct4 and Sox2, are modified by O-GlcNAc. Loss of O-GlcNAc on threonine 228 of Oct4 alters its promoter specificity disrupting transcription of dozens of genes, including some that are involved in neuronal differentiation (e.g. Nanog, NR5A2) (Jang et al. 2012; Wang et al. 2012).

Later in development, O-GlcNAc plays a role in brain morphogenesis and patterning. Loss of OGT decreases brain size, while overexpression of OGT blurs the organization and distinction of hind-, mid- and forebrain regions in zebra fish (Webster et al. 2009). Likewise, when OGT was deleted from neurons specifically by crossing floxed OGT mice with mice expressing Cre recombinase under the

synapsin I promoter, viability until term was reduced. The pups that survived failed to develop any locomotor activity and died within ten days (O'Donnell et al. 2004). In postmitotic neurons, the O-GlcNAc glycosylation of CREB underlies both axonal and dendritic growth and may, at least partly, explain the impaired development of brain function (Rexach et al. 2012). O-GlcNAc is also known to affect axonal branching (Francisco et al. 2009).

## 5.2 O-GlcNAc Underlies Learning and Memory

Learning and memory are fundamental properties of the brain. Over the past decades it has been shown that the brain is a highly malleable organ that dynamically responds to challenges in the environment. It is believed that memories are encoded by neurons through changes in their synaptic communications with other neurons, so-called synaptic plasticity. A prevailing model for how such changes occur is long-term potentiation (LTP) and depression (LTD), occurring mainly at the post-synapse (see, e.g., Hanley 2008; Lynch 2004). Multiple disorders involving mental retardation such as Fragile X and Rett syndrome have been linked to proteins underlying synaptic plasticity (Verpelli and Sala 2012; Grant 2012). OGT is present in the synapse and many postsynaptic proteins involved in LTP and LTD carry O-GlcNAc (see Sects. 4.1 and 4.2). Using electrophysiology, it was further established that O-GlcNAc cycling is necessary for normal expression of LTP; pharmacological inhibition of OGT diminished LTP whereas OGA inhibition elevated LTP (Tallent et al. 2009). O-GlcNAc cycling also regulates LTD (Taylor et al. 2014). The effects on LTP and LTD may relate to relocation of the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptor, the glutamate neurotransmitter receptor responsible for most fast synaptic transmission in the central nervous system (CNS). O-GlcNAc modifies CaMKII in close proximity to a phosphorylation site that regulates its activity (see Sect. 4.2). CaMKII, in turn, is one of the major pathways that can induce the synaptic insertion of the AMPA receptor (Tallent et al. 2009; Shepherd and Huganir 2007). Interestingly, an OGT-dependent increase in AMPA receptor conductivity was absent in AMPA receptors where the main CaMKII phosphorylation site was mutated to alanine (Kanno et al. 2010). Related to OGT's effect on AMPA receptors, OGT also regulates synapse number (Lagerlöf et al. 2017).

O-GlcNAc cycling is important for dynamic synaptic function at the presynapse. Acute OGA inhibition *in vivo* decreases paired-pulse facilitation (PPF). PPF is a measurement of vesicle release of neurotransmitter. O-GlcNAc may regulate PPF via synapsin I. The presynaptic protein synapsin I is extensively modified by O-GlcNAc and inhibition of OGA increases the phosphorylation of synapsin I (Tallent et al. 2009). O-GlcNAc's effect on synapsin is partly mediated by O-GlcNAcylation of threonine 87 (Skorobogatko et al. 2014).

The studies investigating the effect of O-GlcNAc on learning and memory have so far relied heavily upon pharmacological manipulation of OGT and OGA. Unfortunately, some of the drugs used are known to cause off-target effects

in cells. This may explain some apparent contradictions in published results (e.g. Tallent et al. 2009 argues that O-GlcNAc does not affect basal synaptic transmission whereas Kanno et al. 2010 does see a large effect on basal AMPA receptor function). Genetic deletion of OGT confirmed the critical regulation of AMPA receptors and synaptic plasticity by O-GlcNAc signaling (Lagerlöf et al. 2017). While this study shines light on the fundamental regulation of AMPA receptors by OGT, knocking out OGT takes several days. This makes the results difficult to compare with data based on pharmacology. For future research, it will be important to develop mouse models of O-GlcNAc function and focus on mechanisms for the way in which O-GlcNAc may underlie learning and memory. One good example is a study that compared wildtype CREB with an O-GlcNAc loss-of-function mutant of CREB. This study overexpressed these proteins *in vivo* and showed that the O-GlcNAc mutant enhanced CREB-dependent transcription of several synaptic plasticity associated genes as well as short-term memory in mice (Rexach et al. 2012).

The strong links between O-GlcNAc and learning and memory were put in immediate clinical relevance in 2017. Then several groups identified several families with members with intellectual disability where mutations in *OGT* were identified as cause. These discoveries have paved the way for identification of a whole new class of congenital disorders of glycosylation (CDT) – OGT-CDT (Pravata et al. 2020).

### 5.3 *Impaired O-GlcNAc Cycling Contributes to Neurodegenerative Disease*

Neurodegenerative disease is a collection of disorders that are characterized by general cognitive decline and loss of neurons and neuronal synapses. They become more common with age but early-onset variants exist (Koffie et al. 2011; Schulz-Schaeffer 2010). Impaired O-GlcNAc cycling is implicated in the development of several types of neurodegenerative disease, including Alzheimer's, Parkinsonism and Huntington's (Arnold et al. 1996; Dias and Hart 2007; Wang et al. 2012; Lee et al. 2020; Hart et al. 2011). The loci for the genes encoding OGT and OGA are linked to Parkinsonian dystonia and Alzheimer's disease, respectively. In addition, OGA transcripts are alternatively spliced in the brain in patients with sporadic Alzheimer's (see Sects. 3.2, 3.3, and 4).

Two pathological hallmarks of Alzheimer's disease are neurofibrillary tangles and amyloid plaques (Koffie et al. 2011; Dias and Hart 2007). The neurofibrillary tangles consist of paired helical filaments of tau, a protein that is important for microtubuli stability. Tau is extensively modified by O-GlcNAc, including in the region that binds to microtubuli (Arnold et al. 1996; Wang et al. 2010a, b). Results from both *in vitro* and *in vivo* experiments indicated that increasing O-GlcNAc on tau protected it from filament precipitation (Yuzwa et al. 2012). O-GlcNAc may

save tau from precipitating either directly or indirectly by protecting it from hyperphosphorylation (Yuzwa et al. 2012; Yuzwa et al. 2008). Alzheimer's patients present with hyperphosphorylated and hypo-O-GlcNAc glycosylated tau. No O-GlcNAc can be found on the tau tangles isolated from these patients (Liu et al. 2004). Importantly, in mouse models of neurodegenerative disease resulting from expressing mutant forms of tau prone to precipitate, elevating global O-GlcNAc, including O-GlcNAc on tau, by selectively inhibiting OGA pharmacologically, slowed symptom progression (Yuzwa et al. 2012). Interestingly, the main constituent of amyloid plaques, amyloid precursor protein (APP), is also modified by O-GlcNAc (Griffith et al. 1995). The ameliorating effect of increasing O-GlcNAc on Alzheimer's disease may result in part from O-GlcNAc-mediated regulation of necroptosis (Park et al. 2021).

Alzheimer's disease is intimately associated with dysregulated glucose metabolism and insulin resistance (Biessels et al. 2006; de la Monte and Wands 2008). Due to the regulation of O-GlcNAc by nutrient supply and metabolic hormones, OGT has emerged as a key nutrient sensor in cells (see Sect. 3.2). Much of the toxicity associated with excessive intake of glucose is mediated by the hexosamine biosynthesis pathway, the same pathway that produces the O-GlcNAc donor substrate UDP-GlcNAc. Many forms of insulin resistance are also related to OGT (Hart et al. 2007). Overexpression of OGT can independently lead to insulin resistance (Yang et al. 2008). From this perspective, impaired O-GlcNAc signaling represents a model, and pharmacological target, for how metabolic dysfunction may result in neurodegenerative disease (Banerjee et al. 2016).

O-GlcNAc may also be involved in neurodegenerative diseases other than Alzheimer's. For example, in transgenic models where a polyglutamine expansion of huntingtin (the protein that causes Huntington's disease) was overexpressed, deletion of OGT attenuated neuron loss while deletion of OGA exacerbated neuron loss (Wang et al. 2012). Deleting OGT in specific types of neurons in wildtype mice lead to neuron loss in many but not all types (Lagerlöf 2018). In addition,  $\alpha$ -synuclein, the protein found in lesions overrepresented in Parkinson's disease, carries O-GlcNAc in the region known to induce self-aggregation (Wang et al. 2010a, b). It should also be noted that O-GlcNAc in other cell types becomes elevated from many different types of stress and that deletion of OGT makes cells more vulnerable to stress (Zachara et al. 2004; Kazemi et al. 2010). Many recent reports implicate O-GlcNAc signaling as important for stroke outcome; increasing O-GlcNAc attenuates both cell death and motor impairment upon stopping the blood flow to the motor cortex (Lagerlöf 2018).

#### ***5.4 O-GlcNAc Mediates Central Control of Metabolism***

The metabolism in any given cell in the body is coordinated with the activity of other cells in the same tissue, other tissues and the body as a whole. While the coordination occurs through several pathways, the brain has emerged as a critical master

regulator (Schwartz et al. 2000). The brain processes vast amounts of information about everything from current blood glucose concentration to emotional state and long-term goals of the individual. Integrating different types of information puts the brain in a unique position to respond adequately to environmental events that may offset metabolism such as eating a meal or going for a walk on a cold winter day (Rossi and Stuber 2018). One major mechanism on which the brain relies to optimize body metabolism is O-GlcNAc.

In the brain, O-GlcNAc is particularly sensitive to food intake and glucose in a subset of cells called alpha calcium/calmodulin-dependent protein kinase II (aCaMKII) neurons of the paraventricular nucleus of the hypothalamus (PVN<sup>aCaMKII</sup>). The PVN<sup>aCaMKII</sup> become activated upon food intake. Once activated, they turn off further eating. This food-induced activation depends on OGT. When OGT was knocked out in the PVN<sup>aCaMKII</sup> in mice, the cells no longer responded to food intake and the animals ate much more food. It appears that OGT in these cells mediate satiation, the feeling at the end of a meal that limits meal size. Meal size was markedly upregulated in the OGT knockout mice (Lagerlöf et al. 2016). The increased food intake leads to rapid development of obesity including perturbed glucose and insulin homeostasis (Lagerlöf et al. 2016; Dai et al. 2018). Some data suggest that OGT's regulation of meal size shifts the level at which body weight stabilizes rather than continuously pushing body weight higher and higher; one group observed that the body weight difference between the OGT knockout mice and wildtype mice evened out after several months as the normal mice grow heavier as part of their normal behavior (Dai et al. 2018). Determining the level at which body weight stabilizes over long periods of time makes O-GlcNAc a particularly interesting target for weight loss therapy. Most previous attempts at weight loss therapy based on manipulating metabolic signaling pathways have failed because there is no long term effect (Stemmer et al. 2019). O-GlcNAc's regulation of meal size may present unique opportunities to overcome this problem. Surprisingly, the long-term effect of O-GlcNAc is likely at least in part due to a memory-based mechanism. Section 5.2 details the many ways O-GlcNAc cycling mediates learning and memory. Memory was introduced many years ago to potentially be involved in long-term body weight regulation but until a paper by Andersson et al. almost no data on its biological mechanism had been discovered. It turns out that O-GlcNAc incorporates a caloric memory in satiation. This memory affects satiation depending on how many calories that previously have been consumed (Andersson et al. 2021).

While O-GlcNAc regulates food intake in PVN<sup>aCaMKII</sup>, O-GlcNAc affects energy expenditure in agouti-related peptide (agrp) neurons of the arcuate nucleus in the hypothalamus. Fasting increases OGT and O-GlcNAc levels in the agrp neurons, probably depending on stimulation by the hunger hormone ghrelin. OGT in these neurons appears to be necessary for the hunger-induced suppression of so-called browning of retroperitoneal white adipose tissue. In contrast to the effect in PVN<sup>aCaMKII</sup>, deleting OGT in the agrp neurons protects against diet-induced obesity (Ruan et al. 2014).

The contrasting effects of O-GlcNAcylation in agrp neurons and PVN<sup>aCaMKII</sup> show that O-GlcNAc signaling plays multiple roles in the way the brain coordinates



metabolism. These data also show that O-GlcNAc can be regulated very differently in different brain neurons. Genetic data implicate O-GlcNAc in several human metabolic disorders including obesity and type 2 diabetes (Lagerlöf 2018). Identifying mechanisms by which O-GlcNAc signaling regulates distinct circuits will be paramount to harnessing O-GlcNAcylation to treat metabolic disease in humans.

## 6 Summary and Outlook

Every time we think, act and feel information is passed between neurons in the brain. Neurons are extraordinary cells with complex morphology and a lifespan that matches the lifespan of the organism. The posttranslational modification O-GlcNAc, the attachment of  $\beta$ -N-acetylglucosamine to serine and threonine, is expressed in all compartments of the neuron. By the help of only two enzymes, OGT and OGA, O-GlcNAc regulates thousands of proteins underlying diverse cellular processes such as gene transcription and signal transduction. Much recent data clearly shows the importance of neuronal O-GlcNAcylation for a number of human disorders; *e.g.* intellectual disability, Alzheimer's disease and obesity. The big challenge for the future will be to derive a mechanistic picture for how specific O-GlcNAc sites affect protein function and thereby synaptic transmission in specific neuronal circuits. Several new techniques have been developed that improve and simplify the detection of O-GlcNAc. Nevertheless, more tools such as site-specific antibodies for O-GlcNAc are needed to make the O-GlcNAc field more accessible to the broader neuroscience research community. The newly developed OGA PET (positron emission tomography) ligand is another tool that will be instrumental for how to bridge the gap between O-GlcNAc site and clinical phenotype. Understanding the way O-GlcNAc works in the nervous system will not only afford fundamental principles of brain function but also provide novel targets for the treatment of neurocognitive disease.

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# Chapter 10

## Ganglioside Microdomains on Cellular and Intracellular Membranes Regulate Neuronal Cell Fate Determination



Yutaka Itokazu and Robert K. Yu

**Abstract** Gangliosides are sialylated glycosphingolipids (GSLs) with essential but enigmatic functions in brain activities and neural stem cell (NSC) maintenance. Our group has pioneered research on the importance of gangliosides for growth factor receptor signaling and epigenetic regulation of NSC activity and differentiation. The primary localization of gangliosides is on cell-surface microdomains and the drastic dose and composition changes during neural differentiation strongly suggest that they are not only important as biomarkers, but also are involved in modulating NSC fate determination. Ganglioside GD3 is the predominant species in NSCs and GD3-synthase knockout (GD3S-KO) revealed reduction of postnatal NSC pools with severe behavioral deficits. Exogenous administration of GD3 significantly restored the NSC pools and enhanced the stemness of NSCs with multipotency and self-renewal. Since morphological changes during neurogenesis require a huge amount of energy, mitochondrial functions are vital for neurogenesis. We discovered that a mitochondrial fission protein, the dynamin-related protein-1 (Drp1), as a novel GD3-binding protein, and GD3 regulates mitochondrial dynamics. Furthermore, we discovered that GM1 ganglioside promotes neuronal differentiation by an epigenetic regulatory mechanism. Nuclear GM1 binds with acetylated histones on the promoters of *N-acetylgalactosaminyltransferase* (*GalNAcT*; *GM2 synthase*) as well as on the *NeuroD1* genes in differentiated neurons. In addition, epigenetic activation of the *GalNAcT* gene was detected as accompanied by an apparent induction of neuronal differentiation in NSCs responding to an exogenous supplement of GM1. GM1 is indeed localized in the nucleus where it can interact with transcriptionally active histones. Interestingly, GM1 could induce epigenetic activation of the *tyrosine hydroxylase* (*TH*) gene, with recruitment of nuclear receptor related 1 (Nurr1, also known as NR4A2), a dopaminergic neuron-associated transcription factor, to the *TH* promoter region. In this way, GM1 epigenetically

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regulates dopaminergic neuron specific gene expression. GM1 interacts with active chromatin via acetylated histones to recruit transcription factors at the nuclear periphery, resulting in changes in gene expression for neuronal differentiation. The significance is that multifunctional gangliosides modulate lipid microdomains to regulate functions of important molecules on multiple sites: the plasma membrane, mitochondrial membrane, and nuclear membrane. Versatile gangliosides could regulate functional neurons as well as sustain NSC functions via modulating protein and gene activities on ganglioside microdomains.

**Keywords** Carbohydrate · Ganglioside · Glycosphingolipid · Epigenetic regulation · Lipid membrane · Microdomain · Mitochondrion: Neural stem cell · Neural development · Neurogenesis · Neuronal differentiation · Nucleus: Plasma membrane

## Abbreviations

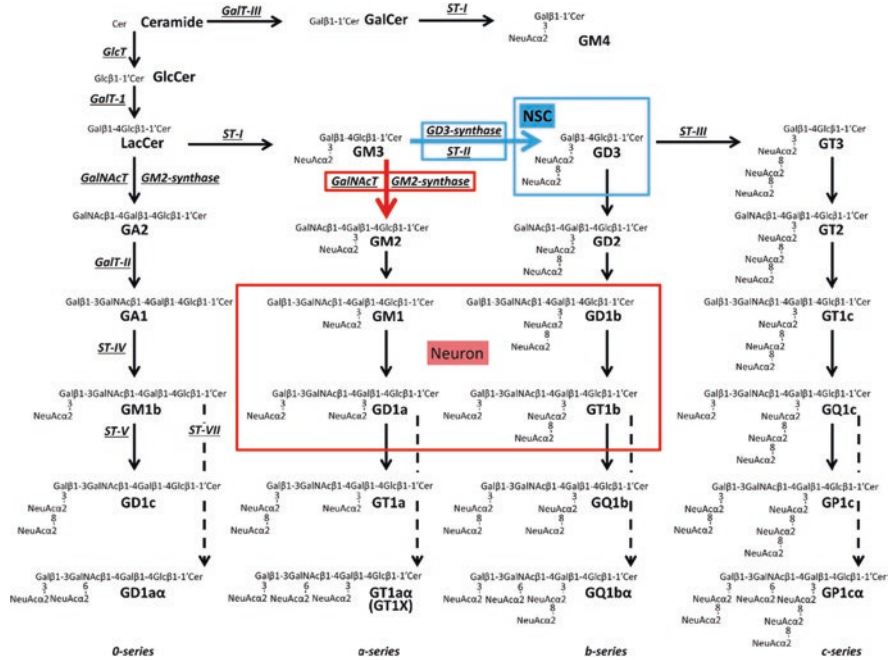
AP-2	Activating protein 2
BrdU	Bromodeoxyuridine
Cer	Ceramide
CNS	Central nervous system
CSF	Cerebrospinal fluid
DCX	Doublecortin
Drp1	Dynamin related protein-1
EGFR	Epidermal growth factor receptor
GalCer	Galactosylceramide
GalNAcT	<i>N</i> -acetylgalactosaminyltransferase
GD3	Disialoganglioside 3
GD3S	GD3 synthase
GFAP	Glial fibrillary acidic protein
GlcCer	Glucosylceramide
GM1	Monosialoganglioside 1
GM2S	GM2 synthase
GM3S	GM3 synthase
GSL	Glycosphingolipid
GT	Glycosyltransferase
LacCer	Lactosylceramide
MS	Mass spectrometry
NEC	Neuroepithelial cell
NPC	Neural progenitor cell
NSC	Neural stem cell
Nurr1	Nuclear receptor related 1, also known as NR4A2
PDGF	Platelet-derived growth factor
PLA	Proximity Ligation Assay
PSA-NCAM	Polysialic acid-neural cell adhesion molecule

RGC	Radial glial cell
SGZ	Subgranular zone
SOX2	SRY (sex determining region Y)-box 2
Sp1	Specificity protein 1
ST	Sialyltransferase
SVZ	Subventricular zone
TH	Tyrosine hydroxylase

## 1 Introduction

Cells and most subcellular organelles are surrounded by biological lipid membranes that define the individual cellular shape and help maintain cellular organization. Biological membranes are highly heterogeneous in structure and there exist diverse microdomains (also known as lipid rafts), which are enriched with sugar molecules, such as glycosphingolipids (GSLs). GSLs are unique amphipathic molecules that contain a hydrophilic carbohydrate portion and a hydrophobic lipid component (Fig. 10.1) (Yu and Itokazu 2014; Yu et al. 2011). Ganglioside synthesis pathways (Fig. 10.1) were initially delineated by Yu and Ando (Yu and Ando 1980). Glucosylceramide (GlcCer) synthase-deficient mice and lactosylceramide (LacCer) synthase-knockout (KO) mice show embryonic lethality (Kumagai et al. 2009; Nishie et al. 2010; Yamashita et al. 1999). These observations indicated that GSLs are essential for development. Gangliosides, sialic acid-containing GSLs, are found in virtually all vertebrate cells but they are particularly abundant in the nervous system (Yu and Itokazu 2014; Yu et al. 2009; Yu et al. 2011). GM3 synthase (GM3S) is a critical enzyme for the synthesis of a-, b-, and c-series gangliosides. Mutation of *GM3S* is associated with human autosomal recessive infantile-onset symptomatic epilepsy syndrome (Simpson et al. 2004) and Rett syndrome-like phenotype (Lee et al. 2016), and an alteration of the *GM2 synthase (GM2S)* gene was reported in patients with hereditary spastic paraplegias (Boukhris et al. 2013) and axonal Charcot-Marie-Tooth disease (Hong et al. 2021). These studies clearly demonstrated that deletions of gangliosides are associated with human diseases. GM2S- and GM3S-double deficient mice, which lack all gangliosides, die soon after weaning at 3 weeks of age (Yamashita et al. 2005), and exhibited sudden death from audiogenic seizures (Furukawa et al. 2014; Kawai et al. 2001). GD3S-KO mice show decreased postnatal neural stem cell (NSC) pools (Wang and Yu 2013) and impaired postnatal neurogenesis (Wang et al. 2014). GM2S-KO mice exhibit impaired movement and have virtually all the neuropathological symptoms of Parkinson's disease (PD) (Ledeen and Wu 2015). A detailed discussion about functional impairment of ganglioside deficiencies can be found in Chap. 14 by Itokazu et al.

Different microdomains regulate protein activities and cellular functions in biological membranes. GSLs help organize microdomains, and regulate growth factor signaling, immune signaling and immune checkpoints, cell-cell interactions including cell adhesion and migration. Growth factor receptors (GFRs) and their signaling



**Fig. 10.1** Metabolic pathways and structure of glycosphingolipids (GSLs), including gangliosides. The nomenclature for gangliosides and their components are based on that of Svennerholm and the IUPAC–IUBMB Joint Commission on Biochemical Nomenclature (1977; Svennerholm 1963). *Cer* ceramide, *CST* cerebroside sulfotransferase (*Gal3st1*, sulfatide synthase), *GalNAc-T* *N*-acetylgalactosaminyltransferase I (*B4galnt1*, *GA2/GM2/GD2/GT2* synthase), *GalT-I* galactosyltransferase I (*B4galnt6*, lactosylceramide synthase), *GalT-II* galactosyltransferase II (*B3galnt4*, *GA1/GM1/GD1b/GT1c* synthase), *GalT-III* galactosyltransferase III (*Ugt8a*, galactosylceramide synthase), *GlcT* glucosyltransferase (*Ugcg*, glucosylceramide synthase), *ST-I* sialyltransferase I (*St3gal5*, *GM3* synthase), *ST-II* sialyltransferase II (*St8Sial1*, *GD3* synthase), *ST-III* sialyltransferase III (*St8Sia3*, *GT3* synthase), *ST-IV* sialyltransferase IV (*St3gal2*, *GM1b/GD1a/GT1b/GQ1c* synthase), *ST-V* sialyltransferase V (*St8sia5*, *GD1c/GT1a/GQ1b/GP1c* synthase), *ST-VII* sialyltransferase VII (*St6galnac6*, *GD1aα/GT1aα/GQ1bα/GP1cα*-synthase). Official symbols of genes are represented in *italics* in this figure legend

are involved in many cellular functions. In carcinoma cells, for example, GM3 (but neither GD3 nor GM1) inhibited epidermal growth factor (EGF)-induced autophosphorylation of EGFR (Bremer et al. 1986). Inhibition was caused by the interaction of the N-linked GlcNAc termini of EGFR with the oligosaccharide portion of GM3 (Hanai et al. 1988). Ganglioside-containing microdomains provide a platform for the initiation of growth factor signaling in neuronal cells. It has been reported that GluR2-containing AMPARs bind selectively to GM1, while AMPAR-trafficking complexes containing Thorase, Nicalin, N-ethylmaleimide-sensitive factor, and its attachment protein  $\gamma$ -SNAP, bind selectively to ganglioside GT1b (Prendergast

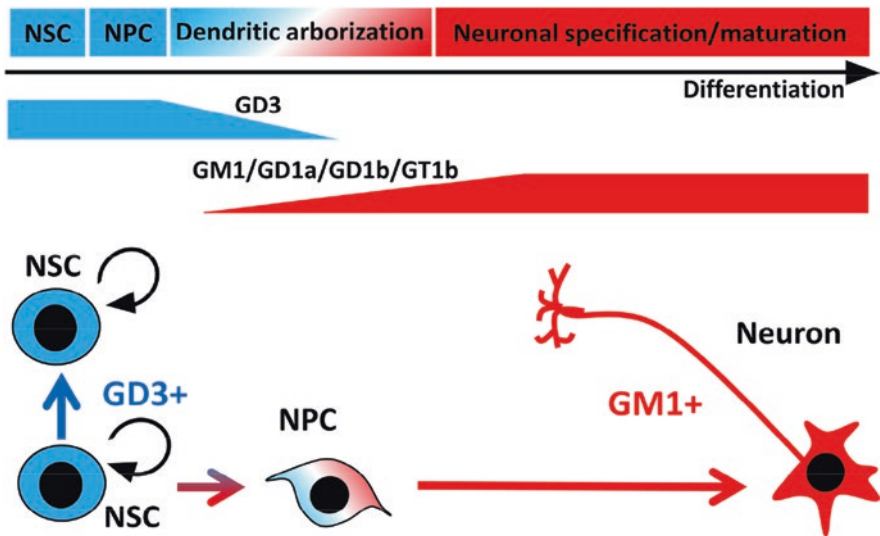
et al. 2014). Specific gangliosides clearly control structures and functions of recently described distinct microdomains for receptor trafficking and for other protein activities. Intriguingly, it has been reported that GM1 ganglioside is bound by the angiotensin-converting enzyme-2 recognized by the spike protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) for COVID-19 infection (Nguyen et al. 2021). Membrane lipids have been revealed to be important not only for separating intracellular from extracellular environments, but also for providing functional platforms for numerous molecular interactions. More specifically, GSLs are essential for maintenance of the biological functions of neuronal cells.

## 2 Ganglioside Multifunction in Cell Membranes and Intracellular Organelles

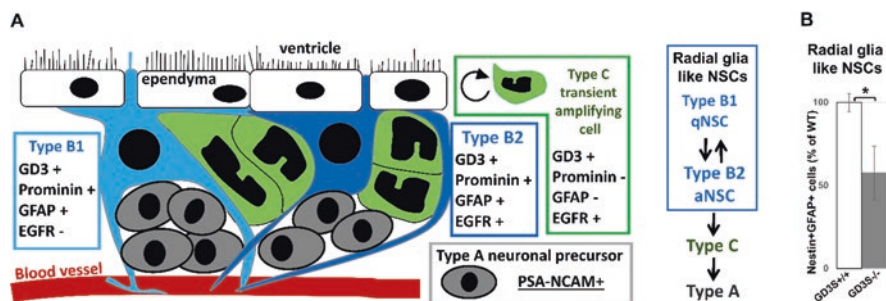
It has been revealed that functional gangliosides regulate the activity of a variety of membrane proteins. Early studies reported that gangliosides have growth factor like activities for neuronal functions (Ferrari et al. 1983; Schwartz and Spirman 1982; Tsuji et al. 1983). Specific gangliosides control protein functions of biological membranes, including plasma membrane, mitochondrial membrane, nuclear membrane, etc. Signaling of neurotrophic factors is regulated by gangliosides (e.g., GD3 for EGF and GM1 for GDNF) (Hadaczek et al. 2015; Itokazu et al. 2018; Wang and Yu 2013). It has also been reported that GM1 in plasma membranes modulates the integrin-FAK signaling pathway to promote neurite outgrowth and cell migration (Itokazu et al. 2014; Palazzo et al. 2004; Wu et al. 2007). We found that GD3 regulates mitochondrial dynamics by mediating dynamin related protein-1 (Drp1) (Tang et al. 2020b). Furthermore, we found that GM1 binds to other mitochondrial proteins to regulate mitochondrial functions (Itokazu et al., unpublished data). With regard to nuclear gangliosides, we demonstrated that nuclear GM1 binds to the neurogenic promoter region to enhance neuronal differentiation (Itokazu et al. 2016b; Tsai et al. 2016). Nuclear receptor related 1 (Nurr1, also known as NR4A2) is an essential transcription factor for differentiation, maturation, and maintenance of midbrain dopaminergic neurons, including activation of the tyrosine hydroxylase (TH) promoter. We discovered that GM1 can recruit Nurr1 to the TH promoter to enhance expression (Itokazu et al. 2021) and that GD3 increased expression of the NSC-associated transcription factor, SRY (sex determining region Y)-box 2 (SOX2) (Itokazu et al. 2019). These are just some examples of how gangliosides can modulate lipid microdomains on membranes, to regulate functions of different molecules on them. In sum, the versatile nature of gangliosides allows them to modulate several aspects, such as, growth factor signaling, mitochondrial function, gene regulation, and altered NSC activities.

### 3 Ganglioside microdomains for Neuronal Cell Fate Determination

NSCs are fundamental cells that are capable of differentiating into various cell types in the nervous system. Gangliosides undergo dramatic qualitative and quantitative developmental changes that are correlated with cellular proliferation, differentiation, and biological functions of nerve cells (Fig. 10.2). For most mammals, neurogenesis commences during early embryonic stages and is almost complete shortly after birth; it continues to occur at a much slower pace and in a limited manner throughout the entire adult life. In the adult brain of mammals, neurogenesis persists primarily in two germinal zones, the subventricular zone (SVZ) of the lateral ventricles (Doetsch et al. 1999; Doetsch et al. 1997) and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Seri et al. 2001; Suhonen et al. 1996). In the adult SVZ, different cell types that have distinct functional roles are present in a NSC niche (Fig. 10.3). Type B cells are radial glial cell (RGC)-like cells and are considered as NSCs. Type B NSCs are slow dividing (duration of cell cycle >15 days) and express glial fibrillary acidic protein (GFAP). The quiescent type B1 radial glial cells are morphologically and phenotypically distinguished from the activated cells (no contacting cilium, EGFR+). Quiescent NSCs express platelet-derived growth factor receptor beta (PDGFR $\beta$ ), and loss of PDGFR $\beta$  was reported



**Fig. 10.2** Neuronal differentiation and concurrent changes in ganglioside expression. The synthesis of GD3 is switched into the synthesis of complex gangliosides (GM1, GD1a, GD1b, and GT1b). During neuronal differentiation, the expression of complex a-series gangliosides, especially GM1, is augmented by a GM1-modulated epigenetic gene regulation mechanism of GalNAcT. Nuclear GM1 modulates transcriptional activity for neuronal differentiation. *NSC* neural stem cell, *NPC* neuronal progenitor cell



**Fig. 10.3** Neural stem cell niche at the subventricular zone (SVZ) on the surface of the lateral ventricle in the adult mouse brain. (a) Type B cells are radial glial cell (RGC)-like cells and have been considered as neural stem cells (NSCs). Gangliosides seem to regulate transition between quiescent type B1 radial glial cells and activated type B2 NSCs. Type C cells are transient amplifying cells that are rapidly proliferating. Type A cells are neuronal precursors that have already committed to becoming neurons expressing PSA-NCAM on the cell surface. (b) Nestin+/GFAP+ radial glia-like NSCs are significantly reduced in the SVZ of GD3S-KO mice (1-month-old). In the 6-month-old SVZ, Nestin+/GFAP+ cells are nearly absent from the GD3S-KO brains (data not shown). Consistent with this observation, the number of Nestin+/GFAP+ radial glia-like cells at the dentate gyrus (DG) of GD3S-KO mice is also significantly reduced. \* $p < 0.05$

to release the NSCs from quiescence (Delgado et al. 2021). Type C cells are transient amplifying cells that are rapidly proliferating (duration of cell cycle about 13 hr) and express the transcription factor Mash1. Type A cells are neuronal precursors that have already committed to differentiate into neurons, and these cells express polysialic acid-neural cell adhesion molecules (PSA-NCAM) and a cell cycle of about 13 hr. (Morshead 2004). Ependymal cells are lined on the wall of the ventricle and have multi-motile cilia, which are important for controlling the flow of cerebrospinal fluid (CSF). Multipotency of the ependymal cells has been reported (Johansson et al. 1999), although this is still not settled (Chiasson et al. 1999; Doetsch et al. 1999; Laywell et al. 2000). Further research reported that ependymal cells have been shown to be the most quiescent type of NSCs whose cell cycle is strictly regulated and reinitiated under specific circumstances. In certain restricted situations, a subpopulation of ependymal cells may develop into neurons, and these cells are considered to be NSCs (Carlen et al. 2009; Coskun et al. 2008). In the SGZ, five types of cells have been described (Filippov et al. 2003). Type 1 cells are considered quiescent neural progenitors that are RGC-like cells and largely equivalent to Type B NSCs in the SVZ. Type 2 cells express Nestin, and this cell type has been classified into two cell populations. Type 2a cells are amplifying neural progenitors that are similar to Type C transient amplifying cells in the SVZ. Type 2b and 3 cells are neuroblasts that express PSA-NCAM and correspond with Type A neuronal precursors (Encinas et al. 2006; Steiner et al. 2006). The other type of cells are mature granule neurons. Recently, it has been reported that Mash1<sup>+</sup> cells do not amplify and are therefore not Type 2a amplifying neural progenitors that can directly differentiate into early neuroblasts without mitosis (Lugert et al. 2012).

With regard to gangliosides, we have shown that GD3 is the predominant ganglioside in NSCs, and it can serve as a convenient cell surface marker of these cells (Nakatani et al. 2010). The interaction of GD3 with EGFR plays a crucial role in maintaining the self-renewal capacity of NSCs by directing the EGFR through the recycling pathway rather than through the degradative pathway after endocytosis (Wang and Yu 2013). Deletion of GD3 in NSCs reduced stem cell pools at the SVZ and dentate gyrus (DG) in the adult mouse brain (Fig. 10.3b) (Wang et al. 2014). Thus, GD3 plays a crucial role in the long-term maintenance of NSC populations in the DG of both the hippocampus and the SVZ of postnatal mouse brain. We have reported that efficient histone acetylation of glycosyltransferase (GT) genes contributes to the developmental alteration of ganglioside expression in mouse brain (Suzuki et al. 2011). Further, we have demonstrated that acetylation of histones H3 and H4 on the *GM2/GD2S* gene promoter leads to recruitment of *trans*-activation factors specificity protein 1 (Sp1) and activating protein 2 (AP-2) during neuronal differentiation (Tsai and Yu 2014). We also found that nuclear GM1 binds with acetylated histones on the promoters of the *GM2/GD2S* gene as well as on the neurogenic transcription factor, *NeuroD1* gene, in differentiated neurons from neuronal progenitor cells (NPCs) (Tsai et al. 2016). More recently, we showed that administration of GD3 augments the expression of the multipotent marker, SOX2, in cells at the SVZ and DG in the adult mouse brain (Itokazu et al. 2019). Our most recent work demonstrated that GM1 enhances neuronal differentiation and up-regulates dopaminergic neuron-specific genes via recruitment of the Nurr1 transcription factor (Itokazu et al. 2021). These examples illustrate the importance of nuclear membrane gangliosides in regulating neural development and neuronal differentiation of NSCs.

## 4 GD3-EGFR

We first discovered that GD3 is the predominant ganglioside species in NSCs (>80%) (Nakatani et al. 2010). The expression of GD3 and Nestin was found in identical cells at the SVZ of postnatal mice. Surprisingly, no significant difference in the proliferation rate and expression of lineage-associated markers was detected between wild-type (WT) and GD3S-KO NSCs that were cultured in the presence of FGF-2 but in the absence of EGF (Yu and Yanagisawa 2007). To clarify whether GD3 modulates the self-renewal capacity or cell fate determination of NSCs, NSCs from GD3-synthase (GD3S)-knockout (KO) mice were cultured with EGF. Intriguingly, GD3S-KO NSCs that were cultured with EGF showed dramatically inhibited cell proliferation (Wang and Yu 2013). The expression of Nestin and EGFR was also strongly down-regulated and the MAPK/ERK pathway signaling was impaired in GD3S-KO NSCs. Additionally, EGFR degradation and the reduction of p-EGFR and p-ERK1/2 in the GD3S-KO NSCs correlated with EGF stimulation. Subsequently a decrease of the MAPK/ERK proliferation pathway was identified in GD3S-KO NSCs. Furthermore, surface expression of membrane

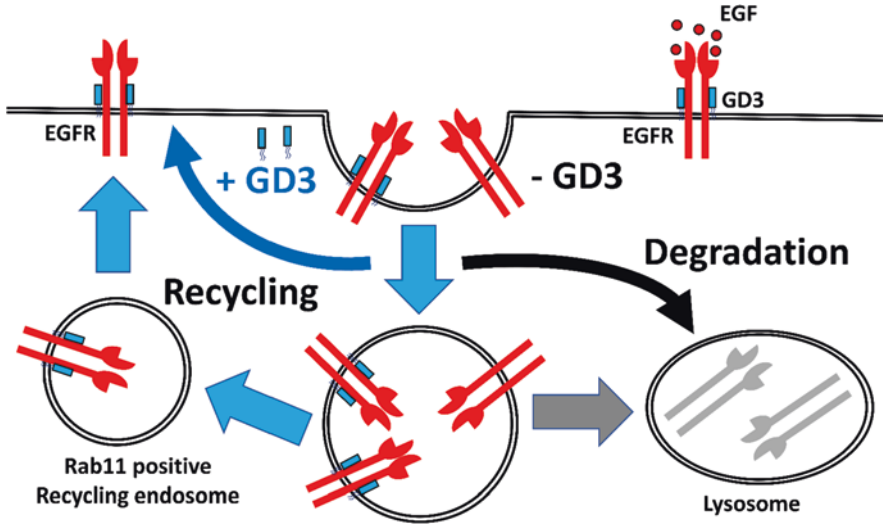
EGFRs is significantly reduced in GD3S-KO NSCs. Correspondingly, GD3 and EGFR were observed to be co-localized in NSCs, and they interacted in the microdomains of the cell surface as well as in intracellular vesicles. Interestingly, EGFR turned out to exist in non-lipid microdomain fractions in GD3S-KO NSCs. One of the most fascinating discovery was that EGFR is localized in GD3 ganglioside-enriched microdomains, and GD3 is essential for the maintenance of the self-renewal capacity in NSCs by recruiting EGFR to the microdomains to sustain the EGF-induced downstream signaling (Wang and Yu 2013).

It is significant to note that GD3 interacts with EGFR, an important mitogen receptor for the self-renewal of NSCs in the microdomain. Endocytosis is a basic cellular process that is used by cells to internalize a variety of molecules. Cells are estimated to internalize, via endocytosis, about half their plasma membrane per hour (Steinman et al. 1983). GSLs are recognized to undergo endocytosis. Once internalized, GSLs can be (1) recycled to the plasma membrane; (2) sorted to the Golgi complex; or (3) degraded in the lysosome. Since EGFR expression is reduced in GD3S-KO NSCs, internalization of its ligand EGF was investigated. It was found that the number of NSCs with internalized biotinylated-fluorescent-EGF was significantly reduced in the absence of GD3. Since it is known that EGFR can be recruited to the endosomes for recycling or sorted to lysosomes for degradation, it was found that in the GD3S-KO NSCs, EGFR exhibited increased co-localization with the lysosome-associated membrane protein 1 (LAMP1) in lysosomes as well as diminished co-localization with a marker of recycling endosomes, Ras-related protein Rab11 (Rab11) (Fig. 10.4). The co-localization with Early Endosome Antigen 1 (EEA1) also showed a mild decrease in the GD3S-KO NSCs. Pursuing the endocytosed EGFR indicated that a large amount of EGFR in GD3S-KO NSCs underwent the endosomal–lysosomal degradative pathway, while a greater proportion of the EGFR was subject to the recycling pathway in the WT NSCs than in GD3S-KO NSCs. Thus, the interaction of GD3 and EGFR in NSCs is responsible for sustaining EGFR surface expression and downstream signaling to maintain the self-renewal of NSCs (Wang and Yu 2013). Our research demonstrated that this interaction functions as: (1) a “platform” to initiate EGFR downstream signaling to induce NSC self-renewal; and (2) a “director” for the recycling of EGFR after endocytosis. In postnatal brain, GD3 is required for the long-term maintenance of NSCs. Deficiency in GD3 leads to developmental and behavioral deficits, such as depression (Wang et al. 2014).

## 5 GD3 Regulates Mitochondrial Dynamics by Interacting with Drp1

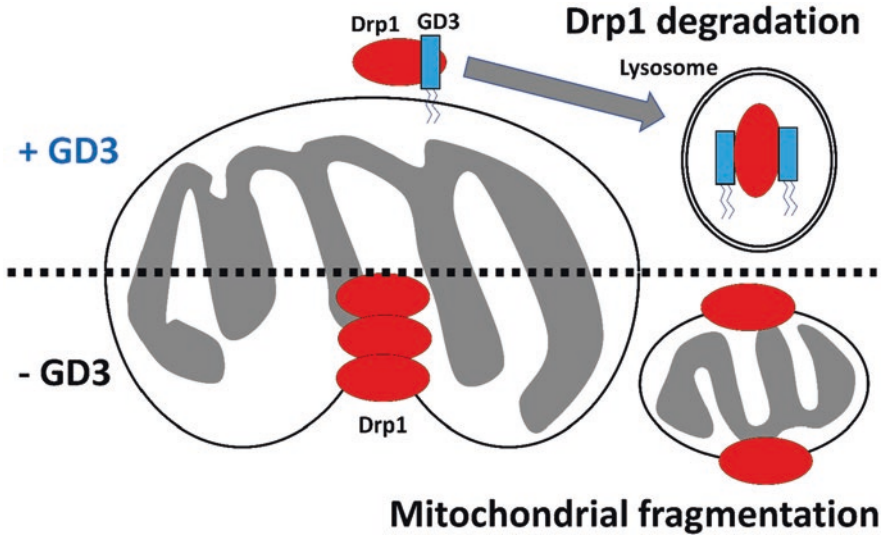
Mitochondrial lipid membranes influence mitochondrial functions, including the electron transport chain activities, nucleotide transport, mitochondrial protein import, membrane properties, ATP synthesis, cell death signaling, and





**Fig. 10.4** Interaction of GD3 with EGFR modulates self-renewal of NSCs. The decrease in EGFR expression is accompanied by an accelerated loss of the self-renewal property in the GD3S-KO NSCs. EGFR (red) is expressed in GD3 (blue)-rich microdomains on neural stem cell (NSC) surface. GD3 regulates the endocytosis of EGFR. With GD3, EGFR is co-localized with a marker of recycling endosomes, Rab11. On the other hand, EGFR showed significantly increased co-localization with the lysosomal marker LAMP1 in GD3S-KO NSCs. The interaction of GD3 and EGFR in NSCs is responsible for sustaining EGFR surface expression and downstream signaling to maintain self-renewal of the cells

mitochondrial dynamics. The morphological changes that occur as NSCs mature to neurons during neurogenesis requires a huge amount of energy (Son and Han 2018). The mitochondrion, the main intracellular organelle for producing adenosine triphosphate (ATP), plays a crucial role in adult neurogenesis (Beckervordersandforth et al. 2017; Son and Han 2018). To understand how GD3 regulates adult neurogenesis, we performed a mass spectrometric (MS) analysis-based proteomic screen for GD3-protein interactions. We first performed affinity chromatography using the anti-GD3 antibody R24 followed by MS analysis. We identified Dynamin-1-like protein (Drp1), a mitochondrial fission protein, as a GD3-interacting protein (Tang et al. 2020b). Drp1 is a GTPase that regulates mitochondrial fission and plays important roles in the regulation of mitochondrial dynamics. To investigate whether GD3 affects mitochondrial dynamics, we examined mitochondrial morphology in the nascent granule neurons. At all stages, mitochondria had a tubular morphology. GD3S-KO neurons had shorter mitochondria with a smaller aspect ratio (major axis/minor axis) than their WT mates, suggesting that mitochondria were fragmented. The neurite mitochondrial index, an index for mitochondrial density, was also significantly reduced in GD3S-KO neurons. Further Western blot analysis demonstrated that Drp1 levels were increased and the activated-Drp1 (phosphorylation of Drp1 at serine 616) was higher in GD3S-KO mitochondria. In contrast, there



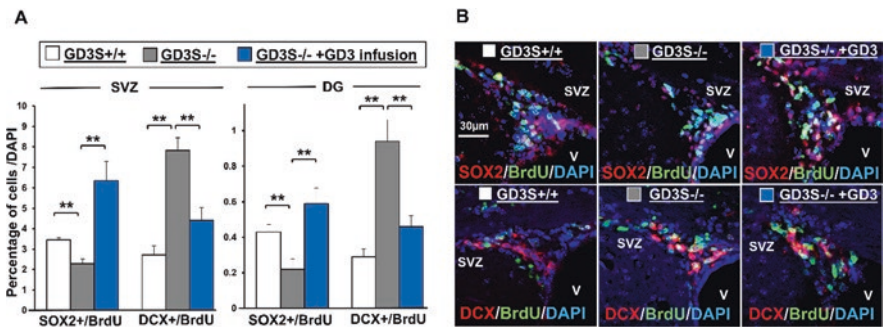
**Fig. 10.5** GD3 regulates mitochondrial Drp1 complex turnover. GD3 was found to be associated with mitochondrial fission protein-Drp1. Without GD3, Drp1 levels are increased and aberrant mitochondrial fragmentation is augmented in newborn neurons (lower cartoon). GD3 seems to regulate the clearance of mitochondrial Drp1 complexes to maintain healthy mitochondria

were no significant changes on the expression of the fusion proteins mitofusion 2 (Mfn2). Image analysis showed that the density and average size of the mitochondrial Drp1 punctate distribution was significantly increased in GD3S-KO neurons. To understand how GD3 regulates the Drp1 protein level, we measured the half-life of Drp1 in control and GD3S-KO neurons and found that the half-life of Drp1 in control cells was about 15 hours. However, it was dramatically increased in GD3S-KO neurons, with no detectable reduction in 20 hours. These findings suggest that GD3 may participate in regulating the clearance of mitochondrial Drp1 complexes. These findings suggest that the loss of GD3 resulted in mitochondrial fragmentation by increased Drp1-induced mitochondrial fissions in newborn neurons. (Fig. 10.5) (Tang et al. 2020b).

## 6 GD3 Amplifies SOX2 Expression and GM1 Promotes DCX Expression

Significantly reduced cellularity is observed in the postnatal SVZ and DG in GD3S-KO mice (Wang et al. 2014). The absolute numbers of bromodeoxyuridine (BrdU) + and SOX2+ cells were reduced in both the SVZ and DG of GD3S-KO mice compared with age-matched WT animals. There was no significant difference in the percentage of BrdU+ versus DAPI+ cells and SOX2+ cells versus DAPI+

cells between different groups. This observation suggested that the reduction of the number of BrdU+ and SOX2+ cells was due to the reduction of the whole NSC pool. To investigate the functional roles of GD3 in postnatal brains, GD3 was intracerebroventricularly (icv) infused into adult (6-month-old) GD3S-KO mice employing a mini-pump for 7 days. In the SVZ of GD3S-KO mice, BrdU positive newly generated and SOX2 positive self-renewal or multipotent cells were less than that in the WT control (Fig. 10.6). On the other hand, GD3 treatment increased SOX2 positive self-renewal or multipotent cells in the SVZ of GD3S-KO mice. In both neurogenic regions, SVZ and DG of GD3S-KO mice, the number of BrdU+/SOX2+ newly generated multipotent cells were significantly increased following GD3 infusion. These data indicate that infusion of GD3 could restore NSCs in both the SVZ and DG to maintain their properties at early NSC stages. Interestingly, GD3S-KO mice had more doublecortin (DCX) + and BrdU+ cells than WT controls and GD3 injection restored normal levels in the SVZ and DG. This data suggests that GD3 maintains the multipotent state of NSCs (SOX2+/BrdU+) and controls their differentiation. In this way, GD3 plays a crucial role in the long-term maintenance of NSC populations in the DG of hippocampus and SVZ of postnatal mouse brains (Itokazu et al. 2019; Itokazu et al. 2018; Wang et al. 2014). Moreover, the impaired neurogenesis in the adult GD3S-KO mice led to depression-like behaviors. Our results provide direct evidence linking ganglioside deficiency to behavioral deficits, and support a crucial role of gangliosides in the long-term maintenance of adult neurogenesis (Itokazu et al. 2018; Wang et al. 2014).



**Fig. 10.6** GD3 promotes SOX2 expression to maintain NSC stages and suppress further differentiation. GD3S-KO mice showed thinner SVZ and DG with reduced cellularity. GD3S-KO mice showed significantly decreased percentage of SOX2 and BrdU double labeled cells versus DAPI+ cells and an increased percentage of DCX and BrdU double labeled cells. On the contrary, intracerebroventricularly (icv) infused GD3 significantly increased the percentage of SOX2 and BrdU double labeled cells versus DAPI+ cells and decreased percentage of DCX and BrdU double labeled cells versus DAPI+ cells compared to GD3S-KO mice. The data in (a) were quantified cell staining photomicrographs of (b). Green, BrdU; blue, nuclear DAPI; and red, SOX2 or DCX. SOX2 maintains stemness such as multipotency and self-renewal. DCX is expressed in cells committed immature neuron stages, including neuronal precursor cells. GD3 infusion can sustain the NSC pool, and prevent them from differentiating in postnatal brains.  $**p < 0.01$

The loss of NSCs is known to occur during normal aging, and it has been hypothesized that an accelerated loss of the NSC pool is one of the mechanisms for transition from normal aging to neurodegenerative diseases, such as Alzheimer's disease (AD). Therefore, sustaining endogenous neurogenesis has been suggested as an important target for treatment and prevention of AD. The 5XFAD transgenic mice with two point mutations in presenilin1 (M146L & L286V) and the Florida (I716V), London (V717I), and Swedish (KM670/671NL) mutations in the amyloid precursor protein have severe pathological phenotypes. The 5XFAD mice are utilized as a preclinical AD mouse model extensively and this model showed a significantly decreased percentage of BrdU and SOX2 double labeled cells versus DAPI+ cells compared to WT mice. To examine the physiological roles of GD3 on postnatal neurogenesis in the DG of AD model mice, GD3 was administered into the 5XFAD mouse brains. GD3 infusion augmented self-renewal and NSCs expressing the multipotent marker, SOX2, in the DG (Itokazu et al. 2019). These data suggest that icv infusion of GD3 could be an effective means to maintain neurogenesis in brains of the 5XFAD mouse AD model.

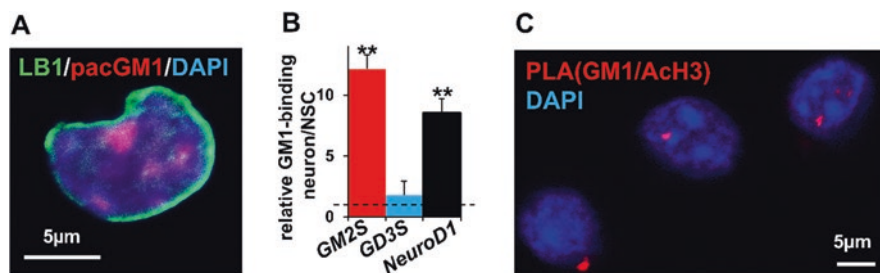
During neuronal differentiation, the concentration of GD3, which is the predominant ganglioside in NSCs, is rapidly decreased. Concomitantly, the levels of "brain-type" gangliosides such as GM1, GD1a, GD1b and GT1b continuously increase in young animals, reaching a plateau during adulthood. These pattern changes follow closely with the up-regulation of GM2/GD2S expression (Ngamukote et al. 2007). The dramatic changes in the expression profile of gangliosides clearly reflect the biological need for GalNAc-containing ganglio-series gangliosides at particular stages of brain development and neuronal differentiation. Throughout neuronal development, GM1-expressing cells are considered as neuronal progenitor cells and neurons. To understand the functional roles of GM1 on postnatal neurogenesis in the DG of the AD model mouse, GM1 was administered into the 5XFAD mouse brains. GM1 increases BrdU+/- DCX+ newly generated immature neuronal cells in 5XFAD mouse brains (Itokazu et al. 2019). As expected, the combinatorial infusion (GD3 and GM1) had a synergistic effect. These results demonstrated that icv infusion of gangliosides GD3 and GM1 simultaneously could enhance neurogenesis in adult mouse brain. For promoting adult endogenous neurogenesis, GD3 restored NSC pools while GM1 enhanced neuronal differentiation by cells in the DG of brains from AD model mice. The combinatorial use of gangliosides (GD3 and GM1) to promote endogenous neurogenesis for the treatment of neurodegenerative diseases could be a new intervention.

## 7 GM1 Binds to Neuronal Gene Promoter Regions

In an initial study, we investigated the epigenetic regulation of two key GTs, GM2S and GD3S, in embryonic, postnatal, and adult mouse brains. Interestingly, the temporal expression patterns of GM2S and GD3S mRNAs are correlated with histone H3 and H4 acetylation (AcH3/AcH4) of the gene's 5' flanking region in chromatin

(Suzuki et al. 2011). These observations suggest that the 5' region of the *GM2S* and *GD3S* genes could be targets for epigenetic regulation by histone modifications. Further, we have demonstrated that acetylation of histones H3 and H4 on the *GM2S* gene promoter leads to recruitment of *trans*-activation factors Sp1 and AP-2. When the cellular histone deacetylase activity was globally inhibited by valproic acid (VPA), more *GM2S* or *GD3S* mRNA was detected, which could be triggered due to a loading boost of the transcription factors AP-2 and Sp1 on the promoter region. Individually knocking down Histone deacetylases 1 (HDAC1) and HDAC2 gene expression increased the levels of AcH3 and AcH4 on the *GM2S* gene (Tsai and Yu 2014). Intriguingly, when both HDAC1 and HDAC2 were knocked down, the expression of *GM2S* mRNA was up-regulated and AP-2- or Sp1-loading was significantly increased, reflecting the elevated level of histone acetylation on the *GM2S* gene. However, this was not the case for the *GD3S* gene. Our results indicated that transcription of *GM2S* and *GD3S* could be regulated by different HDAC isoforms, since double-knockdown by si-HDAC1 and si-HDAC2 led to *GM2S* gene *trans*-activation, but not *GD3S* (Tsai and Yu 2014). In addition, NPCs cultured with GM1 supplementation exhibited a significantly enhanced neurogenic effect (Tsai and Yu 2014). The GM1, but not GD3, enhanced *GM2S* expression of mRNA, while the mRNA level of *GD3S* remained unchanged. The presence of ectopic GM1 resulted in enrichment of the acetylated histones on the gene loci of *GM2S*, but not *GD3S*, which was accompanied by the recruitment of the transcription factors AP-2 and Sp1 within the gene promoter region. This observation suggests the possibility that GM1 generates a positive feedback loop to promote neuronal differentiation and to produce more brain-type gangliosides, such as GM1, GD1a, GD1b and GT1b (Tsai and Yu 2014).

To further study the significance of nuclear GM1, Neuro 2a cells were treated with photoactivatable and clickable (pac) GM1 (pacGM1). PacGM1 in isolated nuclei was visualized using click chemistry-mediated tagging with fluorophores. Figure 10.7a shows that exogenous pacGM1 is indeed delivered into the nucleus. GM1 is co-localized with Lamin B1 (a protein of the nuclear lamina) or Nucleoporin (a protein of the nuclear pore complex) (Tsai et al. 2016) on the nuclear periphery of neurons induced from NPCs. Chromatin immuno-precipitation (ChIP) assay showed that the promoter regions of the *GM2S* and *NeuroD1* genes are associated with GM1 (Fig. 10.7b). *In situ* hybridization assays revealed that GM1 and the promoter region of the *GM2S* gene are in close proximity in the nucleus of a neuron. Recently, Proximity Ligation Assays (PLAs) have been developed to detect the formation of lipid-protein interactions by immunohistochemistry (Itokazu et al. 2021; Kong et al. 2018). Using this technology, we isolated nuclei from WT mice and performed PLAs to detect GM1 and acetylated histone complex. Each PLA probe contains a unique short DNA strand attached to it. If the PLA probes are in close proximity (<40 nm), the DNA strands interact and generate circle-forming DNA used for enzymatic ligation. The ligated DNA is amplified via rolling circle amplification using a polymerase. Several-hundredfold replication of the DNA circle labels complementary oligonucleotide probes that yield a high intensity of

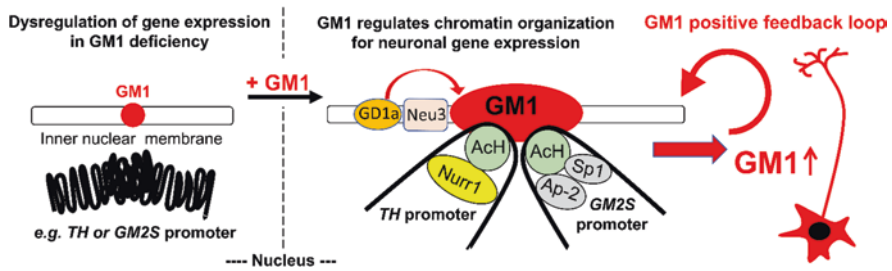


**Fig. 10.7** Nuclear GM1 localizes the nuclear periphery and accumulates on the activated GM2S and NeuroD1 genes in neuronal cells. **(a)** A photoactivatable diazirine ring for the UV-light-induced covalent linkage of photoactivatable GM1 to proteins in close proximity. Photoactivatable and clickable (pac) GM1 (pacGM1) was tagged with TAMRA (Carboxytetramethylrhodamine) on isolated nuclei of Neuro 2a cells. pacGM1 proves that exogenous GM1 (red) can be delivered to the inside of the nucleus. Nuclei were co-stained with Lamin B1 (LB1; green). **(b)** Chromatin immuno-precipitation (ChIP) assay shows that GM1 interacts with GM2S gene and NeuroD1 promoter regions, but not the GD3S gene in the differentiated neurons from NSCs. The amount of specific DNA fragments (GM2S +0, GD3S +0 or NeuroD1 +0) co-precipitated with GM1 was analyzed by quantitative real-time PCR. The data indicated the relative GM1 binding ability in neurons. **(c)** Proximity Ligation Assay (PLA) using anti-GM1 (mouse antibody, MINUS) and acetylated histone H3 anti (rabbit antibody, PLUS) in isolated nuclei from adult mouse brain.  $**p < 0.01$

fluorescence (red). The PLA signals in Fig. 10.7c demonstrate that GM1 is indeed co-localized with acetylated histone H3, *i.e.*, GM1 is localized on active chromatin in the nucleus ( $1.94 \pm 0.297$  PLA signals in nucleus). The result clearly indicates that GM1 is localized in the nucleus where it can interact with transcriptionally active histones (Itokazu et al. 2021; Tsai et al. 2016).

## 8 Nuclear GM1 Promotes Neuronal Gene Expression

GM1 has been reported to have an important role in both neuronal differentiation and neuronal function. Importantly, aged GM2S-KO mice (lacking GM1) present with movement dysfunction and have virtually all the neuropathologies of PD (Ledeen and Wu 2018; Wu et al. 2012; Wu et al. 2020). We determined whether GM1 regulates TH gene expression and found that qPCR analyses of the mRNA level for TH expression were substantially decreased in the substantia nigra pars compacta of GM2S-KO mice. Interestingly, intranasal administration of GM1 for 28 days restored normal TH expression (Itokazu et al. 2021). These data suggested that GM1 is an important regulatory factor in modulating TH gene expression. Next, we analyzed dopaminergic neuron-specific gene expression utilizing Neuro 2a cells after treatment with GM1 or GD3. While TH expression was not detected in untreated cells, it increased dramatically in cells treated with GM1. ChIP assay showed that ectopic GM1 significantly induced epigenetic activation of the TH



**Fig. 10.8** GM1 induces epigenetic activation of the TH or GM2S gene via recruitment of transcription factors on promoter regions. GM1 facilitates binding of acetylated histones (AcH) and transcription factors on promoters to increase their expression via opening the chromatin. The nuclear GM1-lipid domains may serve as a docking site at the nuclear periphery for specific active chromatin for neuronal differentiation and for maintaining neuronal functions

gene, including augmentation of acetylated histone H3. Moreover, GM1 recruited the dopaminergic neuron-associated transcription factor, Nurr1, to the TH promoter region. This result demonstrates that GM1 promotes the interaction of Nurr1 with the TH gene promoter for activating its gene expression. In addition, GM1 also recruited paired-like homeodomain transcription factor 3 (Pitx3), a critical transcription factor for the survival of midbrain dopaminergic neurons (Itokazu et al. 2021). Since transcriptional activity of Nurr1 could be stimulated by GM1, it is possible that nuclear GM1 could modulate nuclear membrane and chromatin structure to enhance gene expression for augmenting dopaminergic neurons. Fundamental cellular processes are governed by changes in chromatin architecture that regulate neuronal gene expression during differentiation and development. Interestingly, our investigations suggest that nuclear gangliosides can modulate epigenetic gene expression for neuronal differentiation and neuronal functions. Ganglioside expression profiles are known to be closely associated with pathogenic mechanisms of neurodegenerative diseases of the central nervous system. Regulating gene expression by nuclear gangliosides is a novel mechanism to control cellular activity to rescue or protect neurons in neurodegenerative diseases (Fig. 10.8).

## 9 Other Gangliosides for NSC Specification

GD3 regulates NSC activities, and GM1s control neuronal differentiation and neuronal functions. Other gangliosides have also been reported to be involved in NSC fate determination. A2B5 antigens, including the c-series gangliosides (Fig. 10.1), are well known markers for progenitor cells in the nervous system and are the first GSL antigens expressed in glial lineage cells (Eisenbarth et al. 1979; Kasai and Yu 1983; Raff et al. 1983; Saito et al. 2001). A2B5 is a monoclonal antibody originally developed using chicken embryonic retina cells as the immunogen (Eisenbarth et al. 1979), and those antigens have been established as the c-series gangliosides,

including GQ1c, GT1c, and GT3 (Kasai and Yu 1983; Saito et al. 2001). A2B5 antibody recognizes the Neu5Ac $\alpha$ 2-8-Neu5Ac $\alpha$ 2-8Neu5Ac $\alpha$ -or  $\alpha$ 2,8-trisialosyl (triSia) structure (Inoko et al. 2010). c-Series gangliosides are phylogenetically conserved and developmentally regulated. The significant expression of c-series gangliosides is in the brain of lower vertebrates, such as fish, and in the brain of mammalian embryos, but not adult brains (Ando and Yu 1979; Freischutz et al. 1994, 1995; Rosner et al. 1988; Yu and Ando 1980). c-Series gangliosides comprise up to 70% of the total gangliosides in adult zebrafish brain (Viljetic et al. 2012). Therefore, the damaged zebrafish brains that largely contain A2B5<sup>+</sup> cells, have high regeneration capacity (Kishimoto et al. 2012). GT3-synthase (GT3S or ST-III), an enzyme for the synthesis for c-series gangliosides (A2B5 antigens), is highly expressed in proliferating NSCs of mammals (Itokazu and Yu 2014; Koon et al. 2015). A population of GFAP-expressing cells is considered to be NSCs as well as astrocytes. A2B5+/GFAP+ cells have a lipid composition distinct from mature astrocytes as it is more analogous to stem or progenitor cells (Itokazu et al. 2016a). During maturation of mammalian brain, the concentration of c-series gangliosides decreases drastically, and this decrease in the synthesis of c-series gangliosides is compensated for by a pathway shift in favor of the accretion of a- and b-series gangliosides (Itokazu et al. 2018; Ngamukote et al. 2007). c-Series gangliosides may modulate the immature stage of stem or progenitor cells, albeit further research is needed to address functional interactions of c-series gangliosides and stem cell or progenitor activities.

Chol-1 $\alpha$  gangliosides (GT1a $\alpha$  and GQ1b $\alpha$ , Fig. 10.1) are minor species in the brain and serve as unique markers of cholinergic neurons (Ando et al. 1992; Hirabayashi et al. 1992). Chol-1 $\alpha$  ganglioside expression is developmentally regulated and their concentrations increase with aging in the rat brain (Derrington and Borroni 1990). It has been reported that the treatment with anti-Chol-1 $\alpha$  monoclonal antibody inhibits the release of acetylcholine from synaptosomes, and remarkably suppressed memory and learning abilities (Ando et al. 2004). Conversely, addition of Chol-1 $\alpha$  gangliosides isolated from synaptosomes induced high affinity choline uptake into synaptosomes and enhanced synthesis of acetylcholine. Accordingly, Chol-1 $\alpha$  gangliosides may contribute to maintaining cognitive functions such as memory and learning. Furthermore, Chol-1 $\alpha$  gangliosides appeared to alleviate the decreased synaptic functions of aged brains (Ando 2014; Ando et al. 1998). Chol-1 $\alpha$  gangliosides were found to be expressed in NSC, *in vitro* (Ngamukote et al. 2007). In total, Chol-1 $\alpha$  antigens may play an important role in cholinergic synaptic transmission and participate in cognitive function, although the detailed mechanisms need to be addressed.

GM3S is a key enzyme involved in the biosynthesis of all major gangliosides (Fig. 10.1). GM3S deficiency causes an absence of GM3 and all downstream gangliosides. We found that GM3S-KO mice were significantly more susceptible to seizures (270% higher in seizure score) than WT mice. In the hippocampal DG, loss of GM3 aggravates seizure-induced aberrant neurogenesis. An increased number of BrdU+/DCX+ immature neurons migrated from the DG to the hilus, and newborn cells were mislocalized in GD3S-KO brains. (Tang et al. 2020a). This result

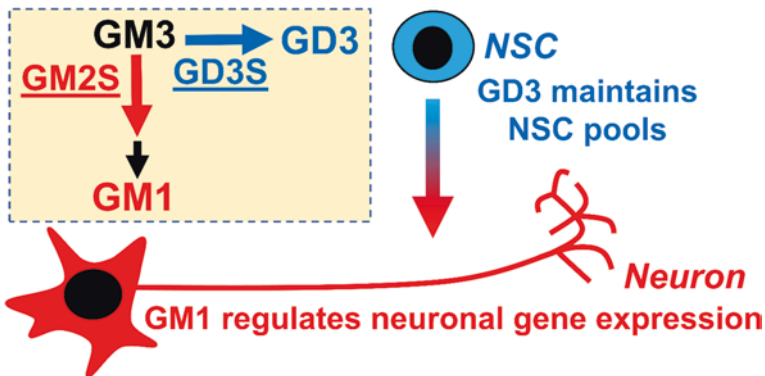


indicates that GM3 and its downstream gangliosides are important regulators of epilepsy and play an important role in placing adult newborn neurons at the right position.

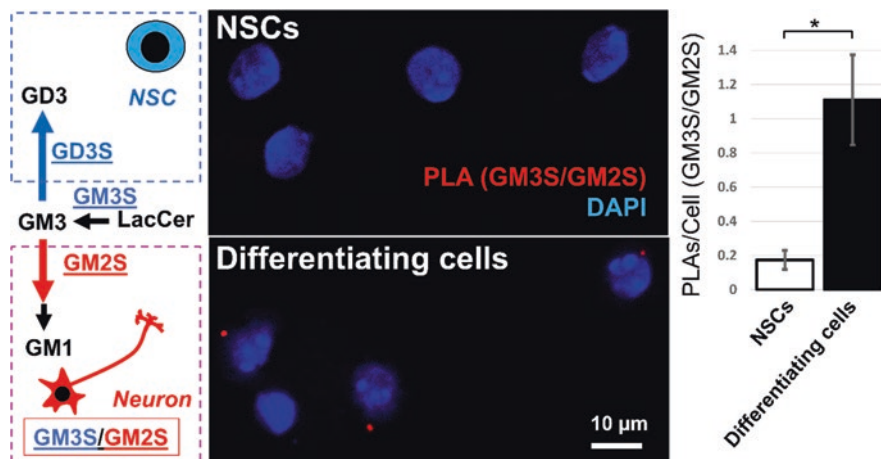
## 10 Future Studies

The simple ganglioside, GM3, is synthesized by attachment of sialic acid to LacCer (Fig. 10.1). This reaction, catalyzed by GM3S, is followed by enzymatic reactions that split ganglioside biosynthesis into two distinct pathways: to GD3 or to GM2/GM1 (Fig. 10.1). Addition of a second sialic acid residue to GM3, a reaction catalyzed by GD3S, results in synthesis of GD3. However, if GalNAc is the next sugar residue added to GM3, a reaction catalyzed by GM2S, ganglioside biosynthesis will exclusively switch to GM1 subsequent to formation of GM2. The switch to GD3 or GM2 (then GM1) is critical for NSCs or neuronal differentiation, respectively. GD3 is a robust ganglioside in NSCs, and loss of GD3 impairs stemness of NSCs. During neural differentiation, however, GD3 is decreased and complex GalNAc containing “postnatal brain type” gangliosides are increased, implying a switch from a decrease in glial cells and an increase in neuronal cells. We refer to this as the ganglioside “pathway switch” (Fig. 10.9).

We are currently analyzing several regulatory mechanisms of GT activities on NSC fate determination. Results from the PLA experiment, shown in Fig. 10.10, demonstrated that GM3S and GM2S are co-localized in differentiating neuronal cells, but not in undifferentiated NSCs. This exciting observation supports the hypothesis that GM3S and GM2S are able to form a protein complex that drives expression of the GTs needed to convert GM3 to more complex gangliosides, such as GM1.



**Fig. 10.9** Ganglioside “pathway switch” from GD3 to complex gangliosides (including GM1) by glycosyltransferases (GTs), has functional roles in determining neural cell fate



**Fig. 10.10** Enzyme complex formation of GTs regulates ganglioside expression and neuronal differentiation and functions. Co-expression of GM3S and GM2S in differentiating cells. NSCs were analyzed by Proximity Ligation Assay (PLA) using antibodies against endogenous GM3S and GM2S. After induction of neural differentiation (by retinoic acid), the PLA signal of GM3S/GM2S dramatically increased. It is now co-localized, in differentiating cells allowing for enzyme complex formation and “pathway switch” of the gangliosides from GD3 synthesis to generate more GM1. \* $p < 0.05$

Despite accumulating evidence obtained using GT-KO mice, the specific roles of gangliosides in determining the stage and cell-lineage determination of NSCs remain unclear. Furthermore, global ganglioside-KO mice, such as the GD3S-KO mice, cannot be investigated to determine if gangliosides regulate adult/postnatal neurogenesis, since their gangliosides are eliminated before birth. To resolve this issue, detailed research on ganglioside conditional KO mice is urgently required. Studies with GD3S/GM2S conditional KO mice are significant and will reveal more about the roles of gangliosides, including those in cell-, stage-, and disease-specific biological functions. We are investigating the molecular mechanisms of ganglioside-modulating receptor activities, including direct interaction of GD3 and growth factor receptors. We are studying the functional links between postnatal neurogenesis regulated by gangliosides, such as GD3, and the behavioral abnormalities of GD3S-KO mice. Further, specific complex formations of GT and gangliosides are predicted to induce GM2S activation leading to synthesis of more GalNAc-containing gangliosides such as GM2 and GM1, and reduced GD3. Ganglioside compositions are partially epigenetically regulated during development and differentiation. The novel epigenetic regulatory mechanisms for GTs will contribute to a better understanding of the “pathway switch” observed during differentiation. Our published data support our hypothesis that up-regulation of complex ganglioside GM1 induces epigenetic maturation of neurons.

Although recent studies of gangliosides have shed light on their roles in modulating signaling pathways during cellular differentiation and reprogramming, mice

deficient in some of these molecules show only subtle phenotypic abnormalities compared with the WT animals in early development. Clearly, the biological functions of one glycoconjugate can be substituted by another, albeit with less efficiency. Nonetheless, aberrant ganglioside expression becomes progressively more serious in the adult stage and pathogenic conditions. The “biological redundancy” can be considered for more pivotal roles of these molecules. Ganglioside expression profiles are connected not only with NSC fate determination but also with respective pathogenic mechanisms of neurodegenerative diseases. Epigenetic and post-translational regulation of cell surface and intracellular ganglioside microdomains will provide additional clues underlying the pathogenic mechanisms, which may be useful in developing novel strategies for disease treatment and neuronal regeneration. Future studies on ganglioside microdomains will prove fruitful in this regard.

**Conflict of Interest** The authors declare no conflicts of interest.

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# Chapter 11

## Gangliosides and Cell Surface Ganglioside Metabolic Enzymes in the Nervous System



Massimo Aureli, Laura Mauri, Emma Veronica Carsana, Dorina Dobi, Silvia Breviario, Giulia Lunghi, and Sandro Sonnino

**Abstract** Gangliosides are a large group of complex lipids found predominantly in the outer layer of the plasma membrane of cells, particularly abundant in nerve endings. Their half-life in the nervous system is short, and their membrane composition and content are strictly connected to their metabolism. The neobiosynthesis of gangliosides starts in the endoplasmic reticulum and is completed in the Golgi apparatus, whereas catabolism occurs primarily in lysosomes. However, the final content of gangliosides in the plasma membrane is defined by other cellular processes.

This chapter will discuss structural changes in the oligosaccharide chains of gangliosides, induced by the activity of plasma membrane-associated glycohydrolases and glycosyltransferases. Some of the plasma membrane enzymes originate from fusion processes between intracellular fractions and the plasma membrane, while, others display a different structure. Several of these plasma membrane enzymes have been characterized and some of them seem to have a specific role in the nervous system.

**Keywords** Gangliosides · Glycosphingolipids · Glycohydrolases · Sphingolipid metabolism · Central nervous system · Neuronal differentiation · Neurodegeneration

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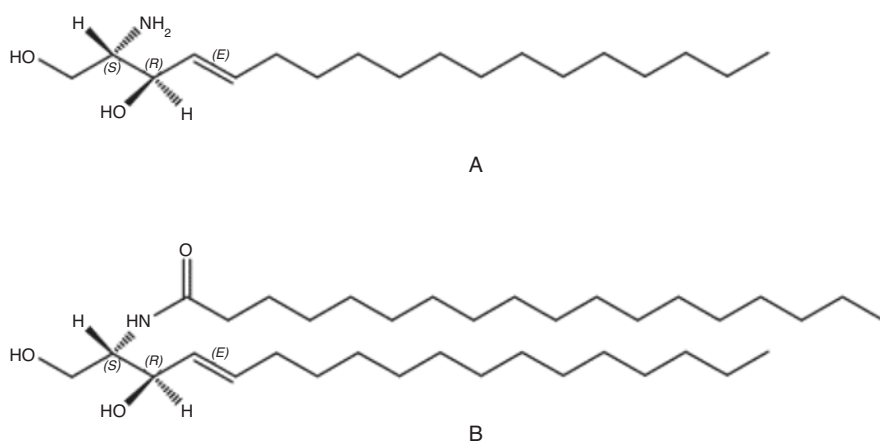
# 1 Gangliosides

Gangliosides are glycosphingolipids (GSLs) containing one or more sialic acid residues. They are components of the external layer of the plasma membrane of all mammalian cells and are particularly abundant in the brain, where they display tenfold higher levels with respect to extra-nervous tissues, representing one-twelfth of the outer layer of glycerophospholipids. Neuronal gangliosides are lipids with strong amphiphilic properties due to their acidic and, in general, large saccharide head group and double-tailed hydrophobic moiety.

## 1.1 Ceramide: The Lipid Portion of Gangliosides

The lipid backbone of gangliosides, as well as the one of all sphingolipids, is ceramide (Fig. 11.1a), which consists of a long-chain amino alcohol connected to a fatty acid by an amide linkage. As a peculiar feature of the nervous system, the amino alcohol can either be 2*S*,3*R*,4*E*,2-amino-1,3-dihydroxy-octadecene, known as sphingosine and C18-sphingosine or 2*S*,3*R*,4*E*,2-amino-1,3-dihydroxy-eicosene, known as eicosasphingosine and C20-sphingosine. The ratio between the two long-chain alcohols is variable: eicosasphingosine is barely detectable in fetal brain and increases gradually with age until it becomes the main component during old age. A small percentage of saturated compounds, sphinganine and eicosasphinganine, has also been identified (Valsecchi et al. 1993, 1996; Chiricozzi et al. 2021a).

In extra-nervous tissues, gangliosides exhibit heterogeneity in their acyl chains, where often a very long fatty acid moiety becomes the major component of the ceramide structure. While in the nervous system, stearic acid (Fig. 11.1b) is the



**Fig. 11.1** Chemical structures of C18-sphingosine (a) and ceramide containing C18-sphingosine and stearic acid (b)

most common fatty acid of the gangliosides accounting for 90–95% of the total fatty acid content. This characteristic of neuronal gangliosides may be necessary for neuronal membrane plasticity.

## 1.2 The Oligosaccharide Portion of Gangliosides

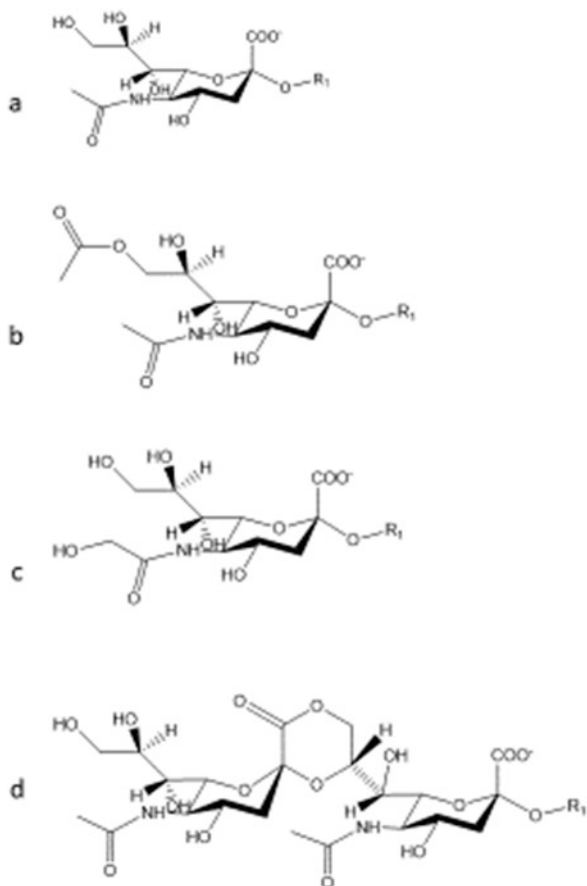
The oligosaccharide portion of neuronal gangliosides widely varies due to the neutral sugar content and the number of sialic acid residues. This property, together with the heterogeneity of the ceramide moiety, makes gangliosides a very large family of compounds. Table 11.1 shows the main ganglioside structures found in the human nervous system, together with trivial and commonly used abbreviations.

Sialic acid is a name that identifies all the derivatives of 5-amino-3,5-dideoxy-d-*glycero-D-galacto-non-2-ulopyranosonic acid*, or neuraminic acid. Among the sialic acids, the 5-*N*-acetyl (Fig. 11.2a), the 5-*N*-acetyl-9-*O*-acetyl (Fig. 11.2b), and the

**Table 11.1** The main oligosaccharide series in the human nervous system

Structure of the oligosaccharide chain	Series
$\beta$ -Gal-	Gal
$\beta$ -Gal-(1-4)- $\beta$ -Glc-	Lac
$\beta$ -GalNAc-(1-4)- $\beta$ -Gal-(1-4)- $\beta$ -Glc-	Gg <sub>3</sub>
$\beta$ -Gal-(1-3)- $\beta$ -GalNAc-(1-4)- $\beta$ -Gal-(1-4)- $\beta$ -Glc-	Gg <sub>4</sub>
$\beta$ -GalNAc-(1-4)- $\beta$ -Gal-(1-3)- $\beta$ -GalNAc-(1-4)- $\beta$ -Gal-(1-4)- $\beta$ -Glc-	Gg <sub>5</sub>
$\beta$ -Gal-(1-4)- $\beta$ -GlcNAc-(1-3)- $\beta$ -Gal-(1-4)- $\beta$ -Glc-	nLc <sub>4</sub>
The main gangliosides from the human nervous system	
Svennerholm nomenclature	IUPAC-IUB nomenclature
GM4	F <sup>3</sup> Neu5AcGalCer
GM3	II <sup>3</sup> Neu5AcLacCer
GD3	II <sup>3</sup> (Neu5Ac) <sub>2</sub> LacCer
GM2	II <sup>3</sup> Neu5AcGg <sub>3</sub> Cer
GD2	II <sup>3</sup> (Neu5Ac) <sub>2</sub> Gg <sub>3</sub> Cer
GM1	II <sup>3</sup> Neu5AcGg <sub>4</sub> Cer
3'-LM1	IV <sup>3</sup> nLc <sub>4</sub> Cer
GD1a	IV <sup>3</sup> Neu5AcII <sup>3</sup> Neu5AcGg <sub>4</sub> Cer
GalNAc-GD1a	IV <sup>3</sup> Neu5AcII <sup>3</sup> Neu5AcGg <sub>5</sub> Cer
GD1b	II <sup>3</sup> (Neu5Ac) <sub>2</sub> Gg <sub>4</sub> Cer
GD1b-lactone	II <sup>3</sup> [Neu5Ac-(2-8,1-9)-Neu5Ac]Gg <sub>4</sub> Cer
GT1b	IV <sup>3</sup> Neu5AcII <sup>3</sup> (Neu5Ac) <sub>2</sub> Gg <sub>4</sub> Cer
<i>O</i> -Acetyl-GT1b	IV <sup>3</sup> Neu5AcII <sup>3</sup> [Neu5,9Ac <sub>2</sub> -(2-8)-Neu5Ac]Gg <sub>4</sub> Cer
GQ1b	IV <sup>3</sup> (Neu5Ac) <sub>2</sub> II <sup>3</sup> (Neu5Ac) <sub>2</sub> Gg <sub>4</sub> Cer
<i>O</i> -Acetyl-GQ1b	IV <sup>3</sup> (Neu5Ac) <sub>2</sub> II <sup>3</sup> [Neu5,9Ac <sub>2</sub> -(2-8)-Neu5Ac]Gg <sub>4</sub> Cer

**Fig. 11.2** Sialic acid structures. **(a)** 5-acetamido-3,5-dideoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid, Neu5Ac. **(b)** 5-acetamido-9-O-acetyl-3,5-dideoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid, Neu5,9Ac<sub>2</sub>. **(c)** 3,5-dideoxy-5-glycolamido-D-glycero-D-galacto-non-2-ulopyranosonic acid, Neu5Gc. **(d)** Sialic acids linked together with ketosidic and ester linkages. R1: saccharides or oligosaccharide chain



5-*N*-glycolyl derivatives (Fig. 11.2c) are the most common ones. Importantly, healthy humans present only the first two sialic acids, in a ratio of 9:1 (Kamerling and Vliegenthart 1975). Also ganglioside lactones, containing polysialyl chains where the sialic acids are linked together with ketosidic and ester linkages (Fig. 11.2d), have been found in human brains (Riboni et al. 1986).

## 2 Gangliosides and Membrane Organization

Major part of the gangliosides are located in confined areas of the plasma membrane known as “lipid rafts” (Sonnino et al. 2006). Lipid rafts are specific membrane domains enriched in sphingolipids and cholesterol, with respect to glycerophospholipids, and contain about 1–4% of the total protein content of the cell (Brown and London 2000; Simons and Toomre 2000; Sonnino et al. 2006). Many lipid raft

proteins are involved in cell signaling events, and this led to the concept that ganglioside–protein interactions are instrumental in signal transduction and cell function (Bremer and Hakomori 1982; Lunghi et al. 2021; Chiricozzi et al. 2019). Protein properties might be affected by specific interactions, however, they could also be modified by the physicochemical properties of the membrane, which are determined by the lipid pattern, lipid amphiphilic and geometric properties and lipid organization. Gangliosides, with their complex and expanded oligosaccharide portion, need a larger interfacial area than glycerolipids. Phase separation by clustering GSLs in a phospholipid bilayer is a spontaneous process driven by minimization of the interfacial free energy. Segregation of amphiphilic molecules with a large hydrophilic head group implies the acquisition of a positive membrane curvature. The interfacial area increases along with the size of the oligosaccharide chain, conferring a more positive membrane curvature and a more pronounced segregation. The geometric properties of each ganglioside inserted into the membrane, depend primarily on the structure of the oligosaccharide portion (Sonnino et al. 1994) and, to a lesser extent, on those of ceramide.

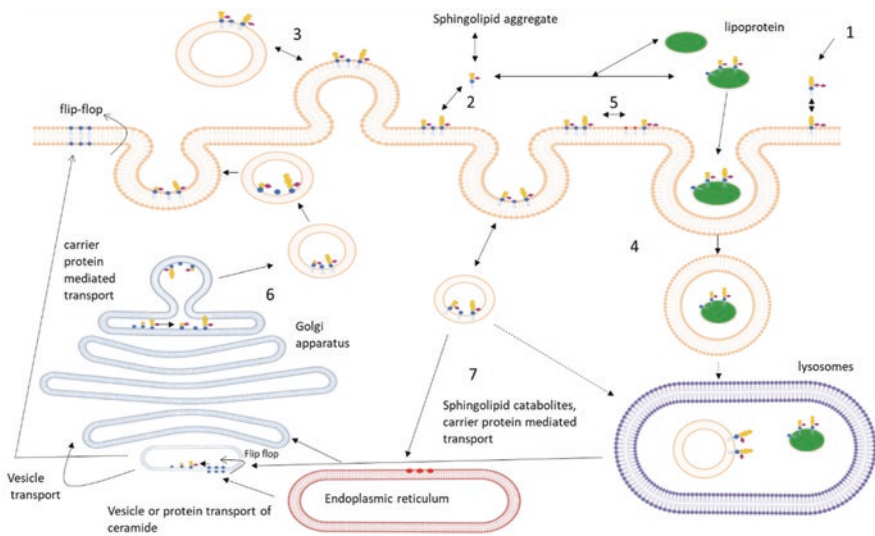
Any variation in either the head group or the ceramide portion can have marked effects on the membrane surface, particularly on lipid rafts, where gangliosides are highly enriched. For instance, in an artificial membrane model, a dramatic effect on membrane curvature and organization, was observed when sialidase was used to catabolize the disialoganglioside GD1a into monosialoganglioside GM1 (Del Favero et al. 2011). Interestingly, this process was also found in senescent neurons and during neurodegeneration, where the sequential hydrolysis of ganglioside to ceramide mediated by plasma membrane-associated GSLs hydrolases occurred (Aureli et al. 2011c).

Ceramide is very hydrophobic and almost insoluble in aqueous solutions, because of the presence of the two hydrocarbon chains. However, in a membrane, it can be considered amphiphilic due to the primary hydroxyl group and the amide planar linkage located at the water–lipid interface. It is claimed that when a large amount of ceramide is produced from complex sphingolipids, it rapidly segregates forming microdomains necessary for cell signaling (Gulbins and Grassme 2002). Ceramide has a very high packing parameter suitable for negative curvature. The removal of the head group from glycosphingolipids and sphingomyelin leads to ceramide-enriched endocytic vesicles as demonstrated in artificial membranes (Holopainen et al. 2000). On the other hand, in a natural membrane this process would require rearrangement of the membrane with the exclusion of some components and the inclusion of others. In this context, the original lipid–protein interactions or the forces exerted by the lipid environment on the protein conformation would change with concomitant modifications of the biological properties of proteins.

### 3 Metabolic Pathways of Gangliosides

The plasma membrane ganglioside content and pattern is due to a dynamic process determined by the balance of neobiosynthesis, catabolism, and complex trafficking in- and outside the cell. Changes in any of these pathways may result in alterations of the plasma membrane ganglioside content that can affect neuronal differentiation and cause neurodegeneration. A general scheme for GSLs metabolism is shown in Fig. 11.3a.

De novo biosynthesis of GSLs requires ceramide, which is synthesized in the endoplasmic reticulum (ER). In contrast to other cells, neurons have two different serine acyl-CoA acyltransferases: one specific for palmitoyl-CoA and the other for stearoyl-CoA. They are expressed in different proportions and in a spatiotemporal



**Fig. 11.3** (a) Scheme depicting glycosphingolipid metabolism. Different metabolic pathways involved in changing plasma membrane glycosphingolipids composition. (1) plasma membrane uptake of extracellular glycolipids shed by neighbour cells; (2) shedding of glycolipid monomers, which can either directly fuse with the membrane, or interact with the extracellular proteins or lipoproteins and are subsequently taken up by the cells and catabolized in lysosomes; (3) release of glycolipid-containing vesicles from the plasma membrane; (4) membrane endocytosis followed by sorting to lysosomes and lysosomal catabolism; (5) biosynthetic modifications by plasma membrane-associated glycosyltransferases and glycosidases; (6) neobiosynthesis of glycosphingolipids and their transport to the cell surface; (7) recycle in the biosynthetic pathway of partially catabolized molecules. (b) Biosynthetic pathway of ceramide in the brain. Ceramide is synthesized in the endoplasmic reticulum. Neurons have two different serine acyl-CoA acyltransferases: one specific for palmitoyl-CoA and the other for stearoyl-CoA. They are expressed in different proportions and in spatiotemporal- dependent manner during neuronal development, and are necessary for the first step in the synthesis of sphinganine and eicosasphinganine, respectively

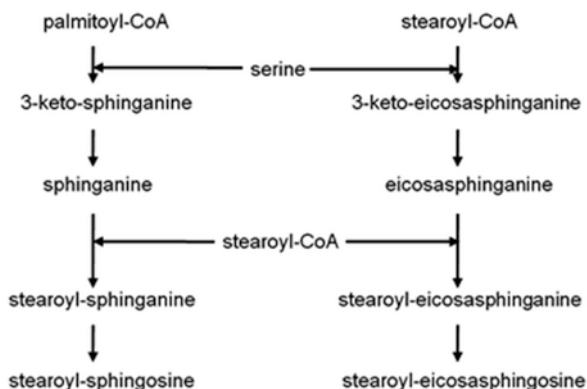


Fig. 11.3 (b)

dependent manner during the development of neurons (Chigorno et al. 1997a) and are necessary for the first step in the synthesis of sphinganine and eicosasphinganine, respectively. The biosynthetic process leading to the production of ceramide is shown in Fig. 11.3b. Ceramide can be transported to the Golgi apparatus, the site of ganglioside synthesis, by either vesicular or non-vesicular (protein mediated) trafficking (Olayioye and Hausser 2012). From the Golgi apparatus, neobiosynthesized gangliosides are transferred to the plasma membrane by a vesicle-mediated transport system, becoming components of the external plasma membrane leaflet.

On the other hand, catabolism of gangliosides takes place in lysosomes, where complex products are degraded into less complex ones and can be released in the cytosol (Kolter and Sandhoff 2005, see chapt. 12).

Among the more hydrophilic gangliosides, a minor portion is released from the plasma membranes into the extracellular environment (Chigorno et al. 2006). Nevertheless, sphingolipids present in the extracellular milieu are, at least in part, taken up by other cells, becoming either components of their membranes, where they may modify the composition, or be directly sorted to the lysosomes (Saqr et al. 1993).

In addition, enzymes involved in catabolism (hydrolases) and biosynthesis (transferases) of sphingolipids are found to be associated with the plasma membrane where they may act on membrane components (Aureli et al. 2011b).

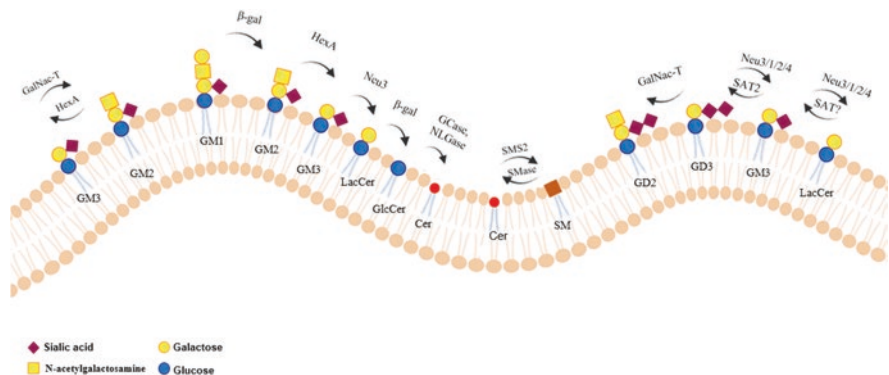
While the steps of the biosynthesis of GSLs are already well defined, little is known about their regulation. Based on observations, biosynthesis is primarily regulated at the transcriptional level by controlling expression of glycosyltransferases and/or transporter proteins. Indeed, changes in the cellular GSL pattern, as those observed during neuronal development, upon oncogenic transformation, and upon the development of drug resistance of tumor cells, are paralleled by changes in the expression of the corresponding glycosyltransferases. However, it is also possible that alterations in the intracellular metabolism and/or in the half-life of each GSL, influence their pattern (Veldman et al. 2002). Indeed, regulation of intracellular

trafficking may be as important as the regulation of enzyme expression, in determining the final GSL composition of the plasma membrane. Dependence of cells on neobiosynthesis and subsequent transport of the GSLs to the plasma membrane could require too much time to modify the plasma membrane GSL composition in response to extracellular signals. In addition, even though the half-life of GSLs may be short in neurons (Prinetti et al. 2001), it is long or very long in the majority of the other cells. In fibroblasts, where the half-life of gangliosides is long (Chigorno et al. 1997b), their major loss is due to shedding into the extracellular environment (Chigorno et al. 2005), rather than to catabolism. Indeed, it has been determined that up to 7–8% of total cell sphingolipids are shed every day by cells in culture (Chigorno et al. 2006), making neobiosynthesis of GSLs a necessity.

The fact that some enzymes involved in GSL metabolism have been found in the plasma membrane, modifies the general concept that glycohydrolases are located only in lysosomes and glycosyltransferases only in the Golgi apparatus. The presence in the plasma membrane of enzymes, involved in GSL metabolism, allowed the gain of information about their activity on natural substrates in living cells, along with identification of the presence of enzyme pairs, such as sialidase and sialyltransferase, that are able to catalyze opposite reactions. This suggests that alterations in plasma membrane GSLs could occur rapidly in response to different stimuli. These changes would only depend on the kinetic properties of the enzymes, which can change very rapidly in response to ligand-triggered stimuli.

#### **4 Plasma Membrane-Associated Enzymes and Ganglioside Pattern**

The concept that GSL biosynthesis and catabolism are specifically associated with the Golgi apparatus and the lysosomes respectively, has been partially overcome by emerging information related to the presence of enzymes involved in GSLs metabolism on the plasma membrane. These enzymes include the sialidases and sialyltransferases,  $\beta$ -hexosaminidases and  $\beta$ -*N*-acetyl-galactosyltransferases,  $\beta$ -galactosidases, and the  $\beta$ -glucocerebrosidase GCase and the non lysosomal  $\beta$ -glucosylceramidase (NL-Gase). Description of the synaptosomal membrane association of sialidases and sialyltransferases goes back to the 70s (Schengrund and Rosenberg 1970; Tettamanti et al. 1972, 1973, 1975; Preti et al. 1980), and leads to the hypothesis that a sialylation–desialylation cycle exists for gangliosides at plasma membrane level and might be involved in defining the functional role of gangliosides in neurons. The sialylation–desialylation cycle anticipates the elucidation of the “sphingomyelin cycle” by 20 years (Venable et al. 1995). In this cycle, the two enzymes, sphingomyelinase and sphingomyelin synthase, modulate cell proliferation and apoptosis playing on the levels of ceramide content. Recent information on the existence of a similar cycle for *N*-acetylgalactosamine, confirms the possibility that the sphingolipid composition of the plasma membrane can be



**Fig. 11.4** Schematic representation of the glycosyltransferases and glycohydrolases associated with the cell surface. The presence of these different enzymes at this level, allows the in situ modification of the cell surface glycolipid composition. The availability of a series of enzyme couples catalyzing the same reaction in opposite directions extends the concept of cycle, originally reported for sphingomyelin, to several other sphingolipids.  $\beta$ -hexosaminidase A (Hex A); UDP-N-UDP-N-acetylgalactosaminyltransferase (GalNAc-T);  $\beta$ -galactosidase Smase( $\beta$ -Gal); sphingomyelinase (SMase); sphingomyelinase2 (SMS2) SAT1

drastically modified. The head group of an amphiphilic compound, together with the volume of the hydrophobic backbone, determines the packing parameter of the monomer inserted into the membrane. In GSLs, this parameter is determined by the ratio between the volume occupied by the oligosaccharide chain and by the ceramide. The enzymatic removal of sugars from the GSL leads to a higher packing parameter resulting in a less curved surface. On the other hand, the enzymatic addition of sugars reduces this packaging parameter and promotes an enhanced curvature. (Sonnino et al. 1994; Brocca and Sonnino 1997). All these aspects allow the formation of “lipid rafts” with specific size and dynamics. In addition, drastic changes in the amphiphilic properties of GSLs, such as the sequential action of hydrolytic enzymes able to produce ceramide from gangliosides, favors the translocation of ceramide from the outer to the inner membrane layer, or the formation of endocytic vesicles. These modifications explain the first step in a process, that causes the shift of ceramide from the plasma membrane to internal cell membranes. Figure 11.4 is a schematic representation showing how the membrane curvature changes in response to enzymatic activities.

## 5 Sialidases, Sialyltransferases and Plasma Membrane Sialidase Neu3

In gangliosides, sialic acid is usually linked to the C3 position of galactose or to the C8 position of another sialic acid residue, by an  $\alpha$ -linkage. Four different sialidases capable of catalyzing the release of ganglioside sialic acid moieties have been



identified. Neu1 is located in lysosomes and it is known from long time to be needed for the catabolism of sialo-compounds. Neu2 is a cytosolic enzyme that seems to be involved in differentiation processes, and is capable of removing the sialic acid linked to the inner galactose of GM1, as well under specific experimental conditions (Tringali et al. 2004). Neu3 is associated with the plasma membrane and has been described to be involved in several processes. Neu4, which is present in humans in two different forms, a short and a long one, has been found associated with mitochondria, ER and lysosomes.(Glanz Victor Yu, *European Journal of Pharmacology* 2018).

Interestingly, it has been demonstrated that the localization of the sialidases can vary according to specific stimuli. Although Neu3 is considered the plasma membrane specific enzyme isoform, small amounts of the other sialidases have also been found to be associated with the plasma membrane or intracellular membranes (Wang et al. 2009; Akita et al. 1997; Lukong et al. 2001; Vinogradova et al. 1998).

Monocyte differentiation up-regulates the expression of lysosomal sialidase Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments (Liang et al. 2006; Nath et al. 2018).

Neu3 (NEU3, EC 3.2.1.18) is the first sialidase to be distinguished from lysosomal isoforms and to be identified at the plasma membrane level. This finding was suggested by enzymatic and immunological studies (Schengrund et al. 1976; Miyagi et al. 1990a, b; Schneider-Jakob and Cantz 1991; Kopitz et al. 1994), as well as by metabolic studies (Riboni et al. 1991; Kopitz et al. 1997a), where a membrane-bound sialidase was purified from human brain grey matter (Kopitz et al. 1997b) and from bovine brain (Hata et al. 1998; Oehler et al. 2002). In 1999, the existence of a specific membrane associated sialidase (coded as Neu3), different from other known sialidases, was proven by cloning its cDNA sequence from human (Wada et al. 1999), bovine (Miyagi et al. 1999) and mouse (Hasegawa et al. 2000). It can be considered an ubiquitous enzyme since it is expressed, albeit at different levels, in the plasma membrane of the majority of healthy and pathological human tissues, such as the human brain (Kopitz et al. 1994), healthy colon, as well as colorectal carcinoma tissues, hepatic tumors, and kidney carcinomas (Monti et al. 2002; Miyagi et al. 2008a, b; Ueno et al. 2006; Kakugawa et al. 2002). In addition, its expression and activity were also assayed in healthy and pathological cell lines, for example in erythroid and erythroleukemic cell lines (Venerando et al. 2002; Tringali et al. 2007a, b), fibroblasts (Chigorno et al. 1986), neurons, neuroblastoma cells (Schengrund and Repman 1982), breast ductal cancer T47D cells, colon carcinoma CaCo<sub>2</sub> cells, colorectal adenocarcinoma HT29 cells, different types of ovarian cancer cells and cervix adenocarcinoma HeLa cells (Kakugawa et al. 2002).

The partial association of lysosomal Neu1 with the plasma membrane is in some cases the result of the fusion processes required to repair plasma membrane damages (Reddy et al. 2001). Nevertheless, other pathways, such as the lysosomal exocytosis due to an excess of intracellular lysosomes, (Samarani et al. 2018) as well as the fusion of mitochondria and ER with the plasma membrane, (Annunziata et al. 2018) are involved in the trafficking of Neu1 towards the cell surface. On the other

hand, even if some evidence suggest its occurrence, it is more difficult to imagine Neu2 traslocation from the cytosol to the plasma membrane (Nath et al. 2018).

It is well-known that in the plasma membrane Neu3 and gangliosides co-localize in Triton X-100 insoluble “lipid rafts” (Kalka et al. 2001). The non random distribution of Neu3 at the cell surface could suggest that the biological effects of this enzyme can be attributed to the local reorganization of GSLs-based signaling units. Remarkably, Neu3 modulates cell surface GSLs composition by *trans* interactions, hydrolyzing substrates on the surface of neighbour cells (Papini et al. 2004). The optimal pH of Neu3, Neu1, and Neu4, ranges between 4.4 and 4.8. This raises the question of how such an acidic pH can be achieved at the cell surface. The proton pumps co-localize with Neu3 and gangliosides and allow the achievement, in a small environment, of the proper pH required for enzyme function and ganglioside hydrolysis. This has been demonstrated in *in vitro* experiments by the inhibition or activation of the cell surface proton pump system (Aureli et al. 2012b).

Neu3 was originally described as an enzyme that specifically hydrolyzes gangliosides (Miyagi et al. 2018). Later, thanks to the metabolic *in vitro* labelling of glycoproteins with [<sup>3</sup>H]*N*-acetylmannosamine, hydrolysis was also observed to occur on sialic acid residues linked to glycoproteins (Valaperta et al. 2006). It is difficult to compare the kinetic properties of glycohydrolases on different substrates, such as glycolipids, oligosaccharides, and glycoproteins. The enzyme  $K_m$  and  $V_{max}$  strictly depend on how the substrate gets presented to the enzyme. Kinetic properties of hydrolases working on gangliosides have been studied in detail. These parameters can vary in several orders of magnitude, from aggregates to free oligosaccharides, due to the different interaction with the substrate and substrate recognition. Similarly, the two parameters are completely different on the same substrate presented as either a homogeneous micelle, micelle containing a detergent, or liposomes with different contents of substrate (Masserini et al. 1982; Venerando et al. 1982).

Since Neu3 is of great interest, several studies have been conducted to understand its role in cellular processes. In colon and renal cancer this sialidase appears to be responsible for acting on the monosialo-ganglioside GM3, maintaining high cellular levels of lactosylceramide (LacCer), that can exert a Bcl-2-dependent anti-apoptotic effect, contributing in this way to the survival of cancer cells and subsequent tumor progression (Kakugawa et al. 2002; Ueno et al. 2006). Neu3, together with plasma membrane associated  $\beta$ -galactosidase and  $\beta$ -glucosidase (see the following paragraphes), can act on gangliosides to produce bioactive ceramide at the cell surface level of cultured human fibroblasts (Valaperta et al. 2006). Expression of any of these three enzymes can affect the expression/activity of the others, for instance overexpression of Neu3 results in an increased expression/activity of both  $\beta$ -galactosidase and  $\beta$ -glucosidase. The concomitant increase of their enzymatic activity catalyzes the conversion of GM3, a substrate for Neu3, to ceramide leading to higher ceramide levels and to a switch from a cell proliferative state to apoptosis.

Nevertheless, the increase of cell surface ceramide is paralleled by a slight reduction in ganglioside GM3, the main ganglioside in fibroblasts. In fact, the overexpression of Neu3 leads to an increased expression of GM3 synthase, the

sialyltransferase (SAT1) responsible for GM3 biosynthesis, allowing for compensation of the reduction of GM3 mediated by Neu3. The augmented expression of SAT1 depletes LacCer, reducing its availability for the biosynthesis of globosides such as globotriaosylceramide, which is abundant in fibroblasts.

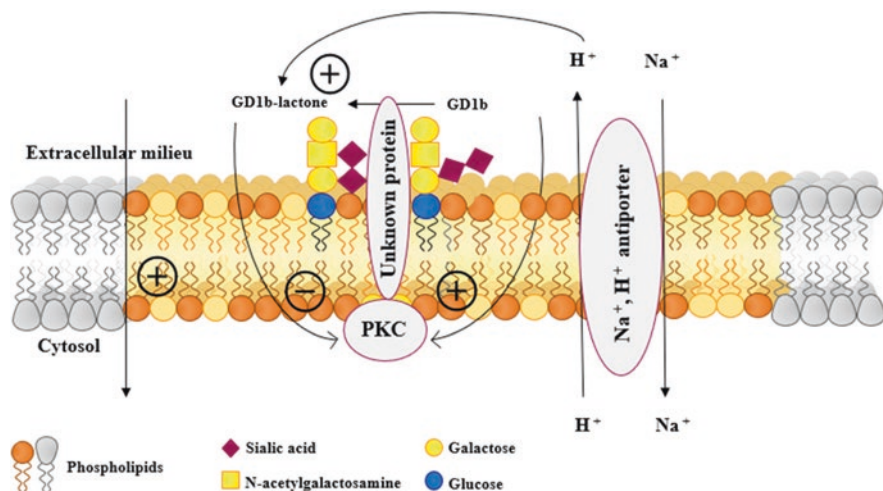
Neu3 catalyzes the hydrolysis of sialic acid acting both on  $\alpha$ 2–8 and  $\alpha$ 2–3 external ketosidic linkages, but it is ineffective on inner  $\alpha$ 2–3 sialyl residues. An increase in Neu3 activity modifies the cell surface ganglioside composition by catalyzing the progressive conversion of GD1a to GM1 and of GM3 to LacCer. These changes have significant effects on neuronal differentiation and apoptosis, both in normal and pathological cells (Kakugawa et al. 2002; Valaperta et al. 2006, 2007). In mouse and human neuroblastoma cells, the pharmacologically induced neuronal differentiation is paralleled by a higher Neu3 expression and activity (Proshin et al. 2002). Neurite outgrowth can also be induced by overexpressing Neu3. In addition, an increase in Neu3 activity, enhances the extension and/or branching of neurites as demonstrated by the exposure of neuroblastoma cells to 5-bromodeoxyuridine (Hasegawa et al. 2000). Conversely, in SK-N-MC neuroblastoma cells, inhibition of Neu3 activity results in the loss of neuronal differentiation markers (Kopitz et al. 1994; von Reitzenstein et al. 2001). However, decrease of Neu3 expression does not prevent induction of neuroblastoma cell differentiation (Valaperta et al. 2006).

In cultured rat granule cells, expression of Neu3 increases during differentiation and is maintained constant during aging (Aureli et al. 2011c). In cultured hippocampal neurons, Neu3 activity regulates the plasma membrane content of GM1 ganglioside, essential for axonal growth and regeneration upon axotomy (Rodriguez et al. 2001; Kappagantula et al. 2014; Chiricozzi et al. 2020, 2021b). In these neurons, Neu3 activity is asymmetrically concentrated at the end of neurites, determining their fate by a local increase in TrkA activity (Da Silva et al. 2005).

Much evidence exists regarding *in situ* sialylation of cell surface gangliosides. The original report on the presence of a synaptosomal membrane sialyltransferase in calf brain (Preti et al. 1980) was confirmed by metabolic studies in chicken embryos (Matsui et al. 1986) and rat brain (Durrrie et al. 1988; Durrrie and Rosenberg 1989). More recently, it has been shown that exposure to dexamethasone markedly increases GM3 ganglioside synthesis by enhancing gene expression and enzyme activity of SAT1. Metabolic studies indicate that this event is localized at the plasma membrane level (Iwamori and Iwamori 2005), thus confirming that glycolipid sialylation is not confined to the Golgi apparatus, contributing to the local modulation of the cell surface GSL pattern.

Direct evidence of expression and activity of plasma membrane-associated sialyltransferases was obtained in epithelial and melanoma cells, where GD3 is produced from endogenous GM3 ganglioside, as well as from exogenously incorporated substrate. GD3 synthesis occurs using CMP-NeuAc synthesized inside the cell prior to transfer outside the cell through an unknown process (Crespo et al. 2010). In addition, it is important to recall that this sialyltransferase displays both *cis* and *trans* activity (Vilcaes et al. 2011).

Given the high concentration of gangliosides in the nervous system, and the very high and progressive increase of Neu3 and total sialyltransferases during neuronal



**Fig. 11.5** Monolactone formation due to the acidic plasma membrane microenvironment. The catalytic protons are generated by the Na<sup>+</sup>/H<sup>+</sup> pump located at the cell surface and activated through the action of protein kinase C (PKC). The local acidic microenvironment is directly responsible for lactone formation

differentiation, it is possible to hypothesize the existence of a sialylation–desialylation cycle on the plasma membrane. This could have a specific role during neuronal cell specialization, especially during cell stages in which it is necessary to build specialized membranes, such as axonal protrusion and elongation, dendritic arborization, and synaptogenesis. These enzymes could also be essential for axon repair and/or synaptic function.

A further modification of the oligosaccharide structure of gangliosides, that involves sialic acid, is represented by the lactonization of molecules containing a disialosyl residue, such as GD1b ganglioside. Ganglioside lactones are present as minor components in the brain of vertebrates (Sonnino et al. 1983; Riboni et al. 1986). GD1b monolactone formation has been studied *in vitro* in the presence of catalytic protons (Bassi et al. 1989) (Fig. 11.5), and it has been demonstrated that lactonization influences the conformation and both the aggregative (Acquotti et al. 1987) and biological properties of GD1b (Bassi et al. 1991). Indeed, GD1b is able to directly interact with several proteins (Prinetti et al. 2000b) and to modulate the activity of several plasma membrane associated protein kinases (Bassi et al. 1991), while GD1b-lactone does not, or does so in a reduced way (Bassi et al. 1991; Sonnino et al. 1990). This suggests that lactonization/delactonization of gangliosides might represent a localized event able to trigger specific ganglioside-mediated cellular events. *In vivo*, GD1b lactonization occurs in neurons through a process that involves the presence of a specific enzyme associated with the plasma membrane (Bassi et al. 1994).

## 6 $\beta$ -Hexosaminidase and *N*-Acetylgalactosaminyltransferase

In neurons,  $\beta$ -hexosaminidase (Hex) is the enzyme that removes *N*-acetylgalactosamine from GM2 and GalNAc-GD1a forming GM3 and GD1a, respectively. Both GM2 and GalNAc-GD1a are minor compounds at the cell surface. GalNAc-GD1a is difficult to be recognized within a ganglioside mixture separated by HPTLC since its chromatographic behavior is similar to that of GD1a (Svennerholm et al. 1973; Acquotti et al. 1994). Indeed,  $\beta$ -hexosaminidase is worldwide associated with the lysosomal hydrolysis of ganglioside GM2.  $\beta$ -Hexosaminidase is a dimeric enzyme that exists in three different isoforms. It has two diverse subunits,  $\alpha$  (528 residues) and  $\beta$  (556 residues), encoded by two different but evolutionarily related genes, HEX A and HEX B (Triggs-Raine et al. 2001). Each subunit has its own active site; the  $\beta$ -subunit hydrolyzes uncharged substrates, whereas the  $\alpha$ -subunit catalyzes cleavage of GalNAc from negatively charged ones (Bearpark and Stirling 1978; Kytzia and Sandhoff 1985). However, dimerization is necessary for the enzyme to become fully functional. Thus, the  $\alpha$ - and  $\beta$ -subunits can form three different  $\beta$ -hexosaminidase isoenzymes: Hex A ( $\alpha\beta$ ), Hex B ( $\beta\beta$ ), and Hex S ( $\alpha\alpha$ ). Only the  $\alpha\beta$  heterodimer, Hex A, is able to remove  $\beta$ -linked GalNAc at the non-reducing end of ganglioside GM2 and GalNAc-GD1a, in the presence of the GM2 activator protein, a specific cofactor of Hex A (Kolter and Sandhoff 2006).

Genetic defects, either in the genes encoding the  $\alpha$ - and  $\beta$ -subunits of Hex A, or in the GM2 activator protein, can result in GM2 accumulation in neural tissues leading to the onset of GM2-gangliosidosis. GM2-gangliosidosis manifests in three forms; Tay–Sachs disease, due to defects in the  $\alpha$ -subunit (TSD, OMIN 2728800), Sandhoff disease, characterized by defects in the  $\beta$ -subunit (SD, OMIN 268800), and the AB variant of Sandhoff disease (OMIN 272750), in which both subunits are affected (Bateman et al. 2011). The massive neuronal accumulation of GM2 is accompanied by progressive neurological deterioration affecting motor, cerebral and spinocerebellar functions.

The presence of active Hex A in the external leaflet of the plasma membrane was discovered in cultured fibroblasts (Mencarelli et al. 2005). Immunological and biochemical characterization of the membrane associated Hex A shares the same structure as the lysosomal isoform. This suggests that a regulated fusion process between lysosomes and the external leaflet of the plasma membrane (Reddy et al. 2001) might be responsible for the transport of lysosomal enzymes to the cell surface, where the enzymes could exert their activity, remodeling the glycolipid content and pattern. It has been demonstrated that during differentiation of murine neuronal stem cells, plasma membrane Hex increases its activity, reaching a peak in fully differentiated cells (Aureli et al. 2011a). On the other hand, analysis of the plasma membrane-associated glycohydrolases in fibroblasts derived from patients affected by different variants of Gaucher disease (GD) demonstrates an increased activity only in cells derived from patients affected by the most severe neuronopathic form of Gaucher disease (GD2) (Aureli et al. 2012a).

Analysis of epithelial cells indicate the presence on the cell surface of an UDP-GalNAc: LacCer/GM3/GD3 *N*-acetylgalactosaminyl transferase able to act on exogenous GM3 (Crespo et al. 2010). Currently, no data are available regarding its activity on endogenously synthesized GSLs. However, its ability to act on exogenous substrates could be important in the plasma membrane remodeling process. In fact, the glycolipid composition of the plasma membrane could also be remodeled by the uptake of glycolipids from the extracellular environment (e.g., from other cells, lipoproteins, or molecules shed by other cells). These compounds, depending on the “cellular request” and on their aggregation, could be endocytosed or could become components of the cell surface, either directly, or after modification by the action of plasma membrane-associated enzymes. The coexistence of Hex and  $\beta$ -hexosaminyl transferase on the cell surface supports the hypothesis of the existence of a glycolipid cycle at the plasma membrane level, which could have important biophysical effects on the membrane itself, and affect events that regulate the “cell social life.”

## 7 $\beta$ -Glucocerebrosidase

$\beta$ -glucocerebrosidase is a hydrolase responsible for the catabolism of glucosylceramide to yield glucose and ceramide. At least three different  $\beta$ -glucocerebrosidase enzymes have been described;  $\beta$ -glucocerebrosidase (GCCase, EC 3.2.1.45) sensitive to inhibition by conduritol B epoxide (CBE) and primarily associated with lysosomes (Neufeld et al. 1996), a cytosolic  $\beta$ -glucosidase (GBA3, EC 3.2.1.21) (Daniels et al. 1981), and a non-lysosomal  $\beta$ -glucosylceramidase (NLGase, EC 3.2.1.45) (Van Weely et al. 1993).

NLGase is able to catalyze the hydrolysis of glucosylceramide both at the cell surface and in the ER (Van Weely et al. 1993; Korschen et al. 2012). In fact, the cellular localization of NLGase is still controversial, since it has been described to be associated with endosomal vesicles, plasma membrane, ER, and the Golgi apparatus. Analysis of data describing its localization, indicate that it depends on the cell type and stage of development. Until now, most of the pieces of information concerning NLGase localization come from studies performed on cells overexpressing NLGase, as a chimeric protein fused to the green fluorescence protein (GFP), therefore, further investigations are needed to define its localization. Database searching of NLGase cDNA sequences revealed apparent orthologs of this enzyme in species ranging from *Drosophila* to *Arabidopsis*, and to vertebrates, indicating that the protein is highly conserved among species and suggesting its functional importance. Study of NLGase expression and activity showed that in humans this enzyme is abundant in the brain, heart, skeletal muscle, and kidney (Matern et al. 2001), whereas in mouse it is mostly represented in the testis and brain (Yildiz et al. 2006).

Interestingly, a study on NLGase knockout (KO) mice presented an abnormal accumulation of glucosylceramide in multiple tissues, including brain, liver, and testis. The KO mice show normal bile acid metabolism and, apparently, no

impairment in the central nervous system (CNS), however, the accumulated glucosylceramide leads to decreased fertility due to the formation of misshapen spermatozoa (Yildiz et al. 2006). More recent studies indicated that mutations in NLGase cause autosomal recessive cerebellar ataxia with spasticity and spastic paraplegia in humans (Martin et al. 2013; Hammer et al. 2013; Malekkou et al. 2018; Gatti et al. 2021). Moreover, antisense morpholino oligonucleotides targeting the NLGase orthologous gene in a zebrafish model lead to abnormal motor behaviour, axonal shortening, and branching of motoneurons (Martin et al. 2013). This condition can be rescued by transfecting cells with human wild-type mRNA for NLGase, but not with mRNA containing the missense mutation found in patients affected by autosomal recessive cerebellar ataxia with spasticity. These data suggest a specific role of NLGase in the control of the cellular glucosylceramide–ceramide balance that could be responsible for the onset of motoneuron defects (Martin et al. 2013). As mentioned before, homozygous NLGase KO mice at 4 months of age, do not show apparent neurological signs, liver dysfunction, or reduced viability, even if the accumulation of glycolipid species has been detected by mass spectrometry analyses in the brain, liver, and testis (Yildiz et al. 2006). It may take longer to observe the neurological phenotype in these mice, as it has been observed in other mouse models of hereditary spastic paraplegia. The latter may reflect the fact that the neurological signs are very slight during the first months of life (Ferreirinha et al. 2004; Soderblom et al. 2010). Impairment of their nervous system could also be masked by the fact that the structure of the corticospinal tract of mice is different from that of humans or zebrafish, or by a compensatory mechanism mediated by other enzyme isoforms during early stages of development (Martin et al. 2013). Of note, NLGase of KO mice lacks only exons 5–10, and retains 50% of normal glucosidase activity. Therefore, it has been hypothesized that the accumulation of glucosylceramide in the ER and/or at plasma membrane level, does not reach the threshold necessary for the development of neurological symptoms.

It has been shown that NLGase activity increases during neuronal differentiation (Aureli et al. 2011a). Indeed, in primary neuronal cultures, cell surface  $\beta$ -glucosidase activity is mainly due to the NLGase enzyme, whose activity increases more than threefold during neuronal differentiation (Aureli et al. 2011c). On the contrary, in murine neuronal stem cells, plasma membrane  $\beta$ -glucosidase activity is largely due to the CBE-sensitive  $\beta$ -glucosidase enzyme, GCase, with a GCase/NLGase ratio of 0.75 in precursors and 2.3 in differentiated cells (Aureli et al. 2011c).

The different behaviors of these enzymes in the two different cell models could be due to the serum-induced murine neuronal stem cell differentiation, which yields only about 10% neurons and about 70–80% glia, whose expression of the two different  $\beta$ -glucosidase enzymes is very different than that of neurons. Indeed, in non-neuronal cell lines, such as human fibroblasts, a GCase/NLGase ratio around 7 has been observed (Aureli et al. 2009). The lack of data on the activity of plasma membrane-associated glycohydrolases in cultures of astrocytes and oligodendrocytes, indicates that further studies are needed. Interestingly, studies on fibroblasts derived from patients affected by different variants of Gaucher's disease (GD), show that the reduction of total GCase activity is paralleled by an increase in

NLGase activity and expression. This is particularly evident in fibroblasts derived from patients affected by GD2, the most severe form of the neuronopathic pathology (Aureli et al. 2012a). Despite all these observations, the link between NLGase and neuronal differentiation, as well as neurodegeneration, are still unclear and require further studies.

Several years ago the presence of a cell surface GCase activity was described (Aureli et al. 2009). Studies on human fibroblasts derived from patients affected by GD showed that GCase plasma membrane-associated activity is significantly reduced with respect to fibroblasts derived from healthy donors, ascribing this reduction to a deficiency in GCase enzyme (Aureli et al. 2012a).

The increase in GCase and NLGase activities on the plasma membrane of human fibroblasts and subsequent increase in ceramide levels are responsible for cell cycle arrest and apoptosis (Valaperta et al. 2006). To date, a multicentre study has demonstrated that GCase mutations represent the most common genetic risk factor for the development of Parkinson's disease (PD) (Sidransky et al. 2009). Importantly, Mazzulli et al. has demonstrated that in the brain and neurons derived from PD patients, the lysosomal accumulation of glucosylceramide, the substrate of GCase, directly influences the abnormal lysosomal storage of  $\alpha$ -synuclein oligomers resulting in a further inhibition of GCase activity (Mazzulli et al. 2011; Wallom et al. 2022). These findings suggest, for the first time, that the bidirectional effect of decreased GCase activity and  $\alpha$ -synuclein accumulation forms a positive feedback loop that may lead to a self-propagation of the disease (Mazzulli et al. 2011). To date, this process has been described for the lysosomal function of GCase, but due to its involvement in the production of pro-apoptotic ceramide at the plasma membrane level, GCase and NLGase associated with the cell surface could contribute to neuronal impairment in neurodegenerative diseases.

In mammalian cells, GCase and NLGase are not just involved in the catabolism of glucosylceramide, but show also transferase activity catalyzing the transglucosylation reaction of glucose from glucosylceramide to cholesterol, forming, glucosylcholesterol, *in vitro* (Akiyama et al. 2013). On the other hand, both GCase and NLGase are able to catalyze the opposing reaction, synthesizing again glucosylceramide starting from glucosylcholesterol (Marques et al. 2016). Interestingly, it has been demonstrated that the increased content of glucosylcholesterol is paralleled by the accumulation of its substrates, glucosylceramide or cholesterol, suggesting that the equilibrium between the synthesis and the catabolism of glucosylcholesterol is strongly dependent on the concentration of the substrates (Marques et al. 2016). The transglucosylation activity of GCase and NLGase represents an interesting intersection between the two major lipid metabolic pathways of sphingolipids and sterols, which could be fundamental in maintaining cell homeostasis. Since GCase and NLGase are widely expressed in the brain, their altered expression does lead to changes in glucosylceramide and glucosylcholesterol metabolism, opening a new scenario on the study of the related pathologies.



## 8 $\beta$ -Galactosidase

Two different  $\beta$ -galactosidase enzymes, involved in GSL metabolism, have been described:  $\beta$ -galactocerebrosidase ( $\beta$ -Gal-ase, GALC EC 3.2.1.46), which catalyzes the hydrolysis of galactose from lactosylceramide, LacCer, and galactosylsphingosine, and the  $\beta$ -galactosidase ( $\beta$ -Gal, EC 3.2.1.23), which catalyzes the hydrolysis of the terminal galactose from GM1 (Li and Li 1999). As it is well known, the loss of function of  $\beta$ -Gal-ase, is responsible for the development of globoid leukodystrophy (Krabbe disease), while deficiency of  $\beta$ -Gal causes GM1 gangliosidosis (Xu et al. 2010). Both sphingolipidoses are characterized by an impairment of the central nervous system, even if the molecular bases are still unclear.

In addition to the other lysosomal enzymes, plasma membrane-associated  $\beta$ -galactosidase activity has been found in several cell lines (Aureli et al. 2011b). The identity of the proteins responsible for the  $\beta$ -galactosidase activity present at the cell surface is still unknown. However, in living human fibroblasts, the presence of a  $\beta$ -galactosidase which displays a *trans* activity on neighbour cells has been verified. The enzyme is active in the absence of detergents or activator proteins, suggesting that on the cell surface there is at least one enzyme with a  $\beta$ -galactosidase-like activity (Aureli et al. 2009). In the same cell model, it has been shown, that the expression of  $\beta$ -galactosidase is upregulated by Neu3 overexpression and correlates with the onset of ceramide-mediated apoptosis (Valaperta et al. 2006).  $\beta$ -galactosidase activity was measured during neuronal differentiation and aging, in both the total cell lysate and the plasma membrane fraction derived from rat cerebellar granule cells. Both activities were upregulated during cell differentiation. As expected,  $\beta$ -galactosidase activity associated with the plasma membrane is lower than the one present in the total cell lysates. While total cell activity remained constant during differentiation, it increased about four fold during aging. In contrast, cell surface activity increases of ten fold during differentiation and then doubles during neuronal senescence (Aureli et al. 2011c). A similar behavior has been described for plasma membrane-associated  $\beta$ -galactosidase activity during differentiation of neural stem cells (Aureli et al. 2011a). To this purpose,  $\beta$ -galactosidase activity has been proposed as a marker of aging and senescence (Coates 2002; Dimri et al. 1995; Severino et al. 2000; Geng et al. 2010). The behavior of the plasma membrane-associated enzyme in rat cerebellar granule cells suggests that  $\beta$ -galactosidase activity could be used as a hallmark of both neuronal differentiation and aging, as well as of apoptosis, in fibroblasts. On the other hand, little is known regarding the functional role of plasma membrane-associated  $\beta$ -galactosidases. It has been hypothesized that they may act as cell surface receptors mediating various cell–cell and cell–matrix interactions which are responsible for cell migration, differentiation, and axonal branching (Evans et al. 1993; Huang et al. 1995). No data are available on their enzymatic properties at plasma membrane level.

## 9 Membrane Dynamics: A Non-canonical Pathway Involved in the Establishment of the GSL Profile at the Plasma Membrane

Recent evidence suggests that the complex network represented by membrane trafficking both inside and outside the cell is involved in defining the final cell GSL composition.

The most common event is the fusion of intracellular vesicles with the plasma membrane. A clear example is given by the pre-synapse, where the fusion of thousand of neurotransmitters-containing microvesicles, induces direct important local changes in the plasma membrane composition. A similar effect is induced by the fusion of lysosomes with the plasma membrane (Blott and Griffiths 2002; Baron et al. 1985; Chambers 1917; Reddy et al. 2001; Rothman 1994; Rao et al. 2004; Raiborg et al. 2015; Arantes and Andrews 2006). This process, an evolutionarily conserved mechanism called lysosomal exocytosis, has important implications in cell homeostasis. Indeed, it is a mechanism that drives the glycohydrolitic enzymes associated with the luminal side of lysosomal membranes, directly to the external leaflet of the cell plasma membrane. Here, thanks to the presence of vacuolar H<sup>+</sup>-ATPase proton pumps and Na<sup>+</sup>/H<sup>+</sup> exchangers, they can exert their activity directly *in-situ* as described above (Aureli et al. 2012b) On the other hand, recent evidence concerning lysosomal storage disorders, suggests that cells activate lysosomal exocytosis upon aberrant accumulation of uncatabolized molecules in the lysosomes, in order to release their content to the extracellular milieu. This process may be beneficial for cells, as a recent paper shows that it is responsible for a dramatic increase in the activity of lysosomal glycosphingolipid hydrolases at the cell surface. These enzymes promote a remodeling of the plasma membrane via aberrant ectopic catabolism of GSLs and production of ceramide, the event responsible for the induction of cell cycle arrest (Samarani et al. 2018).

Another interesting mechanism that could determine changes in GSL composition is shedding. In particular, GSL shedding could be considered an active and controlled process able to modify the sphingolipid composition of neighbor cells in order to condition their fate. The amphiphilic nature of the GSL raises the possibility that multiple equilibriums exist between the lipids inserted in the plasma membrane (where they are present in different grades of segregation) and those present in the aqueous extracellular milieu (Riboni et al. 1997; Sonnino et al. 1994; Koynova and Caffrey 1995). Despite the shedding process being considered a minor event, studies demonstrated that especially tumor cells can shed up to 0.5% of their membrane gangliosides per hour (Li and Ladisch 1991). At the same time acceptor membranes could receive exogenous gangliosides up to 3% of their total membrane lipid content. Interestingly, the aberrant shedding of gangliosides from the neurons may be responsible for glia activation, a common feature of several neurodegenerative disorders (Jou et al. 2006).

New findings suggest that membrane contact sites (MCS) are intracellular components capable of changing the GSL composition of the cells. In particular, these specific areas of the membranes seem to facilitate lipid transport among the different organelles. In these regions, the membrane of two organelles reach a distance of 30 nm and lipids are transposed from one organelle to the other by specific proteins. The ER is considered the hub of these MCS-net since it is widespread into the cell allowing the recruitment of ER membrane in forming multiple contact sites with other organellar membranes, such as those of mitochondria, lysosomes, Golgi apparatus, endosome, and plasma membrane (Levine and Loewen 2006; Levine and Patel 2016; Prinz 2014; Gatta and Levine 2017). In the scenario of the MCS it is reasonable to imagine that ER is the highway for lipid trafficking. Through the ER the cell shares sphingolipids among all intracellular membranes, organelles, and the plasma membrane through a mechanism coordinated by the MCS.

## 10 Conclusions

During neuronal development dramatic changes occur in GSL cell content, along with the reorganization of plasma membrane lipid domains enriched in GSLs (Yu 1994; Prinetti et al. 2001; Yu et al. 2004). While the driving forces of these modifications are not completely understood, some information exists regarding the possibility of a fine-tuning of the cell plasma membrane GSL composition, mediated by the synergic activity of the different plasma membrane-associated glycohydrolases (Valaperta et al. 2006; Aureli et al. 2009).

In cultured rat cerebellar granule cells, the increase in total ceramide content (eight fold from the 2nd to the 17th day in culture) and in that belonging to the sphingolipid-enriched domains (ten fold from the 2nd to the 17th day in culture) has been observed (Aureli et al. 2011c). A parallel reduction in the endogenous content of both sphingomyelin and gangliosides is present in sphingolipid-enriched domains of senescent cells with respect to fully differentiated neurons (Prinetti et al. 2000a). The increase in ceramide content could be explained by the well-known ceramide-sphingomyelin cycle that is known to correlate with apoptotic phenomena (Venable et al. 1995). As found in human fibroblasts, the increased activity of the plasma membrane-associated glycohydrolases during cell aging, supports the speculation that the augmented ceramide in the plasma membrane could be derived from cell surface GSL catabolism (Valaperta et al. 2006). As reported (Rodriguez et al. 2001; Da Silva et al. 2005), the sialidase Neu3 is able to influence extension and symmetry of axons in neuronal cells, possibly by inducing a local change in plasma membrane sphingolipid composition at the axonal cones. All these data support the idea that modulations of the activities of other plasma membrane-associated glycohydrolases during neuronal differentiation could affect the differentiation itself. In addition, they participate in defining the curvature of specific plasma membrane areas, such as synapses or the negative curvature of the membrane near the axon protrusion, by a rapid *in situ* modification of GSL components. An example of the latter is

provided by the geometry of synapses. It is typically characterized by an alternative presence of plasma membrane regions with negative or positive curvature, that correlate respectively to an enrichment of simple sphingolipids or of more complex GSLs (Sonnino et al. 1994; Brocca and Sonnino 1997). On the other hand, an aberrant increase in cell surface glycohydrolases can promote the formation of apoptotic ceramide, leading to the onset of neuronal impairment. For these reasons, the balance between glycosylation and de-glycosylation at the cell surface could be a very important mechanism for maintaining appropriate neuronal physiology.

**Conflict of Interest** All the authors declare that they have no conflict of interest.

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# Chapter 12

## Neuronal Ganglioside and Glycosphingolipid (GSL) Metabolism and Disease



### Cascades of Secondary Metabolic Errors Can Generate Complex Pathologies (in LSDs)

Roger Sandhoff and Konrad Sandhoff

**Abstract** Glycosphingolipids (GSLs) are a diverse group of membrane components occurring mainly on the surfaces of mammalian cells. They and their metabolites have a role in intercellular communication, serving as versatile biochemical signals (Kaltner et al, *Biochem J* 476(18):2623–2655, 2019) and in many cellular pathways. Anionic GSLs, the sialic acid containing gangliosides (GGs), are essential constituents of neuronal cell surfaces, whereas anionic sulfatides are key components of myelin and myelin forming oligodendrocytes. The stepwise biosynthetic pathways of GSLs occur at and lead along the membranes of organellar surfaces of the secretory pathway. After formation of the hydrophobic ceramide membrane anchor of GSLs at the ER, membrane-spanning glycosyltransferases (GTs) of the Golgi and Trans-Golgi network generate cell type-specific GSL patterns for cellular surfaces. GSLs of the cellular plasma membrane can reach intra-lysosomal, i.e. luminal, vesicles (ILVs) by endocytic pathways for degradation. Soluble glycoproteins, the glycosidases, lipid binding and transfer proteins and acid ceramidase are needed for the lysosomal catabolism of GSLs at ILV-membrane surfaces. Inherited mutations triggering a functional loss of glycosylated lysosomal hydrolases and lipid binding proteins involved in GSL degradation cause a primary lysosomal accu-

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Dedicated to Professor Kunihiko Suzuki on the occasion of his 90th birthday.

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mulation of their non-degradable GSL substrates in lysosomal storage diseases (LSDs). Lipid binding proteins, the SAPs, and the various lipids of the ILV-membranes regulate GSL catabolism, but also primary storage compounds such as sphingomyelin (SM), cholesterol (Chol.), or chondroitin sulfate can effectively inhibit catabolic lysosomal pathways of GSLs. This causes cascades of metabolic errors, accumulating secondary lysosomal GSL- and GG- storage that can trigger a complex pathology (Breiden and Sandhoff, *Int J Mol Sci* 21(7):2566, 2020).

**Keywords** Ganglioside · Glycosphingolipid · Glycolipid · Metabolism · Membrane-surface · Development · Neuron · Receptor · Topology · Ganglio-series · Glycosyltransferase · Organelle · Secretory pathway · Endosomal pathway · Lysosome · Catabolism · Degradation · Intra-lysosomal luminal vesicle (ILV) · Hydrolase · Sphingolipid-binding protein (SAP) · Sphingolipid-transfer protein · Lysosomal storage disease (LSD) · Secondary storage · Neurodegenerative disease · Genetic disease · Parkinson · Alzheimer · Frontal lobe dementia

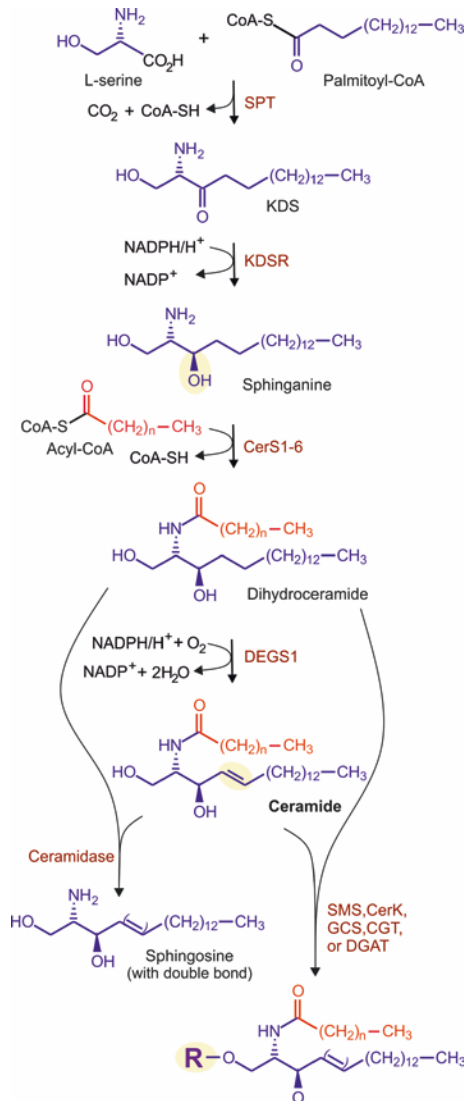
## Abbreviations

ASA	Arylsulfatase A
ASM	Acid sphingomyelinase
BMP	Bis(monoacylglycero)phosphate
CAD	Cationic amphiphilic drug
Cer	Ceramide
CerS	Ceramide synthase
CERT	Ceramide transfer protein
Chol	Cholesterol
CNS	Central nervous system
ER	Endoplasmic reticulum
FA	Fatty acid
GalCer	$\beta$ -galactosylceramide; Ganglioside names are abbreviated according to Svennerholm (1962, 1994) as recommended by IUPAC (Chester 1997)
GBA1	lysosomal $\beta$ -glucocerebrosidase
GlcCer	$\beta$ -glucosylceramide
GM2AP	GM2 activator protein
GRN	Granulin
GT	Glycosyltransferase
Hex A/B/S	$\beta$ -hexosaminidase A/B/S
KDS	3-keto-dihydrospingosine
KDSR	Keto-dihydrospingosine reductase
LacCer	Lactosylceramide
NEU	Neuraminidase
NPC	Niemann–Pick disease type C protein

PA	Phosphatidic acid
PD	Parkinson disease
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PiP	Phosphatidyl inositol phosphate
PL	Phospholipid
PM	Plasma membrane
PS	Phosphatidylserine
Sa	Sphinganine
Sap	Saposin
SAP	Sphingolipid activator protein
SL	Sphingolipid
SM	Sphingomyelin
SM4g	Seminolipid, i.e. 3-sulfogalactosyl-1-alkyl-2-acylglycerol
SM4s	3-sulfogalactosylceramide
So	Sphingosine
S1P	Sphingosine 1-phosphate
SPT	Serine palmitoyltransferase
TGN	Trans Golgi network

## 1 Historical Aspects of Structure, Location and Function of GSLs

Sphingolipids were identified and analyzed in extracts of postmortem brains in 1884 by Johannes L. W. Thudichum (1884). He named the previously unknown basic key structures “sphingosine”, “ceramide” and “sphingomyelin” and identified some small GSLs, glucocerebroside and galactocerebroside, now called glucosyl- and galactosylceramide (For their biosynthesis see Fig. 12.1). Gangliosides (GGs) were isolated and identified as acidic glycolipids of postmortem human brain tissue obtained from infantile patients suffering of amaurotic idiocy by Ernst Klenk (Klenk 1939, 1942). They are enriched in ganglion cells and contain N-acetylneuraminic acid, now also named sialic acid, as an acidic component. As anionic and amphiphilic GSLs, gangliosides form huge micelles in aqueous solution. The first structure of a complex brain GG, GM1a (Fig. 12.2b), became known in 1963 (Kuhn and Wiegandt 1963), allowing the identification of GSL and GG structures accumulating in ganglioside storage diseases (Jatzkewitz and Sandhoff 1963; Sandhoff et al. 1971; Wiegandt 1995). The investigation and analysis of lysosomal storage diseases was a pacemaker for the elucidation of cellular GSL metabolism, its intracellular pathways and locations, its enzymes and the essential cofactors for lysosomal GSL degradation (Conzelmann and Sandhoff 1978; Kolter and Sandhoff 1999; Mehl and Jatzkewitz 1964). This allowed identification of the catabolic blocks caused by inborn errors in GSL and GG storage diseases (Sandhoff et al. 1971). The



**Fig. 12.1** Sphingolipid neo-biosynthesis in mammals. Serine palmitoyl transferase (SPT) initiates sphingolipid biosynthesis by condensing activated fatty acids (most commonly palmitic acid) with L-serine. After reduction by keto-dihydrosphingosine reductase (KDSR), sphinganine is produced, which can be converted to various dihydroceramides by one out of six ceramide synthases (CerS1-6) in mammals. The CerS have different specificities towards activated fatty acids of various chain length. The sphingosine double bond is then introduced by a dihydroceramide desaturase (DEGS1) and subsequent cleavage by ceramidases will release sphingosine, which can be used for sphingolipid synthesis by the salvage pathway. (Dihydro)ceramides are then substrates for sphingomyelin synthases (SMS, R: phosphorylcholine), ceramide kinases (CerK, R: phosphate), glucosylceramide synthase (GCS, R:  $\beta$ -glucosyl), galactosylceramides synthase (ceramide galactosyltransferase or CGT, R:  $\beta$ -galactosyl), or diacylglycerol acyltransferases (DGAT, R: acyl) adding corresponding head groups to the primary hydroxyl group







distribution and pattern of GSL and GG are species-, cell type- and organelle specific.

## 2 Significance and Function of Neuronal GGs

In mammals, most cells of the visceral organs and the skin generate GSLs of the globo-series on their surfaces (see GSL pattern of fibroblasts in Fig. 12.2a). This is also true for neuronal stem cells which, however, substitute them by a molecular switch during neuronal differentiation with the expression of the ganglio-series of GGs (Russo et al. 2018). GGs then become the most abundant GSLs on the neuronal plasma membrane of the developing and ageing nervous system. It has been speculated that the specific combination of hydrophobic and hydrogen bonding elements of GGs may even hide an interaction code, e.g. with signaling receptors, for determining neuronal functions (Kaltner et al. 2019; Lunghi et al. 2021). It is noteworthy that globo-series GSLs repress the epigenetic regulator of neuronal gene expression AUTS2, which otherwise would activate the promotor of GM3 synthase, the first and rate-limiting enzyme of ganglioside-biosynthesis (Russo et al. 2018). Other cells also express different GG during differentiation. For example, male murine germ cells switch between GGs of the a- and the 0-series (Fig. 12.3), when they cross the blood-testis barrier during differentiation (Rabionet et al. 2015; Sandhoff 2010; Sandhoff et al. 2005). This switch also depends on increased activity of GM3 synthase. Another shift occurs in small intestinal epithelial cells during suckling-to-weaning transition (Yoneshige et al. 2010). It was also demonstrated that different GSLs support differentiation of human myeloid HL-60 cells into either monocytes/macrophages or granulocytes (Nojiri et al. 1986, 1988; Sandhoff 1993) as well as the activation of different T cell types (Inokuchi et al. 2015).

GGs are abundant sialic acid containing GSLs of the nervous system (Posse de Chaves and Sipione 2010), where they are enriched in neuronal plasma membranes (Rahmann 1983), especially in nerve endings. They form cell-type-specific patterns on the surfaces of neuronal cells that change with differentiation (Kotani et al. 1993, 1994, 1995; Kotani and Tai 1997). Neuronal GGs contain a ceramide anchor composed mainly of C18-sphingosine with little C20-sphingosine in combination almost exclusively with a saturated stearyl residue. The percentage of C20-sphingosine containing GGs increases steadily with differentiation and age throughout life (Sonnino and Chigorno 2000), but the potential benefit or function of this process remains to be elucidated. GGs, together with high levels of sphingomyelin and cholesterol, stabilize neuronal plasma membranes (Jennemann et al. 2005; Mori et al. 2012), while their hydrophilic glycan head groups on the cellular surfaces contribute to cell-to-cell adhesion processes (Handa and Hakomori 2012; Schnaar 2016). As amphiphilic lipids, GGs and GSLs are anchored onto the outer leaflet of plasma membranes with a hydrophobic ceramide moiety and correspondingly onto anti-cytosolic leaflets of cellular organelles. Thus, the glycan moiety of GGs is part of the glycocalyx at the cellular surface.

GGs are GSL that contain one or more of up to 50 different sialic acid residues, placing negative charges onto cellular surfaces (Schauer 2016). They can serve as ligands for lectins and modulate the activity of membrane proteins, such as insulin receptor, leptin and EGF receptor and serve as binding sites for surface proteins on neighboring cells (Allende and Proia 2014; Furukawa et al. 2012; Hakomori and Handa 2015; Inokuchi 2010; Ledeen et al. 2012; Lipina and Hundal 2015; Nordstrom et al. 2013; Ohmi et al. 2012; Regina Todeschini and Hakomori 2008). These interactions depend on the structure of the hydrophilic oligosaccharide head group on the cell surface and that of the hydrophobic ceramide anchor within the outer leaflet of the plasma membrane (PM) bilayer, both of which can vary significantly with cell type. Ceramide anchors of neuronal GGs of the A- and B- series contain almost exclusively a saturated fatty acid residue, the stearyl moiety (Kishimoto and Radin 1966) ( $n = 16$  in Fig. 12.1), whereas GM3 and many GGs of visceral organs, like liver, spleen and intestine, prefer a variety of long to very long chain fatty acids in their ceramide moieties (Hara et al. 1984; Iwamori et al. 1984; Jennemann et al. 2010, 2012a; Keranen 1976; Nakamura et al. 1987; Riboni et al. 1992). The acyl chain length may also affect uptake, endosomal and intracellular trafficking of GM1 in cell culture as observed by the incorporation of synthetically prepared GM1 molecules containing different acyl chain lengths (Chinnapen et al. 2012; Saslowsky et al. 2013). In *C. elegans*, glycolipids with a C22-acyl chain are required for proper membrane localization of clathrin and clathrin-dependent autophagic lysosome reformation, which subsequently activated TOR and suppressed longevity (Wang et al. 2021a). CerS2 deficiency, which decreases sphingolipids with very long acyl chains (C22 and C24) in favor of those with long acyl chains (C16 and C18), reduced the rate of clathrin-mediated endocytosis in isolated mouse astrocytes apparently through indirect oxidative stress mediated mechanisms (Volpert et al. 2017). Whether the acyl-chain length of GGs would also affect vesicular transport mechanisms in neurons and eventually longevity is not yet clear.

Neuronal stem cells express globo-series GGs. However, when separated from blood and the rest of the body by the blood brain barrier during neuronal differentiation, they substitute them with the expression of ganglio-series GGs of the A- and B-series (Russo et al. 2018). These GGs carry a hydrophilic tetraosyl moiety with a varying number of sialic acids. The head group of axonal GGs like GM1a, GD1a, GT1b stabilize the axon – myelin interaction by binding the myelin-associated glycoprotein of the surrounding inner myelin sheet (Collins et al. 1997; Schnaar 2016; Yang et al. 1996).

As indicated by the analysis of mutant mice and patients with inherited defects in GG biosynthesis pathways, GGs are important for stability of neuronal structures and signaling. Although the molecular mechanisms facilitating these GG functions are still poorly understood (Proia 2003), it became clear that complex GGs GD1a and GT1b are specific ligands for the myelin associated glycoprotein MAG (Schnaar 2019) and support the interaction of axonal membrane with myelin sheaths at the node of Ranvier (McGonigal and Willison 2021). The importance of sialylation for this interaction, which establishes the terminal Neu5Ac $\alpha$ 2,3Gal $\beta$ 1,3GalNAc-binding domain for MAG on GD1a and GT1b, was further underlined by

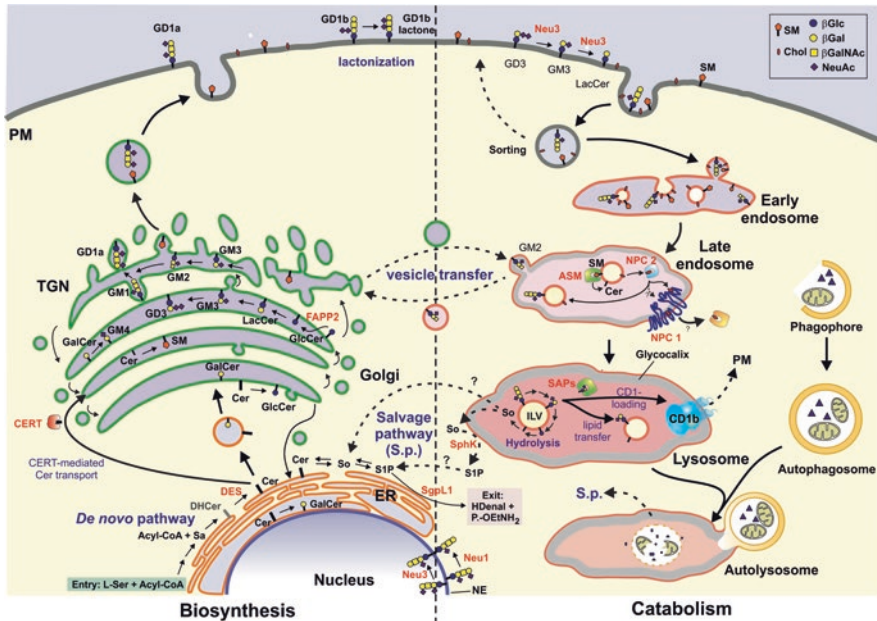
investigations on St3gal2/3 double-null mice, which displayed among other things disruptions at nodes of Ranvier (Yoo et al. 2015). The complex a- and b- series GGs play a central role in preventing dysfunction of the CNS in aging (of mice). The systemic elimination of complex GG of both series in GalNAc-transferase (GalNAc-T) deficient mice (which then overexpress the simple GGs GM3 and GD3, see Fig. 12.3) leads to an age-dependent neurodegeneration. Mice that express only GG GM3, however, suffer from a markedly accelerated neurodegeneration (disintegration of axons and disruption of node of Ranvier architecture) and reduced survival. Reintroduction of both a- and b-series specifically into neurons of GalNAc-T-deficient mice is sufficient to rescue the age-dependent neurodegenerative phenotype. Similarly, reconstituting neuronal a-series GGs but not b-series GGs – such as GD3 – in GM3-only mice by reintroducing neuronal GalNAc-T expression, abrogated the adult lethal phenotype (McGonigal et al. 2021). These results support recent findings that structural glycosphingolipids may be more relevant to achieve the centenarian condition than signaling sphingolipids (Pradas et al. 2022).

The b-series ganglioside GD3 and its key biosynthetic enzyme, GD3-synthase (GD3S), also seem to be relevant in the regulation of phagocytosis in an ischemic stroke model. Both were upregulated in the microglia of mouse hippocampus from 2 to 7 days after global cerebral ischemia, whereas the phagocytic capacity of the GD3S-KO microglia exhibited decreased amoebic morphology, reduced engulfment of neuronal material, and lower expression of the phagolysosome marker CD68. Also, microglia isolated from GD3S-KO mouse brain at 2 days after global cerebral ischemia were less neurotoxic to co-cultured hippocampal neurons than the WT-global cerebral ischemia microglia. Moreover, the impaired phagocytic capacity of GD3S-KO microglia could be partially restored by pre-treatment with exogenous ganglioside GD3 (Wang et al. 2021b).

The b-series GGs apparently play a critical role in regulating the structure and function of the mouse visual system. The absence of these b-series GGs in the retinas of GD3S-KO mice triggered a reduction of retinal ganglion cell density, of axons in the optic nerve and a 15% reduction of photoreceptor nuclei, a 30% reduction of light responsiveness and a reduced visual acuity and contrast sensitivity (Abreu et al. 2021).

A negative function of GGs is that they also provide effective binding sites and receptors for lectin-type bacterial exotoxins such as botulinum toxins, shiga, cholera and tetanus toxin (Cuatrecasas 1973; Dong et al. 2007; Eidels et al. 1983; Hamark et al. 2017; Lingwood 1999; Melton-Celsa 2014; Sandvig et al. 2014; Van Heyningen and Miller 1961) and viruses (Schneider-Schaulies et al. 2021).

Various aspects of glycosphingolipid metabolism and function have been reviewed recently (Ashida and Li 2014; Aureli et al. 2014; Breiden and Sandhoff 2019b; Dunn et al. 2019; Furukawa et al. 2014; Ladisch and Liu 2014; Quinville et al. 2021; Sandhoff and Sandhoff 2018; Schengrund 2015; Schneider 2014; Seyfried et al. 2014; Yu and Itokazu 2014). This review is an update and adaption of our previous review (Sandhoff and Sandhoff 2018).



**Fig. 12.4** Cellular compartmentalization and trafficking in GSL and GG metabolism modified from (Sandhoff and Sandhoff 2018) and (Breiden and Sandhoff 2020). Ganglioside biosynthesis and secretion starts at the endoplasmic reticulum (ER) and finalizes in the lumen of the Golgi system before gangliosides are carried to the plasma membrane by vesicular transport (anabolism, left side). Besides vesicular transport, some sphingolipids such as Cer and GlcCer are also transported between the ER and the Golgi system by lipid transfer proteins like CERT and FAPP2, respectively (Yamaji and Hanada 2015). Upon endocytosis and incorporation into intraendolysosomal luminal vesicles (ILVs), GG are degraded into monosaccharides, free fatty acids, and sphingoid bases (catabolism, right side), which are recycled for sphingolipid synthesis by the salvage pathway (modified after Sandhoff et al. 2018). Functional defects of any catabolic step cause an accumulation of the undegradable substrates in the lysosomes. The increasing lysosomal storage can trigger a reduced ability of lysosomes to fuse with autophagosomes, attenuating autophagy. ASM acid sphingomyelinase, Cer ceramide, CERT ceramide transfer protein, DES dihydroceramide desaturase, DHCer dihydroceramide, FA fatty acid, GlcCer glucosylceramide, ER endoplasmic reticulum, FAPP2 (PLEKHA8) four-phosphate adaptor protein 2 (pleckstrin homology domain containing A8), LacCer lactosylceramide, NE nuclear envelope, NEU neuraminidase, NPC Niemann–Pick disease type C protein, PM plasma membrane, Sa sphinganine, SAP sphingolipid activator protein, SgpL1 S1P lyase, SM sphingomyelin, So sphingosine, S1P sphingosine-1-phosphate, SphK sphingosine kinase, TGN trans Golgi network, HDenal hexadecenal, P-OEtNH<sub>2</sub> phosphoryletanolamine

### 3 Intracellular Pathways of GG and GSL Metabolism

Metabolic steps of GSL and GG metabolism occur at intracellular membranes and are intimately connected with intracellular trafficking steps between organellar membranes of the secretory and endocytic pathways (Fig. 12.4) (De Duve and Wattiaux 1966; Kolter and Sandhoff 1999). The main routes of GSL and GG biosynthesis (Figs. 12.1 and 12.3) and catabolism (Fig. 12.5) proceed in a stepwise



manner through the same membrane-bound lipophilic intermediate structures, which occur, however, at different organellar membranes of different intracellular routes: mainly the secretory pathways in case of biosynthesis and endocytotic pathways in case of degradation (Fig. 12.4). Biosynthesis starts with the formation of sphingoid bases and the hydrophobic ceramide anchor at the cytoplasmic leaflet of ER membranes (Stoffel 1971). Ceramides are substrate for various enzymes including cerebroside galactosyltransferase at the site of the ER and glucosylceramide synthase at the cytosolic site of the early Golgi. The products GlcCer and to some extent GalCer are then substrates for the luminal activity of glycosyltransferases (GTs) localized in Golgi and trans Golgi network (TGN) membranes of the secretory pathway. Remodeling and trimming of GGs has been observed by sialidases and other hydrolases of the PM and endosomal membranes, and by GTs at the TGN and PM, whereas the constitutional catabolism of GSLs and GGs takes place at intraendosomal and intralysosomal vesicles (ILVs) of late endosomes and lysosomes (Fig. 12.4) (Breiden and Sandhoff 2019b; Sandhoff et al. 2018).

#### **4 Emerging Concepts of GSL & GG Metabolism at Organellar Membranes**

The metabolism of amphiphilic, membrane-bound glycolipids is catalyzed at the membrane-water interphase

- (a) By membrane-spanning GTs in case of biosynthetic pathways or
- (b) By soluble catabolic hydrolases with the essential help of soluble lipid-binding protein cofactors at the surface of ILV membranes.

Concentration of the reaction partners within the plane of the same membrane should increase the reaction rate of the biosynthetic processes. Lipid substrates and membrane-bound enzymes should interact within the plane of the membrane by diffusion following a two-dimensional form of the Michaelis-Menten equation (Scheel et al. 1982).

Indeed, biosynthetic reaction rates are significantly increased by concentrating membrane-bound enzymes, the GTs or multi-GTs complexes, and membrane-bound GSL substrates together within the same organellar membranes of the secretory pathways, thereby avoiding needless molecular waste in the cellular environment. Catabolic reaction rates are sped up by binding cationic, positively charged lysosomal hydrolases and lipid binding proteins by forces of enzyme-substrate affinity and above all by electrostatic attraction to the negatively charged surfaces of the GSL-substrate containing ILVs in the lysosomal compartment (Breiden and Sandhoff 2019b).

## 5 Emerging Topology of Glycolipid Biosynthesis

The Golgi is the main glycosylation site of the cell. It harbors a stack of discontinuous cisternae that contain biosynthetic glycosyltransferases (GTases) in specific cisternae along the cis-trans axis of the organelle. A matrix protein, GRASP55, regulates the polarized location and distribution of the GTases in the Golgi and controls GSL biosynthesis (Pothukuchi et al. 2021).

Synthesis of sphingoid bases and the rather hydrophobic dihydroceramides and ceramides occurs at the cytosolic leaflet of the ER membranes and is catalyzed by enzymes that are integral membrane proteins, bound to the same ER membranes as their lipid substrates. Generated (dihydro)ceramides are then transferred to the cytosolic leaflet of Golgi membranes to serve as substrates of the glucosylceramide synthase. From here, the generated glucosylceramides are transferred to the luminal leaflet of the Golgi membranes by ATP-binding cassette transporters. At the luminal side they can be converted to lactosylceramide (LacCer). Multiple ABC transporters apparently provide distinct but overlapping GlcCer and LacCer pools at the luminal surface of Golgi membranes for anabolism of different GSL series by metabolic channeling (Budani et al. 2021). LacCer can be formed by one of two lactosylceramide synthases (Lannert et al. 1994; Nishie et al. 2010; Tokuda et al. 2013). LacCer itself is substrate for six transferases guiding GSL synthesis into different GSL series. Two of them, the GM3-synthase as well as the GA2-synthase, will guide anabolism into ganglio-series GSLs/GGs. These integral membrane-spanning GTs exhibit their catalytic activity at the luminal site of the Golgi and TGN membranes. The lumenally oriented GSLs/GGs reach the plasma membrane by vesicular transport and are therefore mainly located in the extracellular leaflet of the PM (van Meer and Hoetzel 2010; van Meer et al. 2008).

Contrary to the above mentioned neuronal GGs, the smallest ganglioside, GM4, is derived from galactosylceramides, just as is the case for sulfatide (SM4s) (Fig. 12.3). All three lipids, GalCer, SM4s, and GM4, are typical components of myelin and are produced by oligodendrocytes in the central nervous system. GM4 hardly occurs in neurons but has been detected in low concentrations in myelin (Ledeer et al. 1973; Yates 1986), erythrocytes, kidney, and intestine (Iwamori et al. 1984; Tadano and Ishizuka 1980). Its biosynthesis along the secretory pathway has been discussed before (Sandhoff and Sandhoff 2018).

## 6 Generation of Cell-Type-Specific Ganglioside Patterns

There are several factors contributing to the generation of cell type specific patterns on the surface of mammalian cells, which may be summarized as combinatorial biochemistry. Initially surprisingly, most of the enzymes involved in biosynthetic



pathways of GGs and GSLs have a rather poor specificity for their lipid and glycolipid substrates, e.g. ceramide synthases accept sphingoid bases and activated fatty acids of different chain length, saturation and hydroxylation grade. Likewise GTs exhibit a rather low substrate specificity. GalNAc-transferase for example converts LacCer as well as the GGs GM3, GD3, and GT3 into higher GGs of the ganglio-series (Pohlentz et al. 1988) (Fig. 12.3). In this way, the combinatorial activity of only a few promiscuous GTs, the variable levels of which are mostly controlled by transcriptional regulation, allows for the formation of cell type specific GSL patterns on cellular surfaces (Kolter et al. 2002). The combinatorial action of GTs is mirrored by the interplay of ceramide synthases with the cell type specific machinery for synthesis of sphingoid bases and fatty acids to define their ceramide anchor pattern (Kihara 2016). Whereas the sphingoid base C18-sphingosine is present in all cell gangliosides, C20-sphingosine containing gangliosides are rather minor, or absent. They appear, however, during differentiation of neurons and their ratio increases in the brain throughout the life span (Sonnino and Chigorno 2000). During neural differentiation, dynamic changes occur in the pattern of carbohydrate-rich molecules, including in the ganglioside composition. GG GD3 is the predominant ganglioside species in neural stem cells and modulates their proliferation as well as their long-term maintenance (Itokazu et al. 2018).

Cell specific GG and GSL patterns and metabolism have even been observed for different types of neurons, including granule neurons, pyramidal neurons and Purkinje cells (Furuya et al. 1996; Kotani et al. 1992, 1994; Molander-Melin et al. 2004; Tai et al. 1999; Taniike et al. 1995). Cell type- and cell-specific expression of GG and GSL are obviously obtained by a combination of several factors. Major players are (a) the cell type specific expression of often promiscuous biosynthetic enzymes (SPT subunits, ceramide synthases and GTs) and (b) the availability of local acyl-CoA donors and glycolipid acceptors at the organellar membranes of the secretory pathway. Of course, further well-known factors like pH value, ionic strength, ion composition and the availability of soluble enzyme substrates like activated sugar nucleotides in the lumen of the Golgi and TGN compartment are also of great importance.

Emerging factors like membrane fluidity, lipid and protein composition of organellar membranes, and nutritional state will presumably increasingly be recognized as important for the regulation of biosynthetic and remodeling steps in GG and GSL metabolism. For example, the anionic lipid phosphatidylglycerol (PG) stimulates GG biosynthesis in detergent-free in vitro assays (Yusuf et al. 1983a, b) and an increase of membrane fluidity as triggered by the addition of general anesthetics (e.g. halothan, Xenon gas) or a series of fatty acids with declining acyl chain lengths stimulates desialylation of oligosialo-GGs by membrane bound sialidases (Scheel et al. 1982). Finally, high fat or the so called western diet may cause increased production of C16-sphingolipids (Hla and Kolesnick 2014) including for example an incorporation into ganglioside GM3, which in turn can downregulate insulin receptor activity thereby supporting metabolic syndrome and type 2 diabetes mellitus (Lipina and Hundal 2015).

## 7 Enzyme Catalysis at Membrane Surfaces

Biosynthetic steps are catalyzed by integral membrane-bound enzymes, which can interact with their lipid substrates by diffusion within the plane of the membrane. This is the case for enzymes needed for sphinganine (Sa) and ceramide formation in the cytosolic leaflet of the ER and their membrane-bound lipid substrates, whereas the soluble serine substrate can interact with the membrane-bound SPT through the aqueous space (Fig. 12.4) (Braun et al. 1970; Braun and Snell 1968; Kolter and Sandhoff 1999; Stoffel et al. 1968a; Williams et al. 1984). GTs are also membrane spanning proteins, which interact with their lipid substrates by lateral diffusion within the plane of the membrane, and directly with the water-soluble activated sugar nucleotides in the lumen of the Golgi and TGN.

The kinetics of lipid substrate turnover by membrane-bound GTs at the lipid-water interphase, however, has been only poorly studied, mostly in the presence of detergents, which profoundly obscure the physiological interaction between membrane-bound substrate and enzyme. A model case has been investigated, however, for the interaction of plasma membrane-bound sialidases (presumably Neu3) and the radiolabeled GG GD1a in isolated synaptosomal membranes (Durrrie et al. 1988; Ohman 1971; Preti et al. 1980; Saito et al. 1995; Tettamanti et al. 1980). As expected, GG hydrolysis does not follow the well-known Michaelis-Menten kinetics, but its two-dimensional version. A reproducible Michaelis-Menten constant was obtained only when the substrate concentration was presented as amount of GD1a per membrane-surface available in the incubation assay and not as amount of GD1a per incubation volume, as usually done for solutes (Sandhoff and Pallmann 1978; Scheel et al. 1982).

Analysis also revealed a stimulation of GG hydrolysis by sialidase in isolated neuronal membranes with increasing membrane fluidity (Sandhoff and Pallmann 1978; Scheel et al. 1982, 1985) modulated by the addition of free FAs with different chain lengths or general anesthetics like halothane or Xenon gas (Sandhoff and Pallmann 1978; Scheel et al. 1985). The concept of two-dimensional kinetics, however, does not apply to the lysosomal GG catabolism, driven mostly by water-soluble, protonated and positively charged mammalian exo-hydrolases in the lysosomal compartment. Their interaction with membrane-bound substrates is mainly facilitated by electrostatic attraction to the surface of negatively charged anionic ILV-membranes carrying GSL and other lipid substrates. Degradation of GSLs, GGs, SM and other complex lipids is mediated by hydrolases at ILV-surfaces in cooperation with essential small promiscuous lipid binding proteins, the sphingolipid activator proteins (SAPs) (Breiden and Sandhoff 2019b).

## 8 GSL Biosynthesis and Salvage Pathways

### 8.1 Ceramide Synthesis

De novo synthesis of sphingolipids (SLs) starts with the condensation of an activated fatty acid (mainly palmitoyl-CoA) with L-serine at the cytosolic leaflet of the ER membrane (Figs. 12.1 and 12.3). The rate limiting step is catalyzed by the serine-palmitoyl-CoA-transferase (SPT) (Hornemann et al. 2009), which is regulated in a feedback loop by inhibitory ORM proteins in yeast (Breslow et al. 2010; Han et al. 2010), and ORMDL proteins in mammals (Gupta et al. 2015; Siow and Wattenberg 2012). Interestingly, the chain length of the product 3-keto-sphinganine also known as 3-keto-dihydrosphingosine (KDS) is influenced by the presence of either of the two small subunits SPT<sub>ssa</sub> and SPT<sub>ssb</sub> (Han et al. 2009; Zhao et al. 2015). KDS is reduced to Sa by the 3-ketodihydrosphingosine reductase (KDSR) with the help of NADPH; this, in turn, is a substrate of ER-bound ceramide synthases (CerSs). They convert sphingoid bases, e.g. Sa and sphingosine (So), from de novo and salvage pathways to the respective dihydroceramides and ceramides at the ER. In mammals, there are 6 genes leading to different CerS isoforms, which are cell-type specifically expressed. In combination with the available acyl-CoA profile, the expressed CerS pattern leads to characteristic ceramide anchor profiles of SLs and GGs in different cell types and organs (Levy and Futerman 2010; Morell and Radin 1970; Pewzner-Jung et al. 2006; Rabionet et al. 2014; Sandhoff 2010; Sassa et al. 2016; Venkataraman and Futerman 2002). The activities of CerS are regulated at the transcriptional and the protein level (D'Angelo et al. 2007; Yamaji and Hanada 2015). The ceramide anchors of neuronal GGs contain mainly stearic acid due to high expression levels of CerS1 (Jiang et al. 1998; Riebeling et al. 2003; Sambasivarao and McCluer 1964). Enzyme activities of CerS2-6 are regulated in several ways (Wegner et al. 2016), e.g. by phosphorylation. They contain a HOX-domain (Venkataraman and Futerman 2002), which may have a regulatory role as a nuclear DNA-binding protein as shown for the single CerS of drosophila (Sociale et al. 2018; Voelzmann et al. 2016). Transcripts of several genes involved in lipid metabolism and cell division in mouse liver are also regulated by CerS2 activity, suggesting a role of very long acyl chain ceramides in the nucleus for the transcriptional regulation of target genes (Bickert et al. 2018; Pewzner-Jung et al. 2010).

Dihydroceramide (DHCer) formed by the acylation of Sa is subsequently converted to ceramide (e.g. in neurons) by the dihydroceramide desaturase 1 with the cosubstrates NAD(P)H and oxygen by generating a trans-double bond in the 4-position of the sphingoid base (Fig. 12.1) (Geeraert et al. 1997; Michel et al. 1997). DHCer can also be converted to phytoceramides, especially in intestinal tissue (Enomoto et al. 2006; Omae et al. 2004; Ternes et al. 2002).

## 8.2 *Ganglioside Synthesis and Function*

Synthesis of complex GSLs and GGs occurs by the stepwise addition of monosaccharides (Fig. 12.3) to ceramides at the membranes of the Golgi and TGN (Daniotti and Iglesias-Bartolome 2011; Kolter and Sandhoff 1999) (Fig. 12.4). Ceramides reach the Golgi membranes either by vesicular transport or by the inter-organelle transfer protein CERT, preferentially transporting ceramides with C14 to C30 acyl chains (Yamaji and Hanada 2015).

GSL formation is initiated by the addition of glucose to ceramide by UDP-glucose ceramide glucosyltransferase, the glucosylceramide (GlcCer) synthase at the cytosolic leaflet of early Golgi membranes (Futerman and Pagano 1991; Ichikawa et al. 1998; Jeckel et al. 1992) and thus GlcCer may be present on the cytosolic leaflet of cell membranes contrary to all other GSLs and GGs. Generated glucosylceramide can be translocated to the luminal face of Golgi membranes (D'Angelo et al. 2007), where it is converted to lactosylceramide (LacCer) by one of two lactosylceramide synthases (Nishie et al. 2010; Tokuda et al. 2013).

LacCer is the precursor of different GSL series, i.e. ganglio-, asialoganglio-, sulfoganglio-, globo-, isoglobo- and (iso)lacto series (Fig. 3 and Tab. 1 of reference Sandhoff and Sandhoff 2018). These series are formed by cell type specific glycosyltransferases, integral membrane proteins at the Golgi and TGN membranes, which are organized in distinct multi-enzyme complexes (Daniotti et al. 2017; Giraudo and Maccioni 2003).

In neurons, the biosynthesis of a- and b-series gangliosides starts with the transfer of sialic acid from CMP-sialic acid to the galactosyl residue of LacCer in 2,3 linkage to generate the simple ganglioside GM3 (Ishii et al. 1998) (Figs. 12.3 and 12.4). Further sialylation of GM3 yields GD3 and finally GT3 (Kono et al. 1996; Yoshida et al. 1995), the precursors of b- and c-series gangliosides, respectively.

As suggested by Saul Roseman, biosynthesis of GG is often mediated by multi-glycosyltransferase complexes (Bagatolli and Gratton 2000; Roseman 1970; Simons and Gerl 2010; Simons and Toomre 2000; Yusuf et al. 1983b, 1984) located in Golgi and TGN membranes (Fig. 12.4), which stabilize enzymes and improve glycolipid synthesis (Maccioni 2007; Spessott et al. 2012). GG glycosyltransferases (GGTs) are type II membrane glycoproteins (Martina et al. 2000), some of them are S-acylated at conserved cysteine residues, which may be involved in the formation of homodimers through disulfide bonds (Chumpen Ramirez et al. 2017).

Enzymatic steps for the synthesis of GGs had already been identified in the 1960s and 70s (Kaufman et al. 1968; Keenan et al. 1974). It was expected that the formation of a- and b-series gangliosides would be catalyzed by different GTs. Kinetic competition experiments between the lipid acceptors GM3 (of the a-series) and GD3 (of the b-series), however, proved that both lipid substrates competed for the same active site of a quite unspecific GalNAc-transferase (Pohlentz et al. 1988). All GTs involved in the biosynthesis of complex gangliosides turned out to be rather

promiscuous for their lipid substrates (Iber and Sandhoff 1989; Iber et al. 1991, 1992), resulting in the assembly line given in Fig. 12.3. Further modifications of the formed gangliosides (see Fig. 12.3) by different forms of sialylation, O-acetylation and lactonization have been reviewed (Sandhoff et al. 2018). In vivo labeling of lipids from 14-day-old rat brain with ( $^{35}\text{S}$ )-sulfate also allowed the identification of sulfated, sialic acid containing lipids, gangliosides in intact brain tissue, e.g. in oligodendrocytes (Farrer and Quarles 1997). Biosynthesis of gangliosides can be promoted by a low-carbohydrate ketogenic diet via transcriptional regulation of genes related to ganglioside biosynthesis and downregulation or even suppression of the GM2AP-gene, coding for the GM2-activator protein, an essential cofactor for lysosomal ganglioside catabolism (Okuda 2019).

GG biosynthesis is cell-type specific and regulated at the transcriptional and posttranslational protein level (Gupta et al. 2015; Han et al. 2010; Harmon et al. 2013; Hornemann et al. 2009; Kishimoto and Radin 1966; Kolter et al. 2002; Pewzner-Jung et al. 2006; Sandhoff 2010, 2012; Sandhoff and Pallmann 1978; Sassa et al. 2016; Siow and Wattenberg 2012; Tettamanti et al. 1980; Yusuf et al. 1983b) as well as by a sequential organization and coordination of glycosyltransferases with the traffic of biosynthetic intermediates through the membranes of the secretory pathway (Yu et al. 2004). The recent development of high resolution mass spectrometry for the analysis of complex ganglioside patterns of the human motor cortex identified an unexpected complexity of at least 83 different gangliosides, exhibiting a higher degree of sialylation than known before, including fucogangliosides and glycan chains elongated by either-acetylation and /or acetate anion attachment (Ica et al. 2021).

Gangliosides are enriched in the outer leaflet of neuronal plasma membranes. Though their detailed organization is still elusive, a model based on dynamic simulations proposes a dynamic membrane structure composed of transient domains with liquid-ordered character that form and disappear on the microsecond time scale. Nano-domains consisting of gangliosides were observed in the outer leaflet (Ingolfsson et al. 2014). These domains can be small and transient or larger and more persistent and their location can correlate with PIP- and cholesterol rich domains on the inner leaflet of the membrane (Ingolfsson et al. 2017), suggesting the existence of lipid cross-talk between leaflets. Using a freestanding, planar lipid membrane system, Hyun-Ro Lee and Siyoung Q Choi analyzed sphingomyelinase (SMase of *Bacillus cereus*) mediated membrane remodeling in vitro (Lee and Choi 2021). Their results indicated that SMase activation at the surface of the sphingomyelin and GM1 containing leaflet of the lipid bilayer can induce GM1 clustering by at least 2 different spatio-lateral scales. First, ceramide rich domains are derived from less ordered phases, which grow to a few micrometers in size. Second, SMase triggers a reduction of the area covered by lipid aggregates (“rafts”) thereby condensing GM1 clusters to nanosized objects. The obtained data suggest that similar GM1 condensation processes may occur also in cellular plasma-membranes, which might facilitate and modify signal transmission processes.

GSL expression presents a great potential for regulation of neural development and progression of neurological pathology (Bottai et al. 2019). Different authors

recently reviewed the role of neutral GSLs as neuroinflammatory signaling molecules in neurodegeneration (Mutoh 2021) and the physiology of GGs and of anti-GG antibodies in human diseases (Cuttillo et al. 2020) as well as the role of GGs in peripheral pain mechanisms (Santha et al. 2020). GSLs may also play a role in neuronal polarity (Cui et al. 2019).

Neuronal development is associated with dynamically changing GG patterns. Neuronal stem/progenitor cells express stage-specific antigen-4 (SSEA-4), a complex ganglioside of the globoseries (Barraud et al. 2007; Brochner and Mollgard 2016; Kannagi et al. 1983), which is overexpressed in some cancers, linked to disease progression, and defines spontaneous loss of the epithelial phenotype in human solid tumor cells (Sivasubramaniyan et al. 2015). The simple GGs GM3 and GD3 are associated with neural tube formation and neural stem cell proliferation. They decline with neural differentiation, the time point at which complex ganglioseries GGs increase to reach adult levels during axonal/dendritic arborization and synaptogenesis (Olsen and Faergeman 2017). Adult neuronal complex GGs mainly contain a neutral tetraacylceramide backbone (i.e. GM1a, GD1a, GD1b, GT1b) but almost no detectable GGs with a triaacylceramide backbone (i.e. GM2, GD2). Especially GD2 is a target of tumor therapy as it has limited expression in normal tissues but is overexpressed in a variety of tumors (Nazha et al. 2020) including neuroblastoma, which develops from neural crest cells (Schengrund 2020).

The roles of gangliosides as modulators of receptors (Ledeen and Wu 2018a) and in inflammation (Furukawa et al. 2018) have recently been reviewed. Neurostatin, (a derivative of GD1b O-acetylated on the outer sialic acid) and other O-acetylated GGs were described as neuroprotective regulators and can reduce microglia activation (Yanguas-Casas et al. 2019). Different classes of gangliosides are expressed in nociceptive primary sensory neurons and play a role in peripheral pain mechanisms (Santha et al. 2020). Endothelial cells and cancer cells express their own specific ganglioside pattern, especially ganglioside GM3. At least in human breast cancer, GM3 synthase is highly expressed in immune cells and in vascular endothelial cells. In GM3-synthase-KO mice, tumor growth and angiogenesis is increased, suggesting that ganglioside GM3 attenuates tumor angiogenesis (Suzuki et al. 2021). GM3 in the plasma membrane can interact with a transmembrane segment of the EGF receptor (EGFR) laterally as detected by FRET (Förster resonance energy transfer) technology in lipid bilayers. It suppresses cell growth by preventing the autophosphorylation of epidermal growth factor and the formation of active EGFR dimers (Nakano et al. 2021).

## 9 Inherited Errors of GG Biosynthesis

Blocks in GG biosynthesis, as generated in GM3-only KO mice and other mouse models indicate **no** feedback inhibition due to accumulating intermediates (Sandhoff 2012). With respect to defects in GG biosynthesis, mouse models and inherited human defects do not necessarily correlate: Inherited defects of ganglioside GM2

biosynthesis cause severe infantile epilepsy and spastic paraplegia (Harlalka et al. 2013), whereas mice lacking major gangliosides due to a GM2/GD2 deficiency develop manifestations of Parkinsonism (Wu et al. 2011). Human defects in GM3 synthesis (Fragaki et al. 2013; Simpson et al. 2004) cause infantile-onset symptomatic epilepsy syndrome or refractory epilepsy and mitochondrial dysfunction, while GM3 synthase-deficient mice appeared rather normal but are deaf (Yoshikawa et al. 2009, 2015). Congenital disorders of ganglioside biosynthesis have been reviewed recently (Dunn et al. 2019; Li and Schnaar 2018; Trinchera et al. 2018).

## 10 Remodeling and Recycling of Cell Surface Gangliosides

Complex GSLs and GGs reach the cell surface by vesicular transport. They are enriched in the extracellular leaflet of PMs, GGs especially at synaptic surfaces, where they undergo lateral associations and trans interactions (Mori et al. 2012). GG interactions may be regulated by PM located sialidases, like the PM bound Neu3 (Rodriguez-Walker and Daniotti 2017) and sialyltransferase activity, both of which are present in synaptosomal membranes (Durrie et al. 1988; Ohman 1971; Preti et al. 1980; Saito et al. 1995; Tettamanti et al. 1980).

Polysialogangliosides expressed on cellular surfaces can serve as ligands for lectins and substrates for enzymes (Sonnino and Prinetti 2016). Their composition can be adjusted to changing membrane functions by desialylation mostly catalyzed by Neu3 (Miyagi and Yamaguchi 2012; Pan et al. 2017; Shiozaki et al. 2015) and by recycling. Radiotracer techniques and ESR spectroscopy studies on cultured GM2 gangliosidosis fibroblasts demonstrated that the exogenously added radiolabeled GG GM2 can be incorporated into the PM and directly glycosylated to form radiolabeled GGs GM1 and GD1a despite a complete cellular block in GM2 degradation, thus excluding transfer through the endo-lysosomal compartment. A direct glycosylation of labeled GGs GM2 and GM1 was also observed in normal control cells, presumably after trafficking from endosomes to the TGN compartment (Schwarzmann et al. 1983; Sonderfeld et al. 1985) (Fig. 12.4), which may involve the retromer complex (Lucas et al. 2016).

Intracellular trafficking, especially of GG GM1 having unsaturated or shorter ceramide anchors (Chinnapen et al. 2012; Saslowsky et al. 2013), was observed as an important aspect of metabolism and cellular uptake of cholera toxin. Recycling of fluorescently labeled glucosylceramide was also observed from the PM through early endosomes and back (Kok et al. 1989) as well as the glycosylation of non-degradable glucosylceramide analogs involving trafficking from proximal to distal Golgi cisternae to form complex gangliosides (Schwarzmann et al. 1995). Gangliosides were also identified in the nucleus, their metabolism and functions, however, remain mostly unknown to date. GGs GM1 and GD1a are located in both membranes of the nuclear envelope together with two neuraminidases (Ledeen and Wu 2011).

## 11 Concepts of the Constitutive Degradation of Gangliosides and Glycosphingolipids at ILVs

### 11.1 Location and Topology of Sphingomyelin, Glycosphingolipids and Ganglioside Catabolism

The constitutive degradation of membrane lipids like sphingolipids (SLs) and phospholipids (PLs) takes place at the surface of ILVs in the lysosomal compartment. SLs and GGs of the PM can reach ILV membranes by pathways of endocytosis (Fig. 12.4). The concept is supported by biochemical and immune-electro-histochemical studies on the endocytosis of biotinylated and radiolabeled ganglioside GM1 (Mobius et al. 1999b) and allows one to understand mechanisms of drug induced phospholipidosis. Most steps of membrane lipid catabolism are catalyzed by rather promiscuous hydrolases (lipases, phospholipases, glycosidases), for SLs it is often with the essential help of lipid binding proteins, the SAPs.

### 11.2 Endocytosis of Gangliosides

Desialylation of complex polysialylgangliosides is catalyzed by membrane bound sialidases, Neu1, Neu4 and Neu3, to generate ganglioside GM1 (Smutova et al. 2014; Timur et al. 2015). This redundancy may explain why complex polysialogangliosides do not accumulate in the brain tissue of 2 months old mice deficient in either Neu1, Neu3, Neu4 or Neu3 and Neu4 (Pan et al. 2017). Desialylation is an initial step in ganglioside degradation in mammalian tissues (Monti et al. 2010) and can take place at the plasma membrane or later in the endosomes and lysosomes. The lysosomal sialidase Neu1 is a member of a multi-enzyme complex together with protective protein/cathepsin A (a stabilizing protein for sialidase Neu1), and the GM1 cleaving  $\beta$ -galactosidase (Nicoli et al. 2021; Sandhoff et al. 2018). Inherited defects of Neu1 leading to sialidosis cause accumulation of sialylated metabolites (d'Azzo et al. 2015), including an amyloidogenic processing of an over-sialylated amyloid precursor protein in lysosomes and an extracellular release of A $\beta$ -peptides (Annunziata et al. 2013), whereas genetic defects in protective protein/cathepsin A trigger a storage of GM1 besides oligosaccharides and other glycolipids (Bonten et al. 1996; d'Azzo and Bonten 2010).

Lysosomes degrade a variety of macromolecules and complex lipids and release their components into the cytosol as nutrients for use in energy metabolism and biosynthetic salvage pathways (Kolter and Sandhoff 2005). Macromolecules and complex lipids can reach the lysosomes and the intra-lysosomal luminal vesicles (ILVs) by endocytotic pathways (Kolter and Sandhoff 2005), and probably by phagocytosis and autophagy as well (Florey and Overholtzer 2012). Metabolic studies and immuno-electron microscopic observations indicate that PM-bound radio- and biotin-labeled ganglioside GM1 reaches intralysosomal luminal vesicles (ILVs)



for final degradation (Mobius et al. 1999a) (Fig. 12.4). Released components like So, fatty acids, and monosaccharides can leave the lysosomes to reach the ER and the cytosol. Released So can be incorporated again into newly synthesized SLs and GGs (Gillard et al. 1996; Sonderfeld et al. 1985; Tettamanti 2004; Tettamanti et al. 2003), but may also be phosphorylated to generate SIP for final degradation of the sphingoid base by SIP-Lyase (Merrill Jr. et al. 1997; Stoffel et al. 1968b).

### ***11.3 Generation of ILVs During Endocytosis and GSL-Catabolism***

As proposed (Furst and Sandhoff 1992), ILVs are generated by an inward budding of the endosomal membrane and budding off of ILVs into the luminal space, which is catalyzed by ESCRT proteins (Wollert and Hurley 2010) (Fig. 12.4). Components of the endosomal membrane sorted into ILVs can be catabolized by the digestive juice in lysosomes. ILV degradation will destabilize and destroy the bilayer structure of ILV membranes and transiently generate micellar and other amphiphilic aggregates, which either may dissolve or fuse with new incoming ILVs.

Catabolism of GSLs, SLs and GGs in ILVs can be blocked by genetic defects affecting lysosomal proteins (Kolter and Sandhoff 2005), causing lysosomal lipid and membrane storage diseases. An inherited defect of prosaposin, the precursor of four lipid binding proteins, the saposins A, B, C and D, triggers an excessive accumulation of GSLs, SLs and intra-lysosomal ILVs with an average diameter of 90 nanometers within the lumen of late endosomes and lysosomes (see insert in Fig. 12.5). The blocked lipid catabolism can be restored and the pathological lipid and ILV accumulation can be completely reversed by feeding nanomolar concentrations of the missing prosaposin to patients' cultured fibroblasts (Bradova et al. 1993; Burkhardt et al. 1997; Harzer et al. 1989; Mobius et al. 1999a; Sandhoff et al. 2018; Schnabel et al. 1992). ILVs are obviously the main location for degradation of SLs including GGs and membranes.

Progranulin acts as a key regulator of lysosomal function. It is a large glycoprotein, proteolytically cleaved in the lysosomal compartment or in the extracellular matrix to generate up to eight small granulin glycopeptides. In 2006, the discovery of the heterozygous mutation of the granulin (GRN) gene leading to progranulin haploinsufficiency in patients with frontotemporal dementia stimulated the search for its many properties and functions (Wikipedia 2021). Progranulin enhances memory in normal aging and Alzheimer's disease mice (Farr et al. 2021). Delivering progranulin to neuronal lysosomes protects against excitotoxicity (Davis et al. 2021). Both, glucocerebrosidase and progranulin are possible targets in the treatment of Parkinson's disease (Rodrigues and Kale 2021).

Homozygous GRN gene mutations cause a neuronal ceroid lipofuscinosis with accumulation of auto-fluorescent lipofuscin, impairment of lysosomal activity, microgliosis and brain degeneration (Nguyen et al. 2013; Paushter et al. 2018).

Defective lysosomal lipid catabolism is a common pathogenic mechanism for dementia. Defective lipid clearance from endo-lysosomes is a central driver of Alzheimer's disease. Impaired cholesterol turnover or sphingolipid catabolism is sufficient to produce the pathological hallmarks of dementia (Lee et al. 2021; Wang et al. 2021c). Progranulin mutations result in impaired processing of prosaposin and reduced glucocerebrosidase activity (Valdez et al. 2020). GRN variants were identified that may contribute to lifetime risk of multiple neurodegenerative diseases. Expression quantitative trait locus analyses and genome-wide association studies identified functional associations between increasing genetic risk in the GRN region and decreased expression of the GRN gene in Parkinson's, Alzheimer's and amyotrophic lateral sclerosis. GRN expression mediates neuroinflammation functions related to multiple neurodegenerative diseases (Nalls et al. 2021). The lysosomal storage disorder caused by GRN loss of function can be rescued with a brain penetrant progranulin biologic (Logan et al. 2021).

#### ***11.4 Maturation of Intra-Lysosomal Luminal Vesicles (ILVs) and Lipid Sorting***

Whereas ILVs can be attacked by the lysosomal juice, the lysosomal perimeter membrane is protected by a thick glycocalyx, which covers its luminal surface and prevents hydrolases and SAPs from reaching and digesting the perimeter membrane. The coat consists of integral glycoproteins, heavily N-glycosylated with almost digestion-resistant poly-lactosamine units (Eskelinen et al. 2003). Defects in the biosynthesis of the lysosomal perimeter membrane glycoproteins, however, increase their turnover (Eskelinen et al. 2003) and may attenuate their barrier function. Most of the degradation products released within the lysosomal compartment need membrane carriers to move from the lysosol to the cytosol (Eskelinen et al. 2003; Sandhoff et al. 2018), where they can be used for biosynthesis of new macromolecules or as fuel for energy metabolism.

The degradation of complex lipids by lysosomal enzymes is substantially facilitated by a lipid sorting process leading to a maturation of ILVs at the level of late endosomes (Gallala and Sandhoff 2011; Kolter and Sandhoff 2005): membrane stabilizing lipids of the plasma membrane, such as sphingomyelin (SM) and cholesterol, are removed. Acid sphingomyelinase (ASM) cleaves SM and other phospholipids, releasing ceramide and diacylglycerol (Breiden and Sandhoff 2021; Oninla et al. 2014; Quintern et al. 1987). Reconstitution studies in vitro suggest that the decrease of inhibiting SM levels and the concomitant increase of stimulating ceramide levels are prerequisites for an effective secretion of the non-degradable cholesterol from the ILVs by the transfer- and sterol-binding glycoprotein NPC2 (Abdul-Hammed et al. 2010). The anionic lysophospho-glycerolipid bis(monoacylglycerol)phosphate (BMP) contributes to a negative surface charge on the ILV membranes that attracts and binds the protonated NPC2 protein facilitating

its cholesterol transfer function. Reversely, increasing SM levels in ILVs of ASM deficient cells in NPA & B disease inhibit cholesterol secretion from the lysosomal compartment (Abdul-Hammed et al. 2010; Enkavi et al. 2017; Oninla et al. 2014). In addition to BMP, other anionic phospholipids like phosphatidic acid (PA), PG and phosphatidylinositol (PI) were also found, using in vitro studies, to stimulate cholesterol transfer by NPC2. BMP itself is a lysosome-specific lipid as it is a transient intermediate of PG degradation within the ILVs. As BMP is catabolized rather slowly, for example by ASM (Breiden and Sandhoff 2021), significant BMP levels can build up within lysosomes (Abdul-Hammed et al. 2010; Gallala and Sandhoff 2011; Sandhoff et al. 2018).

In summary, lowering SM and cholesterol levels and generating anionic BMP during maturation of ILVs appear to be essential to reach physiological rates of GSL and SL degradation in the endo-lysosomal pathway (Sandhoff 2013).

### ***11.5 BMP Formation Contributes Significantly to the Quintessential Negative Surface Charge on ILVs***

Reaching ILVs in the lysosol, membrane lipids are usually readily degraded in active lysosomes. The catabolism of PG, however, generates an intermediate, the anionic bis-lysolipid BMP, which is only slowly catabolized and therefore increases to make up to 40-60 mol% of ILV-phospholipids (Gallala and Sandhoff 2011; Kobayashi et al. 1999; Mobius et al. 2003; Sandhoff et al. 2018). Like other phospholipids (PA, PG and PI), BMP is negatively charged, even at pH values as low as pH 4 (Oninla et al. 2014; Wilkening et al. 1998). Reconstitution studies in vitro reveal it to generate a negative surface potential on ILVs (Oninla et al. 2014). This potential should force binding and concentration of lysosomal enzymes and SAPs on the surface of ILVs by electrostatic interaction as these proteins are protonated in the acidic lysosol with pH values around 4–5 (Kolzer et al. 2004; Oninla et al. 2014). Importantly, surface bound lysosomal enzymes like ASM (Hurwitz et al. 1994), acid ceramidase (Elojeimy et al. 2006), hexosaminidases A and B (HexA, HexB) and other glycoproteins are partially protected against premature proteolytic digestion in the lysosome. Compensation of the negative surface charge of ILVs favors release of these enzymes and activator proteins and subsequently their proteolytic digestion. This is observed when feeding cationic amphiphilic drugs (such as desipramine) to cultured fibroblasts, which causes a drop in lysosomal degradation activity and triggers a phospholipid storage disease, i.e. an induced phospholipidosis (Hurwitz et al. 1994; Kolzer et al. 2004; Lullmann et al. 1978; Sandhoff et al. 2018). The catabolism of membrane lipids by the concerted action of lipases and glycosidases is supported by the mixture of five known lipid binding and membrane disturbing SAPs and will destroy the lipid bilayer and topology of ILVs. Micelles and smaller lipid aggregates may well be formed as intermediates of ILV degradation.

### ***11.6 Regulation of GG Catabolism and the Removal of Inhibitory Lipids from Intraendosomal Vesicles and ILVs***

The plasma membrane stabilizing lipids SM and cholesterol are inhibitors of GSL and GG catabolism in the lysosomes and are therefore removed from ILVs during endocytosis by a NPC2-mediated cholesterol efflux and an ASM mediated SM digestion. The inherited NPC2-deficiency and the reduced ASM activity in the lysosomes of Niemann-Pick disease type C2 patients may well cause a lysosomal cholesterol and sphingomyelin accumulation (Vanier 1983, 2015), which could strongly inhibit the BMP-enhanced hydrolysis of GM2 and glucosylceramide (Abdul-Hammed et al. 2017; Anheuser et al. 2015). In wild type cells, however, hydrolysis of sphingomyelin by ASM generates stimulatory ceramide, which enhances cholesterol transfer by NPC2 tremendously (Oninla et al. 2014). Sphingomyelin degradation is obviously an initial step required for physiological secretion of cholesterol from the late endosomal compartment, which in turn is a prerequisite for lysosomal GSL and GG digestion (Sandhoff et al. 2018). The secondary storage of LacCer and ganglioside GM3 in NPC patients (Vanier 2015) might also be explained by a cholesterol mediated inhibition of several SAPs, which are important for the digestion of LacCer and ganglioside GM3, Sap A (Locatelli-Hoops et al. 2006), Sap B (Rommel et al. 2007) and GM2 activator protein (GM2AP) (Anheuser et al. 2015). The clinical course of the disease can be improved by reducing GSL storage with Miglustat, a drug inhibiting glucosylceramide synthase, the first step of GSL biosynthesis (Bowman et al. 2015; Patterson et al. 2015).

### ***11.7 Catabolism of Gangliosides at ILVs***

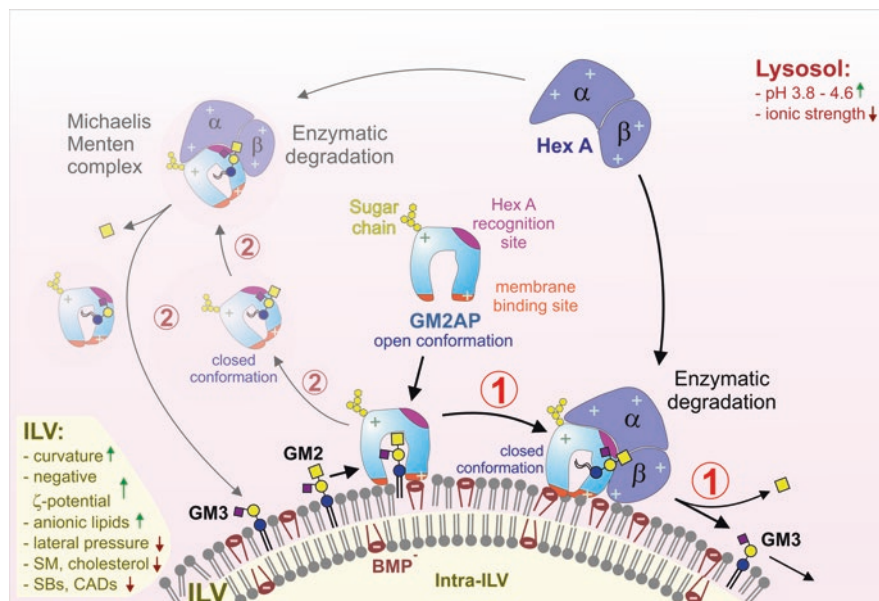
Catabolism of gangliosides, SM and other SLs and their lipophilic catabolites proceeds at the surface of ILVs (Figs. 12.4 and 12.5) (Kolter and Sandhoff 2005). In contrast to GG biosynthesis at the membranes of the secretory pathways, no membrane spanning enzymes are directly involved in lysosomal phospholipid and GG catabolism. The lipids of the ILVs are catabolized by soluble lysosomal glycoproteins, lysosomal hydrolases and lipid-binding sphingolipid activator proteins (SAPs).

Stepwise degradation of complex gangliosides in mammalian tissues starts with the removal of terminal sialic acid units from the oligosaccharide chain by neuraminidase(s) to generate GM1 (Fig. 12.5). GM1 degradation continues with the removal of the terminal galactose by GM1- $\beta$ -galactosidase, supported by the GM2 activator protein or saposin B to yield GM2 (Sandhoff et al. 2018; Wilkening et al. 2000). Thereafter, the terminal N-acetylgalactosamine residue is split off by  $\beta$ -hexosaminidase A with the help of the GM2 activator protein, to form GM3

(Fig. 12.5), which can be degraded to LacCer by an  $\alpha$ -sialidase together with Sap B. In mouse, the first two steps can be bypassed by the removal of sialic acid from GM1 and GM2, producing the corresponding asialo-derivates GA1 and GA2, respectively. The latter are degraded to form LacCer by a  $\beta$ -galactosidase and  $\beta$ -hexosaminidase A and B, respectively, with the assistance of the GM2 activator protein (Sandhoff et al. 2018; Sango et al. 1995; Seyrantepe et al. 2018). Consequently, HexA-deficiency in mice causes a very mild gangliosidosis and does not correlate with the infantile form of Tay-Sachs disease, which is caused by this deficiency in humans (Phaneuf et al. 1996; Sango et al. 1995). Additional deficiency of Neu3 in mice on top of the loss of HexA function blocks this bypass quite efficiently resulting in strong GM2 gangliosidosis (Seyrantepe et al. 2018). Sap B or Sap C-assisted action of  $\beta$ -galactosidase degrades LacCer to GlcCer, whose glucosyl residue is removed by Sap C-assisted  $\beta$ -glucosidase. Finally, ceramide is cleaved by acid ceramidase aided mainly by Sap D resulting in the sphingoid base (mainly sphingosine) and a free fatty acid (Sandhoff et al. 2018). The speed of hydrolytic steps of GM2 catabolism is supported by several factors, such as low pH values (e.g. only a narrow range of pH 3.8–4.5 allows hydrolysis of membrane bound GM2 by HexA and GM2AP (Bierfreund et al. 1999)), surface tension, and electrostatic binding of protonated and positively charged enzymes and SAPs to negatively charged surfaces of ILVs (Fig. 12.6). The optimal combination of all these regulating and essential factors restricts ganglioside catabolism almost exclusively to the ILVs in the lysosol and protects cells from spontaneous self-degradation. Most regulating mechanism of ganglioside homeostasis, however, are still poorly understood, e.g. the influence of ethanol on brain gangliosides and their turnover. In rats, even maternal alcohol consumption can impact the ganglioside content and the ganglioside catabolizing hydrolases in the brain of the offspring (Prasad 1992). The pups exposed to alcohol developed a brain with a reduced wet weight, protein, and DNA content, but with an increased concentration and content of gangliosides as assayed by the total ganglioside sialic acid content. Region-specific alterations in the ganglioside pattern were observed.

The salvage pathway, which utilizes lysosomal degradation products for biosynthesis, e.g. sphingosine for the formation of the growth factor sphingosine-1-phosphate, plays an important role in many cells and may explain that ablation of acid ceramidase blocks cell cycle progression and fertilization of Oocytes (Eliyahu et al. 2012; Lai et al. 2017).

A schematic overview of these and other degradation steps and corresponding inherited diseases, which are described elsewhere in more details is given in Fig. 12.5 (d'Azzo et al. 2019; Desnick et al. 2019; Gieselmann and Ingeborg 2019; Grabowski et al. 2019a, b; Gravel et al. 2019; Kolter and Sandhoff 2006; Levade et al. 2019; Patterson et al. 2019; Sandhoff et al. 2019; Schuchman and Desnick 2019; Schuchman et al. 2019; Suzuki et al. 2019; Valle et al. 2021; Wenger et al. 2019).



**Fig. 12.6** Model of GM2 degradation by human  $\beta$ -hexosaminidase A (Hex A), and the cofactor GM2AP. GM2AP contains a hydrophobic cavity lined by surface loops and a single short helix. The cavity is suitable for the ceramide anchor of GM2 and other lipids. In its open conformation, GM2AP binds to the membrane using the hydrophobic loops (orange) and penetrates to a certain extent into the hydrophobic region of the bilayer. The lipid recognition site of the activator then interacts with the substrate, and its ceramide portion can move inside the hydrophobic cavity. At this point, the lipid-loaded activator may change to its closed conformation, thus the complex becomes more water soluble and can stay (1) or leave the membrane (2), exposing GM2 to the water-soluble Hex A for degradation (modified after Sandhoff and Sandhoff 2018). Besides the cofactor GM2AP, lysosomal degradation is stimulated (green arrow pointing upwards) by the acidic lysosomal pH leading to a net positive charge of hydrolases and activator proteins as well as increasing curvature, negative  $\zeta$ -potential, and anionic lipids of ILVs, whereas increasing lysosomal ionic strength, too high lateral pressure, and the presence of sphingomyelin (SM), cholesterol, free sphingoid bases (SBs), and cationic amphiphilic drugs (CADs) inhibit (red arrow pointing down) lysosomal lipid (GM2) catabolism. The lysosomal-specific anionic lipid BMP serves the net negative charge, reduced lateral pressure, and increased negative  $\zeta$ -potential of ILVs. The ILV-associated effects suggest digestion taking place mainly at the ILV surface (1) in vivo

### 11.8 SAPs Are Essential Cofactors for Lysosomal Ganglioside Catabolism

Soluble lysosomal hydrolases hardly attack lipophilic membrane components of ILVs directly due to a solubility barrier between the aqueous and the lipid phase, also known as the lipid phase problem: Hydrophobic lipids or lipid moieties of SLs like their ceramide residues are insoluble in aqueous phases, whereas hydrophilic molecules like water soluble proteins, carbohydrates or oligosaccharide chains of GSLs and GGs are insoluble in lipid phases.

Therefore the catabolism of GSLs and GGs by water soluble enzymes needs the help of membrane perturbing lipid binding proteins, the SAPs (saposin A, B, C and D, and GM2AP), small glycoproteins with amphiphilic properties, some with lipid transfer and others with fusiogenic functions at low pH values (Kolter and Sandhoff 2005). Their inherited defects cause rare, but fatal, often degenerative brain diseases (Kolter and Sandhoff 2006; Sandhoff 2012; Sandhoff and Harzer 2013) with a clinical picture quite similar to that of the respective enzyme deficiencies (Abdul-Hammed et al. 2010; Sandhoff and Harzer 2013; Schulze and Sandhoff 2014).

The GM2 activator protein (GM2AP) and the dimeric saposin B (Sap B) are promiscuous lipid binding and intervesicular lipid transfer proteins (Breiden and Sandhoff 2019b). GM2AP is an essential cofactor for the catabolism of GM2 by hexosaminidase A (HexA) and of GM1 by beta-galactosidase (Figs. 12.5 and 12.6). Its inherited deficiency causes a fatal GM2 gangliosidosis (variant AB of GM2-gangliosidosis) with a clinical picture almost indistinguishable from Tay-Sachs disease. GM2AP can extract GGs from vesicular membranes at low pH values, forming a stoichiometric and soluble GG-protein complex, which is recognized by HexA as substrate, forming a Michaelis-Menten complex (Fig. 12.6). Furthermore, it facilitates the degradation of GA2 (Liu et al. 1997) and of its sulfated derivative, SM2a, which appeared in the liver of a Tay-Sachs patient (Sandhoff et al. 2002). Film balance experiments show that amphiphilic GM2AP inserts into lipid monolayers only when the lateral surface pressure is below a critical value of about 25 mN/m (Giehl et al. 1999), a value significantly below that of most biological membranes, which ranges between 30–35 mN/m. Due to their small diameter of around 90 nanometer and high curvature, ILVs are more susceptible to an enzymatic attack, as demonstrated for the cleavage of membrane bound ceramide by acid ceramidase in cooperation with saposin D (Linke et al. 2001). These membrane factors, lateral pressure and membrane curvature obviously ensure that GM2AP interacts preferentially with ILVs (Anheuser et al. 2019b). Besides well-known factors like pH value and ionic strength, GM2AP facilitated hydrolysis of membrane bound GM2 by HexA is strongly stimulated by anionic lipids in the GM2-carrying membranes like BMP, PA, PG, PI, fatty acids, and also by ceramide, lyso-phosphatidylcholine and diacylglycerol. The stimulatory role of these lipids argues for degradation of GM2 directly at the surface of ILVs by the GM2AP/HexA complex *in vivo* (Fig. 12.6). In contrast, hydrolysis of membrane bound GM2 is effectively inhibited by membrane stabilizing lipids like SM and cholesterol, cationic lipids like sphinganine and sphingosine and CADs like desipramine, chlorpromazine, imipramine and chloroquine, as well as mucopolysaccharides as primary storage compounds in mucopolysaccharidoses like chondroitin sulfate, dermatan-sulfate and hyaluronan (Anheuser et al. 2019a). These factors also affect the ability of GM2AP to solubilize and mobilize membrane lipids, but hardly affect the hydrolysis of water-soluble synthetic substrates like 4-methyl-umbelliferyl- $\beta$ -D-N-acetylglucosaminide-6-sulfate (MUGS), which are often used for the diagnosis of HexA deficiency in patients with inherited GM2 gangliosidosis.

The homodimeric saposin B is also a promiscuous lipid binding and transfer protein. It is an essential cofactor in the lysosomal hydrolysis of sulfatide SM4s by

arylsulfatase A, binding sulfatides in soluble stoichiometric complexes, which are recognized by arylsulfatase A as substrates (Fischer and Jatzkewitz 1975; Mehl and Jatzkewitz 1963). On the other hand, saposin C (Ahn et al. 2003; Rossmann et al. 2008; Schwarzmann et al. 2015) and D (Graf et al. 2017) are lipid binding proteins, which can fuse lipid vesicles at low pH values, thereby eventually bringing newly formed ILVs together with already processed, hydrolase loaded and BMP containing ILVs. The four saposins A, B, C and D are generated in the lysosomal compartment by proteolytic cleavage of their precursor protein, prosaposin (Kolter et al. 2005). They share a similar folding pattern containing four helices stabilized by three conserved disulfide bridges. They are also involved in loading lipid antigens for presentation by the Cluster of Differentiation 1 (CD1) receptor protein (Fig. 12.4) (Garrido-Arandia et al. 2018).

All hydrolases and SAPs are N-glycosylated and glycosylation regulates their function, as has been shown for Sap B. The un-glycosylated mutant Sap B causes a fatal sphingolipid storage disease just as in patients with classical metachromatic leukodystrophy, despite the presence of the un-glycosylated protein in the lysosomal compartment and its ability to stimulate the enzymatic hydrolysis of sulfatides *in vitro* even better than the glycosylated wild type Sap B. However, un-glycosylated Sap B is unable to extract membrane lipids at acidic pH, which is essential for SL degradation at ILVs *in vivo* (Rommel et al. 2007). An inherited deficiency of prosaposin, the precursor of four SAPs, the saposins A, B, C and D, triggers a perinatal fatal disease. Here, ILVs, ceramide and several GSLs accumulate (Fig. 12.5). In addition, there is a loss of the water permeability barrier of the skin, since SAPs are required in the extracellular processing of barrier lipids, which is required for maturation of water barrier-essential extracellular lipid lamellae between corneocytes (Breiden and Sandhoff 2014; Doering et al. 1999; Hulkova et al. 2001; Sandhoff et al. 2018). Further information on the function of SAPs and corresponding diseases is given in (Sandhoff et al. 2018).

### ***11.9 Membrane Lipid Modifiers Regulate GSL and Ganglioside Catabolism***

In contrast to membrane spanning GTs catalyzing the biosynthesis of GGs at the ER-, Golgi and TGN-membranes, water-soluble hydrolases are the main players in the catabolism of GGs. Due to their insolubility in the lipid phase of the substrate carrying membranes, the soluble hydrolases cannot interact properly with GSL and GG substrates that have short oligosaccharide chains of up to four monosaccharides and are incorporated in the ILV-membranes. They need the support of homo-dimeric SAPs as lipid binding, membrane disturbing and lipid extracting glycoproteins to allow physiologically relevant catabolic rates. The lipid-splitting activity of soluble lysosomal hydrolases depends heavily on posttranslational modifiers such as SAPs, stimulating and inhibiting membrane lipids, pH-values, electrostatic attraction to ILVs, their curvature etc.

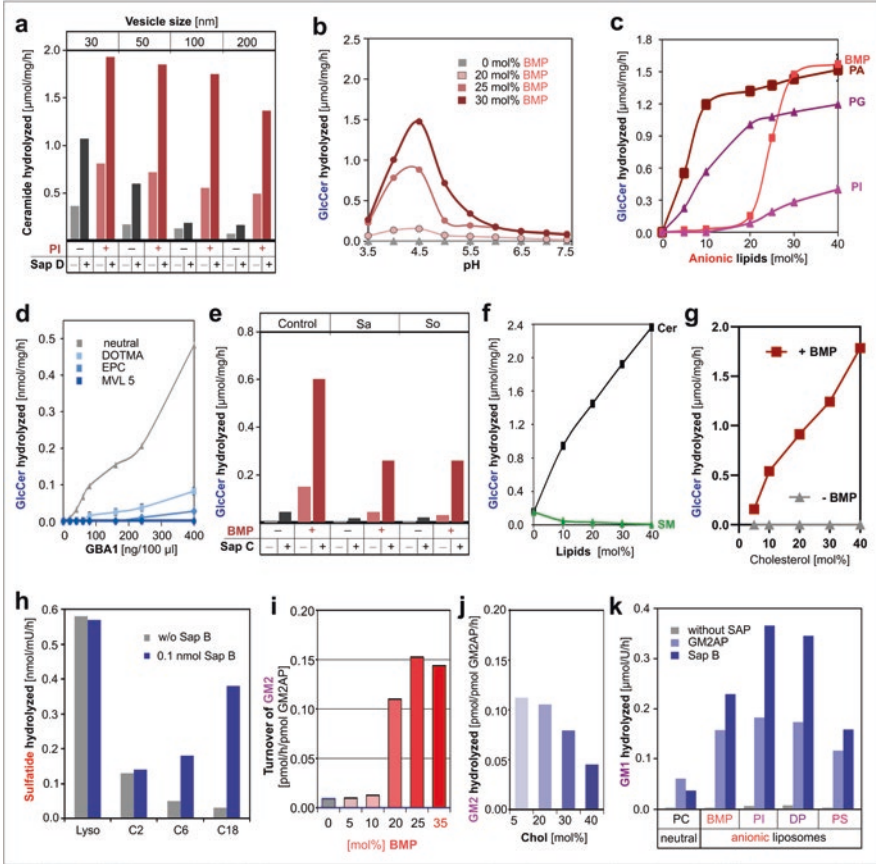


Some SAPs, (Sap B and GM2AP) can bind lipids at low pH values, lift and extract them – like sulfatides and ganglioside GM2 – to form stoichiometric soluble complexes, which they present as Michaelis Menten substrates to their respective hydrolases for degradation (Fischer and Jatzkewitz 1978; Wendeler et al. 2006). Other SAPs, Sap A, C and D, disturb membrane structures, trigger vesicle fusion and are needed for the catabolism of GalCer, GlcCer and ceramides, respectively (Abdul-Hammed et al. 2017; Anheuser et al. 2015; Kolter and Sandhoff 2005; Sandhoff 2016; Schwarzmann et al. 2015). However, GSLs and GGs with long hydrophilic oligosaccharide chains of more than five monosaccharides extending far enough from the membrane surface can be attacked directly by a hydrolase as observed for GG IV<sup>4</sup>-GalNAc-GD1a, which is hydrolyzed by HexA (Meier et al. 1991).

Despite the presence of activator proteins, membrane-bound complex GSLs like GM1 and GM2 are hardly hydrolyzed at all by their respective hydrolases in the absence of ceramide and anionic lipids, such as bis(monoacyl-glycerol)phosphate (BMP), phosphatidic acid, phosphatidylglycerol, PI, or PS, and even less so in the absence of both, SAPs and anionic lipids (Fig. 12.7) (Anheuser et al. 2015; Sandhoff et al. 2018; Werth et al. 2001). Addition of ceramide and anionic lipids to substrate carrying liposomal membranes can stimulate the catabolic rate immensely *in vitro*. Quantitative data on the synergetic stimulation of ganglioside GM1 hydrolysis by  $\beta$ -galactosidase in the presence of SAPs (GM2AP and Sap B) and anionic lipids are given in Fig. 12.7.

A model of GM2 degradation by human  $\beta$ -hexosaminidase A, stimulated by GM2AP, is presented in Fig. 12.6. Results of reconstitution experiments using liposomes to mimic ILVs of the late endosomal/lysosomal compartment show the essential role of anionic membrane lipids like BMP, PI, and PG in the vesicular GM2-carrying membranes to achieve physiologically relevant catabolic rates of GM2 degradation (Anheuser et al. 2015, Sandhoff et al. 2018, Werth et al. 2001) (Fig. 12.7). *In vivo*, however, the lipid composition of ILVs within endosomes and lysosomes is changing constantly due to ongoing lipid-digestion and sorting out of cholesterol along the endocytic pathway. Therefore, *in vivo* rates of GG catabolism should also change continuously depending on the momentary conditions within the lysosomes.

The degradation of sphingolipids with short glycan chains is also regulated by the surrounding lipids of the substrate carrying vesicular membrane. Glucosylceramide is the primary storage lipid in the lysosomes of Gaucher patients and a secondary one in Niemann-Pick disease types A, B, and C. Glucosylceramide can be cleaved by  $\beta$ -glucosidase in the absence of SAPs when anionic lipids are present (Abdul-Hammed et al. 2017; Sandhoff et al. 2018; Wilkening et al. 1998). Anionic lipids stimulate its hydrolysis up to 1000-fold (Fig. 12.7), mostly by transglucosylation (to generate Glc-Chol) while cationic lipids inhibit its degradation, especially sphingoid bases and the minor cytotoxic storage compound glucosyl-sphingosine (Sarmientos et al. 1986), a biomarker for Gaucher disease (Murugesan et al. 2016). Since sphingoid bases are the final product of lysosomal SL degradation, they need to be exported from lysosomes efficiently. Ceramides, fatty acids,



**Fig. 12.7** (Glyco)sphingolipid degradation is regulated by (a) vesicle size, (b) pH, (c–g and i–k) lipid composition of the vesicles, and (h) acyl chain length of lipids. The presence of anionic lipids [(b, c, e, g, i, k) BMP; (a, c, k) PI; (k) PS; (c) PA and PG] and (f) ceramide enhances the degradation of GSLs. Sphingolipid activator proteins stimulate the hydrolysis of GLSs: (a) Sap D the hydrolysis of ceramide by acid ceramidase, (h) Sap B the hydrolysis of micellar sulfatide by the arylsulfatase A, (k) Sap B and GM2AP the hydrolysis of GM1 by  $\beta$ -galactosidase, and (e) Sap C the hydrolysis of GlcCer by GBA1. The presence of cationic substances [(d) cationic lipids or cationic amphiphilic drugs], (j) cholesterol, (f) sphingomyelin, and (e) sphingoid bases sphingosine and sphinganine inhibits the digestions of GSLs. Liposomes in panels j and f contained additional 20 mol% BMP. GBA1 and many other beta-glucosidases are also transglucosidases that catalyze the transfer of the glucose moiety from glucosylceramide to cholesterol generating 1-O-cholesteryl-beta-D-glucopyranoside (GlcChol) (Akiyama et al. 2013, Marques et al. 2016). Such transglucosylation can speed up GlcCer degradation supporting the strong turnover observed in panel f in the presence of 5% cholesterol and g; Cholesterol is an acceptor substrate for Gba1 in g but not for HexA in j, which would explain the seemingly contrary results obtained in these two experiments. Figure 12.7 was modified according to references (Breiden and Sandhoff 2019b; Sandhoff and Sandhoff 2018). Abbreviations: BMP bis(monoacylglycero)phosphate, Cer ceramide, Chol cholesterol, DOTMA 1,2-di-O-octadecenyl-3-trimethylammonium propane, DP dolicholphosphate, EPC 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine, GBA1 lysosomal  $\beta$ -glucocerebrosidase, GlcCer glycosylceramide, GM2AP GM2 activator protein; MVL 5 multivalent cationic lipid, PA phosphatidic acid, PG phosphatidylglycerol, PI phosphatidylinositol, PS phosphatidylserine, Sa sphinganine, SAP sphingolipid activator protein, Sap saposin, SM sphingomyelin, So sphingosine

monoacylglycerol, and diacylglycerol also stimulate glucosylceramide hydrolysis in the presence of anionic lipids while sphingomyelin, sphingosine, and sphinganine play strong inhibitory roles, thereby explaining the secondary storage of glucosylceramide in Niemann-Pick diseases (Abdul-Hammed et al. 2017; Sandhoff et al. 2018).

## 12 GSL and GG Metabolism and Disease

Dysfunctional genes in SPT involved in sphinganine biosynthesis can lead to neurodegeneration and sensory and autonomic neuropathy type 1 (HSAN1) (Penno et al. 2010). These mutations, either in subunit SPTLC1 or in subunit SPTLC2, cause a higher incorporation rate for other amino acids than L-serine, mainly L-alanine and cause increased levels of 1-deoxy-SLs, mainly 1-deoxy-dihydroceramides, which cannot be converted to sphingomyelin or GSLs/GGs and appear to cause mitochondrial dysfunction (Alecu et al. 2017). The big subunits of SPT are associated with a small subunit, either SPTssa or SPTssb, which confer distinct acyl-CoA substrate specificities. In Stellar mice, a mutation in SPTssb results in increased production of C20-sphingoid bases and corresponding GGs in the brain causing neurodegeneration (Zhao et al. 2015). A human correlate is so far unknown.

Downstream of SPT, KDSR reduces KDS to sphinganine. Mutations of this gene have recently been reported to cause a spectrum of keratinization disorders (Boyden et al. 2017; Takeichi et al. 2017), although the overall production of normal epidermal ceramides appeared to not be dramatically affected. Some of these mutation however lead to a metabolic bypass of KDSR: a substantial amount of otherwise not observed ketodihydroceramides is synthesized by ceramide synthases directly from KDS. These ketodihydroceramides were detected in the stratum corneum of the skin, but not in blood of the patients (Pilz et al. 2022). These finding might also explain the absence of systemic failures.

In contrast to KDSR, ceramide synthases comprise a family of six genes in mammals, CERS1-6. In humans, mutations of CERS3, which is expressed mainly in stratified epithelia and male germ cells, were described to cause autosomal recessive ichthyosis (ARCI) (Eckl et al. 2013; Radner et al. 2013) and CerS3-deficient mice die after birth due to breakdown of the lipid based skin barrier (Jennemann et al. 2012b; Rabionet et al. 2014). In mice, germ cell specific deletion of CerS3 affects SLs, including complex GGs and GSLs of the 0- and a-series resulting in a differentiation arrest of spermatids and male infertility (Rabionet et al. 2015). A homozygous nonsynonymous mutation in CERS1, abundantly expressed in neurons, causes progressive myoclonus epilepsy. Expressed *in vitro*, the mutant CERS1 did not cause additional ceramide production in contrast to the expression of the wild type CERS1 gene. How this mutation affects neuronal GG pattern in the patients, however, remains to be elucidated (Vanni et al. 2014). Corresponding

mouse models revealed reduced levels of neuronal ceramides (Zhao et al. 2011), sphingomyelins and GGs with stearic acid, leaving about half the neuronal GG levels (Ginkel et al. 2012). Deficiencies of CerS2 (Ali et al. 2013, 2015; Barthelmes et al. 2015; Bickert et al. 2018; Imgrund et al. 2009; Kim et al. 2017; Marsching et al. 2014; Oertel et al. 2017; Park et al. 2013a, b, 2014, 2015; Petrache et al. 2013; Pewzner-Jung et al. 2010; Rieck et al. 2017; Saroha et al. 2017; Shin et al. 2021; Zigdon et al. 2013), of CerS4 (Ebel et al. 2014; Peters et al. 2015), of CerS5 (El-Hindi et al. 2020; Gosejacob et al. 2016), and of CerS6 (Barron et al. 2021; Ebel et al. 2013; Eberle et al. 2014; Hammerschmidt et al. 2019; Helke et al. 2018; Turpin et al. 2014) as well as that of glucosylceramide synthase (Jennemann et al. 2005, 2010, 2012a; Nordstrom et al. 2013; Rabionet et al. 2015; Watanabe et al. 2010; Yamashita et al. 1999) were analyzed so far only in genetically engineered mice and are reviewed briefly elsewhere (Sandhoff et al. 2018).

Inherited defects in GG biosynthesis cause a severe infantile epilepsy syndrome (GM3-S deficiency) (Boccutto et al. 2014; Fragaki et al. 2013; Simpson et al. 2004) and progressive hereditary spastic paraplegia (GM2-S deficiency) (Harlalka et al. 2013). A wide spectrum of defects has been described in mutant mice (Sandhoff et al. 2018) and it will be a matter of time until corresponding inherited human diseases will be identified.

Ganglioside degradation can be blocked at almost every catabolic step, either by an inherited deficiency of a hydrolase or the respective activator protein. The rare and fatal gangliosidoses are dominated by neuronal ganglioside, GSL and lyso-GSL storage, triggering neurodegeneration (Kolter and Sandhoff 2006; Regier et al. 2016; Sandhoff and Harzer 2013). GG and GSL storage diseases have been briefly reviewed in (Rosenberg and Pascual 2020; Sandhoff et al. 2018).

### **13 Cascades of Metabolic Errors in LSDs and in Defective GSL Biosynthetic Pathways Generate Complex Pathologies**

Some membrane lipids such as SM and Cholesterol emerge as inhibitors of lysosomal lipid catabolism (Breiden and Sandhoff 2020). As primary storage compounds, insoluble lipids and soluble storage compounds like chondroitin 6 sulfate can trigger cascades of secondarily accumulating sphingolipids inducing complex pathologies in LSDs. In reconstitution experiments, primary storage compounds were identified as strong inhibitors of SAPs (GM2AP, SapA & SapB), which are essential for the catabolism of many GSLs. They were furthermore identified as inhibitors of specific catabolic steps in lysosomal ganglioside catabolism and cholesterol release from the late endosomal compartment. In particular, accumulating SM triggers a massive secondary accumulation of cholesterol, and SM and cholesterol together induce increasing levels of ganglioside GM2 and glucosylceramide in

Niemann-Pick disease type A, B, and C (Anheuser et al. 2019b; Oninla et al. 2014). Chondroitin-6-sulfate effectively inhibits GM2 catabolism in mucopolysaccharidoses like Hurler, Hunter, Sanfilippo and Sly syndrome and causes a secondary neuronal ganglioside GM2 accumulation, triggering neurodegeneration (Anheuser et al. 2019a). Secondary storage of simple monosialo-gangliosides, GM2 and GM3, and lactosylceramide can cause neurological forms of several mucopolysaccharidoses (Viana et al. 2020).

Accumulating metabolic modifiers within the lysosomal compartment can dysregulate cellular metabolism. These include SM, cholesterol, positively charged toxic lyso-sphingolipids (glucosylsphingosine (Sarmientos et al. 1986), galactosylsphingosine), other cationic bases like sphingosine and sphinganine and the primary storage compound chondroitin-6-sulfate in MPS, Hurler, Hunter, Sanfilippo and Sly syndrome (Anheuser et al. 2019a).

Furthermore, secondary ganglioside, GSL and lipid accumulation is known in many cellular and lysosomal storage diseases, often without known molecular basis (Grassi et al. 2019; Hornemann 2021). Secondary lysosomal storage of minor GGs and GSLs like GM2, GM3, glucosylceramide and/or lactosylceramide were observed in prosaposin deficiency, muco-lipidoses, glyco-proteinoses, neuronal ceroid lipofuscinoses and in hereditary spastic paraplegia (Breiden and Sandhoff 2020).

Juvenile neuronal ceroid lipofuscinosis is caused by mutations of the CLN3 gene. A genomic deletion in the CLN3 gene triggered a significant increase of the GG GM3 levels, apparently caused by a reduction in activity of GM2 synthase that converts GM3 to GM2 in cerebellar precursor cell lines. A significant reduction of the major  $\alpha$ -series GGs GM1a and GD1a in brain cells seems to be a consequence of the blockade of the biosynthetic pathway (Somogyi et al. 2018).

In a form of Batten disease, carrying CLN3 mutations, the levels of various soluble, lysosomal proteins, which are involved in the degradation of diverse macromolecules and sphingolipids, were also reduced. In particular, GM3 and lactosylceramide appeared to accumulate while hexosylceramides further downstream in catabolism decreased (Schmidtke et al. 2019).

A secondary but deleterious GG-accumulation can also be caused by an inhibition of lysosome membrane recycling in human hereditary spastic paraplegia 11 (SPG11) and corresponding mouse models (Boutry et al. 2018). The primary lysosomal accumulation of GG GM1 in GM1-gangliosidosis can spill out slowly, contaminate and interfere with the function of membrane contact sites, for example between lysosomes and mitochondria, leading to neuronal cell death and neurodegeneration (Annunziata et al. 2018).

Long-term treatment of patients, animals or cultured cells with cationic amphiphilic drugs (CADs) can also lead to an intralysosomal accumulation of phospholipids, GGs and GSLs. CADs are trapped in the lysosomal compartment, where they interfere with negatively charged ILVs, the major platforms of cellular degradation of complex lipids, attenuating their catabolism (Breiden and Sandhoff 2019a).

## 14 Gangliosides and Glycosphingolipids as Secondary Storage Compounds

GGs are the primary and dominant storage compounds in GM1 and GM2 gangliosidoses (Sandhoff and Harzer 2013). Small GGs like GM2 and GM3 and small GSL like GlcCer and LacCer, however, can also accumulate as secondary storage compounds in different forms of Niemann-Pick disease (Vanier 1983) and other LSDs. A review on sphingolipid metabolism and the disruption of sphingolipid homeostasis was published recently (Quinville et al. 2021). The lysosomal catabolism of small GSLs is apparently inhibited by primary storage compounds (Breiden and Sandhoff 2020), generating a secondary ganglioside, GSL and lipid accumulation. For instance in Niemann Pick type A and B diseases (NPA and NPB, respectively), caused by defects in acid sphingomyelinase, the primarily accumulating sphingomyelin (SM) strongly inhibits the sterol transfer protein NPC2 and thereby the secretion of cholesterol from the late endosomal and lysosomal compartment (Abdul-Hammed et al. 2010; Oninla et al. 2014). The combined primary and secondary storage lipids in Niemann Pick diseases, SM and cholesterol, respectively, subsequently inhibit the catabolism of GM2 (Anheuser et al. 2015), GlcCer (Abdul-Hammed et al. 2017), and the function of several SAPs, GM2AP (Anheuser et al. 2015), Sap A (Locatelli-Hoops et al. 2006) and B (Remmel et al. 2007) together, and thereby the catabolism of further GSLs (Breiden and Sandhoff 2020).

Soluble primary storage compounds like chondroitin-6-sulfate and other mucopolysaccharides can also inhibit GG-catabolism and trigger a secondary ganglioside accumulation in Hurler, Hunter, Sanfilippo and Sly syndrome by impacting for instance the interaction between GM2AP and the surface of GM2-carrying ILV-membranes (Anheuser et al. 2019a).

## 15 Accumulation of Lyso-Sphingolipids, Likely Attenuating Lysosomal SL-Catabolism

Lysosomal SL-storage is often accompanied by increasing levels of cationic lyso-sphingolipids in the endo-lysosomal compartment. Their analysis can be applied to diagnose patients with sphingolipidoses using LC-MS/MS (Polo et al. 2017). Though the pathways of their generation are poorly understood, recent evidence points to an active role of acid ceramidase in the deacylation of lysosomal glycosphingolipids, glucosylceramide in Gaucher disease and globotriaosylceramide in the case of Fabry disease, to generate lysosphingolipids (Ferraz et al. 2016). Besides other factors deregulating lysosomal functions (Hoglinger et al. 2015; Lloyd-Evans et al. 2008), accumulating lyso-sphingolipids may well have a pathological potential by attenuating lysosomal lipid degradation. It is expected that lipophilic sphingoid bases and lysolipids can easily integrate into the lipid bilayers of the ILVs and get their free amino-groups protonated in the acidic environment of the lysosomes.

Cationic lysolipids in the membranes of ILVs should reduce the negative surface charge of the SL-substrate carrying ILVs and reduce their electrostatic capability to attract protonated catabolic hydrolases. As observed for cationic amphiphilic drugs, the increasing desorption of enzymes from the substrate-carrying ILV-surfaces (Breiden and Sandhoff 2019a) should attenuate the lysosomal degradation of many SLs.

Krabbe disease is caused by an inherited deficiency of galactosylceramidase (Igisu and Suzuki 1984), which normally degrades both, the lysolipid galactosylsphingosine and galactosylceramide, the latter with the help of the lipid binding protein, saposin A (Matsuda et al. 2001). The monogenic deficiency of galactosylceramidase causes predominantly a toxic accumulation of the cationic lysolipid galactosylsphingosine (also called psychosine) probably exclusively in the myelin forming oligodendrocytes of the brain, triggering their dysfunction and death, followed by an arrest of myelination and myelin degeneration (Suzuki 2003). It was postulated, that the accumulation of toxic psychosine is responsible for the rapid loss of oligodendrocytes in Krabbe disease, which explains the initially surprising absence of galactosylceramide storage despite its blocked degradation. (Suzuki 1998).

## 16 GSL Metabolism and Parkinson Disease

The neuropathology of Parkinson disease (PD) is characterized by fibrous inclusions of Lewy-bodies in the degenerating dopaminergic neurons of the substantia nigra, which are enriched in aggregates of alpha-synuclein ( $\alpha$ -Syn).  $\alpha$ -Syn binds GG GM1 and other SLs, inhibiting fibril formation. The pathological changes lead to motor dysfunctions. Reduced GM1 levels due to a heterologous disruption of GM2/GD2 synthase in mice present an accurate PD phenotype including elevation of  $\alpha$ -Syn aggregates, motor impairment and non-motor symptoms characteristic of PD, all of which can be corrected by GM1 replacement therapy (Ledeen and Wu 2018b; Wu et al. 2020). GM1 triggers expression of neuroprotection genes in astrocyte-neuron co-cultures, a novel mechanism mediated by astrocytes, thereby exerting its metabolic and neuroprotective effects (Finsterwald et al. 2021). On the other hand, neuroinflammation in PD seems to be linked with a dysregulation of GSL metabolism, especially with an impaired lysosomal catabolism of GSLs and SLs (Abdelkarim et al. 2018; Belarbi et al. 2020; Kim et al. 2018; Nelson et al. 2018). Elevated levels of lysosomal proteins such as SAPs, Sap C and LAMP1, have also been found in Alzheimer disease mouse brains during (A beta) plaque growth (Sharoar et al. 2021). Mutations in the GBA1 gene coding for the glucocerebrosidase (GCase) are the most common genetic risk factor for the development of PD. Pharmacological inhibition of the GCase and the D409V GBA1 mutation enhance the accumulation of GSLs and insoluble phosphorylated  $\alpha$ -Syn. Accumulation of GlcCer with very long acyl chains ( $\geq$ C22) appeared to especially promote pathological  $\alpha$ -syn aggregation (Fredriksen et al. 2021). On the other hand,

inhibition of glucosylceramide synthase (GCS) by a novel inhibitor (benzoxazole 1) attenuates  $\alpha$ -Syn pathology and lysosomal dysfunction in preclinical models of synucleinopathy, suggesting that reduction of GSLs by GCS inhibition may impact progression of synucleinopathy (Cosden et al. 2021). Most PD patients show significant deficiency of GGs, including GM1, in the brain. Supplementation of GGs, such as GD3 and GM1 via intranasal infusion could reduce neurotoxic proteins and restore functional neurons via modulating chromatin status by nuclear GGs (Itokazu et al. 2021). Recent evidence has shown that Parkinson disease can be reversed also by feeding a hydrophobic isoprenoid, farnesol, to several mouse models of PD. Farnesylation of the protein PARIS has been reported to prevent the build-up of damaging reactive oxygen species and thereby the loss of dopamine-releasing neurons. Thus, farnesol treatment may turn out to be beneficial for PD patients (Jo et al. 2021).

Therapeutic approaches for lysosomal GG-storage diseases have been summarized recently (Blumenreich et al. 2021; Nicoli et al. 2021; Platt et al. 2018). It is important to know that white matter deficits, well known components of gangliosidosis pathology, are partially resistant to correction by gene therapy (Maguire and Martin 2021).

## 17 Conclusions and Perspectives

Gangliosides (GGs) and glycosphingolipids (GSLs) are especially enriched in neuronal plasma membranes. Their functional significance became more visible recently by studies of their metabolic pathways in cell cultures and animals including those affected by monogenetic diseases. As GGs, GSLs and their lipophilic metabolic intermediates are poorly soluble amphiphilic lipids, they are bound to cellular membranes. Therefore, biosynthetic and catabolic pathways occur mostly at membrane surfaces either of the secretory pathway organelles or at intraluminal vesicles, the ILVs, within endosomes and lysosomes of the catabolic pathway. Though we know only a little about the complex regulation of individual metabolic steps, it seems to be clear that the main biosynthetic and catabolic pathways can be regulated at genetic as well as posttranslational levels. Many of the known biosynthetic steps involve an interaction between membrane-bound sphingolipids (SLs) and glycosyltransferases within the plane of the lipid bilayer following a kind of two-dimensional Michaelis-Menten kinetic (Scheel et al. 1982). On the other hand, the major catabolic steps of GGs and GSLs are catalyzed by protonated hydrolases with the help of lipid binding proteins (SAPs), basically soluble cationic glycoproteins in the lumen of the lysosomes. They are – besides other still unidentified forces – electrostatically attracted to the SL-substrate carrying negatively charged ILVs to enable SL catabolism. The dominant role of electrostatic attraction for the lysosomal degradation of SLs, SM, GSL and glycerophospholipids has been summarized recently (Breiden and Sandhoff 2019a). A negative surface potential is generated on ILV-membranes by a high content of anionic phospholipids, especially of



the ILV-specific BMP and a few other anionic phospholipids, that exists even at low lysosomal pH values (Oninla et al. 2014). The negative surface potential of ILVs, however, can be strongly reduced by feeding CADs (cationic amphiphilic drugs) to cultured cells that integrate into ILV-membranes (Oninla et al. 2014). The mostly antidepressant CADs have not been identified as specific inhibitors of the ASM-catalyzed degradation of SM to ceramide, but as protonated cationic lipophilic molecules, that reduce and eventually compensate the negative surface of the ILVs in the lumen of lysosomes. This concept, often ignored in the medical literature, is supposed to be valid for the interaction of many lysosomal hydrolases with their SL- and PL-substrates carried by the ILV-membranes. We believe that terms like “functional inhibitors of acidic sphingomyelinase” disguise the major general impact of CADs on lysosomal functions and may hamper correct interpretation of scientific results. Detailed molecular studies on the molecular action of anti-depressive CADs in the brain are still missing. Whereas most glycerophospholipids can be degraded by different catabolic pathways, lysosomal SL-catabolism follows a stepwise and strictly sequential one. As a consequence, monogenetic functional defects of lysosomal SL catabolism typically trigger an accumulation of its substrates, generating a storage disease and modifying cellular metabolism. Lysosomal SL-catabolism is regulated by genetic and by posttranslational modifiers as outlined in Figs. 12.5, 12.6, and 12.7. Surprisingly, lipids of the SL-substrate carrying membranes are not only essential building blocks of their ILVs-membrane, but can also effectively stimulate SL-hydrolysis – like anionic lipids – or reduce SL-substrate turnover – like cationic sphingolipid bases. This novel concept of intra-lysosomal regulation can easily explain a secondary accumulation of GSLs and a few other lipids in LSDs. They accumulate despite the absence of any genetic defects in their catabolic pathways. An interesting illustration is the secondary accumulation of cholesterol (Chol) and several GSLs in ASM-deficient Niemann-Pick disease. This is due to the primary storage of SM, which specifically inhibits the NPC2-protein, which exports Chol from the lysosomal compartment. Both, SM and the strongly accumulating Chol, effectively trigger a cascade of secondary and tertiary accumulations by inhibiting the enzymatic hydrolysis of GlcCer and GM2 ganglioside as well as the action of GM2AP and of several other lipid binding proteins. Cascades of secondarily accumulating lipids aggravate the clinical course of the disease. Therefore, ASM appears to be a key player for the regulation of cellular homeostasis. GM2-catabolism is also inhibited by primary storage compounds in some mucopolysaccharidoses, Hurler-, Hunter-, Sanfilippo- and Sly-disease, especially by chondroitin-6-sulfate, accumulating GM2 and triggering a neurodegenerative disease. Dysregulation of the cellular lipid composition and cascades of accumulating lipids may well dysregulate cellular metabolism, which may especially affect patients with obesity, Alzheimer and Parkinson disease.

In recent years, several neuronal GSL-functions have been uncovered in the brain. GGs and GSLs are involved in metabolic, neurologic, neurodegenerative, psychiatric, and psychological features of the CNS. In many studies, however, the molecular and cellular basis of their involvement, for example in Alzheimer,

Parkinson and Frontal lobe dementia, remains unknown, which leaves important starting points for further studies.

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# Chapter 13

## Gangliosides in Neurodegenerative Diseases



Robert Ledeen and Suman Chowdhury

**Abstract** The main purpose of this chapter is to summarize the chief findings on ganglioside changes/interactions with some of the neurodegenerative disorders. For the latter we have focused on three diseases that have seen especially intensive study in that regard: Parkinson's, Alzheimer's, and Huntington's diseases. Parkinson's disease (PD) has received the most intensive study with revelation of systemic deficiency of GM1 in brain and all peripheral tissues that have been analyzed to date; this pointed to GM1 replacement as a promising therapy which proved only partially successful when tried for reasons that are discussed. Huntington's disease resembles PD in also manifesting GM1 deficiency, which did, however, respond to GM1 replacement therapy – apparently due to GM1 being administered directly into the brain. Alzheimer's disease was more complex in relation to gangliosides, with b-series (GD1b, GT1b) apparently depressed along with a-series. GM1 administered in brain appeared to induce improvement, but in a limited number of patients. We summarize studies showing why GM1 is of critical importance in neuronal function, and we also briefly point to a few additional neurological disorders in which one or more ganglioside changes have been implicated.

**Keywords** Neurodegenerative disorders · Alzheimer's disease · Parkinson's disease · Huntington's disease · Gangliosides · GM1 essentiality

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## Abbreviations

AD	Alzheimer's disease
APP	Amyloid precursor protein
BBB	Blood-brain-barrier
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CtxB	Cholera toxin B
PPMP	D-Threo phenyl-2-palmitoylamino-3-N-morpholine-1-propanol
Gal-1	Galectin-1
GD3S	GD3 synthase
GDNF	Glial cell-line-derived neurotrophic factor
GBA	Glucocerebrosidase
HPTLC	High performance thin-layer chromatography
HTT	Huntingtin
HD	Huntington's disease
mHTT	Mutated HTT
NCX	Na <sup>+</sup> /Ca <sup>2+</sup> exchanger
NGF	Nerve growth factor
NTFs	Neurotrophic factors
NURR1	Nuclear receptor related 1
PD	Parkinson's Disease
PBMC	Peripheral blood mononuclear cells
PNS	Peripheral nervous system
pRet	Phosphorylated Ret
pMAPK	Phosphorylated mitogen-activated protein kinase
PMCA	Plasma membrane Ca <sup>2+</sup> ATPase
SERCA	Sarcoplasmic endoplasmic reticulum Ca <sup>2+</sup> -ATPase
SNpc	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
VDAC1	Voltage dependent anion channel
αSyn	α-synuclein
Aβ	β-amyloid

## 1 Introduction

Gangliosides are receiving growing interest in relation to neurodegenerative disorders owing at least in part to their well-recognized modulatory roles in neuronal function and pathology. Their potential role as therapeutic agents has further enhanced this interest. How this all developed can perhaps be best understood in its historical context, beginning with Klenk's seminal discovery of their presence in the brains of patients with lysosomal storage diseases – principally Tay-Sachs and

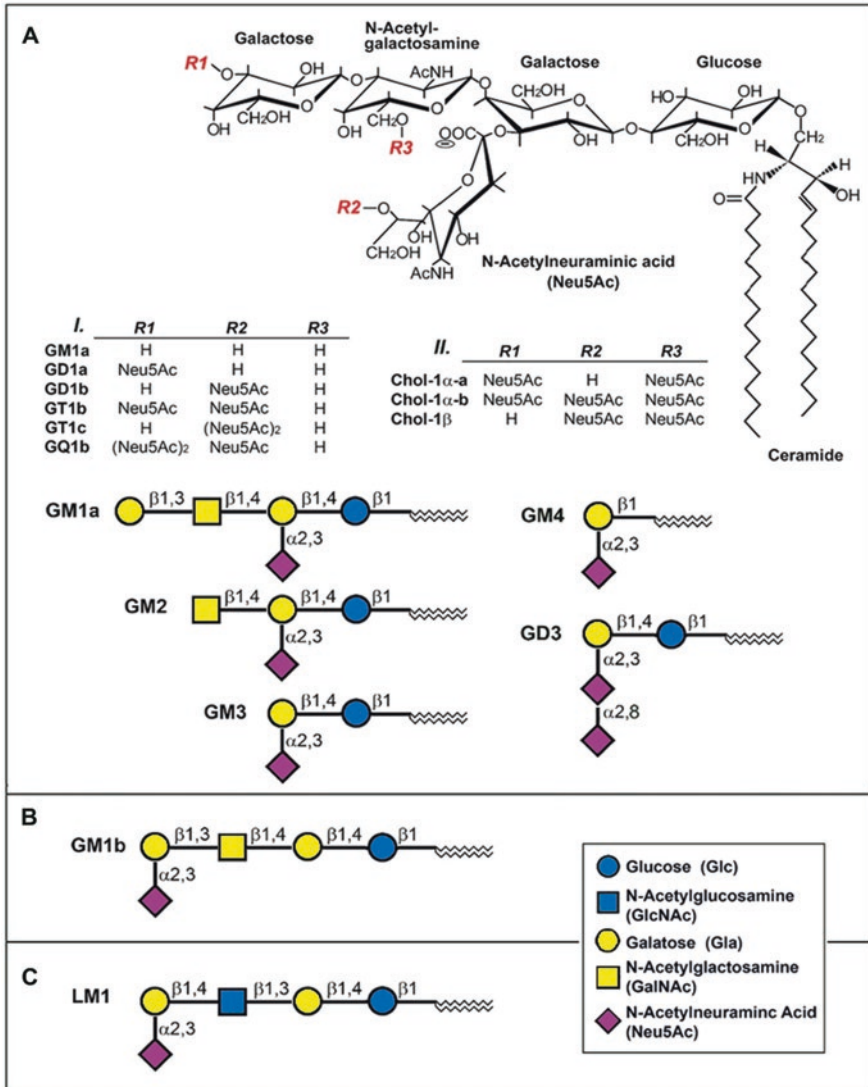
Niemann-Pick. He introduced the term “ganglioside” in relation to what was thought might be a special association with “gangliozellen” (neurons) (Klenk 1942). This proved partially appropriate in that gangliosides are most abundant in neurons, but they are now known to occur, albeit often at low levels, in virtually every vertebrate cell and body fluid subjected to careful analysis.

Klenk went on to isolate and characterize similar substances from the normal brain, thus opening the door to further study of what came to be recognized as a large group of complex glycolipids. Gangliosides are a subgroup of this now burgeoning field defined by the presence of one or more sialic acids in the oligosaccharide chain. This is the defining carbohydrate, also known as neuraminic acid, which has the pyranose conformation with carboxyl group axial to the ring and adjacent to the  $\alpha$ -ketosidic linkage (Yu and Ledeen 1969) (Fig. 13.1). Sialic acids exist in multiple substituted forms which are limited to 5-N-acetyl and 5-N-acetyl-9-O-acetyl derivatives in humans but are more varied in other species (Varki and Schauer 2009).

The structure of the major monosialo-ganglioside of the brain was determined by Kuhn and Wiegandt (Kuhn and Wiegandt 1963) and this ganglioside eventually acquired the currently recognized designation of GM1; that symbol first appeared in a landmark study by Svennerholm which demonstrated thin-layer chromatography as an effective tool for depicting the major gangliosides of brain (Svennerholm 1963). These included, in addition to GM1, the oligosialo-gangliosides GD1a, GD1b, and GT1b (Fig. 13.1). These are the major gangliosides of mammalian neurons, accompanied by a large and somewhat diverse group of minor gangliosides, depending to some extent on the neuronal type. Examples of the latter are the chol-1 $\alpha$  family specific to cholinergic neurons (Ando et al. 1992; Giuliani et al. 1990; Hirabayashi et al. 1992). These have a role in acetylcholine release from cholinergic neurons and in learning and memory (Ando et al. 2004). These specific markers of cholinergic neurons are significantly increased in the frontal lobes of Alzheimer’s patients, in contrast to substantial decline of the major neuronal gangliosides (see below). It is of course possible that additional gangliosides specific to other neurotransmitter-associated neurons will be identified in the future.

Gangliosides occur in all vertebrates but with some variation in structures, those with more sialic acids being more abundant in the lower vertebrates. Sialic acid-containing glycosphingolipids have also been found in some invertebrates but of significantly different structures from those found in vertebrates (Merrill Jr 2011). A summary of gangliosides from all vertebrate tissues lists 188 structures (Yu et al. 2007), compiled over a decade ago and possibly in need of updating. Penta, hexa, and even hepta-sialo ganglioside forms have been found in lower vertebrates. O-Series gangliosides such as GM1b, with sialic acid attached to terminal galactose (Fig. 13.1) are present to a minor degree but become elevated with suppression of the ganglio-series (Shevchuk et al. 2007). This structural diversity refers to the oligosaccharide chain exclusive of the hydrophobic ceramide unit, and in that regard, it may be noted that glycans have become recognized as efficient vehicles for information storage and transmission (viz the “Sugar Code”) with potentially greater coding capacity than the genetic code itself (Gabijs 2011). This pertains to the





**Fig. 13.1** Structures of gangliosides. (a) I. Gangliotetraose (ganglio-series) gangliosides. II. Alpha series. Lower portion shows abbreviated depictions of GM1 (GM1a) and four minor gangliosides of brain. (b) O-Series: GM1b. (c) PNS ganglioside: LM1. (Figure is adapted from Ledeen and Wu 2018a with permission granted by Springer Nature, Copyright 2018 Springer Nature)

carbohydrate identities, their arrangement in the oligosaccharide chain, and the position and conformation of attachment sites. The ceramide portion, which contains two long-chain hydrophobic units, also manifests structural diversity including the presence of a few fatty acids in addition to stearate (18:0), the predominant form in brain – all of which are joined in amide linkage to the amino group of

sphingosines. The asymmetric carbons at C2 and C3 of sphingosine have the 2S, 3R configurations (Carter et al. 1947). Mammalian brain gangliosides are unique in containing C20 sphingosine in addition to the C18 form which characterizes virtually all other sphingolipids. The C20 form is absent or very low at birth but increases progressively with development (Sonnino and Chigorno 2000). The double bond of sphingosine has the trans configuration, in contrast to the cis double bonds that characterize fatty acids in general. A minority of gangliosides lack this double bond of sphingosine. The ceramide unit is widely viewed to function as a membrane anchor for sphingolipids but has been shown to also dictate glycosphingolipid nanodomain assembly and function (Arumugam et al. 2021).

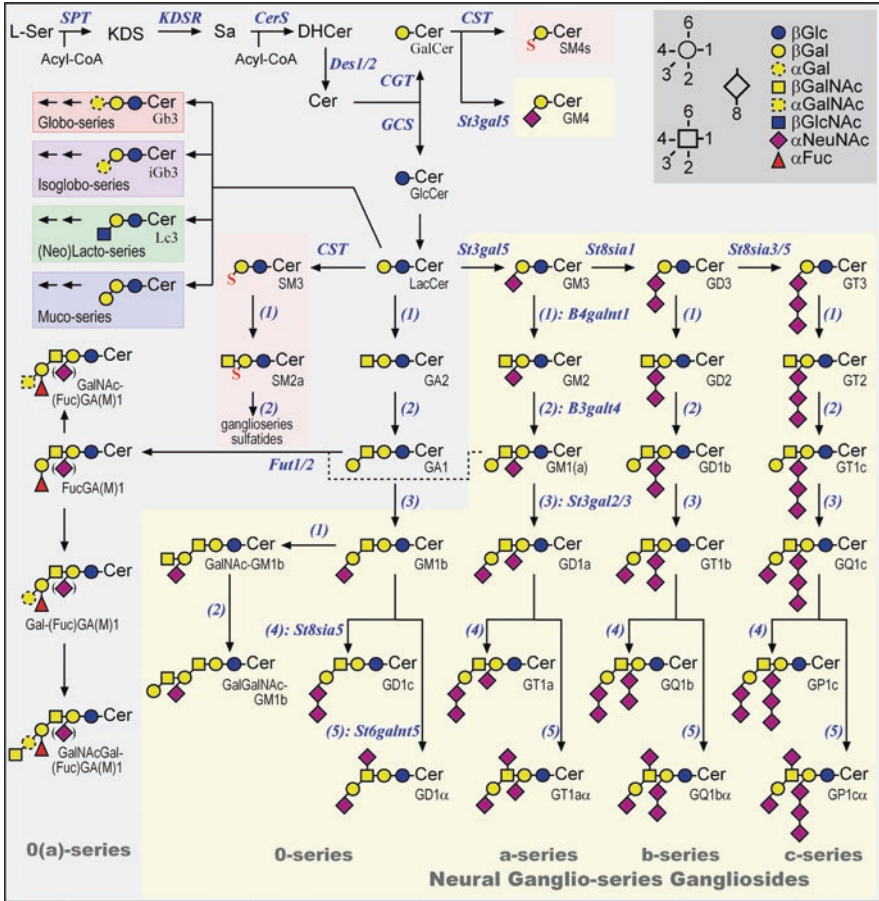
Gangliosides of peripheral neurons are less well characterized due to the common practice of analyzing whole nerves – including glia in combination with neurons. However, ganglio-series gangliosides have been detected along with high levels of GM3, GD3, and LM1 (Fig. 13.1), the latter three likely deriving from the PNS glia (Ohsawa 1990). The fact that these three are generally not encountered in nerves of the central nervous system (CNS) likely relates to the different cell types involved in myelin formation, these being Schwann cells in the peripheral nervous system (PNS) vs oligodendrocytes in the CNS. Monoclonal antibodies have been used to localize major gangliosides in the PNS – principally axons – with implications concerning selective nerve damage in various conditions (Gong et al. 2002). For more comprehensive summaries of nervous system gangliosides and their relation to neurodegenerative diseases, the reader is referred to what follows in addition to recent reviews of this subject (Ariga et al. 1998; Fazzari et al. 2022; Ledeen and Wu 2018a; Schengrund 2015; Schnaar 2016; Sipione et al. 2020).

## 2 Essentiality of a-Series Gangliosides

The essential role of a-series gangliosides in neuronal function is well-illustrated by inborn conditions resulting in the deletion of neuronal gangliosides. One such condition involves functional impairment of the *ST3GAL5* (GM3 synthase) gene, resulting in absence of GM3, GD3, and all ganglio-series gangliosides; the O-series (e.g., GM1b) are spared and possibly increased in some cases. This leads at a very early age to the infantile-onset symptomatic epilepsy syndrome together with severe refractory epilepsy and general neurological deterioration (Boccutto et al. 2014; Fragaki et al. 2013; Simpson et al. 2004). This genetic lesion is prevalent in the Amish religious community (Tell 2016) and also occurs in non-Amish patients with similar phenotypes (Heide et al. 2021; Wang and Yu 2013). Skin dyspigmentation and auditory defects occur frequently (Boccutto et al. 2014; Wang et al. 2013; Yoshikawa et al. 2015). Interestingly, none of these abnormalities, except the hearing loss, occurs in the *ST3GAL5*-null mouse (Yoshikawa et al. 2015). Additional studies indicate these pathologies can be attributed to the deletion of the a-series (see below).

A somewhat less severe condition involving hereditary spastic paraplegia occurs with loss of function of the *B4GALNT1* (GalNAc transferase) gene, responsible for the synthesis of GM2, GD2, GT2 and indirectly all downstream gangliosides; GM3 and GD3 are retained and even elevated (Bhuiyan et al. 2019; Boukhris et al. 2013; Harlalka et al. 2013; Wakil et al. 2014). In addition to spasticity of lower limbs, this condition can include cortical atrophy, peripheral neuropathy, and psychiatric disorders – depending on the nature of the genetic defect. Mutations that allow partial preservation of GalNAc transferase activity result in later onset and milder symptoms (Bhuiyan et al. 2019). *B4GALNT1*-null mice manifest several aspects of the human pathology (Chiavegatto et al. 2000; Sheikh et al. 1999; Sugiura et al. 2005; Takamiya et al. 1996; Wu et al. 2011). Such mice, devoid of ganglio-series gangliosides, showed several symptoms of Parkinson's disease (Wu et al. 2011) and interestingly, monoallelic *B4GALNT1* (+/–) mice with only partial deficiency of the ganglio-series showed similar Parkinson's Disease (PD) symptoms and were proposed as a PD model (Wu et al. 2012) (see below). These reductions in ganglioside expression are the result of alterations in the stage-specific expression of glycosyltransferase genes (Fig. 13.2), which can also be altered by epigenetic influences. An example of the latter is histone acetylation, such as that of histones H3 and H4 which were shown to promote or inhibit (in the absence of acetylation) the genes involved in the synthesis of GM2 and GD3 (Tsai and Yu 2014). Figure 13.2 illustrates the important finding of multi-substrates for single enzymes, viz. that the enzyme that synthesizes GM2 (*B4GALNT1*) also synthesizes GD2 (from GD3) and GT2 (from GT3); this was based on the landmark studies of K. Sandhoff and coworkers showing that similar patterns apply for the other members of the ganglio-series (Sandhoff and Sandhoff 2018).

Although both the above *ST3GAL5* and *B4GALNT1* mutations cause deletion of the b- and c-series as well as the a-series gangliosides it is the latter that is apparently responsible for the manifested pathologies – based on such studies as that of McDonald and coworkers (Bernardo et al. 2009) showing that elimination of GD3 synthase (and consequently all b- and c-series gangliosides) actually improved symptoms in transgenic mice – likely due to elevation of GM1. Such mice were shown to undergo normal development and experience normal life span (Kawai et al. 2001). Apparently only in regard to neural stem cells, which require GD3 interaction with the EGF receptor (Wang and Yu 2013), was pathology evident as seen in the progressive loss of such cells (Wang et al. 2014). The key role of a-series gangliosides in neuronal function calls attention to the important finding of Svennerholm and coworkers that these gangliosides (GM1 and GD1a) decrease progressively in brain with age (Svennerholm et al. 1994). This decrease was especially pronounced for GD1a (Svennerholm et al. 1989), generally considered to function as a reservoir for GM1 with the aid of Neu3 neuraminidase whose membrane location is close to that of GD1a (Miyagi and Yamaguchi 2012). GM1 appears unique among vertebrate sialo-glycoconjugates in being resistant to the more prevalent forms of neuraminidase, nature's apparent device (via GD1a and Neu3) for maintaining and increasing its availability where needed. Thus, in regard to the above genetically-induced pathologies, primary attention is directed to GM1, which in turn begs the question of why GM1 is so essential to neuronal function and viability.



**Fig. 13.2** Pathways of combinatorial ganglioside biosynthesis. (Figure is adapted from Sandhoff and Sandhoff 2018 with permission granted by author (K. Sandhoff) and FEBS letters, Copyright 2018 Federation of European Biochemical Societies)

### 3 Functional Roles of Neuronal Gangliosides: GM1 and Receptors

GM1 has been termed a “true factotum of nature” (Ledeen and Wu 2015) in view of the many neuronal functions it regulates – and fails to regulate (due to deficiency) in certain neurodegenerative diseases (Chiricozzi et al. 2020; Ledeen and Wu 2015). Effective functioning of several macromolecules, especially proteins, requires correct tertiary and quaternary structures, as seen in the neurological disorders manifesting misfolded proteins (see below). GM1 has a major role in preventing such misfolding by stereospecific association with the proteins in question, a notable example being GM1 association with  $\alpha$ -synuclein ( $\alpha$ Syn) which maintains this

protein in its helical, non-aggregating conformation – of critical importance in PD (Bartels et al. 2014; Martinez et al. 2007) (see below). An example of GM1 modulation of quaternary structure is its promoted coalescence of the Ret and GFR $\alpha$ 1 proteins that comprise the protein components of the receptor of the glial cell-line-derived neurotrophic factor (GDNF) (Hadaczek et al. 2015); GM1 is an additional and essential component of that receptor (see below). An example of charge-charge interaction – likely involving the sialic acid of GM1 – was GM1 modulation of a  $\delta$ -opioid (excitatory) receptor possessing a positively charged amino acid in a key position, but failure to do so in the absence of such amino acid charge (Wu et al. 1998). The  $\delta$ -opioid receptor in CHO cells was converted from its normal inhibitory form to excitatory mode by application of nM concentrations of GM1 (Wu et al. 1997). Similar dual responses were observed for the  $\mu$ - and  $\kappa$ - receptors. Another example of a G-protein coupled receptor modulated by GM1 is the  $\beta$ 1-adrenergic receptor in Sf9 cells which inhibits isoproterenol-induced cAMP formation (Saito et al. 1995). Ganglioside-protein interaction was well illustrated in cross-linking studies with photoactivable, radioactive GM1 derivatives having an active azide group (Mauri et al. 2003; Prioni et al. 2004).

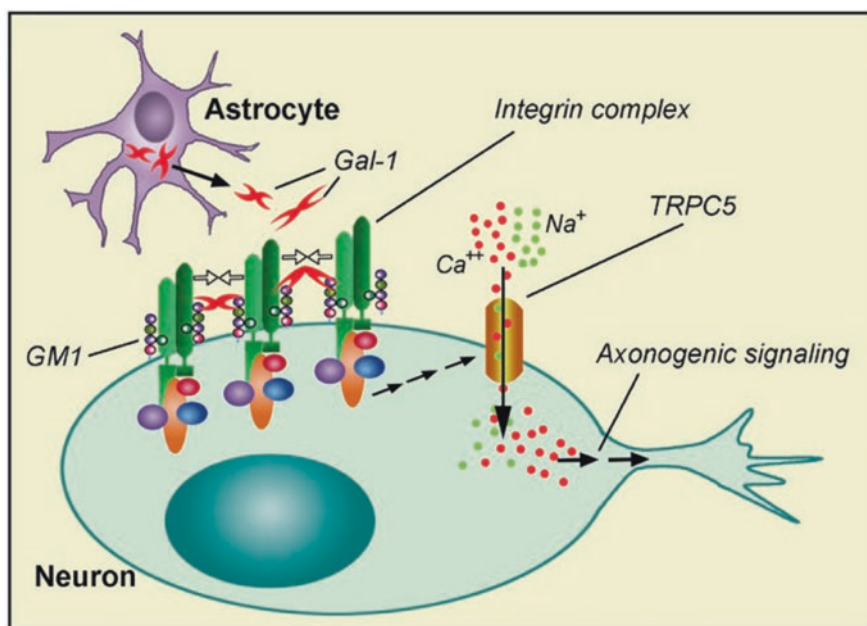
The above are examples of GM1-protein interaction in the cis-mode (occurring in the same membrane), while similar interactions occur in the trans-mode involving ganglioside promotion of cell-cell interactions (Hakomori et al. 1998; Lopez and Schnaar 2009). Gangliosides themselves can be the target of interactive proteins, as in the cross-linking of membrane-associated GM1 by extracellular Galectin-1 (Gal-1) which activates TRPC5 channels to activate Ca<sup>2+</sup> influx (Wu et al. 2016). A notable example of trans interaction among neuronal receptors functionally dependent on GM1 are the neurotrophic factors (NTFs) essential for life-long viability in addition to initial differentiation. GM1 contributes to this by high-affinity association with the NTF receptors – such as TrkA, the tyrosine kinase receptor for nerve growth factor (NGF) (Mutoh et al. 1995; Rabin and Mocchetti 1995). Such colocalization depends on glycosylation of the Trk protein (Mutoh et al. 2000). A similar high-affinity arrangement was observed for GM1 and TrkB, the receptor for brain-derived neurotrophic factor (BDNF) (Pitto et al. 1998). Significantly, TrkA expressed in cells lacking GM1 failed to appear on the cell surface, a problem remedied by stable transfection of GM1 synthase (Mutoh et al. 2002).

## 4 Functional Roles of Neuronal Gangliosides: GM1 and Ion Transport

Ion transport across lipid-rich neuronal membranes is especially important in cells manifesting excitability and involving rapid electrical signaling. GM1 especially has been studied for its prominent role in Na<sup>+</sup> and Ca<sup>2+</sup> transport. Evidence of GM1 association with Na<sup>+</sup> channels came in the observation that current densities of both

tetrodotoxin-sensitive and insensitive  $\text{Na}^+$  channels were significantly decreased by cholera toxin B subunit (CtxB – specific for GM1) (Qiao et al. 2008). Evidence also came from GM1 stabilization of paranodal junctions in myelinated nerve fibers of mice lacking GM1 (Susuki et al. 2007).

In addition to its well-known pivotal role in neurotransmission,  $\text{Ca}^{2+}$  has many other critical roles in neuronal function, several of which are mediated by GM1 through association with a variety of neuronal regulatory proteins. These control  $\text{Ca}^{2+}$  movement in both directions across the plasma membrane as well as movement between intracellular compartments (Fig. 13.3). The importance of such regulation became evident in studies with cerebellar granule neurons subjected to high (toxic) levels of glutamate (De Erausquin et al. 1990) and other cells deficient in ganglio-series gangliosides exposed to depolarizing levels of potassium (Wu et al. 2001). Calcium homeostasis and neuronal viability were restored by GM1, and or membrane-permeable analogs of GM1 (LIGA4, LIGA20) (De Erausquin et al. 1990). L-type  $\text{Ca}^{2+}$  channels situated in lipid rafts were negatively modulated by GM1 (Carlson et al. 1994). Activation of low-threshold voltage-dependent T type channels accounted for one  $\text{Ca}^{2+}$  influx mechanism (Wu et al. 1996), while another involves GM1 crosslinking by agents such as CtxB (Milani et al. 1992). The latter involves the TRPC5 channel which is normally activated by crosslinking of GM1



**Fig. 13.3** Galectin-1 (Gal-1) was shown to be an effector of axonogenesis in cerebellar granule neurons and NG108–15 cells by cross-linking GM1 ganglioside and its associated glycoprotein  $\alpha 5\beta 1$ -integrin. The resulting signaling led to a transient increase in intracellular  $\text{Ca}^{2+}$  by opening TRPC5 channels. (Figure is adapted from Wu et al., 2016 with permission granted by Journal of Neurochemistry, Copyright 2015 John Wiley and Sons)

via Gal-1 in neurons (Wu et al. 2007) and T cells (Ledeen et al. 2012; Wang et al. 2009). GM1 cross-linking, in turn induces co-crosslinking of  $\alpha 5\beta 1$  integrin heterodimer which is associated with GM1 and this induces tyrosine autophosphorylation of a focal adhesion kinase with which it is associated; this is followed by activation of phospholipase C $\gamma$  and phosphoinositide-3-kinase, all of this occurring at an early stage of neurite outgrowth (Wang et al. 2009). Calcium efflux from neuronal cytoplasm is also influenced by GM1 via plasma membrane Ca $^{2+}$  ATPase (PMCA); isoform 2 of PMCA is mainly neuronal in location and is activated by oligosialo-gangliosides (e.g., GD1b) (Burette et al. 2003; Jiang et al. 2014). GM1 also promotes Ca efflux via Na $^{+}$ /Ca $^{2+}$  exchanger (NCX); NCX showed association with GM1 in the plasma membrane though less strongly than for NCX of the nuclear membrane (Xie et al. 2002) (see below). Loss of these several regulatory functions of GM1 were proposed to account for the deleterious effects in neurons with reduced ganglio-series gangliosides (Xie et al. 2002) and likely accounts for some of the neuron loss in neurodegenerative diseases.

Gangliosides also modulate Ca $^{2+}$  flux within the neuronal cytoplasm and were proposed to have a role in the unfolded protein response – an example of the ER stress mechanism (D’Azzo et al. 2006; Tessitore et al. 2004). GM2 was reported to inhibit the transfer of Ca $^{2+}$  from the cytosol to ER via the sarcoplasmic endoplasmic reticulum Ca $^{2+}$ -ATPase (SERCA) mechanism in a mouse model of ganglioside storage disease (Pelled et al. 2003). The latter study also reported a reduced rate of Ca $^{2+}$  uptake in normal brain microsomes by applied GM2 and to a lesser extent by GM1 and GM3.

Reports on the presence of gangliosides in the nucleus, with important functions related to Ca $^{2+}$  regulation, came as a surprise to some in view of the widespread belief at that time that gangliosides were limited in location and function to the plasma membrane. However, the evidence was quite clear that both a-series gangliosides occur in the nuclear membrane and their upregulation coincides with the onset of critical Ca $^{2+}$  regulatory mechanisms in the nucleus. This Ca $^{2+}$  effect involved GM1 in high affinity association with a nuclear NCX (Xie et al. 2002). This neuroprotective effect of GM1-NCX lost function in *B4gant1*-null mice lacking GM1, which was restored by application of GM1 and even more efficiently by LIGA20, the above-mentioned membrane-permeable analog of GM1 (Wu et al. 2004). This GM1-NCX complex, located in the inner nuclear membrane, was able to mediate the transfer of nuclear Ca $^{2+}$  from nucleoplasm into the nuclear envelope and hence the ER with which it is continuous. Calcium movement by this mechanism was shown to function as an alternative to the SERCA pump for transferring cytoplasmic Ca $^{2+}$  to the ER – which depends on cytosolic Ca $^{2+}$  being substantially in equilibrium with nucleoplasmic Ca $^{2+}$  via the nuclear pores. An additional nuclear transporter, Na $^{+}$ /K $^{+}$ -ATPase (Galva et al. 2012), is involved in creating the Na $^{+}$  gradient which is the driving force for the Ca $^{2+}$ -gating mechanism. Neuraminidase occurs in both nuclear membranes together with the a-series gangliosides. The high-affinity association of GM1 with nuclear NCX was attributed to charge-charge interaction between negatively charged GM1 and positively charged amino acids in the alternative splice region of NCX (Xie et al. 2002). This interaction does not

occur in the plasma membrane NCX, thus explaining the lower affinity of that association. The nuclear GM1-NCX complex is not limited to neurons but occurs in other cell types as well (Xie et al. 2004a, b).

## 5 Parkinson's Disease and Gangliosides

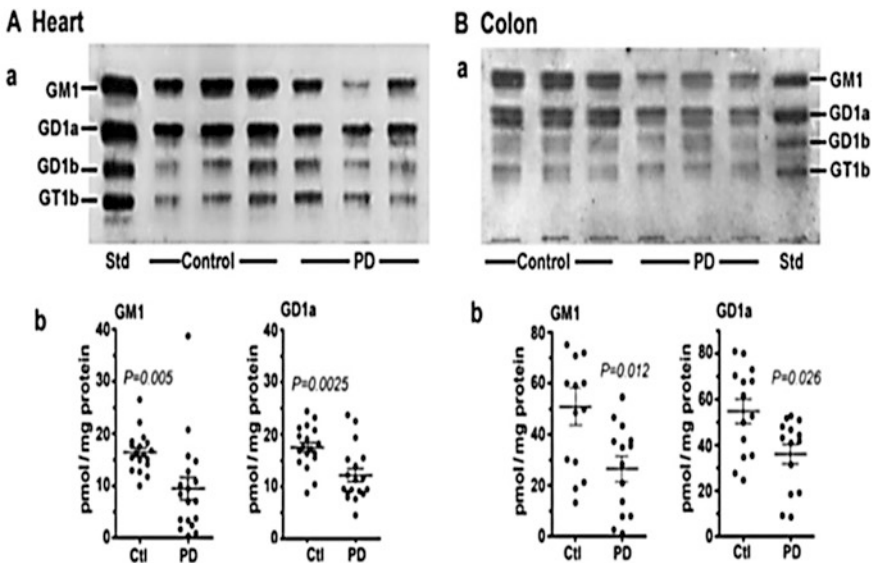
Parkinson's disease is the most common of the synucleinopathies, a group that also includes dementia with Lewy bodies and multiple system atrophy. Their principal neuropathology is the presence in neurons or glia of elevated and aggregated  $\alpha$ Syn – i.e., Lewy bodies and Lewy neurites. This is a structural protein localized to axon terminals and, to some extent, the nucleus. Ganglioside GM1 has been thought to have an important role in preventing such fibrillation (see below). Whereas gangliosides in general have received considerable attention in regard to several neurological diseases, the three neurodegenerative conditions considered in some detail below were found to be most intimately involved with gangliosides – both etiologically and therapeutically. This pertains to a-series gangliosides in particular, with a primary focus on GM1, although other ganglioside changes have also been noted. Parkinson's disease in particular has seen this perception develop over the years following its early limited consideration as merely a prototypic movement disorder. The current view of PD as a progressive, multisystem disease with both motor and non-motor dysfunctions has drawn attention to neuron loss in both the CNS and PNS stemming from the failure of key neuro-supportive functions affecting virtually all neurons throughout the body. This was featured in Braak's 6-stage hypothesis including an early prodromal phase occurring many years prior to overt motor symptoms and a final stage many years later frequently accompanied by cognitive dysfunction (Braak et al. 2003, 2006). That GM1 has a key role in the regulation of many such functions has become evident through the efforts of several researchers (Chowdhury and Ledeen 2022; Fazzari et al. 2022; Hadjiconstantinou et al. 1986; Magistretti et al. 2019; Schneider and DiStefano 1995; Schneider 2014; Yu et al. 2011), and this has occurred in tandem with growing recognition of the pathology-inducing effects of GM1 deficiency.

The critical role of GM1 deficiency became evident in studies of *B4galnt1*-null mice in which ganglio-series deficiency resulted in impaired movement and several of the key neuropathological symptoms of PD – depletion of striatal dopamine (DA), loss of tyrosine hydroxylase (TH)-positive DA neurons in the substantia nigra pars compacta (SNpc), aggregation and elevation of  $\alpha$ Syn, and reduced pRet expression in TH+ neurons (Hadaczek et al. 2015; Wu et al. 2011). Of major interest was the fact that heterozygotes *B4galnt1*(+/-) with diminished but still considerable gangliosides showed quite similar PD symptoms (Hadaczek et al. 2015; Wu et al. 2012). This finding gained significance with the concomitant findings of GM1 deficiencies in actual PD tissues – such as nigrostriatal neurons of the SNpc (Wu et al. 2012) and the occipital cortex of PD brains (Hadaczek et al. 2015).

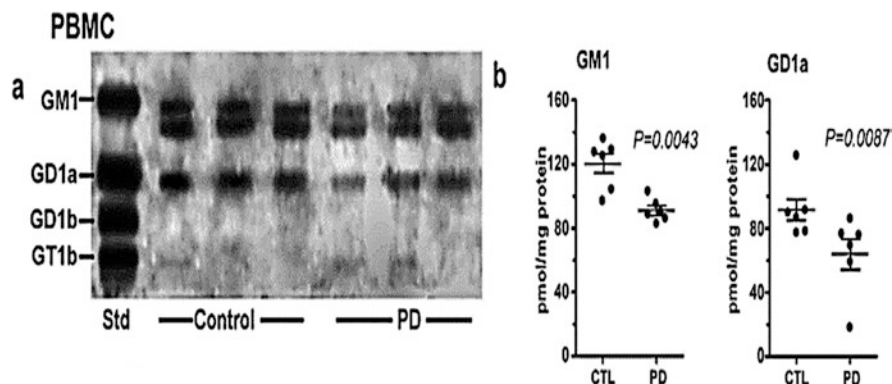


This provided incentive for similar study of other PD tissues, especially considering that a number of peripheral sites manifest PD symptoms, such as the gastrointestinal and cardiovascular systems (Braak et al. 2003, 2006). Employing high performance thin-layer chromatography (HPTLC), a simple and reliable method for quantifying individual gangliosides (Scandroglio et al. 2009; Wu and Ledeen 1988), revealed clear deficiency of a-series gangliosides (both GM1 and GD1a) in heart and colon of sporadic PD patients (Fig. 13.4) (Ledeen et al. 2022). The same study revealed similar findings for skin – often overlooked as among the peripheral tissues affected in PD despite frequent early occurrence of seborrheic dermatitis (Gregory and Miller 2015). Of considerable interest was GM1 deficiency in peripheral blood mononuclear cells (PBMC) (Fig. 13.5), white blood cells without direct neuronal involvement. GM1 deficiency was more pronounced in PBMC of PD patients with the glucocerebrosidase (GBA) malfunction and based on these findings GM1 deficiency was proposed as a potential method for early diagnosis of the two forms of PD (Alselehdar et al. 2021).

Although GM1 deficiency appears to be the operative defect in sporadic PD for the reasons outlined above, additional ganglioside changes have been observed in PD – such as decrease of GT1b (along with GD1a) in the substantia nigra of male PD patients (Seyfried et al. 2018). Another study found decreased levels of all four ganglio-series gangliosides along with an increase in glucosylceramide in



**Fig. 13.4** Gangliosides in heart and colon from PD patients and age-matched controls. (a) Heart ( $n = 18$  in each group), (b) Colon ( $n = 14$  in each group); In (a, b), subpanel a is HPTLC, and subpanel b is densitometry quantification showing the statistical difference between PD and controls, determined by Mann–Whitney rank sum U test. (Figure used is adapted from Ledeen et al. 2022 with permission granted by Glycoconjugate J., Copyright 2021 Springer)



**Fig. 13.5** Gangliosides in PBMCs from PD patients and age-matched controls. These were analyzed with HPTLC (n = 6 in each group). Subpanel **a** is HPTLC, and subpanel **b** is densitometry quantification showing the statistical difference between PD and controls, calculated by Mann-Whitney rank sum U test. (Figure is adapted from Ledeen et al. 2022 with permission granted by Glycoconjugate J., Copyright 2021 Springer)

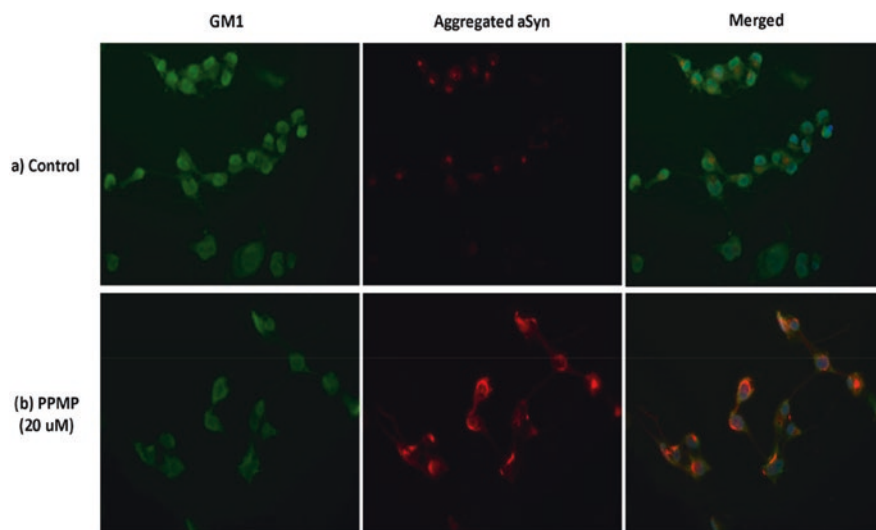
postmortem PD substantia nigra; the same four gangliosides were decreased in CSF of PD patients while only a-series were decreased in PD serum (Huebecker et al. 2019). The meaning of these additional ganglioside changes in relation to PD neuropathology is not clear.

Considering the key role of GM1 in promoting neurotrophic and neuroprotective activities of NTFs through association with NTF receptors (TrkA, TrkB), it was perhaps not surprising to find a similar role for GM1 in relation to GDNF, an NTF essential for preserving the long-term viability of catecholaminergic neurons – including DA neurons of the SNpc (Pascual et al. 2008). Subnormal GM1 impaired GDNF signaling in the SNpc of GM1-deficient mice and PD subjects, as seen in reduction of phosphorylated Ret (pRet) and phosphorylated mitogen-activated protein kinase (pMAPK), which was remedied by GM1 analog application (Hadaczek et al. 2015). Formation of the Ret/GFR $\alpha$ 1 complex, the protein components of the GDNF receptor, was diminished by subnormal GM1, the extent of this failure being proportional to the magnitude of the GM1 deficit. Significantly, GM1 proved to be an integral component of this receptor – similar to GM1 in TrkA and TrkB. The fact that the latter receptors are also dependent on GM1 association may explain the late onset of some PD symptoms – e.g., cognitive dysfunction mediated by such NTFs as NGF and BDNF – the delayed onset of which may result from the high affinity association of GM1 with these receptors (Pitto et al. 1998; Mutoh et al. 1995).

Another critical function of GM1 in relation to PD pathophysiology is its interaction with  $\alpha$ Syn, a protein localized in the nucleus and synapse whose cellular function is not entirely known. It was described as an almost entirely soluble, monomeric protein (Maroteaux and Scheller 1991) prone to aggregate under adverse conditions with the formation of Lewy bodies and Lewy neurites. Such Lewy aggregates constitute a hallmark of PD pathology (Spillantini et al. 1997) and were a major guide

to Braak in developing his six-stage hypothesis (see above). The question then arises as to what causes such aggregation in PD and prevents it in the normal state, and we have pointed to evidence suggesting that GM1 association with  $\alpha$ Syn is the mechanism holding this protein in its non-aggregating, helical conformation (Ledeen and Wu 2018b). This was based on reports showing that GM1 interacts with  $\alpha$ Syn with specificity and high affinity (Martinez et al. 2007), an association enhanced by N-terminal acetylation of  $\alpha$ Syn (Bartels et al. 2014). We have proposed this as the function of the small pool of cytosolic, soluble GM1 (Ledeen et al. 1976; Sonnino et al. 1984) which was shown to bind soluble proteins (Sonnino et al. 1984).

The gradually increasing deficiency of GM1 in this small soluble pool could conceivably lead to gradual increase in  $\alpha$ Syn fibrillation and Lewy formations. Preliminary support for this proposal was seen in  $\alpha$ Syn aggregation following D-Threo phenyl-2-palmitoylamino-3-N-morpholine-1-propanol (PPMP)-induced GM1 reduction in NG108-15 cells (Fig. 13.6) (Ledeen et al. 2022 supplement). This is supported by animal studies with the above-mentioned mice with monoallelic inactivation of the *B4galnt1*(GM2 synthase) gene in which partial reduction of GM1 resulted in brain and body-wide fibrillation of  $\alpha$ Syn; such aggregates were largely dispersed by GM1 and the LIGA20 analog (Hadaczek et al. 2015; Wu et al. 2012, 2020). Alpha-synuclein aggregates were also dispersed by GM1 application to rats with the  $\alpha$ Syn model of PD (Schneider et al. 2019). These animal results led to speculation that PD may have a similar genetic cause, although no evidence for this was found in genome-wide association studies (Bonifati 2007; Nalls et al. 2019). This was perhaps surprising in view of the in-situ hybridization study which



**Fig. 13.6** Fluorescent image analysis of double-labeled NG108-15 cells treated as described above. Note increase in aggregated  $\alpha$ Syn resulting from PPMP-induced reduction in GM1. (Figure is adapted from Ledeen et al. 2022 with permission granted by Glycoconjugate J., Copyright 2021 Springer)

revealed that neuromelanin-containing neurons of PD substantia nigra show significantly reduced expression of *B3GALT4* (GM1 synthase) and *ST3GAL2* (GD1a synthase) genes (Schneider 2018) (*B4GALNT1*– GM2 synthase was apparently not included in this study). We have speculated that the reduced GM1 expression may derive from epigenetic effects known to influence ganglioside synthesis (Itokazu et al. 2017; Tsai and Yu 2014). Also worthy of consideration is the possibility that the disease condition could cause a-series reduction considering the high susceptibility of ganglioside synthesis to negative influences.

Examples of such extraneous influences include environmental toxins (Goldman 2014) and the gut microbiota (Keshavarzian et al. 2020). The studies of Svennerholm and coworkers demonstrated that a major part of GM1 deficiency is due to the aging process itself in that GM1 and GD1a decline progressively with age (Svennerholm et al. 1989, 1994). Moreover, they found considerable variation in the level of the a-series among individuals of the same age, thus pointing to the possibility that the minority of individuals who start life with GM1 and GD1a in the low normal range could reach a point in midlife where these fall below the threshold required to maintain neuron function and viability. Considerable attention has been given to the possible influence of defective lysosomal hydrolases – such as the above-mentioned glucocerebrosidase, the most prevalent of these, which suppressed GM1 expression (Alselehdar et al. 2021); in addition, potentially damaging variants of more than 50 less prevalent lysosomal hydrolase genes have been reported in PD cases (Robak et al. 2017). It is noteworthy that the Amish community has one of the world's highest incidences of PD (Racette et al. 2009), which may relate to this population's high incidence of mutated *ST3GAL5* (GM3 synthase) (Tell 2016) with the likelihood of that causing GM1 deficiency in first degree relatives of the severely afflicted *ST3GAL5*-null children.

The many reports showing successful GM1 therapy with animal models of PD suggested the use of GM1 replacement therapy for PD patients. This idea was put to the test by Jay Schneider and coworkers who carried out a five-year open label clinical trial that showed GM1-treated PD patients suffered less movement disability after 5 years of treatment than at baseline (Schneider et al. 2010). A subsequent controlled, delayed-start phase II trial resulted in reduced movement impairment and slowing of PD symptoms over the 2-year trial period (Schneider et al. 2013). From this the investigators concluded that GM1 may have symptomatic and potentially disease-modifying effects on PD. Failure to obtain more decisive effects with GM1 might be attributed to the limited ability of GM1 to cross the blood-brain-barrier (BBB) and neuronal plasma membrane as demonstrated with fluorinated (<sup>18</sup>F)F-GM1 administered intravenously to monkeys that resulted in less than 0.5% entering the brain (Revunov et al. 2020). This problem might find solution with membrane-permeable analogs similar to those mentioned above, while another possible solution is the use of the oligosaccharide unit of GM1 (OligoGM1), devoid of ceramide, that was shown to readily cross the human BBB by a paracellular route (Di Biase et al. 2020). The latter may explain the ability of this oligosaccharide to resolve the PD symptoms of the *B4galnt1*(+/-) mouse PD model (Chiricozzi et al. 2019). Yet another possibility is to bypass the BBB, as suggested in a recent study

employing intranasal infusion of GM1, together with GD3, to A53T  $\alpha$ Syn overexpressing mice (Itokazu et al. 2021). Beneficial results included reduction of  $\alpha$ Syn aggregates and restored expression of TH in the SNpc along with the voltage dependent anion channel (VDAC1) in the outer mitochondrial membrane. Also observed was restoration of the essential nuclear transcription factor nuclear receptor related 1 (NURR1). However, while it is clear that intranasal infusion might well benefit PD pathologies originating in the brain, it is not known what therapeutic benefit this would afford to PD pathologies occurring in the periphery.

## 6 Alzheimer's Disease and Gangliosides

Alzheimer's disease (AD) pathology is characterized by intraneuronal deposition of hyperphosphorylated tau and axonal microtubule-associated protein that occurs as neurofibrillary tangles, and also  $\beta$ -amyloid ( $A\beta$ ) protein formed from amyloid precursor protein (APP) which forms extracellular plaques and has been well studied in relation to GM1 association (see below). Alzheimer's disease is the most common of the large group of tauopathies that includes progressive supranuclear palsy, corticobasal degeneration, and Down's syndrome among many others (Galpern and Lang 2006). Alzheimer's disease pathology is believed to be linked to gangliosides and ganglioside metabolism in a number of ways (Ariga et al. 2008; Grimm et al. 2012; Schengrund 2015). On the negative side, GM1 was found to dramatically increase the production of  $A\beta$ , the above-mentioned component of extracellular senile plaques in AD pathology (Selkoe 2004). This accords with cell studies showing that inhibition of glycosphingolipid synthesis markedly reduced the secretion of APP which also decreased secretion of  $A\beta$ ; these results were reversed by ganglioside addition to the cultured cells (Tamboli et al. 2005).  $A\beta$  bound to GM3 was shown to inhibit GD3 synthase (GD3S), the enzyme leading to the formation of the b-series (GD1b, GT1b). Similar inhibition of GD3S was produced by APP intracellular domain, the other cleavage product of APP (Grimm et al. 2012). Both effects would be expected to reduce the b-series, which was in fact the finding in some studies. One report showed a decrease of GD1b and GT1b in the early-onset form of AD but with little ganglioside change in the late-onset form except for mild deletion of the a-series in the temporal lobe (Svennerholm and Gottfries 1994). Another study found near normal levels of total gangliosides in the frontal cortex but some decrease of GD1b and GT1b (Brooksbank and McGovern 1989). Two studies of several AD regions reported a general decrease of the ganglio-series gangliosides with the b-series preferentially affected (Crino et al. 1989; Valdes-Gonzalez et al. 2011). Yet another study reported all ganglio-series to be depleted in the nucleus basalis and in the frontal and temporal cortex (Kracun et al. 2002). Thus, the analytical results tended to favor reduction of the b-series but were otherwise quite variable, apparently depending on the stage of AD, the region analyzed, and perhaps the method of analysis. In addition to metabolic considerations, reduction of b-series would be consistent with the well-established loss of axon terminals in AD and the

high levels of GD1b and GT1b in those structures (Avrova et al. 1973; Breckenridge et al. 1972; Svennerholm and Gottfries 1994).

To add to the confusion, the AD mouse model APP/PSEN1 mentioned above, upon deletion of GD3S and hence the b-series, showed near-complete elimination of both aggregated and unaggregated A $\beta$  along with associated neuropathology (Bernardo et al. 2009). This triple mutant also recovered from the major memory impairments shown by the double mutant. The authors suggested these several improvements might be attributed to the elevation of GM1 and GD1a, or to the removal of the b-series, especially GD3 in view of its prominent role in apoptotic pathways – such as that induced by A $\beta$  (Copani et al. 2002).

A key feature of AD is selective loss of cholinergic neurons especially in the basal forebrain (Davies and Maloney 1976), which suggested NGF involvement in AD (Iulita and Cuello 2014). However, the presence of elevated pro-NGF, the precursor to NGF, together with impaired NGF signaling presented a paradox; that was resolved in the demonstration of inhibited conversion of pro-NGF to mature NGF along with enhanced NGF degradation in AD (Iulita and Cuello 2014). Also, to be considered is the possible deficiency of GM1, demonstrated in some of the above-mentioned studies, in view of the dependence of NGF activity on GM1 association with TrkA (Mutoh et al. 1995; Rabin and Mocchetti 1995), although such deficiency was not consistently reported. In regard to selective loss of cholinergic neurons, it was of interest that AD patient sera were found to contain antibodies to GQ1b $\alpha$ , a chol-1 antigen-specific to that cell type (Ariga et al. 2013). It was also of interest that chol-1 $\alpha$  gangliosides specific to cholinergic neurons (e.g., GQ1b $\alpha$ , GT1a $\alpha$ ) increase in AD brain (Fukami et al. 2017) – perhaps a self-recovery mechanism.

A number of additional studies have linked GM1 to AD in either positive or negative mode, an example of the latter being GM1 bound to A $\beta$  acting as a proposed seed for fibrillogenesis of soluble A $\beta$  (Hayashi et al. 2004; Yanagisawa 2007). Also, GM1 associated with A $\beta$  (1-40) was found to be cytotoxic to mouse embryonic neural stem cells (Yanagisawa et al. 2010). More positively, GM1 was shown to inhibit A $\beta$ -induced release of proinflammatory cytokines IL-1 $\beta$ , IL-6, and TNF $\alpha$  (Ariga and Yu 1999) in contrast to its toxicity when associated with A $\beta$ . Another positive effect was the ability of GM1 to improve the viability of PC12 cells transfected with a mutant gene expressing APP following exposure to A $\beta$  (25-35) or hydrogen peroxide (Sokolova et al. 2007). More direct clinical evidence for GM1 therapeutic efficacy was the finding of Svennerholm and coworkers in their treatment of five patient with early onset AD who received continuous infusion of GM1 into the frontal horns of the lateral ventricles via a programmed minipump over 1 year; all five patients showed significant mental, emotional, and physical improvement (Svennerholm et al. 2002). On the other hand, an earlier clinical trial in which AD patients were administered GM1 via intramuscular injection produced no improvement in cognitive symptoms (Flicker et al. 1994). Svennerholm's results, despite the small patient size, point to the desirability of continuing clinical efforts to employ GM1 therapeutically for AD pending the availability to improved methods to surmount the BBB.

These positive benefits of GM1 are somewhat perplexing in view of the above-mentioned failure to find consistent deficiency of GM1 and GD1a in relevant AD regions, as was found in PD brain. However, it is well to realize that total tissue levels may be less meaningful than functionally available ganglioside, and in AD this might well be diminished through association with A $\beta$ . If as outlined above, an important intraneuronal function of GM1 is association of  $\alpha$ Syn, the common occurrence of  $\alpha$ Syn aggregates in AD (Galpern and Lang 2006) would suggest deficiency of functionally available GM1 in AD neurons. Such “hidden” deficiency could conceivably be corrected by GM1 replacement therapy, as might have occurred in the above-mentioned clinical study of Svennerholm et al. (Svennerholm et al. 2002). These and additional relevant topics are discussed in informative reviews on ganglioside involvement in AD (Ariga et al. 2008; Schengrund 2015; Sipione et al. 2020).

Also worthy of consideration are the ideas put forth by Galpern and Lang on the significant clinical and pathological overlap between PD, the most common synucleinopathy, and AD, the most common tauopathy (Galpern and Lang 2006). They point to the chemical analysis of Lewy bodies showing that tau and  $\alpha$ Syn often colocalize and in addition that Lewy bodies are frequently found in the brains of AD patients. It is well known that PD patients often manifest dementia and that AD patients often suffer parkinsonian movement disabilities. The disease, dementia with Lewy bodies, with symptoms of both conditions, is cited as evidence of a continuum among these diseases. Hence it may not be unreasonable to hope that discovery of a disease-altering therapy for one might in fact apply to both conditions.

## 7 Huntington’s Disease and Gangliosides

Huntington’s disease (HD) is another protein misfolding condition that is caused by an autosomal dominant monoallelic mutation in the huntingtin (HTT) gene. This results in expansion of a CAG triplet repeat which causes expansion of a polyglutamine stretch in the N-terminal region of the mutated HTT (mHTT) protein leading to aggregation as the result of misfolding (Roos 2010). Expression of mutant mHTT causes significant neuronal dysfunction and patients with this condition suffer chorea, loss of muscle coordination and finally psychiatric and cognitive dysfunctions. It was perhaps unexpected in a genetic disorder of this type to find ganglioside abnormalities such as significant GM1 deficiency similar to that found in PD (see above). Certain genes involved in ganglioside synthesis were shown to be suppressed in HD caudate nucleus, including *B4GALNT1*, *ST3GAL5*, *ST8SIA3*, and *ST3GAL2* (Desplats et al. 2007). The same study reported similar findings in the striatum of HD transgenic (R6/1) mice, consistent with deficient GM1 in the striatum. The HD patient study showed deficiency of GM1 but with high variability, while GD3 showed 162% elevation – likely reflecting suppression of the competing a- and b-series pathways. Reduction of GM1 in fibroblasts of HD patients (Maglione et al. 2010) suggested the possibility of systemic GM1 deficiency, similar to that in

PD (see above). Interestingly, GM1 was elevated in HD cerebellum, a region not generally involved in HD (Denny et al. 2010)– possibly an indication of a brain recovery effort. Additional mouse models of HD have shown GM1 and other ganglioside depletion in brain, such as the YAC128 mouse that showed down-regulation of *B3GALT4* (Maglione et al. 2010). The pathophysiological consequences of GM1 reduction in HD brain have been extensively studied by Sipione and coworkers, as seen in alleviation of motor, cognitive, and psychiatric-like symptoms along with general neurodegeneration through administration of GM1 to HD mice via chronic intraventricular infusion (Di Pardo et al. 2012). This mode of GM1 restoration was described as resulting in dramatic therapeutic and disease-modifying effects, with reversal of motor, cognitive and other symptoms with suppression of neurodegeneration and normalization of neurotransmitter levels (Alpaugh et al. 2017; Di Pardo et al. 2012). Those workers proposed that the therapeutic benefits derive at least in part from direct GM1 interaction with mHTT, as seen in GM1 reduction of soluble and aggregated mHTT in HD mice without affecting mHTT transcription or wild type HTT levels (Gu et al. 2009). The underlying mechanism was thought to possibly involve phosphorylation of mHTT at Ser13 and Ser16, shown to be associated with reduction in mHTT aggregation (Gu et al. 2009).

## 8 Gangliosides and Additional Neurological Disorders

As mentioned, the above three neurodegenerative conditions have received intensive studies by several group in regard to ganglioside involvement, but that does not exhaust the list of neurological conditions that have been shown to involve ganglioside changes. For example, amyotrophic lateral sclerosis was found to express significantly elevated GM1 and GM3 in the spinal cord, suggesting possible impairment of ganglioside catabolism (Dodge et al. 2015). Patients with West syndrome, a genetic infantile epilepsy disorder, showed diminished levels of GM1 and GD1a in the CSF (Izumi et al. 1993). The influence of stroke on gangliosides has been examined in a number of animal models, such as middle cerebral artery occlusion, which showed decrease of the complex gangliosides; the latter together with elevation of GM2 and GM3 was potentially attributed to catabolism of the complex gangliosides in the lysosome (Whitehead et al. 2011). Large clinical trials with stroke patients, in which GM1 was administered peripherally, showed only modest, if any, improvement (Alter et al. 1994; Argentino et al. 1989; Lenzi et al. 1994). Finally, patients with acute traumatic spinal cord injury showed significantly faster recovery than controls when treated intravenously with GM1, although the final extent of recovery was not different from controls (Geisler et al. 2001). For more detailed discussion of these additional neurological disorders in relation to gangliosides, see (Sipione et al. 2020). All in all, there is evidence to indicate a variety of ganglioside involvements in neurodegenerative disorders with promise of GM1 therapeutic benefit, especially those characterized by GM1 deficiency, once satisfactory means are



found to overcome (or bypass) the hindrances afforded by the BBB and neuronal plasma membrane.

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# Chapter 14

## Functional Impairment of the Nervous System with Glycolipid Deficiencies



Yutaka Itokazu, Takahiro Fuchigami, and Robert K. Yu

**Abstract** Patients with nervous system disorders suffer from impaired cognitive, sensory and motor functions that greatly inconvenience their daily life and usually burdens their family and society. It is difficult to achieve functional recovery for the damaged central nervous system (CNS) because of its limited ability to regenerate. Glycosphingolipids (GSLs) are abundant in the CNS and are known to play essential roles in cell-cell recognition, adhesion, signal transduction, and cellular migration, that are crucial in all phases of neurogenesis. Despite intense investigation of CNS regeneration, the roles of GSLs in neural regeneration remain unclear. Here we focus on the respective potentials of glycolipids to promote regeneration and repair of the CNS. Mice lacking glucosylceramide, lactosylceramide or gangliosides show lethal phenotypes. More importantly, patients with ganglioside deficiencies exhibit severe clinical phenotypes. Further, neurodegenerative diseases and mental health disorders are associated with altered GSL expression. Accumulating studies demonstrate that GSLs not only delimit physical regions but also play central roles in the maintenance of the biological functions of neurons and glia. We anticipate that the ability of GSLs to modulate behavior of a variety of molecules will enable them to ameliorate biochemical and neurobiological defects in patients. The use of GSLs to treat such defects in the human CNS will be a paradigm-shift in approach since GSL-replacement therapy has not yet been achieved in this manner clinically.

**Keywords** Carbohydrate · Ganglioside · Glycoconjugate · Glycolipid · Glycosphingolipid · Regeneration · Neural development · Neural stem cell · Neurodegenerative disease · Neurogenesis · Neurological disorder · Mental health disorder

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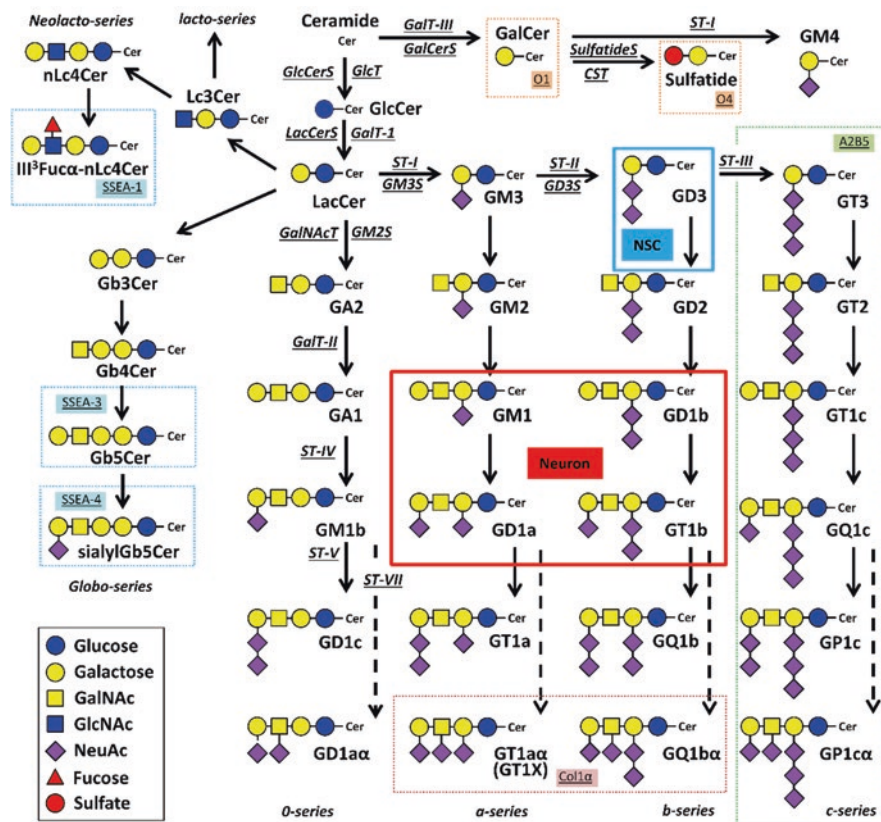
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## Abbreviations

AD	Alzheimer's disease
BrdU	Bromodeoxyuridine
Cer	Ceramide
CNS	Central nervous system
DG	Dentate gyrus
GalCer	Galactosylceramide
GalNAc	<i>N</i> -acetylgalactosamine
GlcCer	Glucosylceramide
GSL	Glycosphingolipid
LacCer	Lactosylceramide
NSC	Neural stem cell
PD	Parkinson's disease
PNS	Peripheral nervous system
SGZ	Subgranular zone
ST	Sialyltransferase
SVZ	Subventricular zone

## 1 Introduction

In the nervous system of vertebrates, lipids are the most abundant organic compounds, and a variety of lipids control the biophysical nature of lipid membranes. Cells and most subcellular organelles are surrounded by biological lipid membranes that define the individual cellular shape and help maintain cellular organization. It was hypothesized that distinct lipid microdomains enriched in glycosphingolipids (GSLs) provide functional platforms and modulate protein activities associated with the biological membrane (Hakomori et al. 1998; Simons and Ikonen 1997). GSL microdomains are considered to be multiple signaling centers, cellular protein sorting machines, and important in membrane trafficking pathways. Biological membranes are highly heterogenous in structure with diverse microdomains which are enriched with gangliosides, a group of GSLs containing sialic acid. Different microdomains regulate protein activities and cellular functions in stage-, cell-, and cellular site-specific manners. Those microdomains regulate growth factor signaling, immune signaling and immune checkpoints, and cell-cell interactions including cell adhesion and migration. A detailed discussion about ganglioside microdomains and neuronal cell fate can be found in Chap. 10 by Itokazu and Yu. GSLs are unique amphipathic molecules containing a hydrophilic carbohydrate portion and a hydrophobic ceramide. Lipid portions are in the membrane and their carbohydrate heads are on surface of the membrane. Glycosylation of ceramide with glucose forms Glucosylceramide (GlcCer) (Fig. 14.1). Further glycosylations make more complex GSLs, including gangliosides. Ganglioside synthesis pathways (Fig. 14.1) were



**Fig. 14.1** Metabolic pathways and structure of glycosphingolipids (GSLs), including gangliosides. The nomenclature for gangliosides and their components are based on that of Svennerholm and the IUPAC–IUBMB Joint Commission on Biochemical Nomenclature (1977; Svennerholm 1963). *Cer* ceramide, *CST* cerebroside sulfotransferase (*Gal3st1*, sulfatide synthase), *GalNAc-T* *N*-acetylgalactosaminyltransferase I (*B4galnt1*, GA2/GM2/GD2/GT2 synthase), *GalT-I* galactosyltransferase I (*B4galT5* & *B4galT6*, lactosylceramide synthase), *GalT-II* galactosyltransferase II (*B3galT4*, GA1/GM1/GD1b/GT1c synthase), *GalT-III* galactosyltransferase III (*Ugt8a*, galactosylceramide synthase), *GlcT* glucosyltransferase (*Ugcg*, glucosylceramide synthase), *ST-I* sialyltransferase I (*St3gal5*, GM3 synthase), *ST-II* sialyltransferase II (*St8Sial1*, GD3 synthase), *ST-III* sialyltransferase III (*St8Sia3*, GT3 synthase), *ST-IV* sialyltransferase IV (*St3gal2*, GM1b/GD1a/GT1b/GQ1c synthase), *ST-V* sialyltransferase V (*St8sia5*, GD1c/GT1a/GQ1b/GP1c synthase), *ST-VII* sialyltransferase VII (*St6galnac6*, GD1α/GT1α/GQ1bα/GP1cα-synthase). Official symbols of genes are represented in italics in this figure legend

initially described by Yu and Ando (1980) following their structural characterization of c-series trisialogangliosides. During neural development, changes in expression patterns of GSLs in the nervous system correlate with neurodevelopmental processes (Yu and Itokazu 2014). For instance, the globo-series of GSLs are robustly expressed in fertilized eggs. As cell division proceeds, neolacto-series GSLs are expressed, followed by the ganglio-series of GSLs in the developing brain. In early

embryonic rodent brains, GM3 and GD3 are the predominant GSLs. Then, b-series gangliosides, such as GD1b, GT1b, and GQ1b, start to increase, followed by increases in a-series gangliosides such as GM1, GD1a, and GT1a, while expression of GM3 and GD3 markedly decreases during development. In the adult brain, GM1, GD1a, GD1b, and GT1b became major GSL components. GD3 synthase (GD3S) activity (GM3  $\Rightarrow$  GD3) decreased from late embryonic to adult brains (Yu et al. 1988). In contrast GM2 synthase (GM2S) activity (GM3  $\Rightarrow$  GM2) increased at mid-embryonic development to adult. These changes indicate that ganglioside “pathway switch” from GD3 to the enzymatically catalyzed synthesis of complex gangliosides (including GM1) correlates with their functional roles in determining neural cell fate.

Patients with GM3-synthase (GM3S) mutations have symptomatic epilepsy syndrome with severe neuronal dysfunction (Fragaki et al. 2013; Simpson et al. 2004). Patients have marked increases of the immediate precursor of GM3, lactosylceramide (LacCer), and an increased accumulation of the globosides Gb3 and Gb4, in their plasma and fibroblasts. Mutation of the GM2S gene was found in patients with hereditary spastic paraplegias with additional neurological symptoms (Boukhris et al. 2013; Harlalka et al. 2013). Fibroblasts from affected patients have increases in the levels of GM3, LacCer, and Gb4 when compared with control subjects. Interestingly, the patients have sialylated Gb3 that is not expressed in unaffected individuals. Since patients with defects that affect their ganglioside biosynthetic enzymes exhibit devastating disorders, development of effective therapies is needed to restore ganglioside-dependent neuronal structure and functions.

Because, a majority of patients with neurodegenerative diseases have alterations in their GSL metabolism, those changes may contribute to their accompanying pathogenesis (Ariga 2014). Concentrations of complex gangliosides such as GM1 are significantly lower in the brains of patients with Alzheimer’s disease (AD) than healthy subjects (Ariga 2017). Most patients with Parkinson’s disease (PD) show significant deficiency of complex gangliosides, including GM1, in the brain (Seyfried et al. 2018; Wu et al. 2012). In Huntington’s disease (HD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS), alterations in GSL expression are observed in the central nervous system (CNS), primarily in the form of decreased complex ganglioside levels (Ariga 2014; Yu et al. 1982). The importance of complex gangliosides in the prevention of neurodegenerative diseases has been shown in animal models. In APP/PSEN1/GD3S-KO mice, increased GM1 (163.8%) may prevent AD development (Bernardo et al. 2009). Importantly, amyloid-beta peptides (A $\beta$ s) could not bind to the cells of GD3S-KO mice which have a significantly increased concentration of GM1, and no A $\beta$ -associated neuropathology was found. In contrast, APP<sup>SL</sup>/GM2S-KO mouse brains, which are deficient in GM1, were found to have significantly increased A $\beta$  levels and senile plaques (Oikawa et al. 2009). Furthermore, GM2S-KO mice exhibit impaired movement and have virtually all the neuropathological symptoms of PD (Wu et al. 2011, 2020). In this chapter, we will introduce these deficiency of GSLs in neurological disorders and describe possible therapeutic strategies. Phenotypes causing deficiencies of GSLs in the mouse nervous system are summarized in Table 14.1.

**Table 14.1** Phenotypes/symptoms of GSL synthase-KO mice

Disrupted Synthase		Gene	Lost GSLs	Increased GSL	GSL expression	Phenotypes of the nervous system	Reference
GlcCerS		<i>Ugcg</i>	Major GSLs except GalCer-based GSLs		GalCer, GM4, Sulfatide	Embryonic lethal; cKO (nestin-Cre) died at P11–24	Yamashita et al. (1999) Jennemann et al. (2005)
LacCerS		<i>B4galT5</i> <i>B4galT6</i>	LacCer, gangliosides	GlcCer	GlcCer, GalCer, Sulfatide, GM4	Embryonic lethal; cKO (nestin-Cre) died by P30	Kumagai et al. (2009) Yoshihara et al. (2018)
GalCer		<i>Ugt8</i>	GalCer, GM4 sulfatide	GlcCer	GlcCer-based GSLs such as gangliosides	Some animals died (P8–30), progressive loss of function in the hindlimbs (P60); Died before myelination (P30), smaller size, whole body tremor, loss of locomotor activity	Coetzee et al. (1996) Bosio et al. (1996)
SulfatideS		<i>Gal3st1</i>	Sulfatide		Except for sulfatide	Hindlimb weakness, tremor, progressive ataxia	Honke et al. (2002)
GM3S		<i>Sf3gal5</i>	a-, b-, c-series gangliosides, GM4	GM1b, GD1 $\alpha$ , Sulfatide	0-series, GlcCer, LacCer, GalCer, Sulfatide	Enhanced insulin sensitivity, reduced development of insulin resistance; Attention-deficit hyperactivity disorder (ADHD); Enhancement of osteoarthritis with aging; Healing loss, abnormal auditory structure and function; Hyperactive, susceptible to seizures, seizure-induced aberrant neurogenesis; Autism spectrum disorder, dysregulated inflammation;	Yamashita et al. (2003) Niimi et al. (2011) Sasazawa et al. (2014) Yoshikawa et al. (2015) Tang et al. (2020a) Strekalova et al. (2021)

(continued)

**Table 14.1** (continued)

Disrupted		Gene	Lost GSLs	Increased GSL	GSL expression	Phenotypes of the nervous system	Reference
Synthase							
GD3S		<i>Sf8xial</i>	b-, c-series gangliosides	GM1, GD1a, GM2, GM3	0-, a-series, GlcCer, LacCer, GalCer, Sulfatide, GM4	<p>Impaired regenerative capacity of damaged hypoglossal nerves;</p> <p>Thermal hyperalgesia and mechanical allodynia;</p> <p>Functional impairment of EGF induced neural stem cell (NSC) self-renewal;</p> <p>Reduced NSC pools at SVZ &amp; DG, impaired neurogenesis at OB &amp; DG;</p> <p>Morphological abnormalities, impaired regenerative capacity of sciatic nerve;</p> <p>Lesser bone loss with aging (10 month old);</p> <p>Reduced visual acuity and contrast sensitivity</p>	<p>Okada et al. (2002)</p> <p>Handa et al. (2005)</p> <p>Wang and Yu (2013)</p> <p>Wang et al. (2014)</p> <p>Ribeiro-Resende et al. (2014)</p> <p>Yo et al. (2019)</p> <p>Abreu et al. (2021)</p>
GM2S		<i>B4galnt1</i>	Complex gangliosides including GM1	GD3, GM3	GM3, GD3, GT3, GlcCer, LacCer, GalCer, Sulfatide, GM4	<p>Reduction in the neural conduction velocity;</p> <p>Sterility &amp; aspermatogenic of male;</p> <p>Demyelination, axonal degeneration, and reduced expression of myelin-associated glycoprotein (MAG);</p> <p>Motor deficits, gradual abnormalities with aging;</p> <p>Dysregulated Ca(2+) homeostasis in cultured granule neurons;</p> <p>Parkinsonian pathology: loss of dopaminergic neurons in the substantia nigra pars compacta;</p> <p>Declined GDNF signaling;</p> <p>Urinary and bladder dysfunction;</p> <p>Disrupted membrane microdomains and dispersed prion proteins in the brain</p>	<p>Takamiya et al. (1996)</p> <p>Takamiya et al. (1998)</p> <p>Sheikh et al. (1999)</p> <p>Chiavegatto et al. (2000)</p> <p>Wu et al. (2001, 2004)</p> <p>Wu et al. (2011, 2012)</p> <p>Hadaczek et al. (2015)</p> <p>Gil-Tomme et al. (2019)</p> <p>Kobayashi et al. (2019)</p>

Table 14.1 (continued)

Disrupted		Lost GSLs	Increased GSL	GSL expression	Phenotypes of the nervous system	Reference
Synthase	Gene					
GD3S/GM2S(DKO)		All gangliosides other than GM3	GM3	GM3, GlcCer, LacCer, GalCer, sulfatide, GM4	Phenotypes of the nervous system 50% of KO mice died by 13 weeks of age, a sudden death, lethal seizures by sound, male sterility; Peripheral nerve degeneration, skin lesion primarily on the face; Smaller brain, memory & learning deterioration, progressively motor & sensory dysfunctions with aging; Upregulated inflammatory cytokine genes similar to AD, disrupted microdomain, increased glial numbers	Kawai et al. (2001) Inoue et al. (2002) Tajima et al. (2009) Ohmi et al. (2009, 2011)
GM3S/GM2S(DKO)		All gangliosides	LacCer, SM3	LacCer, GalCer-derivative	Severe neurodegenerative disease leads to death soon after weaning	Yamashita et al. (2005)



## 2 GlcCer Synthase-KO Mouse

GlcCer synthase (GlcCerS, uridine diphosphate (UDP)-glucose:ceramide glucosyl-transferase; *Ugcg*, GlcT-1, EC2.4.1.80), catalyzes the first glycosylation step in glycosphingolipid synthesis, and was originally cloned by Hirabayashi's group (Ichikawa et al. 1996). GlcCerS-knockout (KO) mice die between embryonic day 6.5 and 9.5 (E6.5–E9.5) (Yamashita et al. 1999). Conditional KO mice having a floxed *GlcCerS* gene in combination with the Nestin gene promoter-driven Cre transgene was developed (Jennemann et al. 2005). In these mice, gangliosides were deleted at stage E15.5 by Nestin-promoted GlcCerS gene deletion in neuronal cells. Central nervous system specific KO mice of GlcCerS died from postnatal day 11–24 (P11–P24). Soon after birth, the conditional GlcCerS-KO mice displayed dysfunction of cerebellum and peripheral nerves. Significant loss of axon branching and diminished dendritic complexity were observed. It was found that myelin sheaths were broadened and focally severely disorganized. Deficiency of GSL in conditional GlcCerS-KO brains caused alterations in expression of numerous mRNAs needed for brain development and neuronal homeostasis. These results demonstrated that importance of GSLs not only on membrane properties but also on regulating gene expression for brain development and neuronal differentiation. Cerebellar Purkinje cell-specific GlcCerS-KO mice were bred by crossing L7 gene promoter-driven Cre transgenic mice with GlcCerS-flanked mice (Watanabe et al. 2010).

This conditional GlcCerS-KO mouse line showed numerous axonal swellings, dystrophic morphology of axons, and subsequent neuronal degeneration. In the swollen axons, accumulations of mitochondria, synaptic vesicle proteins, and microtubule-associated motor proteins, were seen. Thus, axonal transport deficits are caused by GlcCer deficiency. Further, loss of GlcCerS disrupted myelin sheaths by causing formation of doubly myelinated axons, axons enveloped by an additional concentric myelin sheath. The excessive paranodal loops and doubly-myelinated sheaths were shaped preceding Purkinje cell loss. On the other hand, mice having a specific absence of GlcCerS in oligodendrocytes, made by crossing mice carrying a gene encoding the myelin-associated enzyme 2', 3' cyclic nucleotide 3' phosphodiesterase (CNP) in combination with a gene promoter-driven Cre and GlcCerS-flanked mice, revealed no phenotype in the CNS (Saadat et al. 2010). Based on these observations, neuronal GlcCer and derivative GSLs are suggested to be necessary for regulating axonal maintenance and stabilizing myelin sheath formation.

### 3 LacCer Synthase-KO Mouse

LacCer is a common precursor and is required for the synthesis of all GSLs of GlcCer derivatives. LacCer is a key intermediate for a majority of GSLs including the ganglioside-, globo-, isoglobo-, lacto-, and neolacto-GSLs. GlcCer is synthesized by GlcCerS by transfer of glucose from UDP-glucose to ceramide, and then LacCer is synthesized by LacCer synthase (LacCerS) catalyzing the transfer of galactose from UDP-galactose to GlcCer. LacCerS is mainly synthesized by beta-1,4-galactosyltransferase 5 (*B4galt5*, EC 2.4.1.274) during early mouse embryogenesis (Kumagai et al. 2009). The *B4galt5*-KO mice die by E10.5 and their growth is delayed by one to 1.5 days from E7.5 (Kumagai et al. 2009; Nishie et al. 2010). The embryonic lethality seen in the  $\beta$ 4GalT5-KO mice is similar to that seen in the GlcCerS-KO mice. Although LacCerS activities are 38% and 52% in *B4galt5* conditional KO (Nestin-Cre) and in *B4galt6* KO mice respectively, LacCerS activity were completely absent in brain of the conditional *B4galt5* and 6 double KO (D-cKO) (Yoshihara et al. 2018). The D-cKO mice revealed growth retardation and motor deficits at 2 weeks and the mice died by the time they were 1-month-old. Axon-oligodendroglial communication was severely impaired in the CNS of the D-cKO mice.

GSLs, including gangliosides, are known to modulate cell proliferation, cell adhesion, cell migration, and cell signal transduction (Todeschini et al. 2007; Yu and Itokazu 2014), and GSLs clustered at cell surfaces interact with transmembrane proteins such as integrins to facilitate cell-cell and cell-extracellular matrix adhesions. For example, focal adhesion kinase (FAK) is a key cytoplasmic tyrosine kinase and important mediator of intracellular signaling by integrins. Intriguingly, the consequence of deletion of FAK signaling is similar to that seen with GSL-KO mice as FAK-KO mice die at about E8.5–10 (Ilic et al. 1995). GlcCerS-KO and LacCerS-KO mice also die at this stage during embryogenesis. GSLs provide specific microdomains needed for essential functions during both embryonic development as well as postnatal life.

### 4 GalCer Synthase-KO Mouse and Sulfatide Synthase-KO Mouse

Galactosylceramide (GalCer) is synthesized by addition of galactose to ceramide with UDP-galactose as the donor in a reaction catalyzed by the enzyme UDP-galactose:ceramide galactosyltransferase (CGT, GalCer synthase; GalCerS, *Ugt8*, EC2.4.2.62). GalCerS-KO mice did not have GalCer, or either its sialylated derivative, GM4, or sulfated derivative, sulfatide, but did have GlcCer in their myelin (Coetzee et al. 1996). These KO mice showed severe generalized tremor, mild ataxia, and conduction deficits induced by reduced insulative capability of the myelin sheath. GalCerS-KO mice developed age-related progressive hind limb

paralysis and extensive myelin vacuolation of the ventral region of the spinal cord. The growth of GalCerS-KO mice is retarded and most of them died before the end of myelinogenesis (P25-P30) (Bosio et al. 1996). After increasing loss of locomotor activity and development of a very conspicuous gait pattern, KO mice develop a whole body tremor. In an open field test GalCerS-KO mice showed minimal activity. Those reports suggest that lack of membrane-stabilizing GalCer and its derivatives altered membrane structure on the molecular level in both the CNS and the peripheral nervous system (PNS). GalCer is the major component of the myelin membrane that is a unique plasma membrane of oligodendrocytes found in the CNS and Schwann cells in PNS and is essential to facilitate saltatory nerve conduction. Oligodendrocyte-specific overexpression of GalCerS induced by proteolipid protein (PLP) promoter, completely restored the multiple phenotypes seen in the GalCerS-KO mice (Zoller et al. 2005). These mice had no change in the total amount of GalCer in their brains supporting the conclusion that specific loss of GalCerS in oligodendrocytes is responsible for the myelin structural and functional deficits seen in mice with a GalCer deficiency.

Sulfatide (sulfogalactosylceramide), a major sulfoglycolipid in the brain, is a major lipid component of the myelin sheath and is synthesized in both oligodendrocytes in the CNS as well as Schwann cells in the PNS. Sulfatide synthase (SulfatideS, 3'-phosphoadenosine-5'-phosphosulfate-cerebroside sulfotransferase, cerebroside sulfotransferase, CST, *Gal3st1*, EC 2.8.2.11) adds sulfate to GalCer. SulfatideS-KO mice that lack sulfatides, display hindlimb weakness before 6 weeks of age and subsequently developed an apparent tremor and progressive ataxia (Honke et al. 2002). SulfatideS-KO mice had milder phenotypes than those of GalCerS-KO mice, though SulfatideS-KO mice displayed abnormalities in paranodal junctions and males were infertile. During oligodendrocyte development, O4 (sulfatide; HSO3-3Gal $\beta$ 1-1'Cer) and O1 antigens (galactosylceramide; GalCer; Gal $\beta$ 1-1'Cer), have been utilized as specific markers to define immature and mature oligodendrocytes, respectively. The O1 and O4 antigens play important roles as modulators of oligodendrocyte development and function as well as major components of the myelin sheath to facilitate nerve conduction. Interestingly, the number of oligodendrocytes is increased in sulfatideS-KO mice, indicating that the O4 antigen, sulfatide, is a critical molecule for the negative regulation of terminal differentiation of oligodendrocytes (Hirahara et al. 2004).

## 5 GM3 Synthase-KO Mouse

In early embryonic CNS, the pattern of ganglioside expression is characterized by the expression of a large amount of simple gangliosides, such as GM3 and GD3. In the later developmental stages, more complex gangliosides prevail, particularly the gangliotetraose gangliosides GM1, GD1a, GD1b, and GT1b, which account for more than 90% of total gangliosides (Yu et al. 2009). GM3S (sialyltransferase I, ST-I, CMP-sialic acid:lactosylceramide alpha2,3-sialyltransferase; *St3gal5*, EC

2.4.99.9) is a critical enzyme for the synthesis of all complex gangliosides. GM3 is a precursor molecule in the synthesis of a-, b-, and c- series gangliosides. ST-1 also catalyzes the synthesis of GM4 from GalCer. GM3S-KO mice are unable to synthesize GM3 ganglioside, other GM3 derived gangliosides (Yamashita et al. 2003), and GM4 (Chisada et al. 2009). The KO-mice have GM1b and GD1 $\alpha$  as major gangliosides in the brain. Hearing ability were absent in GM3S-KO mice and completely lost by P17 of age, showing a deformity in hair cells in the organ of Corti (Yoshikawa et al. 2009). Abnormal distribution and trafficking of stereocilia and kinocilia were observed in cochlear hair cells in GM3S KO mice, suggesting the essential role of GM3 for the proper function of auditory hair cells (Yoshikawa et al. 2015). In the visual system, thinner retina in GM3-KO mouse is observed, although neither histopathological abnormalities in the retina nor abnormal optic nerve functions were found in the KO mice (Hiraoka et al. 2019).

GM3S-KO mice display attention-deficit hyperactivity disorder (ADHD), which includes hyperactivity, reduced anxiety, lower attention, and increased impulsive behaviors (Niimi et al. 2011). Although both male and female GM3S-KO mice showed increased activity and enhanced methylphenidate hydrochloride sensitivity, GM3S-KO male mice had more severe inattention and impulsivity behaviors. GM3S-KO mice have substantial abnormalities in dominant behavior and neutral sociability. GM3S-KO mice also displayed impaired inhibitory learning, anxiety-like and stereotyped behaviors, abnormal social interaction, moderate motor deficits, systemic inflammation, and signs of hypomyelination (Strekalova et al. 2021). These phenotypes in GM3S-KO mice reflect an autism spectrum disorder (ASD)-like syndrome. Molecular analysis showed decreased expression of myelin protein proteolipid protein-1 (Plp1) and abnormal expression of proinflammatory cytokines in the KO-mice. This study suggested that a deficiency in a-, b- and c-series gangliosides in the brain correlates with abnormalities seen in ASD.

To investigate how a deficiency of GM3 is involved in seizure-susceptibility, we induced seizures with chemoconvulsants (including kainate and pilocarpine) in GM3S-KO mice. We found enhanced susceptibility to seizure induced by chemoconvulsants in GM3S-KO mice compared to wild-type (WT) mice. In the hippocampal dentate gyrus (DG), loss of GM3 aggravates seizure-induced aberrant neurogenesis. An increased number of bromodeoxyuridine (BrdU)+/doublecortin+ (DCX+) immature neurons abnormally migrated from the DG to the hilus, and were mislocalized in GM3S-KO brains (Tang et al. 2020a). Although GM3S-KO mice displayed reduced body weight and hyperactivity, naive animals showed no difference in adult neurogenesis in the dentate gyrus or morphological abnormality in the brain. These data indicate that GM3 and its downstream gangliosides are important regulators of epilepsy and play an important role to place adult newborn neurons at the right position.

GM3S-KO mice display higher insulin sensitivity and low grade inflammatory states (Nagafuku et al. 2015; Yamashita et al. 2003), and were protected from high-fat diet-induced insulin resistance. *GM3S* gene-deficiency in obese diabetic mice (KK-Ay; GM3S-KO) significantly ameliorated their obese phenotype compared to that seen with KK-Ay mice, although the KO mice did develop obesity when given

high fat diets (Inamori et al. 2018). Since the GM3S-KO mice were protected from high-fat diet-induced insulin resistance, GM3 ganglioside might be a negative regulator of insulin signaling and a potential therapeutic target in type 2 diabetes.

GM3S-KO mice have exacerbated inflammatory arthritis in the mouse model of rheumatoid arthritis (RA), a chronic systemic inflammatory disorder (Tsukuda et al. 2012). GM3S-KO mice showed severe pathologic osteoarthritis after interleukin-induced inflammation (Sasazawa et al. 2014). GM3-deficiency leads to the expression of matrix metalloprotease (MMP)-13, disintegrin, metalloprotease with thrombospondin type I motifs (ADAMTS)-5 secretion, and induced chondrocyte apoptosis. The expression of GM3S by chondrocytes suppressed the expression of MMP13 and ADAMTS-5, which resulted in reduced cartilage degradation even after interleukin-stimulation. These studies indicate that gangliosides have a suppressive capacity in the development of inflammation. The discrepancy of inflammatory states in GM3S-KO mice suggests that a variety of microdomains with different gangliosides and GSLs in individual T cells at local environments may contribute to the pathogenesis of or resilience to allergic diseases. Since there are no treatments that block the progression of these diseases, functional gangliosides could be an effective therapeutic for the treatment of inflammatory disorders.

## 6 GD3 Synthase-KO Mouse

GD3 ganglioside is a major b-series ganglioside expressed in immature vertebrate neuroepithelial cells (Goldman et al. 1984). GD3 was detected in neural tubes early in development using the GD3-specific monoclonal antibody R24 (Rosner et al. 1992) and GD3 is the predominant ganglioside species (>80%) in embryonic and postnatal neural stem cells (NSCs) (Nakatani et al. 2010). Its postnatal expression levels in the CNS rapidly declines, and becomes undetectable during neural development. The expression of GD3 is limited to NSCs that express Nestin and stage-specific embryonic antigen-1 (SSEA-1), with expression levels drastically decreased during differentiation. GD3 expressing cells do not have neuronal lineage markers such as microtubule-associated protein 2 (MAP 2) and neuron-specific beta-III tubulin (Tuj-1). GD3S (sialyltransferase II, ST-II, alpha-N-acetylneuraminatase alpha-2,8-sialyltransferase; *St8sia1*, EC 2.4.99.8)-KO mice do not express b- and c- series gangliosides (Okada et al. 2002). GD3S-KO mice had impaired regenerative capacity of damaged hypoglossal nerves and their surviving neuron number was significantly reduced to 50% after damage. Apparently, b- and/or c- series gangliosides are critical in the protection and repair of damaged nerves.

Our studies discovered that GD3 regulates the self-renewal of NSCs via epidermal growth factor (EGF) signaling by interacting with EGF receptors (EGFR) in the microdomains of the plasma membrane preventing their degradation (Wang and Yu 2013). Thus GD3 on the cell-surface of NSCs can facilitate signal transduction in response to extrinsic stimuli. The self-renewal capability is greatly reduced in higher passage numbers of NSCs taken from both embryonic and postnatal GD3S-KO

mice. The results support the hypothesis that GD3 has a role in the maintenance of NSCs. Interestingly, the NSC populations and neurogenesis sites of GD3S-KO mice differ little from those of WT mice in two germinal zones, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) in the DG of the hippocampus during the embryonic and early postnatal stages (Wang et al. 2014).

Both 1- and 6-month-old GD3S-KO mice exhibit a progressive reduction in cell number in the thinner SVZ. The cellularity of the DG showed no significant difference in 1-month-old GD3S-KO mice, however, the cell number in the DG of 6-month-old GD3S-KO mice was significantly reduced compared with their WT littermates. This progressive reduction of cellular pool might be caused by impaired maintenance of the NSC population in the GD3S-KO mouse brain. BrdU-labeled cells in the SVZ were reduced in GD3S-KO mice compared with their WT littermates at 1- and 6-months of age. Moreover, SRY (sex determining region Y)-box 2 (SOX2)-expressing cells were also reduced in the SVZ of GD3S-KO mice compared with WT mice at both 1- and 6- months. These observations indicate that a GD3-deficiency leads to a decline of SOX2-positive NSC cells in mice as young as 1 month. The Nestin and GFAP double-positive radial glia-like cells at both the SVZ and SGZ were also significantly reduced in GD3S-KO mice compared with their WT littermates. These results indicate that GD3 is required to maintain the postnatal NSC pool. Most importantly, the impaired neurogenesis in the adult GD3S-KO mice led to depression-like behaviors. Our research provides direct evidence linking ganglioside deficiency to behavioral deficits, and support a crucial role for gangliosides in the long-term maintenance of adult neurogenesis and NSC activities (Itokazu et al. 2018; Wang et al. 2014).

We also found that depletion of GD3 decreased dendritic arborization and spines of nascent granule neurons in the DG, and compromised integration of newborn DG neurons in hippocampal circuitry in the adult brain (Tang et al. 2020b). The novel object recognition task was performed to test hippocampus-dependent memory of GD3S-KO mice. WT mice spent more time exploring the novel object than the familiar one that was used in the training session, while the GD3S-KO mice spent significantly less time exploring the unfamiliar object, implying inferior recognition memory performance. Barnes maze task, a hippocampal-dependent spatial memory test, was also performed. GD3S-KO mice displayed a significant increase in escape latency to find the hidden chamber, showing a spatial memory defect. We identified dynamin-1-like protein (Drp1) that is recruited to the outer mitochondrial membrane and critical for GTP-dependent mitochondrial fission, essential for maintenance of the mitochondrial network, as a GD3-interacting protein. Observation of dsRed-labeled mitochondria demonstrated that mitochondria were highly fragmented in the DG of GD3S-KO mice compared with WT. Consistent with this observation, we found an increased level of Drp1 and significantly increased levels of activated-Drp1 (phosphorylation of Drp1 at serine 616), as well as excessive mitochondrial fragmentation in the GD3S-KO mice. The reduced number of mitochondria in GD3S-KO neurons might cause the fewer arborization of dendrites and spine formation. These findings demonstrated that GD3 has an important role in the

cytoarchitecture of the mature neuron through the regulation of mitochondrial fusion-fission dynamics, which affect hippocampal memory formation.

We reported that GD3 is the major ganglioside in developing and postnatal retina of vertebrates (Seyfried and Yu 1985; Seyfried et al. 1982). Abreu et al. found that a significant reduction of retinal ganglion cells and optic nerve in GD3S-KO mice (Abreu et al. 2021). Cell number of the outer nuclear layer, where photoreceptors are expressed, was also decreased in GD3S-KO mice. Consistent with these results, electrophysiological function and contrast sensitivity was reduced in GD3S-KO mice. These findings suggested that GD3 plays a key role in the optomotor system by maintaining retinal ganglion cells and photoreceptors.

Under particular conditions, GD3S-KO mice showed both motor and sensory dysfunctions. Axonal thickness and myelination of the sciatic nerve was reduced in GD3S-KO mice which leads to a reduced pain threshold with a hyperalgesic response (Handa et al. 2005; Ribeiro-Resende et al. 2014). Notable motor deficits were also observed in GD3S-KO mice at high speeds of the rotarod motor test. Regenerative ability of GD3-KO sciatic nerve was found to be lower with a decreased axonal growth rate after crush injury. Neurite growth of dorsal root ganglia after lesioning was reduced in GD3S-KO whereas exogenous GD3 administration dramatically restored regeneration by increasing and recruiting  $\beta$ 1-integrin to the growing neurites. These results indicate that GD3 and its derivative b- and c-series gangliosides have functional roles during PNS maintenance and regeneration. Meanwhile, in the CNS, GD3S-KO mice show decreased postnatal NSC pools (Wang and Yu 2013) and impaired postnatal neurogenesis leads to depressive symptoms (Wang et al. 2014). Consequently, GD3 has strong potential to restore both CNS and PNS dysfunctions.

## 7 GM2 Synthase-KO Mouse

For synthesis of further complex gangliosides, UDP-*N*-acetylgalactosamine (GalNAc) is added to GM3 (Fig. 14.1) by GM2S (UDP-GalNAc:GM3 *N*-acetylgalactosaminyl-transferase; GalNAcT, beta 1,4-*N*-acetylgalactosaminyl-transferase; *B4galnt1*, EC 2.4.1.92). GM2S is one of the key enzymes needed for synthesis of the major “brain-type” gangliosides, including GM1, GD1a, GD1b, and GT1b. GM2S-KO mice do not express GalNAc-containing gangliosides, and only express GM3 and GD3 as major gangliosides but total amount of brain gangliosides is not different between WT and KO mice (Sheikh et al. 1999; Takamiya et al. 1996). GM2S-KO mice present morphological abnormalities, including axonal degeneration, decreased myelination, and demyelination in the CNS and PNS at 12–16 weeks of age and motor deficits at 8–12 months (Chiavegatto et al. 2000). GM1 is involved in  $\text{Ca}^{2+}$  homeostasis and nuclear GM1 was proposed to function as a modulator of nuclear  $\text{Ca}^{2+}$  during axonogenesis. Cultured cerebellar granule neurons from GM2S-KO mice showed persistent elevation of intracellular  $\text{Ca}^{2+}$  leading to apoptosis, which was averted by exogenous GM1 or a GM1 membrane

permeant analog, LIGA20 (Wu et al. 2001, 2004). GM2S-KO mice exhibit the moderate phenotype of PD including motor disability on aging, gastrointestinal and sympathetic cardiac disorders, and memory dysfunction (Wu et al. 2011, 2012, 2020). The primary and rate-limiting step in the successive biosynthesis of catecholamines including dopamine, is conversion of L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA) catalyzed by tyrosine hydroxylase (TH). Consistently, expression levels of the rate-determining enzyme, TH in the dopaminergic neurons of the substantia nigra pars compacta are reduced in GM2S-KO mice along with an increase in alpha-synuclein (aSyn) levels.

Expression of TH is promoted by glial cell derived neurotrophic factor (GDNF) signaling. GDNF recruits and activates GDNF receptors, such as GDNF family receptor alpha-1 (GFR $\alpha$ -1) and rearranged during transfection (RET). GM2S-KO mice (*i.e.* GM1-deficient mice) showed decreased complex formation of GDNF receptors, suggesting that GM1 is necessary for the GFR $\alpha$ -1-RET complex formation on the cell surface that facilitates intracellular functions induced by GDNF signaling. A GM1 analog, LIGA20, restores the activation of GDNF signaling and the expression of TH in GM1-KO mice despite its possible cytotoxicity (Hadaczek et al. 2015). GM2S-KO mice also exhibit PD-like non-motor symptoms such as detrusor muscle underactivity, which results in decreased voiding compared to that of WT littermates. Enlarged bladders with elevated pro nerve growth factor (NGF) which causes an adverse effect on bladder function, and abnormal bladder nerve myelination were also observed in GM2S KO mice (Gil-Tommee et al. 2019). We also found that mRNA expression of TH is significantly down-regulated in brains of GM2S-KO mice, and intranasally administered GM1 reached the brain and restored TH expression through the recruitment of Nurr1 transcription factor on active chromatin (Itokazu et al. 2021). Those results strongly suggest that PD symptoms due to reduced biosynthesis of dopamine might be restored by administration of GM1.

## 8 Both GD3S/GM2S-KO Mice (GM3 Only Mice)

GD3S/GM2S double KO mice expressed GM3 as the major ganglioside. In contrast to the single mutant mice, GD3S/GM2S double KO mice displayed a sudden death phenotype and were extremely susceptible to induction of lethal seizures by sound stimulus (Kawai et al. 2001). After weaning, GD3S/GM2S double KO mice had an extremely high mortality rate and 50% of KO mice died by 13 weeks of age. Like GM2S-KO mice, male GD3S/GM2S double KO mice displayed sterility due to a defect in sperm maturation. GD3S/GM2S double KO mice showed enhanced susceptibility to lethal seizure. Induced tonic-clonic seizure caused the death of around 90% of GD3S/GM2S double KO mice, although neither GD3S-KO nor GM2S-KO mice died from induced seizures. Whole brains of GD3S/GM2S double KO mice had about a 20% reduction in weight after about 30 weeks of age and progressively poorer motor function after 6–8 months (Tajima et al. 2009).



## 9 Both GM3S/GM2S-KO Mice (Ganglioside Null Mice)

Both GM2S and GM3S double KO mice are unable to synthesize gangliosides, which are the major GSL class in the nervous system (Yamashita et al. 2005). Those mice have increased amounts of LacCer and sulfated LacCer (SM3) in their brains. At 2 weeks of age, GM3S/GM2S double KO mice developed motor dysfunctions including hind limb weakness, ataxia, and tremors. The double KO mice died soon after weaning at 3 weeks of age, exhibited sudden death from audiogenic seizures, and revealed striking vacuolar pathology with axonal degeneration and perturbed axon–glia connections. In animals that survived to 1 month of age, the brains of GM3S/GM2S double KO mice were smaller than those from WT mice (Furukawa et al. 2014). These studies indicated that deletions of gangliosides are associated with human diseases.

## 10 Diseases Caused by Ganglioside Alterations

### 10.1 Diseases Caused by Congenital Mutations of Ganglioside Metabolism

Autosomal recessive lysosomal storage disorders are caused by dysfunctions of lysosomal hydrolases or small lipid-binding proteins. In gangliosidosis, degradation pathways of ganglioside are blocked, and certain gangliosides accumulate. Both GM1 gangliosidosis and Morquio type B disease (Mucopolysaccharidosis type IVB) are caused by mutations in the  $\beta$ -galactosidase gene (GBL1) encoding lysosomal acid  $\beta$ -galactosidase (E.C.3.2.1.23). Lack of  $\beta$ -galactosidase activity leads to accumulation of  $\beta$ -linked galactose-containing glycoconjugates. GM1 gangliosidosis is a severe neuronopathic disorder with progressive neurodegeneration. Accumulation of GM1 in the brain contributes to neurologic symptoms of GM1 gangliosidosis. Morquio type B disease is caused by different mutations in the GBL1 gene from those of GM1 gangliosidosis and major accumulation of galactose containing keratan sulfate and oligosaccharides has been shown. In addition, accumulation of GM2 (Tay–Sachs Disease, Sandhoff Disease, GM2 Activator Deficiency), GlcCer (Gaucher disease), and other sphingolipids leads to lysosomal storage diseases with central nervous system involvement (Sandhoff et al. 2018).

Gangliosides are found in virtually all vertebrate cells but they are particularly abundant in the nervous system (Yu and Itokazu 2014; Yu et al. 2009, 2011). GM3S is a critical enzyme for the synthesis of all gangliosides. Mutation of *GM3S* is associated with human autosomal recessive infantile-onset symptomatic epilepsy syndrome (Simpson et al. 2004), refractory early onset seizures with failure to thrive and psychomotor delay by a respiratory chain dysfunction and mitochondrial membrane potential perturbation (Fragaki et al. 2013), Salt & Pepper syndrome characterized with severe intellectual disability, epilepsy, scoliosis, choreoathetosis,

dysmorphic facial features and altered dermal pigmentation (Boccuti et al. 2014), Rett syndrome-like phenotype (Lee et al. 2016), hearing loss (Yoshikawa et al. 2015), progressive microcephaly, psychomotor stagnation, dyskinetic movements, somatic growth failure (Bowser et al. 2019), mild to severe dystonia with poor visual tracking and quadriparesis, (Wang and Kilbane 2021), skin dyspigmentation of freckle-like hyperpigmented macules on the extremities (Wang et al. 2013). GM3S dysfunction causes refractory epilepsy and mitochondrial dysfunction (Fragaki et al. 2013). The patients have developmental stagnation, blindness, required feeding tubes (poor feeding, vomiting and failure to thrive), seizure activity (started within the first year of life), generalized tonic-clonic seizures, tonic spasms, episodes of eye deviation, startle from sleep, startle myoclonus. Those seizure controls are difficult, requiring multiple medications and, for some cases vagus nerve stimulators. They are unable to sit unsupported, reach or walk and nonverbal. Eye contact and visual function deteriorate, caused by optic atrophy and cortical impairment. Electroencephalograms (EEG) ultimately showed multifocal epileptiform discharges. Furthermore, severe auditory impairment observed in GM3S-deficient patients suggests ganglioside microdomains maintain the structural and functional integrity of cochlear hair cells (Inokuchi et al. 2017). Deficiency of GM3S in patients have much severe phenotypes as compared to GM3S-KO mice. GM3S/GM2S double KO mice more closely reflect human GM3S deficiency. Gangliosides in nervous system have much more important functions and biological significant in human life than murine models.

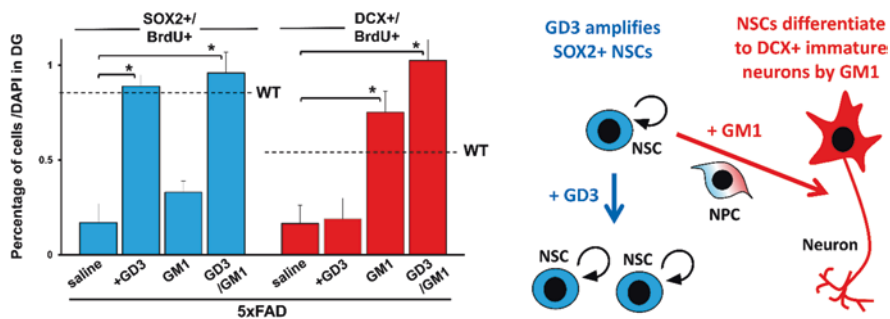
An alteration of the *GM2S* gene was reported in patients of hereditary spastic paraplegias that is characterized as a heterogeneous disorder with progressive spasticity, weakness of the lower limbs, hyperextended knees and gait abnormalities, additional neurological manifestations (Boukhris et al. 2013; Harlalka et al. 2013; Wakil et al. 2014) and axonal Charcot-Marie-Tooth disease, a hereditary motor and sensory neuropathy (Hong et al. 2021). These clinical features of patients are reasonably similar to the abnormal phenotypes detected in GM2S-KO mice. While a GM3S mutation may affect neuronal development, a deficiency in GM2S results in milder phenotypes with slower disease progression.

## 10.2 Ganglioside Deficiency in Neurodegenerative Diseases

Aging is the principal risk factor for the development and progression of neurodegenerative diseases. Gangliosides undergo dramatic qualitative and quantitative developmental changes that correlate with cellular events such as proliferation, differentiation, and neuronal function. In early embryonic brains, the pattern of gangliosides is characterized by the expression of several simple gangliosides, such as GM3 and GD3. GD3 is the predominant ganglioside species in NSCs (Nakatani et al. 2010), while in later developmental stages, more complex gangliosides prevail, particularly GM1, GD1a, GD1b, and GT1b through increases in GM2S levels. Ganglioside metabolism is closely associated with the pathology of

neurodegenerative diseases. For instance, alterations of GD3, GM1 and other ganglioside levels were reported in AD patients and mice (Ariga 2017; Ariga et al. 2008; Barrier et al. 2007; Svennerholm 1994). The potential involvement of gangliosides in AD pathogenesis and disease treatment has been reviewed by us (Ariga et al. 1998, 2008, 2010, 2011; Yu et al. 2012). Progressive imbalance of cell membrane lipid composition is a physicochemical property that changes during normal aging, and further disruptions in it are observed in neurodegenerative diseases. Ganglioside synthesis which occurs in sequential steps is controlled mainly by glycosyltransferases (GTs; ganglioside synthases) found predominantly in the Golgi apparatus. Golgi fragmentation is observed in A $\beta$ -treated primary neurons and in the hippocampus in a transgenic AD [B6C3Tg(APP<sup>swe</sup>,Psen1dE9)85Dbo/J] (Joshi et al. 2014) mouse model. It is expected that GT (ganglioside synthase) activities in different regions of the Golgi apparatus are impaired by the disrupted Golgi structure. Ganglioside composition is altered in the brains of AD patients; major brain-type gangliosides, including GM1, are significantly decreased in the hippocampus in addition to several other areas important for cognition, such as frontal and temporal cortices, parietal cortex, basal telencephalon, and frontal white matter (Ariga 2017; Ariga et al. 2008; Svennerholm 1994). A progressive alteration in brain gangliosides, *e.g.*, reduced cortical GM1, is exhibited by patients and in multiple transgenic mouse models of AD.

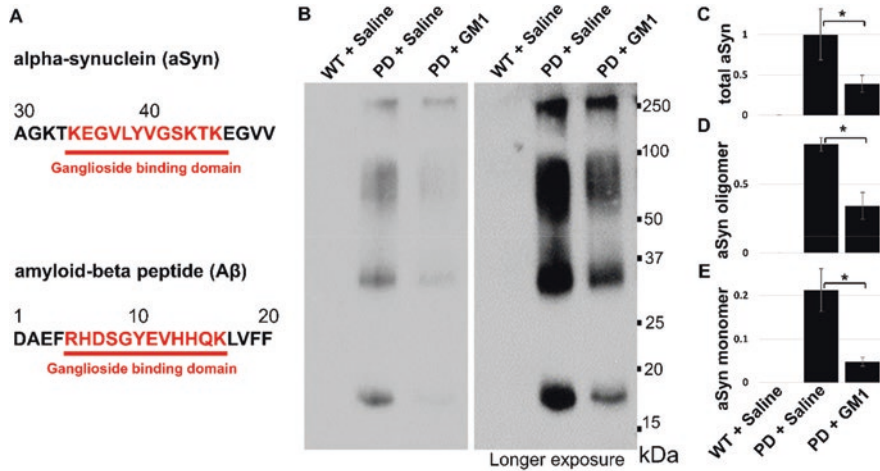
Although the biological significance of adult hippocampal neurogenesis in humans is still under debate, the detection of adult neurogenesis in certain brain regions supports the hypothesis that the adult brain exhibits more plasticity than previously thought, and this affects memory and the pathogenesis of neurodegenerative diseases (Gage 2021). Emerging evidence from AD patients and mouse models supports an active level of neurogenesis in early/moderate onset of the disease, that then becomes sluggish in brains of patients with late/severe AD. Diminished neurogenesis occurs in normal aging, and it is hypothesized that an accelerated loss of the NSC pool is one mechanism for transition from a healthy to an AD brain. Impaired adult hippocampal neurogenesis occurs before signs of cognitive and memory deficits in AD (Berger et al. 2020; Moreno-Jimenez et al. 2019; Tobin et al. 2019). Therefore, promotion of endogenous neurogenesis has been suggested as an important target for treatment and prevention of AD (Choi et al. 2018; Mu and Gage 2011). Essential cellular processes in NSC fate-determination and self-renewal are heavily influenced by cell surface glycoconjugates, including gangliosides. To examine the physiological roles of GD3 and GM1 on postnatal neurogenesis in the brain of AD model mice, gangliosides (GD3 or GM1 and combination) were intracerebroventricularly (icv) administered into the 5XFAD mouse brain (Fig. 14.2). Our data clearly show that GD3 augments self-renewal and cells expressing the multipotent marker, SOX2, in DG (Itokazu et al. 2019). On the other hand, GM1 increases BrdU+/DCX+ newly generated immature neurons in 5XFAD mouse brains. As expected, the combinational infusion of GD3 and GM1 had a synergistic effect. The data evidenced that exogenous gangliosides can restore the NSC pool in AD brains in the 5XFAD mouse model. Remarkably, Lars Svennerholm et al. described that icv administration of GM1 to AD patients could stop the continuous



**Fig. 14.2** GD3 and GM1 restore the number of SOX2+ and DCX+ cells in the AD mouse brain. The 5XFAD transgenic mouse is an AD model with two point mutations in presenilin1 (M146L & L286V) and the Florida (I716V), London (V717I), and Swedish (KM670/671NL) mutations in the amyloid precursor protein. 5XFAD mice showed a significantly decreased percentage of both SOX2/BrdU and DCX/BrdU double labeled cells compared to WT mice. GD3 and GM1 (5 mg/kg bodyweight/day GD3 or GM1) were introduced into the brains of (10-week-old) 5XFAD mice (via icv for 7 days by micro-osmotic pump at a flow rate of 0.5  $\mu$ L/h) and then injected with BrdU to assess its neurogenic potential with co-staining of lineage-associated markers. For the combinational experiment, GD3 was first infused for 7 days and then GM1 was infused for 7 days. ( $n = 3$ ), \* $p < 0.05$ . WT = wild type. GD3 restores the number of SOX2+ cells and GM1 restores DCX+ neuronal cells in the DG of 5XFAD. The combinational infusion (GD3 and GM1) had a synergistic effect. The data demonstrated that GD3 amplifies NSCs and then GM1 promotes neuronal differentiation. WT: levels of wild-type; NSC, neural stem cell; NPC, neuronal progenitor cell

deterioration of nerve processes and increased the turnover of transmitter substances, although the molecular mechanisms underlying the positive effects of GM1 in AD brains were not determined (Svennerholm et al. 2002).

Accumulations of aSyn is a major pathological hallmark in PD, and gangliosides bind to aSyn with high affinity to stabilize an alpha-helical state and inhibit fibrillation (Bartels et al. 2014; Martinez et al. 2007). Importantly, aSyn has a ganglioside binding domain, which has the most critical residues for binding GM1 (Di Scala et al. 2016). As expected, intranasally administered GM1 dramatically reduced aSyn levels, not just monomeric but oligomeric aSyn in substantia nigra pars compacta of a PD mouse model, which is known as Halpha-Syn(A53T) transgenic line G2-3 (referred to as A53T PD mouse) (Fig. 14.3) (Itokazu et al. 2021). Increasing disproportion of the lipid composition of cell is a physicochemical property altered with normal aging, and advanced disruptions to these processes are observed in neurodegenerative diseases, including in PD and AD brains. It is reported that the ganglioside composition is altered in the brains of PD patients, e.g., major “brain-type” gangliosides, such as GM1, are significantly decreased (Seyfried et al. 2018; Wu et al. 2012). In earlier studies, intraperitoneally injected GM1 was shown to restore TH activity and dopamine content in striatum of rodents after surgical and chemical (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPTP) damage (Hadjiconstantinou et al. 1986; Toffano et al. 1983). A lot of rodent research on the effectiveness of GM1 therapy followed with positive results.

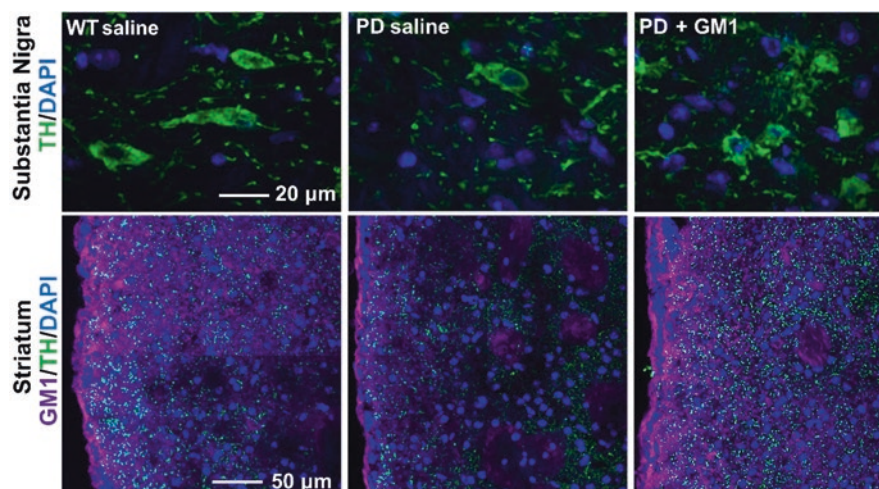


**Fig. 14.3** Intranasally administered GM1 reduced aSyn monomer, oligomers, and aggregates in A53T PD mouse brain. aSyn has a ganglioside binding domain. Intranasally infused GM1 (5 mg/kg/day for 28 days) removed aSyn in A53T PD mouse brain (8-month-old). (a) aSyn and Aβ have functional ganglioside binding domain at K34–K45 (red), and R5–K16 (red), respectively. (b) Brain extracts from substantia nigra of WT and PD mouse brain were loaded for SDS PAGE. aSyn monomer is shown a band around 15–20 kDa. Nasal GM1 infusion significantly reduced monomer, oligomer and total aSyn. Total (c), oligomer (d; 30–260 kDa), and monomer (e) aSyn levels were quantified by imageJ and normalized by actin (data not shown).  $n = 3$  mice/group, male,  $*p < 0.05$

To date, there is no definitive proven disease-modifying therapy for patients with PD. Bovine-derived GM1 has been extensively studied in many PD patients over many years in Jay Schneider et al.'s long-term NIH-funded research (Schneider 2021). The safety profile of GM1 trials were impressive with no serious adverse events reported and no clinically significant abnormalities in blood chemistry, hematology measures, or urinalysis results. There was no antigenicity by ganglioside treatment. Although improvement of movement disorders and potential beneficial mechanisms of action of GM1 in PD were observed, use of GM1 from bovines was abandoned due to concern of possible contamination of GM1 supplies with abnormal prion protein from bovine spongiform encephalopathy (BSE)-affected brains. Another problem is the lack of efficient routes of administering GM1 in a manner that permits it to efficiently cross the blood–brain barrier (BBB). Intravenous or intramuscular administration of GM1 to patients with stroke or spinal cord injury was found to be safe, no adverse effects, and most importantly the patients showed neurological improvement (Magistretti et al. 2019).

Results of our studies of mouse models demonstrate that intranasally administered GM1 was successfully delivered to various brain tissues including the olfactory bulb, cortex, midbrain, germinal zones, and cerebellum (Itokazu et al. 2021). PD is a neurodegenerative disease characterized by the loss of midbrain dopaminergic neurons with a subsequent decrease in the concentration of striatal dopamine.

The expression level of TH was markedly reduced in the substantia nigra and striatum in the brains of A53T PD mice, and intranasally infused ganglioside GM1 dramatically restored it (Fig. 14.4). This suggests that the chronic dopamine depletion seen in PD-model mice and possibly PD patients might be restored by nasal administration of GM1. GDNF downstream transcriptional factors, Nurr1 and Pitx3, are involved in the expression of the TH needed for survival of substantia nigra dopaminergic neurons (Maxwell et al. 2005; Peng et al. 2011). Nurr1 is also essential for the expression of a GDNF receptor, Ret, suggesting a regulatory loop of GDNF signaling (Volakakis et al. 2015). However, GDNF treatment of PD-model cells, rodents, and patients with PD failed to protect midbrain dopaminergic neurons (Decressac et al. 2011; Kambey et al. 2021; Manfredsson et al. 2020; Mesa-Infante et al. 2022; Nutt et al. 2003). Since the expression of GDNF receptors is regulated by GM1 (Hadaczek et al. 2015), it is likely that GDNF alone (without GM1) does not activate further signaling. In our research, GM1 restored nuclear localization of Nurr1 in brains of A53T PD mice and recruited Nurr1 and Pitx3 to the TH promoter to activate gene expression. Further, exogenous GM1 itself induced GM2S expression to augment endogenous GM1 by activating a chromatin-based epigenetic



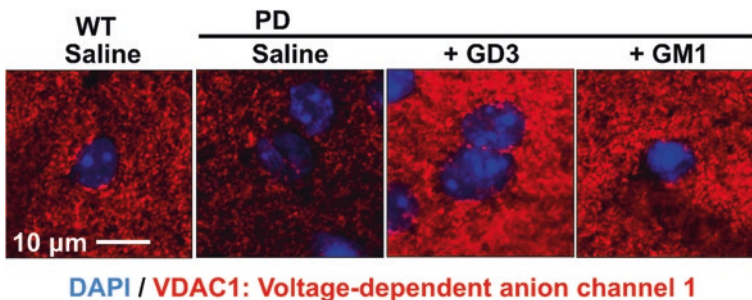
**Fig. 14.4** Intranasally administered GM1 restored dopaminergic neurons in the PD mouse brain. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthesis of dopamine, and it is regularly used as a marker for dopaminergic neurons. Patients with PD exhibit neurodegeneration of dopaminergic neurons in the substantia nigra. We found a markedly reduced expression of TH in the A53T PD mouse brain (8-month-old). Intranasally infused GM1 (5 mg/kg/day for 28 days) increased expression of TH in the substantia nigra in brains of A53T PD mice. (Lower images) The striatal projection of dopaminergic neurons (TH+) are vigorously decreased, on the other hand, intranasal infusion of GM1 recovered the TH+ axons in the striatum. The data shows that GM1 (green) and TH (purple) levels were dramatically restored in substantia nigra and striatum by nasal GM1. This suggests chronic dopamine depletion in the striatum of PD-model mice and possibly of PD patients might be restored by intranasally administration of GM1. Green: TH, Purple: GM1, Blue: nuclear DAPI

mechanism (Itokazu et al. 2016; Tsai and Yu 2014). We propose that nuclear ganglioside microdomains modulate gene transcription during disease progression and regeneration of cells in diseased brains.

Disruption of mitochondrial homeostasis and mitochondrial dysfunction play a vital role in the pathogenesis of several neurodegenerative diseases, and mitochondrial dynamics are critical for hippocampal dependent learning and memory (Simmons et al. 2020; Trinh et al. 2021). Figure 14.5 shows that a major component of the outer mitochondrial membrane, voltage-dependent anion channel 1 (VDAC1) known to regulate mitochondrial functions, is drastically downregulated in an A53T PD mouse brain. Intranasally administered GD3 or GM1 dramatically restored VDAC1 levels in an A53T PD mouse brain. We have shown that GD3 regulates mitochondrial dynamics by mediating Drp1 (Tang et al. 2020b). We also found that GM1 binds to other mitochondrial proteins to regulate mitochondrial functions (Itokazu et al., unpublished data). To cure neurodegenerative diseases, targeting mitochondrial dysfunction with functional gangliosides is another promising approach for the development of future therapies.

## 11 Future Studies

From the foregoing, significance of glycolipid deficiencies in the nervous system can be clearly seen. Mice deficient in some GSLs show only subtle phenotypic abnormalities compared with wild-type animals during early development. Clearly, the biological function of one glycoconjugate can be substituted by another (McGonigal et al. 2019), albeit with less efficiency. Notably, aberrant ganglioside expression becomes incrementally more serious in the adult stage and pathogenic conditions. The “biological redundancy” indicates that GSLs play more pivotal roles than general molecules that lack any biological function overlap in the nervous



**Fig. 14.5** Intranasally administered GD3 and GM1 restored expression of VDAC1, a major component of the outer mitochondrial membrane known to regulate mitochondrial functions. Intranasally infused GD3 and/or GM1 (5 mg/kg/day for 28 days) increased VDAC1 expression in dopaminergic neurons within the substantia nigra pars compacta (data not show) and cortex of an A53T PD mouse brain (8-month-old)

system. Evidently, patients with ganglioside deficiency have more severe clinical phenotypes than those of ganglioside KO mice, although mice completely deleted in gangliosides do have lethal phenotypes. Many patients with neurological disorders suffer from immense pain, immobility, disease progression, and problems with everyday needs. The lack of satisfying medications for these individuals reflects the lack of knowledge of the diseases which in turn underlies the paucity of effective therapies.

Biological membranes are highly heterogenous in structure and there exist diverse microdomains (also known as lipid rafts), which are enriched with GSLs. GSLs are unique amphipathic molecules that contain a hydrophilic carbohydrate portion and a hydrophobic lipid component. GSLs are a class of lipids highly enriched in the nervous system, with a wide variety and complexity. GSLs appear to be essential for neuronal development and neuronal functions. The significance is that GSLs modulate lipid microdomains to regulate functions of important molecules on plasma, mitochondrial, nuclear, and other biological membranes. Multifunctional gangliosides regulate specific cells in distinct stages via modulating protein and gene activities through the GSL microdomains. Despite cumulative evidence obtained by studying ganglioside-KO mice, the specific roles of gangliosides in the stage-, cell-, and disease-specific significance remain unclear. GD3S/GM2S-double KO mouse (GM3 only mouse) has severe phenotypes as described above. Neuronal expression of a-series gangliosides significantly abrogated the lethal phenotype and partially attenuated the neurodegenerative manifestations. Studies with ganglioside conditional KO mice with other disease models will help clarify cell-, stage-, and disease-specific biological functions of gangliosides.

Overall, ganglioside deficiency contributes to development of neurodegenerative diseases and mental health disorders. Adult neurogenesis is suggested to contribute to a healthy life. An adult human generates 700 new neurons daily in the hippocampus, with a gradual decline during aging (Spalding et al. 2013). Adult neurogenesis declines more in patients with neurodegenerative diseases and mental disorders (Berger et al. 2020; Moreno-Jimenez et al. 2019). Human subjects with higher numbers of DCX+ immature neurons tend to have higher cognitive performance (Moreno-Jimenez et al. 2019; Tobin et al. 2019). Reduced numbers of DCX+ cells were observed in patients with mild cognitive impairment and AD, and an accelerated reduction in later stages of pathology. In early to moderate stages of neurodegenerative diseases, GM1 would be sufficient to maintain neuronal functions. On the other hand, at more severe stages of diseases, first GD3 is needed to amplify NSCs and then GM1 to support neuronal differentiation of NSCs. Both GM1 and GD3 can reduce levels of neurotoxic proteins in the brains of people with neurodegenerative diseases. For depression treatment, GD3 may be needed first to increase the number of NSCs. The use of a combination of GD3 and GM1 represents a powerful means to slow down the progression of disease and restore dysfunctional neurons. Since the population of people with age-related neurodegenerative diseases and mental disorders is increasing, GSL research will benefit millions of patients, their families and society.



**Conflict of Interest** The authors declare no conflicts of interest.

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# Chapter 15

## Carbohydrates: Binding Sites and Potential Drug Targets for Neural-Affecting Pathogens



Cara-Lynne Schengrund

**Abstract** A number of viruses that have caused wide spread concern e.g. Ebola, Zika, and SARS-CoV2 (severe acute respiratory syndrome coronavirus 2 also known as COVID 19) have at various times, become newsworthy as a result of being newly discovered, mutations enabling them to more efficiently infect humans or modern modes of transportation moving them to areas with naive, susceptible populations. As more is learned about the mechanisms whereby these pathogens enter human cells it has become increasingly evident that carbohydrates expressed on the surface of either target cells or the pathogens themselves are essential. Variability in carbohydrate structures as well as the presence of carbohydrate binding receptors (lectins) provides a plethora of potential binding interactions by which infection of cells can occur. Identification of specific lipid- or protein-associated carbohydrates essential for infection provides support for research being done to develop carbohydrate related inhibitors of those interactions. This chapter (1) discusses scenarios for how carbohydrates affect the ability of specific infectious agents to interact with neural cells, (2) gives examples of problems that may result from development of antibodies to carbohydrate antigens found on pathogens that are similar to epitopes expressed on mammalian cells, and (3) provides examples of approaches either in use or under consideration for translational uses of this information.

**Keywords** Bacterial toxins · Molecular mimicry · Glycodendrimers · Multivalency · Latency · Lipid rafts · Peptide mimetics

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## Abbreviations

BBB	Blood–brain barrier
Bgp	Borrelia GAG-binding protein
BoNT	Botulinum neurotoxin
CaMKII	Ca <sup>2+</sup> /calmodulin-dependent kinase II
CNS	Central nervous system
COVID-19	Coronavirus disease 2019
CTx	Cholera toxin
DC-SIGN	Dendritic cell-specific intercellular molecule-grabbing nonintegrin
GABA	Gamma-aminobutyric acid
GAG	Glycosaminoglycan
Gb3	Globotriaosylceramide
GBS	Guillain–Barre syndrome
GSL	Glycosphingolipid
H	Hemagglutinin
HIV	Human immunodeficiency virus
Iv	Intravenous
mAb	Monoclonal antibody
MBCD	Methyl- $\beta$ -cyclodextrin
SARS-CoV2	Severe acute respiratory syndrome coronavirus 2
SNAP-25	Synaptic vesicle-associated protein with a mass of 25 kilodaltons
SV2	Synaptic vesicle glycoprotein 2
TeNT	Tetanus neurotoxin
TNF $\alpha$	Tumor necrosis factor-alpha
TTC	Carboxyl terminal half of the heavy chain of tetanus toxin
VAMP-1-2	Vesicle-associated membrane protein-1-2 or synaptobrevin-1 and -2

## 1 Introduction

In Chaps. 1, 3, 4, and 5 the various types of glycoconjugates expressed in the nervous system and the almost limitless diversity they can display when considered from a structural point of view are discussed. Also covered are some of the myriad number of problems that may occur as a result of incorrect glycosylation (e.g. Chap. 8). The variability in carbohydrate structures as well as the presence of carbohydrate-binding receptors (e.g. Chap. 7) on the surface of target cells provides a plethora of potential binding sites for many pathogens. This chapter will present (1) scenarios for how carbohydrates may affect the ability of infectious agents to interact with neural cells, (2) examples of problems that may result from development of antibodies to carbohydrate antigens found on pathogens that are similar to epitopes found on cells, and (3) approaches either in use or under consideration for translational uses of this information.

## 2 Bacterial Toxins: Lessons Learned

Cholera toxin (CT<sub>x</sub>), the heat labile enterotoxin of *Escherichia coli*, and Shiga-like toxin, causative agents for the symptoms of cholera, travelers' diarrhea, and hemolytic-uremic syndrome, respectively, are well-studied examples of carbohydrate-binding agents. Each of the three toxins listed are of the AB<sub>5</sub> type. This indicates that the binding subunit is made up of five identical polypeptide chains while the A subunit, or a part of it, mediates activity of the toxin. In the case of cholera toxin, each of the five identical binding (B) subunits contain components of a single binding site formed by the intersection of one peptide with the next, resulting in five binding subunits. Each binding subunit is able to adhere to the oligosaccharide portion of ganglioside GM1 (see Table 15.1 for composition of oligosaccharides discussed), while the pentameric B subunit can adhere to five (Merritt et al. 2002). While individual protein-carbohydrate interactions are often of low affinity, the adherence of multiple binding subunits present on the AB<sub>5</sub> toxins to multiple carbohydrate residues present on either a single or multiple receptors was shown to result in a much higher binding affinity, one that was greater than the sum of the individual interactions (Lee and Lee 2000). This observation provided an explanation for why "multivalent" carbohydrate inhibitors, termed glycodendrimers, were observed to be more effective inhibitors of the binding of AB<sub>5</sub> toxins than the free oligosaccharide (e.g. Thompson and Schengrund 1997; Kitov et al. 2000) and provided the basis for understanding how pathogens may use cell surface

**Table 15.1** Carbohydrate composition of oligosaccharides discussed

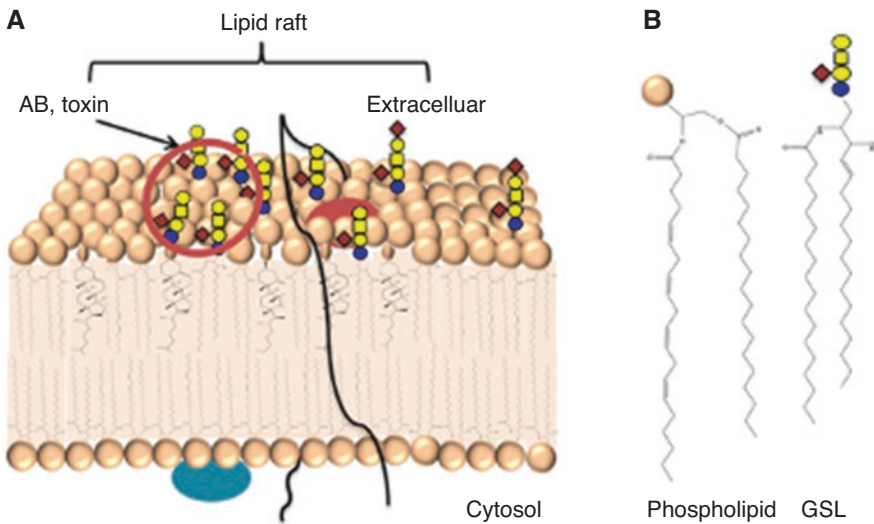
Name	Carbohydrate composition
Gb3	Gal(α1-4)Gal(β1-4)Glcβ1-
GM3	SA(α2-3)Gal(β1-4)Glcβ1-
GM1	Gal(β1-3)GalNAc(β1-4)[SA(α2-3)]Gal(β1-4)Glcβ1-
GD3	SA(α2-8)SA(α2-3)Gal(β1-4)Glcβ1-
GD2	GalNAc(β1-4)[SA(α2-8)(SA(α2-3))]Gal(β1-4)Glcβ1-
GD1b	Gal(β1-3)GalNAc(β1-4)[SA(α2-8)SA(α2-3)]Gal(β1-4)Glcβ1-
GT1b	SA(α2-3)Gal(β1-3)GalNAc(β1-4)[SA(α2-8)SA(α2-3)]Gal(β1-4)Glcβ1-
GQ1b	SA(α2-8)SA(α2-3)Gal(β1-3)GalNAc(β1-4)[SA(α2-8)SA(α2-3)]Gal(β1-4)Glcβ1-
LLG-3 <sup>a</sup>	8-OMe-Neu5Ac(α2-11)Neu5Gc(α2-3)Gal(β1-4)Glcβ1-
Heparan sulfate <sup>b</sup>	[GlcNAc(β1-4)GlcUA/IdoA] <sub>n</sub> -linkage tetrasaccharide
Dermatan sulfate	[IdoA/GlcUA(α1-3)GalNAc] <sub>n</sub> -linkage tetrasaccharide
Hyaluronic acid	[GlcUA(β1-3)GlcNAc] <sub>n</sub>
PGL-1 <i>M. leprae</i>	3,6-di-O-methylGlc(α1-4)2,3-di-O-methylrhamnose(β1-2)3-O-methylmannose

<sup>a</sup>Neu refers to neuraminic acid while SA refers to sialic acid, the general name used when not specifically indicating substituents

<sup>b</sup>Heparin and heparan sulfate have the same disaccharide repeating unit but heparin has more *N*-acetyl groups, and fewer *N* and *O*-sulfates. Dermatan sulfate contains predominantly iduronic acid while hyaluronic acid is not sulfated. For details about GAGs see Chap. 5

carbohydrates as high-affinity binding sites. The finding that protein–sugar interactions are often strengthened as a result of the protein adhering to multiple carbohydrates may explain observations made by Hanashima et al. (2008) when studying Shiga toxin binding to its receptor Gb3 on Vero cells. The toxin adhered to wild-type Vero cells where Gb3 was present in lipid rafts but did not bind mutated Vero cells whose lipid rafts had a lower density of Gb3. For a schematic of a lipid raft, an area of the membrane enriched in cholesterol, glycosphingolipids, and signal transduction molecules (Brown and London 2000; Simons and Toomre 2000) see Fig. 15.1.

Both tetanus neurotoxin (TeNT), blocks release of inhibitory neurotransmitters by neurons causing the spastic paralysis seen in tetani, and botulinum neurotoxin (BoNT), blocks release of acetylcholine by neurons at the neuromuscular junction causing the flacid paralysis seen in botulism, bind gangliosides. In contrast to the aforementioned Txs (CTx, the heat labile enterotoxin of *E. coli* and Shiga-like Tx) their primary ganglioside receptor is GT1b, a ganglioside found primarily on the



**Fig. 15.1** (a) Schematic of the lipid raft portion on the outer surface of a cell's plasma membrane. The increased concentration of glycosphingolipids are indicated by sugars shown by the *circles*, *squares* and *diamonds*, possible adherence of an AB<sub>3</sub> toxin to a number of carbohydrates indicated by the round *red structure*, a transmembrane-raft-associated protein by the *black line* and a surface-associated protein by the *ellipse*. Due to the increase in sphingolipids such as gangliosides present in lipid rafts, lateral mobility within the membrane is reduced relative to that in the nonraft, phospholipid-enriched portions of the membrane. This reflects the ring structure of cholesterol and the presence of sphingosine with its single *trans* double bond and the fact that the fatty acid linked to it to form the ceramide portion of sialylated glycosphingolipids such as gangliosides is usually saturated. (b) Comparison of the hydrocarbon chain composition frequently seen in phospholipids relative to that seen in glycosphingolipids such as gangliosides. Note the *cis* double bonds in the unsaturated fatty acid often found at the two position in a phospholipid. The *trans* double bond and saturated fatty acid found in ceramides (Cer) associated with gangliosides allow for tighter packing of hydrocarbon chains than seen when unsaturated fatty acids are present. Sugars on the GSL chain are glc (●), gal (●), galNAc (■), and sialic acid (◆) (Varki et al. 2015)

plasma membranes of neural cells. While TeNT appears to just bind GT1b (Burns and Baldwin 2014), BoNT/A,B, and E (three serotypes that most commonly affect people) require a protein co-receptor as well. For BoNT/A and E it is synaptic vesicle glycoprotein 2 and for BoNT/B, synaptotagmin (Marhold et al. 2013). The enriched expression of GT1b (Rappaport 1981) on neural cells is a major factor in determining the cell specificity of these neurotoxins.

Despite availability of a vaccine for TeNT, many people in parts of the world lacking readily accessible medical care are not immunized. Due to the low prevalence of botulism in humans, people are not routinely immunized against it. TeNT acts to inhibit CNS neuronal release of inhibitory neurotransmitters which in turn disinhibits inhibition of lower motor neurons allowing the uncontrolled muscle activity that causes the symptoms of tetani. BoNT acts to block acetylcholine release at the neuromuscular junction thereby inhibiting muscle activity. These observations led to testing the ability of BoNT to reduce the uncontrolled muscle activity causing the symptoms seen in tetani. While used in only a small number of people, it was successful (Hassel 2013 for a review). Despite occasional negative results (Yiannakopoulou 2015) the neuronal specificity of BoNT and its effectiveness at inhibiting transmitter release at the neuromuscular junction has resulted in its growing use for treating nerve, muscle, and gland hyperactivity disorders caused by altered behavior of cholinergic neurons (e.g. Peng et al. 2012). As properties of the various domains of the toxin (binding, catalytic, and translocation) have become more completely understood, investigations into their use in the development of new therapies for treatment of problems such as movement disorders and muscle stiffness/spasms have evolved (Rasetti-Escargueil and Popoff 2021). The clinical significance of BoNT has influenced thought about immunizing people against accidental exposure, as that could eliminate its use to treat uncontrolled neuromuscular stimulation. In addition to the toxins discussed, a number of pathogens use carbohydrates as part of the infectious process. Table 15.2 shows examples of pathogens that may cause neural problems and carbohydrates identified as possible ligands needed for their infection of target cells.

### 3 Bacterial Infections

*Borrelia burgdorferi*, the primary spirochete causative agent of Lyme disease in the USA is transmitted by bites from the Ixodes ticks that carry it (Burgdorfer et al. 1982). The spirochetes proliferate in the guts of the ticks, become disseminated, and are then injected into humans where the infected ticks feed on their blood. While there are at least 20 different genospecies of *B. burgdorferi* sensu lato (general sense), *B. burgdorferi* sensu stricto is the most common tickborne disease in the USA (Marques et al. 2021), and one for which there is currently no prophylactic treatment (Steere et al. 2016; Lin et al. 2017). While *B. burgdorferi* can infect a variety of tissues, when it infects the nervous system it is most often seen clinically as meningitis, cranial neuritis, and radiculoneuritis (inflammation of nerve roots and

**Table 15.2** Examples of diseases caused by pathogen-induced utilization of carbohydrates when affecting neural cells

Agent	Cells infected	Common disease	Ligand/anchor
<i>Toxins</i>			
1. <sup>a</sup> Cholera toxin	Submucosal neurons	Cholera	GM1
2. LTIIa and LTIIb of <i>E. coli</i>	Neurons	Potential therapeutic to carry drugs to neurons	LTIIa: GM1 GD1b; LTIIb: GM1 GD1a
3. Botulinum neurotoxin	Neurons (PNS)	Botulism	GT1b
4. Tetanus neurotoxin	Neurons (CNS)	Tetani	GT1b
5. Shiga toxin-2	Myelin sheaths	Neuronal apoptosis	Gb3-Cer
<i>Bacteria</i>			
6. <i>Borrelia burgdorferi</i>	Neurons/Glia	Meningitis and radicular pain	Dermatan sulfate/heparin/Gal-cer
7. <i>Listeria monocytogenes</i>	Meninges/neurons	Meningitis/meningoencephalitis	Heparin/heparin sulfate
8. <i>Mycobacterium leprae</i>	Schwann cells	Leprosy	Laminin $\alpha 2$ binds trisaccharide on PGL-1
9. <i>Staphylococcus aureus</i> (methicillin-resistant)	Microglia	Meningitis/brain abscess	Heparin/heparin sulfate
<i>Bacterial epitope mimicry</i>			
10. <i>Campylobacter jejuni</i>	Peripheral neurons	Peripheral neuropathy	Gangliosides
11. Group A streptococci	Neurons	Sydenham chorea	GlcNAc, Gangliosides
<i>Protozoa</i>			
12. <i>Plasmodium falciparum</i>	Microvasculature	Cerebral malaria	Heparin
13. <i>Toxoplasma gondii</i>	Neurons	Unbalanced synaptic activity and seizures	Sialic acid-containing receptor
<i>Viruses</i>			
14. Adenovirus 37	Neurons	Keratoconjunctivitis	Glycoproteins with a GD1a motif
15. Ebola (Zaire)	Astrocytes	Ebola	Mannose-binding lectins
16. HIV	Microglia/neurons	AIDS	Glc-Cer containing GSLs plus CD4, CCR5/CXCR4
17. Influenza H5 N1	Neurons/microglia	Ataxia, tremor, bradykinesia	$\alpha 2-3/\alpha 2-6$ sialic acid; viral carbohydrates can be bound by lectins
18. JC Polyomavirus	Oligodendroglia/astrocytes	Multifocal leukoencephalopathy	$\alpha 2-6/\alpha 2-3$ sialic acid

(continued)

**Table 15.2** (continued)

Agent	Cells infected	Common disease	Ligand/anchor
19. Rabies	Neurons	Encephalitis/ paralysis	Nicotinic cholinergic receptor
20. Varicella- zoster (herpes zoster virus)	Neurons	Latent—shingles	Heparin/mann-6-P receptors
21. West Nile	Neurons	Encephalitis/flaccid paralysis	DC-SIGNR
22. Dengue type 2/3	Neurons, astrocytes, microglia	Encephalitis and encephalopathy	Neolactotetraosylceramide heparin & heparan sulfate
23. Zika	Neurons		Highly sulphated heparin
24. SARS-CoV-2	Nervous system	For example, encephalitis, encephalopathy, inflammatory- mediated neurological disorders	Sialylated glycosphingolipids
25. Cytomegalovirus	Neurons, glia	Encephalitis	Heparan sulfate
26. Semliki forest virus	Oligodendrocytes and neurons	Encephalitis	Heparan sulfate
23. Ch27. Chikungunya	Neurons (central and peripheral)	Meningoencephalitis, seizures	Sulfated glycosaminoglycans
28. Japanese encephalitis	Neurons and neural stem/ progenitor cells	Encephalitis	Glycosaminoglycans
29. Nipahvirus	Neurons	Encephalitis	Attachment and fusion glycoproteins
<i>Fungi</i>			
30. Cryptococcus neoformans	Endothelial cells between vessels and neuropil	Meningoencephalitis	Hyaluronic acid

<sup>a</sup>References for the above are 1. Fung et al. (2018); 2. Chen et al. (2015); 3. Bercsenyi et al. (2013); 4. Herreros et al. (2000); 5. Fujii et al. (1998), Takahashi et al. (2008); 6. Garcia-Monco et al. (1989), Kaneda et al. (1997) and Fischer et al. (2006); 7. Disson and Lecuit (2012); 8. Ng et al. (2000) and Rambukkana et al. (2002); 9. Naesens et al. (2009); 10. Rees et al. (1995) and Usuki et al. (2006); 11. Kirvan et al. (2003) and Cunningham (2012); 12. Boyle et al. (2010), Ramos et al. (2013); 13. Carruthers et al. (2000), Mendez and Koshy (2017), Wohlfert et al. (2017), Xing et al. (2020); 14. Nilsson et al. (2011); 15. Denizot et al. 2012); 16. Harouse et al. (1995) and Puri et al. (1998); 17. Simon et al. (2011), Liu et al. (2020); 18. Komagome et al. (2002), Neu et al. (2011) and Assetta and Atwood (2017); 19. Rustici et al. (1989); 20. Zhu et al. (1995) and Jacquet et al. (1998); 21. Davis et al. (2006); 22. Li et al. (2017), Aoki et al. (2006), Hidari et al. (2013); 23. Kim et al. (2017), Rosa-Fernandes et al. (2019), Tan et al. (2019); 24. Kim (2020), Achar and Ghosh (2020), Nguyen et al. (2021); 25. Compton et al. (1993); 26. Ferguson et al. (2015); 27. Arpino et al. (2009), McAllister et al. (2020), Silva et al. (2020); 28. Ariff et al. (2013), Wang et al. (2016), Kim et al. (2017); 29. Goh et al. (2000), Dawes and Freiberg (2019); 30. Chang et al. (2004) and Huang et al. (2011)

their accompanying peripheral nerves; Halperin et al. 2007). The ability of the bacteria to infect the CNS means that treatment requires drugs that can cross the BBB. The spirochete has been shown to adhere to extracellular matrix glycosaminoglycans (sulfated and/or carboxylated carbohydrates generally linked to a protein core, see Chaps. 1 and 5) via one of its cell surface glycosaminoglycan (GAG)-binding proteins. It has been hypothesized that pathogen binding to extracellular matrix GAGs serves to concentrate it on the cell surface thereby enhancing its ability to interact with additional receptors and infect cells (Kurre et al. 1999). The recombinant *Borrelia* GAG-binding protein identified as Bgp in studies of strain N40 D10/E9 (Parveen and Leong 2000) was shown to adhere to heparin and to inhibit binding of intact *B. burgdorferi*. Another GAG-binding protein found on *B. burgdorferi* was shown to adhere to dermatan sulfate and heparin (Fischer et al. 2006) as well as laminin (Verma et al. 2009). In addition to its ability to adhere to GAGs, earlier studies done in three different labs showed that the bacteria bound to Gal-Cer and some studies also indicated that different strains adhered to additional GSLs (Garcia-Monco et al. 1992; Backenson et al. 1995; Kaneda et al. 1997). It was also found that when virulent low-passage strains of bacteria were serially subcultured, high passage strains did not bind as well to Gal-Cer and could not infect mice (Kaneda et al. 1997). While this observation indicates the need for interaction with GSLs, the question of whether its ability to interact with GSLs is mediated by one of the GAG-binding proteins or an as yet unidentified protein has not been addressed. The variability in carbohydrates bound coupled with the identification of more than one GAG-binding protein have made developing effective prophylactics for people living in areas infested with Ixodes ticks difficult.

## 4 Viral Infection

Some viruses adhere to carbohydrates on the surface of their target cells while carbohydrates on the surfaces of others are bound by lectins expressed on the surface of the target cells. Human immunodeficiency virus (HIV) is perhaps the most widely recognized human pathogen that utilizes carbohydrates in its binding to human target cells. HIV-induced dementia affects a number of people with HIV (20–30%, Albright et al. 1999) and its CNS effects range from cognitive impairment to central diabetes insipidus (Banks et al. 2001). Gp120 shed from the surface of HIV virus particles is able to cross the BBB as is intact HIV-1 which can be taken up by brain endothelial cells and move across the BBB (Banks et al. 2001). Primary cells infected are brain microglia. Gp120 can induce neuronal cell death by apoptosis (Corasaniti et al. 2001) and induce formation of reactive oxygen species leading to neurodegeneration (Maccarrone et al. 2002). Different strains of HIV-1 have been shown to require globosylceramide in addition to CD4, CCR5, and CXCR4, for effective gp120/gp41-mediated fusion of the virus with its target cells to occur (Harouse et al. 1995). Initial evidence for this was provided by the observation that anti-Gal-Cer antibodies were able to inhibit HIV-1 entry into glioma and human

neuroblastoma cells (Harouse et al. 1991). Subsequent studies indicated that when synthesis of Glc-Cer containing GSLs, needed for Gb3 formation, was inhibited, gp120/gp41-mediated fusion of virus to target cells was inhibited. However, addition of Gb3 to the cells resulted in recovery of fusion (Puri et al. 1998) indicating that GSLs were required. This observation was used to develop effective multivalent carbohydrate inhibitors of HIV-1 fusion with its target cells *in vitro* (e.g. Rosa Borges et al. 2010). Despite the research that has gone into developing drugs that effectively inhibit HIV-1 replication in the plasma and suppress it for long periods of time, they are not effective at killing virus production within monocyte-derived-macrophage CD4 cells (Lee et al. 1999) where it integrates its genes into their chromosomes (Anderson and Maldarelli 2018). Therefore a great majority of patients have to continue on antiretroviral therapy (for a review see Aquaro et al. 2020).

Another example of viral adherence to target cell glycoconjugates is provided by human JC polyomavirus (JCP). It is a double-stranded DNA virus that when present in immunocompromised individuals can cause progressive multifocal leukoencephalopathy due to its infection of oligodendroglia (Stettner et al. 2009). Of its three capsid proteins (VP1–3), VP1 was shown to have a primary role in mediating viral attachment to cells (Goldmann et al. 1999). VP1 was found to adhere to neoglycoproteins containing terminal  $\alpha 2-3$ - or  $\alpha 2-6$ -linked sialic acid moieties with contribution from an internal  $\alpha 2-6$ -linked one as well. It was also shown to bind gangliosides GM3, GD2, GD3, GD1b, GT1b, and GQ1b found on the plasma membranes of brain cells (Bullens et al. 2003). Importance of its adherence to GT1b was confirmed when pretreatment of JCP with GT1b was found to inhibit its ability to infect IMR-32 cells by about 80% (Komagome et al. 2002). While many are non-neuronal, JCP is just one of a number of viruses that recognize sialic acid as part of their binding site (for a general review see Neu et al. 2011). Observations indicating that a human polyoma virus bound to Neu5Ac $\alpha 2,6$ LacNAc (Ströh et al. 2015), supported study of its effectiveness when presented as a mono-, di- or tetravalent glycoside ligand. Results indicated that the tetravalent glycosides were most effective and caused agglutination of the polyoma virions enabling them to be isolated by filtration (Ogata et al. 2020). These observations support further investigation of their utility as a possible treatment.

Interestingly, while most people think of influenza virus as infecting the respiratory system, the H1N1 strain responsible for the 1918 Spanish influenza pandemic induced neurological symptoms as does the very pathogenic avian influenza virus H5N1. The virus has been shown to infect CNS neurons and microglia and to induce neurodegeneration (Jang et al. 2009). After binding of H5N1 to receptors on cranial nerves it is transported axonally to the brain stem. While the binding site on hemagglutinin molecules on human influenza virions recognizes terminal  $\alpha 2-6$  linked sialic acid residues, the H5 of H5N1 can adhere to both  $\alpha 2-6$  and  $\alpha 2-3$  linked sialosyl moieties. The latter may enable it to bind not only to glycoproteins but to gangliosides such as GD1a present on the termini of cranial nerve endings (Simon et al. 2011). The presence of terminal sialic acid residues on both glycoproteins and glycolipids on the outer surface of cell plasma membranes makes them relatively accessible pathogen-binding sites. The presence of plasma membrane-associated



sialidase able to catalyze cleavage of sialyl residues on the plasma membranes of cells permits the cells to modulate sialic acid expression thereby modulating availability of those binding sites (Pshezhetsky and Hinek 2011). While people can and are immunized against influenza, unexpected changes in viral subtype expression [a total of 16 H (H1–16, H is hemagglutinin) and 9 N (N1–9, N is neuraminidase) have been identified in birds (McAuley et al. 2019)], can result in vaccination failing to effectively prevent infection. To develop an effective drug for treating influenza, researchers looked for compounds able to inhibit viral neuraminidase activity significantly more effectively than the human enzyme. Two inhibitors were identified, zanamivir (Relenza) and oseltamivir (Tamiflu). While the drugs can be effective, problems have been noted. (1) Over time naturally occurring changes in amino acids that comprise the active site of neuraminidase (more appropriately named sialidase since it doesn't catalyze removal of neuraminic acid but of one of its derivatives generically called sialic acid) have resulted in drug resistance; and (2) the drug has to be taken within 24–72 h after infection as that is the peak time for viral replication. Timing is essential in order to block enzymatic cleavage of hemagglutinin-bound progeny virions from cell surface sialic acid on host cells. Progeny release is necessary for them to be able to infect other cells. To eliminate drug and vaccine problems, the possibility of developing multi-domain antibodies against hemagglutinin capable of protecting against both influenza A and B is being investigated (Laursen et al. 2018).

Cell surface lectins that can bind mannose rich glycans present on viral cell surface oligosaccharides function as attachment sites for a number of viruses such as West Nile (Davis et al. 2006), Dengue (Fuchs et al. 2010; Misra et al. 2015), and Ebola (Brudner et al. 2013). All three of these viruses may affect neural cells causing problems ranging from encephalitis and flaccid paralysis to widespread cell apoptosis. In the CNS, mannose-binding lectins, able to recognize carbohydrates on Ebola virions, are found on astrocytes while dendritic cell-specific intercellular molecule-grabbing nonintegrin (DC-SIGN) lectins that bind mannosyl-containing oligosaccharides on all three viruses are found on perivascular cells. In contrast to Ebola and West Nile viruses, Dengue is also bound by the mannose macrophage receptor. Identification of lectin involvement in binding of these viruses has led to study of the effectiveness of lectin inhibitors as possible therapeutic agents (e.g. Idris et al. 2016) as well as studies of the use of carbohydrates to block interaction of viral proteins with host cell glycosaminoglycans (Kim et al. 2017).

Currently, the ravages of SARS-CoV-2 have caused a proliferation of studies designed to find a vaccine and/or drugs that might ameliorate its severest effects. This statement is supported by the observation that a search of PubMed listed 232 papers published under *COVID And carbohydrates*, between March 15, 2019, and January 10, 2022. Evidence indicates that lipid rafts are necessary for SARS-CoV-2 infection (Fantini et al. 2020; Sorice et al. 2021) and that in addition to binding of the spike protein to angiotensin-converting enzyme-2 (ACE2) it also binds sialic acid moieties (Wielgat et al. 2020) associated with gangliosides (Nguyen et al. 2021). The association with gangliosides was determined using electrospray ionization mass spectrometry (Wang et al. 2019). The presence of both ACE2 and

gangliosides in lipid rafts (Lambert 2009) supports the finding that lipid rafts are needed. Additional support for the need for lipid rafts is provided by the finding that the spike protein is comprised of three identical protomers (Duan et al. 2020) each of which has a sialic acid binding site with relatively low binding strength (Nguyen et al. 2021). The presence of three binding sites able to bind to gangliosides clustered in lipid rafts could result in stronger multivalent binding (Lee and Lee 1995). These observations support the hypothesis that infection by SARS-CoV2 might be prevented by a multivalent glycan ligand.

## 5 Latency

When a virus infects the CNS and is not cleared by the body it is possible that it will remain in a dormant or latent form (either not undergoing replication or replication is minimal). Varicella zoster, a human alphaherpesvirus best known as the causative agent of chickenpox, provides a well known example of such behavior. It interacts with cells by first binding to heparan sulfate proteoglycan. In order for it to enter target cells it must also be bound by a mannose 6-phosphate receptor (Zhu et al. 1995). While the symptoms associated with chickenpox are generally resolved within a few weeks, the virus may not be entirely cleared from the body. Instead it remains in a latent state in the cell bodies of certain neurons. It may then reappear many years later, frequently in stressed or immunocompromised individuals, in the form of the painful rash characteristic of shingles and/or as encephalomyelitis. Interestingly, in immunocompetent patients meningitis was the CNS presentation seen more often than encephalomyelitis (Pahud et al. 2011). While a shingles vaccine (Shingrix, Zoster Vaccine Recombinant, Adjuvanted) has been developed, the problem of latency upon infection is significant and becomes more so when people become less immunocompetent, a problem that becomes more prevalent with increasing age. The reduction in immunocompetency that can occur as one ages underscores the need for older people who have had chicken pox to get vaccinated.

Varicella zoster isn't the only CNS-affecting virus that exhibits latency. The JC polyoma virus that induces progressive multifocal leukoencephalopathy (PML, brain demyelinating disease), was found in the brains of patients without PML, prior to immunosuppression, supporting the conclusion that it was latent in them (Tan et al. 2010). For a discussion on the role of the immune system in expression of progressive multifocal leukoencephalopathy as a result of JC polyomavirus infection of the CNS see Harypursat et al. (2020). For a review discussing a number of other viruses that exhibit latency see Traylen et al. (2011).

## 6 Neural Problems Induced by Mimicry Between Pathogen and Host

Infection by *Campylobacter jejuni*, a major cause of bacterial diarrhea in the U.S., is perhaps the best known example of the negative effect expression of antibodies to bacterial cell wall oligosaccharides may have on neural function. After apparent recovery from infection by *C. jejuni* a number of patients develop Guillain–Barre syndrome (GBS, Rees et al. 1995), characterized by peripheral neuropathy. In response to the question of how an enteric bacterium that causes diarrhea can affect the nervous system, the answer is that upon infection the body produces antibodies to the lipo-oligosaccharide expressed on the bacterial wall of *C. jejuni*, and in some instances those antibodies recognize gangliosides GM1 and GQ1b (Schwerer 2002) associated with neural cells. Their adherence induces the neurological symptoms associated with GBS. Injection of the lipo-oligosaccharide into rabbits was shown to induce neuropathy similar to acute motor axonal neuropathy as well as anti-ganglioside antibodies (Moran et al. 2005). To hasten recovery from GBS, plasmapheresis can be used to dilute the antibody concentration (Meena et al. 2011). For a recent review on the significance of carbohydrate specific antibodies see Kappler and Hennet (2020).

Another bacteria whose negative effects are enhanced by this type of molecular mimicry are group A streptococci, one of which is *Streptococcus pyogenes*. With respect to carbohydrates, studies of rheumatic heart disease indicate that infection by *S. pyogenes* induces expression of antibodies to the group A carbohydrate, *N*-acetyl- $\beta$ -D-glucosamine (GlcNAc). The antibodies produced can also recognize an epitope found on lysoganglioside GM1 (GM1 lacking a fatty acyl residue, Kirvan et al. 2003). Binding of these antibodies to neuronal cell surface GM1 results in activation of Ca<sup>2+</sup>/calmodulin – dependent kinase II (CaMKII) as well as increased release of dopamine. These changes correlate with the symptoms associated with Sydenham chorea, a disorder of the CNS characterized by involuntary movements and changes in mood. Initial results have shown that iv administration of immunoglobulins ameliorated movement problems but more research is needed in this area (Cunningham 2012).

Molecular mimicry to some viral infections has also been found to induce Guillain-Barre syndrome. Among the viruses discussed in this chapter are Zika (Rivera-Correa et al. 2019), influenza (Babazadeh et al. 2019), cytomegalovirus (Orlikowski et al. 2011), and HIV (Brannagan and Zhou 2003). A small number of patients with SARS-CoV-2 have also been found to develop GBS (Toscano et al. 2020) and there was at least one report that anti-ganglioside antibodies were found (Civardi et al. 2020). The increasing numbers of bacterial and viral infections associated with development of GBS underscores the importance of pathogen cell surface carbohydrates in the bodies immune response.

## 7 Prophylactic Approaches: Under Investigation and in Use

Knowledge about the roles that carbohydrates can have in the interaction of pathogens with their target cells as well as their mechanism of action has led to development of approaches to prevent their interaction with cells, and to the study of some as therapeutic agents, some of which were referred to above. A search of the literature indicated that for viruses shown in Table 15.2 and not included in Table 15.3 there is currently no USA approved prophylactic (vaccine or drug) for that virus. It is interesting that for West Nile virus there is a vaccine approved for use in horses (Seino et al. 2007) but not people. It can be seen in Table 15.3 that use of antigens

**Table 15.3** Examples of methods used to disrupt viral-target cell interactions (not comprehensive and there are some viruses for which specific drugs or vaccines are not yet available)

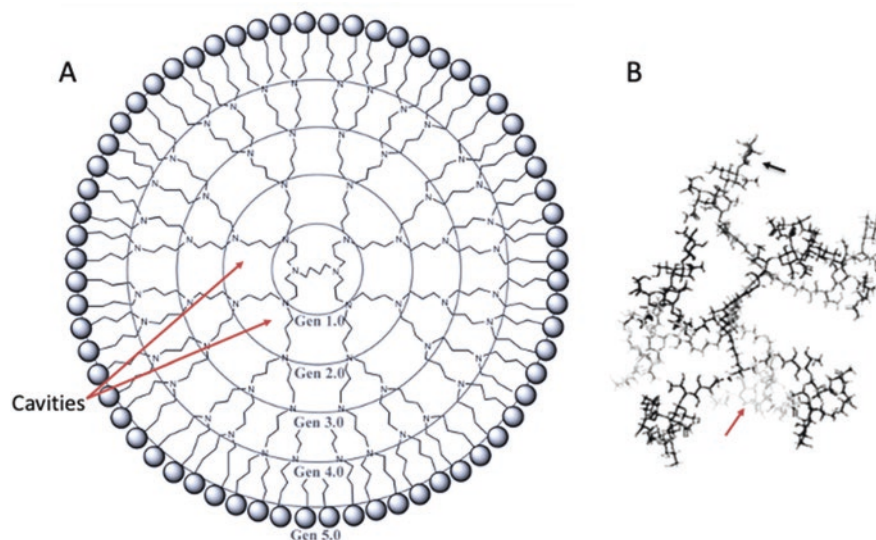
Virus	Drug	Effect on virus	Reference
Influenza	Vaccine to viral antigens	Abs bind hemagglutinin (inhibit infection)	Gomez Lorenzo and Fenton (2013)
	Oseltamivir phosphate (Tamiflu)	Sialidase inhibitor	Liu et al. (2021)
	Zanamivir (Relenza)	Sialidase inhibitor	
	Peramivir (Rapivab)	Sialidase inhibitor	
	Baloxavir marboxil (Xofluza)	Viral polymerase acidic endonuclease inhibitor	
Ebola-Zaire	Immazeb (3 monoclonal Abs, REGN-EB3)	Binds viral glycoprotein	Maxmen (2019)
	mAB114 (Ansuvimab)	Binds viral glycoprotein	
	Ervebo (rVSVΔG-ZEBOV-GP)	Ab binds viral glycoprotein	Wolf et al. (2021)
West Nile	Vaccine <sup>a</sup>	Ab binds viral E-glycoprotein	Ulbert (2019)
HIV-1	Enfuvirtide (fuzeon) <sup>b</sup>	gp41 antagonist	Aquaro et al. (2020)
	HAART (highly active antiretroviral therapy)	Inhibits viral replication	Venanzi Rullo et al. (2019)
Rabies	Vaccine	Abs to viral glycoprotein	Hicks et al. (2012)
Varicella-zoster	Vaccine	Abs to viral glycoproteins (inhibits infection)	Birlea et al. (2013)
Dengue 1–4	Vaccine (dengvaxia) use is problematic	Abs to virus	Paz-Bailey et al. (2021)
SARS-CoV2	Molnupiravir	Inhibits viral replication	Bernal et al. (2021)
	mRNA vaccines (Pfizer-Comirnaty, Moderna-Spikevax)	Inhibit function of viral spike protein	Bettini and Locci (2021)
Japanese encephalitis	Multiple vaccines (use depends on country)	Abs bind different viral proteins	Hegde and Gore (2017) (review)

<sup>a</sup>Not available for human use

<sup>b</sup>Only drug approved as of 2019 for HIV-1 as a fusion inhibitor. Targets the HIV-1 glycoprotein gp41. Distinct from anti-retroviral treatment

to induce antibody formation to viral proteins has been a major approach for preventing infection by viruses listed in Table 15.2. While many of the prophylactic approaches for viral infections rely on vaccines that induce antibodies, for many years antibiotics were the primary approach for treating bacterial infections. The appearance of antibiotic resistant strains of *Streptococcus pneumoniae* provided the needed impetus to develop vaccines directed to its capsular polysaccharides (Austrian 1989). Investigations into the development of glycoconjugate vaccines for other bacteria are ongoing (Mettu et al. 2020).

As information about the use of carbohydrate-recognition sites for cell attachment/fusion by different pathogens and their ability to function as antigens, was acquired, it was used to develop inhibitors of a variety of interactions. Many of the molecules used to carry them were branched in structure and given the name dendrimer. More specifically, the term dendrimer refers to the fact that the three dimensional molecules used have branching structures the termini of which can be derivatized. In the discussion of cholera and shiga toxin, carbohydrates were the terminal moieties of the glycodendrimers used. The advantage of using dendrimers is that it allows for use of a wide variety of molecules generally defined by the purpose they will serve. The dendrimer shown in Fig. 15.2 consists of five generations. It started with a diaminobutane core (2 arms) to which additional branches were added via repetitive reactions to yield a fifth generation dendrimer with 64 arms (Kensinger et al. 2004). For a discussion of the different types of dendrimers and



**Fig. 15.2** Dendrimer schematic. (a) A fifth generation DAB-Am poly(propylene imine) dendrimer with 64 termini (CAS nomenclature; modified from Kensinger et al. 2004); and (b) predicted lowest energy confirmation of an octa(propyleneimine) oligo-GMI-PITC dendrimer (modified from Thompson and Schengrund 1997). Arrows in (a) indicate cavities, red arrow (gray portions) in (b) indicates moieties below the plane, the black arrow (blackest structures) those above

approaches used to synthesize them see Sandoval-Yañez and Castro Rodriguez (2020) and Mousavifar and Roy (2021). The cavities that can be clearly seen in the 2-dimensional structure shown can be used to carry drugs to cells targeted by molecules carried at the end of each arm.

Crystallographic data indicating that Shiga-like toxins contained a pentameric binding subunit with three carbohydrate-binding sites per subunit arranged so that all 15 binding sites are on the same surface (Ling et al. 1998) was used in development of decavalent “starfish” dendrimers. These were comprised of a core molecule of glucose derivatized with five dimers of Gb3, one of which was linked to each glucose hydroxyl (Kitov et al. 2000).  $IC_{50}$ s obtained for Shiga-like toxins 1 and 2 binding to the dendrimers were close to  $10^{-10}$  and  $10^{-9}$  M, respectively. Examples of similar studies of multivalent saccharide ligands indicated that (1) an average of 7 oligo-GM1 moieties linked to the 8 arms of a second generation dendrimer prepared from a diaminobutane core effectively inhibited the binding of cholera toxin to GM1 on target cells (Thompson and Schengrund 1997), (2) multivalent presentation of oligo-GM3 or oligo-Gb3 inhibited binding of primary isolates of HIV to target cells (Rosa Borges et al. 2010), and (3) multibranched dendritic polymers derivatized with mannosyl residues inhibited binding of Ebola virus to DC-SIGN (Lasala et al. 2003). When considering possible ways in which carbohydrates might be used as either drugs or vaccines consideration must be given to the fact that while DNA encodes synthesis of the glycosyl transferases needed to synthesize oligosaccharides, it does not serve as a template for their synthesis *per se*. This means that while a mRNA-based vaccine can elicit an immune response to the protein it encodes, *it does not* elicit one to an oligosaccharide or the glycosylated portion of the pathogen’s glycoprotein.

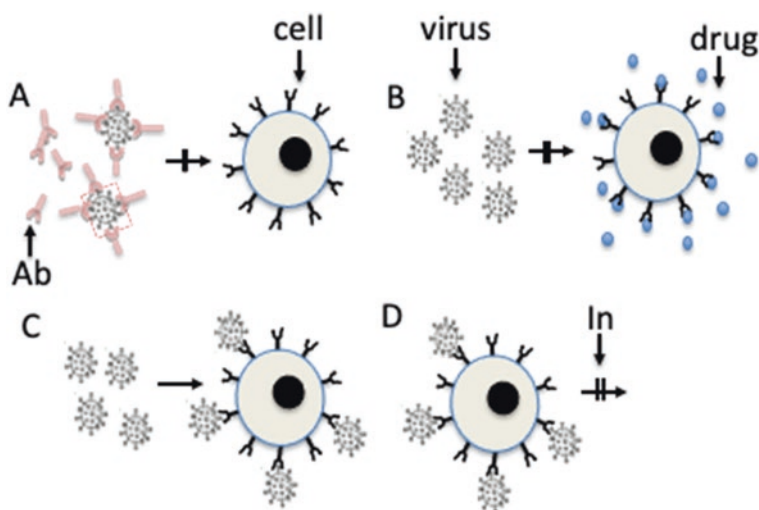
While use of multivalent ligands has confirmed the need for specific carbohydrates for adherence and subsequent fusion of a pathogen to its target cells, the use of these compounds to treat neural infections is problematic. For those using more complex carbohydrates as ligands, there is the need to obtain the carbohydrates in sufficient quantities and at costs that would make their use feasible. Another is targeting them to appropriate sites. To overcome some of the difficulties encountered using chemical methods to synthesize oligosaccharides, chemoenzymatic approaches are being used by a number of investigators (e.g. Champion et al. 2009; Muthana et al. 2009; for a comprehensive review see Li et al. 2019). In order to obtain enzymes capable of catalyzing a specific reaction investigators have used molecular approaches to engineer more active enzymes or enzymes with altered specificity. This approach was used to synthesize the neurotogenic starfish ganglioside LLG-3 (Rich and Withers 2012). To avoid the need to synthesize oligosaccharides, natural products have been evaluated for their effectiveness at inhibiting pathogen–host cell interactions. An example of this approach was the observation that cyanovirin-N, a high mannose oligosaccharide-binding protein found in blue-green algae, could adhere to sugars on the surface of HIV and Ebola thereby reducing their ability to bind and infect target cells and eliminating the need for “multivalent” oligosaccharide inhibitors (Barrientos et al. 2003).

Instead of dealing with the complexities of obtaining quantities of specific oligosaccharides, or preparing recombinant lectins, investigators have started looking for peptide mimetics. Phage display libraries, used to identify peptides (Matsubara 2012), were used to identify a peptide that could bind to a mAb (14G2a) that recognized GD2 (Horwacik et al. 2011). GD2 is an antigen associated with most neuroblastomas as well as other cancers. For children with neuroblastoma, anti-GD2 antibodies are used for diagnosis and to follow treatment response. Effective peptide mimetics of GD2 should make it possible to use them instead of GD2 for preparation of anti-GD2 antibodies. Since GD2 is one of the gangliosides identified as a binding site for JC virus, it is possible that this peptide or others mimicking the oligosaccharide portions of other GSLs might be effective inhibitors of pathogen–cell interactions. The Baker lab (Cao et al. 2022) is working to identify peptides that might function as “miniantibodies” by developing software programs able to identify peptides that can bind to specific sites on target proteins. As proof of concept they identified and produced “miniproteins” able to bind and neutralize the spike protein on SARS-CoV-2 as well as protect mice genetically engineered to be susceptible to the virus (Case et al. 2021).

In addition to the use of multivalent ligands to inhibit binding of pathogens bound by cell surface lectins (Chap. 7), investigators are looking at the possibility of using recombinant lectins as possible drugs. The hypothesis for this approach is that they would bind the virus before it interacts with target cells. An example of their potential effectiveness was provided by the observation that a sevenfold or more increase in the concentration of serum recombinant human mannose binding lectin allowed mice to survive after injection with lethal concentrations of Ebola (Michelow et al. 2011). Positive results with the use of lectins, supports research being done to develop synthetic carbohydrate receptors that might be effective against heavily glycosylated virions (Bravo et al. 2021).

Studies of the antibody response to strain HS<sub>19</sub> of *C. jejuni* that led to the molecular mimicry seen in Guillain–Barre syndrome indicated that antibodies produced in response to the infection recognized the oligosaccharide portion of GD3 reflecting the fact that the terminal 3 sugar moieties of GD3 were similar to part of the bacterial lipo-oligosaccharide. The presence of GD3 on the Schwann cell surface and in the nodes of Ranvier of the sciatic nerve (Usuki et al. 2006) makes it an available target when the body responds by producing that particular anti-lipo-oligosaccharide antibody. Using phage display Usuki et al. (2010) identified a peptide that when injected into rats is able to restore peripheral nerve function in those previously induced to develop Guillain–Barre syndrome by injection of the GD3-like lipo-oligosaccharide. This observation supports the hypothesis that the peptide might provide an additional approach for treating people with Guillain–Barre syndrome. Identification of effective peptide mimetics provides an approach for obtaining quantities of specific mimetics at prices that may be more realistic than those currently associated with obtaining similar quantities of specific carbohydrates.

A different approach has been used to inhibit cell to cell spread of influenza virus. In order for newly synthesized virions to be released from the cell surface its sialidase (N1) catalyzes cleavage of sialic acid residues from both the newly synthesized virions and the cell surface thereby removing binding sites for the hemagglutinin (H). This releases newly formed virions from the cell surface, a necessary step if they are to move and infect more cells. While N1 associated with different strains of human influenza was found to preferentially catalyze cleavage of  $\alpha$ 2–3 sialic acid linkages, it could also catalyze cleavage of those linked  $\alpha$ 2–6 albeit somewhat more slowly (Mochalova et al. 2007). The requirement for sialidase activity led to analysis of its sialic acid-binding site, knowledge of which was then used in development of inhibitors of its action. Inhibitors of N1 currently in use, e.g. Relenza and Tamiflu, were developed by modifying sialic acid with either a positively charged guanidinium or ammonium substituent on C4. The problem with these drugs, especially Tamiflu, is that resistant strains are starting to appear. To counteract this, mechanism-based difluoro-inhibitors having the same substituents at C4 but with fluorines at C2 and 3 were developed and found effective at suppressing viral replication and prolonging survival of mice exposed to the virus intranasally (Kim et al. 2013). A schematic showing the various approaches currently under investigation to prevent association and fusion or dissociation of virions from their target cells is shown in Fig. 15.3.



**Fig. 15.3** Schematic of prophylactic approaches. (a) Effect of an antiviral antibody on binding of the virion to its target cell; (b) inhibition of viral binding by a drug designed to block cell surface binding sites; (c) binding in the absence of a prophylactic; and (d) inhibition of release of newly synthesized virions from infected cells



## 8 Targeting Drugs to the CNS

Targeting drugs to the CNS requires their transport across the blood–brain barrier (BBB) in which tight junctions between cerebral endothelial cells restrict access (see Bigbee, Chap. 2). Research has shown that this problem may be circumvented by linking the drug to a vector that can bind to luminal surface receptors on cerebral endothelial cells and be transcytosed across the BBB. The fact that the carboxyl-terminal portion of the heavy chain of tetanus toxin (TTC) can be retrogradely transported and enter motor neurons led to its use as a carrier of proteins such as superoxide dismutase, and glial-derived neurotrophic factor and brain derived neurotrophic factor to them. This approach is seen as a possible means for delivering drugs needed for treatment of motor neuron diseases resulting in muscle paralysis (Calvo et al. 2013). In addition to the use of TTC, antibodies and peptides have also been used to target drug-carrying nanoparticles across the BBB. For example, the anti-transferrin antibody, OX26, has been found to be an effective vector for this purpose (e.g. Bao et al. 2012) as has the GM1 binding peptide, G23, although it is targeted to the lungs as well (Stojanov et al. 2012). While much of the research done with nanoparticles [e.g. dendrimers, liposomes, and neoglycoliposomes (for a review see Mousavifar et al. 2021)] as drug carriers has been aimed at targeting cancer therapeutics, as microorganisms become more resistant to drugs currently available, and new neural pathogens evolve, it may become necessary to develop new drugs, such as those that inhibit the carbohydrate–protein interactions needed for infection, as well as approaches for targeting them to the CNS.

As our understanding of how viruses interact with cells has progressed, investigators have started to look at how viruses able to infect neural cells could be used to deliver genes to patients suffering from neurological diseases. The identification of different cell receptors for adenoviruses has allowed for development of specific adenovirus-based gene delivery systems (Cupelli and Stehle 2011). Adeno-associated viral vectors have proven useful as depending upon viral capsid type, they can (1) infect a number of different CNS cell types, (2) they induce relatively few side-effects, (3) gene transfer is efficient, and (4) transgene expression is long-lasting (for a review see Lentz et al. 2012). In addition to using viruses as gene delivery systems, a harmless strain of *E. coli* having a mutation that resulted in truncation of its lipopolysaccharide (LPS) was genetically manipulated to express specific glycosyl transferases able to catalyze addition of saccharides to the outer core region of the LPS to yield oligosaccharides recognized by the Shiga-like toxin receptor. When the modified bacteria were used to inhibit infection in mice by Shiga toxin *E. coli* expressing bacteria they were found to be 100% effective (Paton et al. 2010). While the modified *E. coli* were used to treat an enteric infection, the two examples presented indicate that as more is learned about how various pathogens act, we may be able to use them to help treat/prevent disease. Interestingly this type of receptor mimicry is being considered as a possible therapeutic approach for treating agents considered as biothreats (Thomas 2010).

Due to the difficulties of preparing drugs that can be transported across the BBB, different routes of administering drugs are being used/tested. Nasal administration of a drug permits it to avoid the gastrointestinal tract or clearance by the kidneys, and to enter the brain directly via olfactory and trigeminal sensory neurons. Potential detriments to this approach include amount of drug that can be given and its interaction with mucous and mucocilliary clearance. For a more detailed discussion of the pros and cons of delivery methods see Dong (2018).

A novel approach was recently described in which drugs were given via intrathecal injection of the glymphatic system, a network that promotes fluid exchange between cerebral spinal fluid and CNS interstitial fluid. It serves to both clear molecules from and transport them to the CNS. Because this system is active during sleep (Xie et al. 2013), lilius et al. (2019) hypothesized that intrathecal co-administration of drugs and dexmedetomidine (sedative) would increase drug uptake by the CNS. Upon testing in rats, they found this to be correct. It is a drug delivery possibility to consider when looking for appropriate prophylactics for treatment of diseases caused by carbohydrate based interactions.

## 9 Conclusions

From the foregoing discussion, it can be seen that a number of pathogens initiate their interaction with target cells by either binding to a cell surface carbohydrate residue or by having a carbohydrate on their surface bound by a cell surface lectin. In many instances these interactions are strengthened by the fact that they are “multivalent”, information being used in the development of effective inhibitors of their binding. As more is learned about steps involved in the binding and release of pathogens, the use of ligands targeted to block specific steps in the infectious process should increase as should our ability to use this knowledge in the treatment of people with neural diseases. The need for development of methods for treating viral diseases in general is underscored by the recent computer identification of over 100,000 new viruses including nine that are coronaviruses (Edgar et al. 2022).

**Compliance with Ethics Requirements** Cara-Lynne Schengrund declares that she has no conflict of interest and that she has used no human subjects in work cited that was done in her laboratory.

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## Chapter 16

# Antibodies to Glycolipids in Guillain-Barré Syndrome, Miller Fisher Syndrome and Related Autoimmune Neurological Diseases



Susumu Kusunoki

**Abstract** Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS) are acute immune-mediated neuropathies, often preceded by an infection. Anti-glycolipid antibodies are frequently detected in patients' sera in the acute-phase. In particular, IgG anti-GQ1b antibodies are positive in as high as 90% of MFS cases. Anti-glycolipid antibodies are useful for the diagnosis of GBS and MFS. In addition, those antibodies may be directly involved in the pathogenetic mechanisms by binding specifically to the regions where the target glycolipid antigen is densely localized. This was proven by the development of animal models of anti-glycolipid antibody-mediated neuropathies. The presence of antibodies that specifically recognize a new conformational epitope formed by two gangliosides (ganglioside complex) in the acute-phase sera of some GBS patients suggested existence of a carbohydrate-carbohydrate interaction between glycolipids. Further intensive research is needed to clarify this point.

**Keywords** Guillain-Barré syndrome · Miller Fisher syndrome · Ganglioside · Neuroimmunology · Peripheral nerve · Autoantibody

## Abbreviations

AIDP	Acute inflammatory demyelinating polyneuropathy
AMAN	Acute motor axonal neuropathy
BBE	Bickerstaff brainstem encephalitis
CIDP	Chronic inflammatory demyelinating polyneuropathy

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CNS	Central nervous system
Gal-C	Galactocerebroside
GBS	Guillain-Barré syndrome
GSC	Ganglioside complex
MFS	Miller Fisher syndrome
MMN	Multifocal motor neuropathy
PNS	Peripheral nervous system
SGPG	Sulfated glucuronyl paragloboside

## 1 Introduction

There are a group of neurological diseases caused by autoimmune mechanisms. Guillain-Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP) and IgM paraproteinemic neuropathy affect the peripheral nervous system (PNS) through autoimmune mechanisms and are therefore categorized as autoimmune neuropathies.

GBS is an acute neuropathy, in which weakness in the four limbs is the predominant symptom. Miller Fisher syndrome (MFS) is a variant of GBS, in which ophthalmoplegia and ataxia are the predominant symptoms. GBS and MFS share several characteristics: frequent presence of antecedent infection involving the respiratory or gastrointestinal tract, an acute and self-limited clinical course, and albuminocytological dissociation in cerebrospinal fluid.

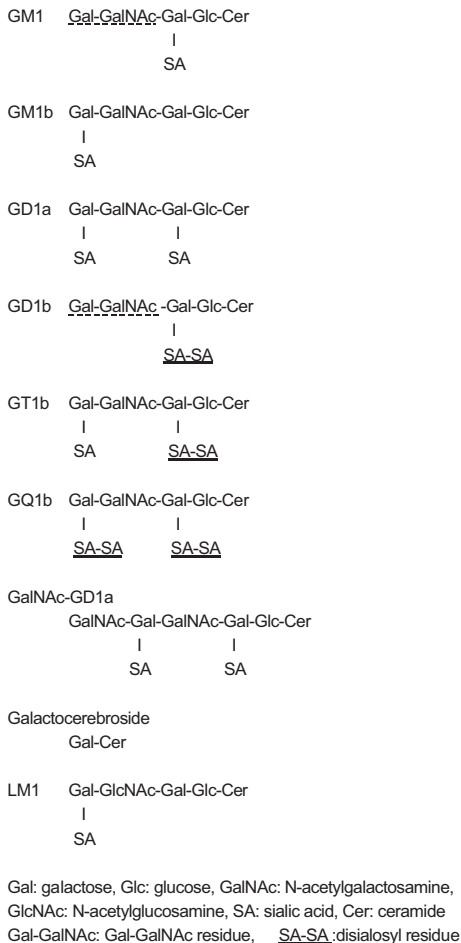
Anti-glycolipid antibodies are frequently present in the acute phase sera from patients with GBS and MFS (Kusunoki 2000; Kaida and Kusunoki 2010). There are diversities in the carbohydrate sequences of the glycolipids (Fig. 16.1). Gangliosides are the glycolipids with sialic acid(s) included in the carbohydrate sequence. Each glycolipid has a unique distribution within the PNS. Anti-glycolipid antibodies can be useful markers for diagnosing both GBS and MFS. Because glycolipids are localized in the plasma membrane with their carbohydrate portions extended to the extracellular space, anti-glycolipid antibodies may bind to those epitopes and cause neuropathy. The antibody titers are highest in the acute phase and decrease with clinical improvement (Kusunoki 2000). It indicates that the elevation of the anti-glycolipid antibody titer may not be a result of damage to the peripheral nerve but may be directly associated with the pathogenetic mechanisms.

This chapter focuses primarily on the anti-glycolipid antibodies in GBS and MFS. In addition, the anti-glycolipid antibodies in CIDP and diseases affecting the central nervous system (CNS) are briefly described in the last part.

## 2 Antibodies to Glycolipid Antigens in GBS and MFS

The effectiveness of plasma exchange in the treatment of GBS was established in the 1980's. It suggested that autoantibodies binding to the peripheral nervous system are probably involved in the pathogenetic mechanisms of GBS.

**Fig. 16.1** Carbohydrate sequences of glycolipids



Antibodies to the carbohydrate epitopes in autoimmune neuropathies were first reported for the IgM paraproteinemic neuropathy (Braun et al. 1982). The IgM M-protein was shown to bind to myelin-associated glycoprotein (MAG). After that, a unique glycolipid was also reported to be recognized by the same IgM M-protein because the sugar chains containing the sulfated glucuronyl epitope were shared by MAG and the glycolipid, later identified as sulfated glucuronyl paragloboside (SGPG) (Ariga et al. 1987). Subsequently, the frequent presence of anti-GM1 IgM antibody in multifocal motor neuropathy (MMN), considered as a clinical variant of CIDP, was reported (Pestronk et al. 1988). These reports indicated that the antibodies against carbohydrate epitopes are involved in the pathogenesis of autoimmune neuropathies.

Following the above reports, the presence of anti-glycolipid antibodies in the acute-phase sera from GBS patients was reported in 1988 (Ilyas et al. 1988). In that paper, anti-glycolipid antibodies were detected in 5 of 26 cases of GBS. IgG

anti-LM1 antibodies were detected in sera from one patient, IgG anti-GD1b antibodies in sera from two, and IgM antibodies to GD1a and GT1b from two. Since then, the association between anti-glycolipid antibodies and GBS has been extensively investigated (Kusunoki 2000; Kaida and Kusunoki 2010).

## 2.1 *IgG Anti-GM1 Antibody*

IgG anti-GM1 antibodies in GBS were first reported in 1990 (Yuki et al. 1990), and subsequently many papers reporting their presence in GBS were published (Kusunoki 2000; Kaida and Kusunoki 2010).

PNS myelin was originally considered to be the only target affected in GBS and acute inflammatory demyelinating polyneuropathy (AIDP) had been used as a synonym for GBS before the 1980s. Around 1990, the presence of a GBS subtype in which the primary target is the axonal membrane, rather than myelin, became recognized and that subtype was referred to as acute motor axonal neuropathy (AMAN). The patients with IgG anti-GM1 antibodies had predominantly AMAN type phenotype and frequently had a preceding *Campylobacter jejuni* infection.

The lipooligosaccharide from GBS patients that had an infection with *C. jejuni* preceding development of AMAN due to the presence of IgG anti-GM1 antibodies, was shown to have a carbohydrate sequence similar to that of GM1 (Yuki et al. 1993). The production of the anti-GM1 antibodies was therefore considered to be due to immune reactions to the carbohydrate epitopes of *C. jejuni*, suggesting a “molecular mimicry” mechanism.

## 2.2 *IgG Anti-GQ1b Antibody*

In 1992, we reported the detection of the IgG anti-GQ1b antibodies in sera drawn from patients in the acute-phase of MFS (Chiba et al. 1992). The antibodies were present in sera from each of the 6 MFS patients examined in the first paper. Additional studies showed that IgG anti-GQ1b antibodies were present in sera from about 90% of the patients with MFS. IgG anti-GQ1b antibodies were also frequently detected in diseases which had pathogenetic mechanisms similar to those of MFS, such as GBS with ophthalmoplegia and/or ataxia, atypical MFS showing only ophthalmoplegia or ataxia, and Bickerstaff brainstem encephalitis (BBE), which has clinical characteristics of MFS and central nervous system involvement (as described below in Sect. 7.1). The elevation of serum anti-GQ1b IgG antibody titers is therefore closely associated with acute ophthalmoplegia and/or ataxia caused by pathogenetic mechanisms similar to those underlying GBS (Chiba et al. 1993). The discovery of the anti-GQ1b antibody strongly supported the idea that the anti-glycolipid antibodies are specifically associated with the pathogenetic mechanisms of GBS and related disorders. Biochemical analyses of ganglioside fractions from human cranial nerves and spinal nerve roots (ventral and dorsal roots) showed that the cranial nerves innervating



extraocular muscles (oculomotor, trochlear and abducens nerves) had a relatively higher content of GQ1b than the other cranial and peripheral nerves (Chiba et al. 1997). A monoclonal anti-GQ1b antibody specifically immunostained paranodal myelin of cranial nerves innervating ocular muscles (Chiba et al. 1992, 1993) and a subset of large primary sensory neurons (Kusunoki et al. 1999a). The binding of the anti-GQ1b antibodies to those regions may be involved in the pathogenesis of ophthalmoplegia and ataxia experienced by MFS patients.

Respiratory infections most frequently precede the neurological onset of IgG anti-GQ1b-induced symptoms in positive patients, while an antecedent infection by *C. jejuni* is found in some. The production of IgG anti-GQ1b antibodies in GBS and MFS preceded by *C. jejuni* infection was also shown to be due to the molecular mimicry mechanism. A genetic polymorphism related to the production of polysaccharides by *C. jejuni* was reported to determine the antibody reactivity (e.g. anti-GM1 or anti-GQ1b) (Koga et al. 2005).

The possible association of anti-GQ1b antibody and respiratory paralysis in GBS was subsequently reported (Kaida et al. 2004a). Binding of the antibody to the distal motor nerve terminals of neuromuscular junctions (Plomp et al. 1999) may be related to respiratory failure, as described in Sect. 3.

### 2.3 *IgG Anti-GD1b Antibodies*

GD1b has both a galactosyl-N-acetyl galactosaminyl (Gal-GalNAc) epitope and a disialosyl epitope (Fig. 16.1). When an antibody binds to the Gal-GalNAc epitope, that antibody can also bind to GM1 because GM1 also has the Gal-GalNAc epitope. Patients expressing an antibody to Gal-GalNAc mostly exhibit a clinical phenotype similar to that of the anti-GM1 antibody-positive patients, pure motor or AMAN type GBS. In contrast, when the patients have anti-GD1b antibody without anti-GM1 reactivity, they have different clinical features. It was reported that GBS patients having IgG anti-GD1b antibodies without other anti-glycolipid antibodies had sensory disturbances and none had the primary axonal form (Miyazaki et al. 2001). GD1b was shown to be localized in large neurons in dorsal root ganglia (DRG) and paranodal myelin of human PNS (Kusunoki et al. 1993). Clinical features of GBS patients with monospecific anti-GD1b IgG antibodies may be related to the localization of GD1b in the human PNS. The association of antibodies specific to GD1b and ataxia is described in Sects. 3 and 5.

### 2.4 *IgG Antibodies Against GalNAc-GD1a and GM1b*

Gangliosides such as GM1, GD1a, GD1b and GT1b are the major gangliosides in the nervous system and therefore antibodies against those antigens were examined in early studies. However, as GQ1b, a relatively minor component, proved to be an important target antigen, we considered that other minor unidentified gangliosides

might also be targets for serum antibodies. Serum antibody activity against a crude ganglioside fraction from bovine brain was examined using thin-layer chromatogram immunostaining. As a result, the IgG antibodies in sera from some GBS patients specifically and strongly bound to an antigen that migrated just below a large amount of GD1a. Interestingly, unlike GD1a, that antigen was resistant to treatment with *Clostridium perfringens* neuraminidase, (catalyzes hydrolysis of GD1a to yield GM1). Therefore, after removing GD1a by treatment with *Cl perfringens* neuraminidase, the antigen was isolated by column chromatography and identified as GalNAc-GD1a using fast atom bombardment-mass spectrometry (Kusunoki et al. 1994).

Using a similar methodology, GM1b, another minor ganglioside component, was also shown to be a target antigen for IgG antibodies found in sera from acute-phase GBS patients (Kusunoki et al. 1996a). Both IgG anti-GalNAc-GD1a antibodies and IgG anti-GM1b antibodies were mostly detected in sera from patients with pure motor or AMAN type GBS.

## 2.5 Antiglycolipid Antibodies in AIDP

As described above, anti-glycolipid antibodies, particularly anti-ganglioside antibodies, are frequently detected in the acute-phase sera from patients with AMAN. In contrast, anti-glycolipid antibodies have been detected less frequently in sera from AIDP patients. Identification of the target molecule recognized by antibodies found in sera from patients with AIDP is a question researchers need to address. Towards that end, a few reports have been published about anti-glycolipid antibodies being associated with AIDP.

### 2.5.1 Anti-galactocerebroside (Gal-C) Antibody

During routine antibody assays on sera from patients with autoimmune neuropathies, we found an association between anti-Gal-C antibody and infection by *Mycoplasma pneumoniae* (Kusunoki et al. 1995). As described in the section about animal models, rabbits sensitized with Gal-C were reported to be affected with a demyelinating neuropathy (Saida et al. 1979). This makes it apparent that for the role of anti-Gal-C antibodies and neuropathy, an animal model was developed first and antibodies in sera from patients described later.

Pre-incubation of the patients' sera with *M pneumoniae* reagent, derived from *M pneumoniae* and used for the serological diagnosis, significantly inhibited anti-Gal-C antibody activity in GBS patients after mycoplasma infection. *M pneumoniae* was shown to express several glycolipids recognized by the anti-Gal-C antibody (Kusunoki et al. 2001). The association between the presence of anti-Gal-C antibodies and the demyelinating type of GBS was reported (Samukawa et al. 2016). This observation supported the previous suggestion that when GBS is preceded by

infection with *M pneumoniae*, the immune reaction against *M pneumoniae* may induce production of anti-Gal-C antibodies and that those antibodies may be involved in the pathogenesis of demyelinating disease (Kusunoki et al. 2001). This is another example of molecular mimicry in GBS.

### 2.5.2 Anti-LM1 Antibody

LM1 was shown to be the most abundant component in the glycolipid fraction from human peripheral nerve myelin. It was reported that some patients had IgG antibodies against LM1. GBS cases with such antibody activities had primarily AIDP symptoms (Kuwahara et al. 2011). Therefore, LM1 is among the target antigens in the sera from patients with GBS, mainly AIDP.

Anti-glycolipid antibodies in GBS and MFS, localization of glycolipid antigens and associated clinical features are listed in Table 16.1.

## 3 Animal Models

The antibodies to glycolipids, including gangliosides, are useful diagnostic markers of GBS. In contrast, it was an issue of controversy whether anti-ganglioside antibodies were pathogenetic autoantibodies in GBS. Now, at least some of the anti-glycolipid antibodies are considered to participate in the pathogenesis because anti-glycolipid antibody-mediated *in vivo* animal models have been developed.

**Table 16.1** Anti-glycolipid antibodies in GBS and MFS (localization of glycolipid and associated clinical features)

Glycolipid	Class	Localization of antigen	Clinical features
GQ1b	IgG	Paranodal myelin of cranial nerves III,IV,VI some neurons in DRG	MFS, GBS with ophthalmoplegia and/or ataxia
		n.d.(within CNS)	BBE
GD1b(monospecific)	IgG	Paranodal myelin	AIDP
		Large neurons in DRG	Ataxic GBS
GM1	IgG	Nodes of Ranvier	AMAN, Pure motor GBS
			Pure motor GBS
GD1a	IgG	n.d.	AMAN
GalNAc-GD1a	IgG	Periaxonal membrane	AMAN, Pure motor GBS
GM1b	IgG	n.d.	AMAN
LM1	IgG	Myelin in PNS	AIDP, CIDP
Gal-C	IgG	Myelin in PNS and CNS	AIDP, encephalomyelitis

*n.d.* not determined

An animal model with apparent neurological symptoms and signs was first reported by Nagai et al. They sensitized rabbits with GM1 and GD1a (Nagai et al. 1976). In that paper, descriptions of the neurological symptoms and signs, serological examinations, and pathological investigations could not be evaluated in detail. However, we can say that their work paved the way for later research of autoimmune neuropathies with anti-ganglioside antibodies.

Saida et al. sensitized rabbits with Gal-C, a major glycolipid both in the central and peripheral nervous systems. Flaccid paresis and hypesthesia of four limbs were observed in 13 of 31 sensitized rabbits (Saida et al. 1979). Some of the rabbits showed respiratory paresis. Electrophysiological studies showed multifocal conduction block and reduction of motor conduction velocities. Multifocal demyelinating lesions with macrophage infiltration in the PNS were shown pathologically. Intraneural injection of rats with rabbit anti-Gal-C serum induced demyelinating neuropathy, which was shown to be complement mediated. This animal model, reported in 1979, is the first established animal model of an autoimmune neuropathy mediated by an anti-glycolipid antibody. Although the pathogenetic roles of anti-Gal-C antibodies were clearly shown by these works, clinical relevance was not fully recognized at that time because a significant association between anti-Gal-C antibody and human diseases had not been reported. As described above, it wasn't until 1995 when the presence of anti-Gal-C antibodies in GBS subsequent to mycoplasma infection was reported that the association was made (Kusunoki 1995).

Gal-C, which has no sialic acid, is not a ganglioside but a neutral glycosphingolipid. Even after publication of the animal model of demyelinating neuropathy caused by anti-Gal-C antibodies, there was no established animal model of anti-ganglioside antibody-mediated neuropathy. One reason for the difficulty of developing such an animal model was the difference in the distribution of individual gangliosides from species to species. It was reported that IgM M-protein binding to gangliosides with a disialosyl residue, such as GD1b, was associated with sensory ataxic neuropathy. GD1b is densely localized in the large neurons in human DRG (Kusunoki et al. 1993), suggesting that GD1b could be a target molecule for autoantibodies in sensory ataxic neuropathy. Because GD1b ganglioside is similarly localized in rabbits, we sensitized rabbits with GD1b ganglioside and were able to successfully develop an animal model with robust neurological signs (Kusunoki et al. 1996b). Muscle power was not affected but the rabbits showed awkward movements. Pathologically, there was axonal degeneration in the dorsal root and dorsal column of the spinal cord, showing involvement of large primary sensory neurons mediating deep sensation, whereas the ventral root, consisting of motor nerves, was completely intact. The affected rabbits were diagnosed with sensory ataxic neuropathy pathologically as well as clinically. Titers of anti-GD1b antibodies were increased with no lymphocytic infiltration observed in the affected areas, indicating that the autoantibodies play crucial roles. Further examinations showed that IgG antibodies monospecific to GD1b were the main causative factor in this animal model (Kusunoki et al. 1999b). Passive transfer of the antiserum from the affected rabbits to control rabbits caused neuropathological changes similar to those

observed in the affected animals (Kusunoki et al. 1999c). This rabbit model of GD1b-induced sensory ataxic neuropathy (GD1b-induced SAN), was the first established animal model of an anti-ganglioside antibody-mediated autoimmune neuropathy (Kusunoki et al. 1996b). Apoptosis, identified by TUNEL assay and immunohistochemistry using an anti-caspase 3 antibody, was shown to occur in a subset of DRG neurons from affected rabbits. TUNEL positivity was found in large diameter neurons. Therefore, apoptosis of the large primary sensory neurons subsequent to the binding of the GD1b-specific antibodies was considered to be one important pathogenetic mechanism of GD1b-induced SAN (Takada et al. 2008).

Rabbits sensitized with either a bovine brain ganglioside mixture (BBG) or purified GM1 were reported to develop acute motor neuropathy (Yuki et al. 2001). The affected rabbits had anti-GM1 antibodies. Pathological findings showed axonal degeneration with neither lymphocytic infiltration nor demyelination. This study indicated that anti-GM1 antibodies are a causative factor and this model should be a useful animal model for investigation of AMAN. Immunohistochemical investigation of the nodes of Ranvier of a BBG-induced motor axonal neuropathy model showed that voltage-gated sodium channel clusters were disrupted or disappeared from nodes in the acute phase. The deposition of IgG and complement products was observed (Susuki et al. 2007). Combined the results supported the conclusion that BBG-induced motor axonal neuropathy is a complement-mediated disease.

Results obtained using the mouse hemi-diaphragm model indicated that anti-GQ1b antibodies were able to bind and disrupt presynaptic motor nerve terminals at the neuromuscular junction (NMJ) (Plomp et al. 1999). Mice injected intraperitoneally with anti-GQ1b antibody followed by an intraperitoneal injection of normal human serum, as a source of complement, showed breathing difficulties (Halstead et al. 2008). Patients with pure MFS do not develop respiratory weakness, but anti-GQ1b antibody-positive GBS patients more frequently need mechanical ventilation than anti-GQ1b-negative patients (Kaida et al. 2004a). Therefore, this mouse model can be said to be a model of GBS with respiratory insufficiency. It was also reported that eculizumab, which blocks the formation of human C5a and C5b-9, protected mice from respiratory paralysis in this model by preventing complement-mediated damage at motor nerve terminals (Halstead et al. 2008). This work provides us with the rationale for the clinical trials of anti-complement therapy for GBS. The clinical trials of eculizumab have so far presented hopeful results. Anti-complement therapy can possibly be a novel therapeutic method for severe and intractable GBS.

#### **4 Antibodies to a Combination of Ganglioside and Phospholipid**

Glycosphingolipids are localized in the plasma membrane, where they are closely surrounded by phospholipids. Therefore, possible effects of phospholipids on the activity of anti-glycolipid antibodies were examined.

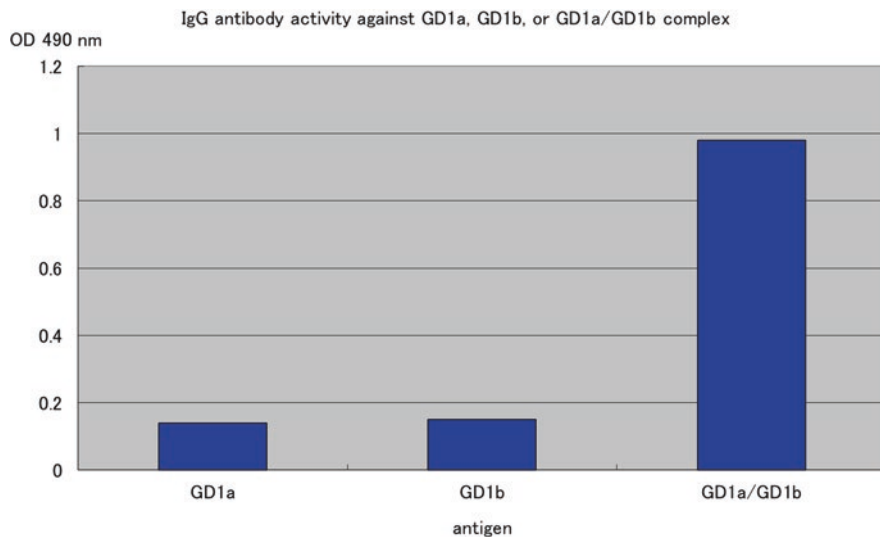
The effect of phosphatidic acid (PA) on the antibodies was investigated first. The results showed that PA had an enhancing effect on the activity of IgG anti-GM1 antibodies in GBS. Some patients' sera showed no antibody activity to GM1 alone but strong binding activity to a mixture of GM1 and PA by ELISA (Kusunoki et al. 2003). Subsequently, the effects of phospholipids other than PA on the IgG anti-GM1 antibody activities were examined. The results showed that phospholipids such as phosphatidyl inositol (PI) and phosphatidyl serine (PS) as well as PA had enhancing effects, but sphingomyelin (SM) was inhibitory. As for IgG anti-GQ1b antibodies, the enhancing effects of PA, PI, and PS were not significant while SM inhibition was seen. (Hirakawa et al. 2005).

Possible explanations for the inhibitory effect of SM on the binding activity of IgG antibodies to ganglioside epitopes is that the polar head group of SM alters the local charge thereby inhibiting antibody binding. To investigate the pathogenetic role of anti-glycolipid antibodies in autoimmune neuropathies, more attention should be focused on the effects of phospholipids that surround the glycolipids in cell membranes of the nervous system.

## 5 Antibodies to Ganglioside Complexes in GBS

Gangliosides tend to form clusters in the plasma membrane (Hakomori 2002). In the clusters, the carbohydrate structure of a ganglioside may interact with that of another to form a novel epitope. Some GBS patients have serum antibodies that specifically recognize novel glycoepitopes formed by two individual ganglioside molecules. We named such antibodies as "anti-ganglioside complex (GSC) antibodies" (Kaida et al. 2004a, b).

How we found the presence of anti-ganglioside complex antibodies is as follows. We investigated serum from a patient with acute severe flaccid tetraparesis by an enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatogram (TLC)-immunostaining. ELISA results were negative for each of the gangliosides tested (GalNAc-GD1a, GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, and GQ1b). However, we found an unidentified immunoreactive band in the position just below GD1a on TLC of a crude ganglioside fraction from bovine brain. Upon examination of the binding activity of that serum IgG to a mixture of GD1a and GD1b by ELISA, the serum IgG was found to bind strongly to the well coated with a mixture of GD1a and GD1b (GD1a/GD1b). In TLC-immunostaining using a developing solvent of chloroform/ methanol/0.2%CaCl<sub>2</sub>-2H<sub>2</sub>O (50:45:10), positive staining was present in the lane in which both GD1a and GD1b were developed, but not in the lanes in which GD1a or GD1b were developed separately. Immunostaining was only present in the overlapping portion of GD1a and GD1b (Fig. 16.2). When another developing solvent (C/M/0.2%CaCl<sub>2</sub>-2H<sub>2</sub>O, 30/65/10) was used that completely separated the GD1a from GD1b, the immunostaining disappeared (Kaida et al. 2004b). Mixing GD1a and GD1b may produce a new conformational glycoepitope which is



**Fig. 16.2** ELISA result for a serum with IgG antibodies against a GD1a/GD1b complex. The antibody activities were expressed as optical density (OD) values. IgG in this serum had strong reactivity against a mixture of GD1a and GD1b (GD1a/GD1b) but only a subtle reaction with either one alone

different from that of GD1a or GD1b alone and that the antibody specifically recognizes.

The anti-GD1a/GD1b-positive serum antibodies also recognized GM1/GD1a, GM1/GT1b, and GD1b/GT1b. All of the 4 antigens are combinations of a ganglioside with a terminal galactosyl epitope and one with a sialyl-galactosyl epitope (Fig. 16.1). Therefore, it can be said that the antibodies recognized the combined epitope of carbohydrate sequences.

Most of the sera from MFS patients have IgG antibodies to GQ1b, as described above. However, when binding of the anti-GSC antibodies were examined, some MFS sera had higher binding activities against a combination of GQ1b and another ganglioside than against GQ1b alone. MFS sera could be categorized into 3 patterns: those specific to (1) GQ1b alone, (2) GQ1b/GM1, and (3) GQ1b/GD1a (Kaida et al. 2006).

The reactivity of the monospecific anti-GD1b IgG against a mixture of GD1b and another ganglioside was also examined. If the interaction of two gangliosides creates new epitopes with conformational changes, the binding activity of the antibody highly specific to one ganglioside may be decreased by the addition of another ganglioside. The results showed that the reactivity of the IgG antibody monospecific to GD1b in the routine antibody assay was not affected by the addition of GM1, GM2, GM3 and GA1 (Group A), but strongly inhibited by the addition of GD1a, GD3, GT1a, GTb, GQ1b and GalNAc-GD1a (Group B) (Kaida et al. 2008). Thus, the GD1b may interact with Group B gangliosides (but not with Group A

gangliosides) to form a novel epitope, not recognized by the highly specific anti-GD1b antibody. This result provides indirect evidence for the carbohydrate-carbohydrate interaction between two different gangliosides or their aggregation may provide a better ligand for antibody binding. In addition, the percent reduction of antibody binding was different between patients with ataxia and those without: binding after addition of Group B gangliosides was significantly more reduced in ataxic patients than in those without ataxia. This not only suggests that the anti-GD1b IgG antibodies in ataxic patients may be more specific to GD1b, it further confirmed the association between anti-GD1b antibody and sensory ataxic neuropathy.

The number of glycolipid complexes that are able to be examined by standard ELISA is limited. It is possible that there are antibodies to still unidentified glycolipid complexes that are present in acute-phase sera from GBS patients. To investigate antibody activities to a number of glycolipid complexes more efficiently, a combinatorial glycoarray method was recently developed (Rinaldi et al. 2013). Using this method, we found 4 of 100 patients with GBS had IgG antibodies that only recognized a GQ1b/sulfatide complex, not adhering to GQ1b or sulfatide alone. All of the four patients had ophthalmoplegia (Morikawa et al. 2016).

In another study, of 63 patients with GBS with ophthalmoplegia, 31 patients had IgG antibody activities not only to GQ1b and GSCs containing GQ1b (GQ1b-related antibodies) but also to GD1b and GSCs containing GD1b (GD1b-related antibodies). It was found that those patients with both GQ1b-related antibodies and GD1b-related antibodies required mechanical ventilation more frequently (Yoshikawa et al. 2018). Those antibody activities can possibly be used as a prognostic biomarker of GBS.

## 6 Anti-glycolipid Antibodies in CIDP

IgM anti-GM1 antibodies are detected in the sera from patients with multifocal motor neuropathy (MMN), a clinical variant of CIDP. In contrast, anti-glycolipid antibodies are usually not detected in sera from patients with typical CIDP.

Recently, we found some CIDP patients, as well as AIDP patients, had IgG antibodies against LM1 and LM1-containing GSCs. Those CIDP patients with IgG antibodies to LM1-related antigens frequently had ataxia. In addition, they did not show cranial nerve involvement (Kuwahara et al. 2013). It may be explained by the localization of LM1 in the peripheral nerve; LM1 is a major glycolipid antigen in the peripheral nerve innervating extremities but is scarcely detected in cranial nerves. Nerve biopsy finding from a CIDP patient with IgG anti-LM1 antibody showed deposition of complement on the myelin (Koike et al. 2020). IgG anti-LM1 antibody could be related to the complement-mediated demyelination seen in CIDP.

It is possible that some minor unidentified glycolipid antigens or GSCs are the targets for CIDP sera. Further investigation is necessary.



## 7 Anti-glycolipid Antibodies in Diseases Affecting the Central Nervous System

Anti-glycolipid antibodies reported so far are mostly associated with autoimmune neuropathies that affect the PNS. Considering that glycolipids are even more abundant in the CNS than PNS, it is possible that anti-glycolipid antibodies are also involved in the pathogenesis of CNS diseases. The following are two such examples.

### 7.1 *Anti-GQ1b Antibodies in Bickerstaff Brainstem Encephalitis*

Bickerstaff brainstem encephalitis (BBE) is an acute self-limited autoimmune encephalitis. Ophthalmoplegia, ataxia and impaired consciousness are the triads of BBE. IgG anti-GQ1b antibodies are frequently detected in sera from BBE patients and BBE is considered to be a disease related to MFS. Recently, clinical characteristics of anti-GQ1b antibody-positive and -negative BBE were investigated. Results showed that preceding upper respiratory infection and sensory disturbance were more common, the cell count or protein concentration was lower in the cerebrospinal fluid, abnormal findings upon brain MRI were less, and the consciousness disturbance disappeared earlier in anti-GQ1b antibody-positive BBE (Yoshikawa et al. 2020). Therefore, BBE with IgG anti-GQ1b antibodies has homogeneous features, suggesting important pathogenetic roles of anti-GQ1b antibodies. Characteristics of the anti-GQ1b antibodies in BBE are not different from those in MFS (Yoshikawa et al. 2018), suggesting that some other factors may be needed for the development of consciousness disturbance.

### 7.2 *Anti-Gal-C Antibodies*

Gal-C is a major glycolipid in myelin in both the PNS and CNS. The spectrum of clinical characteristics and the antibodies to glycolipids including Gal-C were recently investigated in neurological diseases following *M.pneumoniae* infection (Kuwahara et al. 2017). Of the 46 patients studied, 30 were affected with GBS, 17 with CNS diseases (one patient was affected with both GBS and CNS disease). The patients with CNS diseases were significantly younger than those with GBS. Anti-Gal-C antibodies were the most frequently detected antibodies. In the patients with CNS diseases, IgM antibodies to Gal-C were more common than IgG antibodies. In contrast, in GBS, IgG antibodies to Gal-C were more common than IgM antibodies. It can be speculated that IgM anti-Gal-C antibodies are produced in younger patients affected for the first time with *M. pneumoniae*. Because the blood-brain barrier is not yet mature in younger patients, the antibodies produced might more easily access the CNS and cause inflammatory disease.

More intensive investigation of pediatric cases may be necessary to reveal the possible roles of anti-glycolipid antibodies in CNS diseases.

## 8 Future Perspectives

### 8.1 *More Efficient Use of Anti-glycolipid Antibodies as Diagnostic and Prognostic Markers*

The detection of a certain IgG anti-glycolipid antibodies in the acute-phase sera from patients with neuropathy strongly suggests the diagnosis of GBS. However, locations in which antibody assays can be performed is limited and hence they are not yet included in routine laboratory examinations. Development of easy and rapid assay methods is needed for the efficient use of anti-glycolipid antibodies in the daily clinical treatment of GBS.

The use of anti-glycolipid antibodies as a prognostic marker is also required in neurology. IgG antibodies to GD1a were previously shown to be associated with AMAN type GBS (Ho et al. 1999). Most recently, the association between IgG anti-GD1a antibodies and poor outcome, inability to walk independently at 6 months after disease onset, was reported (Yamagishi et al. 2020). mEGOS (modified Erasmus GBS outcome score) is a clinical score of GBS and has been reported as a useful prognostic marker. The combination of serum IgG anti-GD1a antibodies and a high mEGOS was found to provide a more accurate prediction of poor prognosis than mEGOS alone (Yamagishi et al. 2020).

### 8.2 *To Determine the Clinical Significance of GSCs and the Anti-GSC Antibodies*

Considering the characteristic formation of clusters of gangliosides in the plasma membrane (Hakomori 2002), anti-GSC antibodies might cause nerve dysfunction more efficiently than those specific to a single ganglioside. Future studies on GSCs in basic glycobiology should provide us with an understanding of the possible roles of GSCs on the cell membrane and clinical relevance of anti-GSC antibodies.

## 9 Conclusion

Anti-glycolipid antibodies are frequently present in sera from patients during the acute phase of GBS, MFS and related diseases. Discovery of the antibody activities to glycolipid complexes broadened the perspectives of research in these diseases.

Further intensive research in this area is needed to clarify the pathogenetic mechanisms and develop useful biomarkers for the improvement of the clinical management of GBS, MFS and related autoimmune neurological diseases.

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