Chapter 17
N-Glycosylation in Regulation of the Nervous System

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Abstract Protein N-glycosylation can influence the nervous system in a variety of ways by affecting functions of glycoproteins involved in nervous system development and physiology. The importance of N-glycans for different aspects of neural development has been well documented. For example, some N-linked carbohydrate structures were found to play key roles in neural cell adhesion and axonal targeting during development. At the same time, the involvement of glycosylation in the regulation of neural physiology remains less understood. Recent studies have implicated N-glycosylation in the regulation of neural transmission, revealing novel roles of glycans in synaptic processes and the control of neural excitability. N-Glycans were found to markedly affect the function of several types of synaptic proteins involved in key steps of synaptic transmission, including neurotransmitter release, reception, and uptake. Glycosylation also regulates a number of channel proteins, such as TRP channels that control responses to environmental stimuli and voltage-gated ion channels, the principal determinants of neuronal excitability. Sialylated carbohydrate structures play a particularly prominent part in the modulation of voltage-gated ion channels. Sialic acids appear to affect channel functions via several mechanisms, including charge interactions, as well as other interactions that probably engage steric effects and interactions with other molecules. Experiments also indicated that some structural features of glycans can be particularly important for their function. Since glycan structures can vary significantly between different cell types and depend on the metabolic state of the cell, it is important to analyze glycan functions using in vivo approaches. While the complexity of the nervous system and intricacies of glycosylation pathways can create serious obstacles for in vivo experiments in vertebrates, recent studies have

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indicated that more simple and experimentally tractable model organisms like *Drosophila* should provide important advantages for elucidating evolutionarily conserved functions of N-glycosylation in the nervous system.

Keywords Glycosylation • Sialylation • N-Glycan • Neural transmission • Neural excitability • Ion channel • *Drosophila*

Abbreviations

β4GalNAcTA	β1,4-*N*-acetylgalactosaminyltransferase A
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASIC	Acid-sensing ion channel
CDGs	Congenital disorders of glycosylation
ConA	Concanavalin A
CSAS	CMP-sialic acid synthetase
DSiaT	*Drosophila* sialyltransferase
GABA	γ-Aminobutyric acid
GalNAc	*N*-Acetylgalactosamine
GnTI	*N*-Acetylglucosaminyltransferase I
iGluR	Ionotropic glutamate receptor
LacNAc	*N*-Acetyllactosamine
nAChR	Nicotinic acetylcholine receptor
NCAM	Neural cell adhesion molecule
NMDA	*N*-Methyl-D-aspartate
NMJ	Neuromuscular junction
Para	Paralytic
PSA	Polysialic acid
Sia	Sialic acid(s)
SV2	Synaptic vesicle protein 2
TRP	Transient receptor potential

17.1 Introduction

N-Linked glycan modifications of proteins exist in all three domains of life, Eukarya, Bacteria, and Archaea (Abu-Qarn et al. 2008). N-Glycosylation is especially abundant in eukaryotic cells, where it represents one of the most frequent and ubiquitous posttranslational protein modifications (Stanley et al. 2009; Moremen et al. 2012). In human cells, the majority of N-glycosylation sequon-containing proteins likely acquire N-glycans in the secretory pathway (Apweiler et al. 1999). Although N-glycosylation is not a prerequisite for the viability of mammalian cells in cultured conditions (Gottlieb et al. 1975; Stanley et al. 1975), the biosynthesis of N-glycans

appears to be essential for cell communication as defects in N-glycosylation result in embryonic lethality (Ioffe and Stanley 1994; Metzler et al. 1994). The best known functions of N-glycosylation concentrate on promoting protein folding and mediating quality control within the secretory pathway inside the cell (Helenius and Aebi 2001). While biological functions of N-glycans outside of the cell are significantly less understood, they are involved in many essential processes, including cell communication and adhesion. It is more challenging to study these functions because they are less amenable to cell culture approaches and require in vivo analyses that are commonly complicated by pleiotropic effects and complex regulation of glycosylation pathways. The repertoire of N-glycan structures present on a protein can be very heterogeneous at the tissue and cellular level. Their biosynthesis is intimately linked to cell metabolism, reflecting a dynamic read-out of a physiological state of the cell (Dennis et al. 2009).

Many extracellular functions of N-glycans depend on interactions with specific lectins, proteins that bind particular carbohydrate structures (Varki et al. 2009). Glycoprotein-lectin interactions are known to affect a multitude of cell adhesion and signaling processes. These interactions are also involved in building a functional molecular landscape of cell surfaces (Sharon 2007; Dennis et al. 2009). Moreover, N-glycosylation can promote glycoprotein functions via stabilizing steric interactions that protect from proteolysis ((Wittwer and Howard 1990), reviewed in (Wormald and Dwek 1999)). All these functional outcomes of N-glycosylation are pertinent to the development and physiology of different organs and tissues, including the nervous system. In this review, we will focus on several novel paradigms of neural functions of N-glycans. Our goal is not to provide an extensive review of experimental data in this field. Instead, we will concentrate on the discussion of a number of recent studies that unraveled some interesting functional mechanisms underlying these paradigms.

17.2 N-Glycosylation in Neural Development

The critical involvement of N-glycosylation in development of the nervous system is evident from the studies of human congenital disorders of glycosylation (CDGs). They revealed that genetic defects in the N-glycosylation pathway are almost always associated with severe neurological abnormalities (reviewed in (Freeze et al. 2012)). Gene inactivation experiments in mice have shed light on the in vivo functions of several key glycosyltransferase genes and their glycan products in the nervous system (Lowe and Marth 2003). For example, brain-specific inactivation of GlcNAcT-I, a glycosyltransferase that mediates the biosynthesis of hybrid and complex N-linked carbohydrates, was found to result in severe neurological defects, including abnormal locomotion, tremors, and paralysis (Ye and Marth 2004). However, pleiotropic effects of glycosylation on the development and physiology commonly obstruct conclusive analyses and interpretation of phenotypes produced by knockouts that affect core structures. On the other hand, mutations affecting

more specialized and some terminal structures of glycans have proven to be more amenable to study. Phenotypes of such mutations demonstrated the involvement of certain N-glycan structures in specific regulatory events. Thus, genetic inactivation of ST8Sia II and ST8Sia IV polysialyltransferases that modify N-glycans of the neural cell adhesion molecule (NCAM) with polysialic acid (PSA) unveiled the prominent role of PSA in the nervous system (Weinhold et al. 2005; Angata et al. 2007; Hildebrandt et al. 2009). PSA is a long polymer of α2,8-linked sialic acid residues that can be attached to the termini of glycans on some glycoproteins, including N-glycans of NCAM. The PSA structure was shown to regulate brain development, neurite outgrowth and targeting, and to affect synaptic plasticity, learning, and memory (for reviews on the structure and functions of NCAM-PSA see (Muhlenhoff et al. 1998, 2009; Rutishauser 2008; Colley 2010)). Remarkably, the most severe phenotypes associated with PSA deficiency, including early postnatal lethality, defects in major axonal tracts and progressive hydrocephalus, result from the gain-of-function effect of NCAM that lacks proper PSA modification, and the genetic inactivation of NCAM rescues all gross morphological defects in the brain of PSA-deficient mice (Weinhold et al. 2005). Another notable example of a N-glycan structure that plays a specialized role in neural development is represented by poly-*N*-acetyllactosamine oligosaccharides (PLN). The synthesis of PLN in the developing olfactory system depends on the activity of β1,3-*N*-acetylglucosaminyltransferase (β3GnT2) that initiates and participates in the elongation of PLN on the terminal β1-linked galactose residues of N-glycans (Zhou et al. 1999). Targeted genetic inactivation of the β3GnT2 glycosyltransferase results in numerous abnormalities in the olfactory system in mice, including defects of axonal guidance and failure of glomeruli formation. The primary cause of this phenotype appears to be the hypoglycosylation of adenylyl cyclase 3. This enzyme generates cAMP, a key signaling molecule that functions in olfactory axon targeting, and the loss of PLN dramatically downregulates the activity of adenylyl cyclase and the production of cAMP (Henion et al. 2011).

These examples likely correspond to just the tip of the iceberg of numerous yet unknown important roles of N-glycosylation in the development of the nervous system. The nervous system is regulated by a broad spectrum of N-glycosylated proteins, including cell surface and extracellular matrix (ECM) glycoproteins participating in cell adhesion and signaling (Kleene and Schachner 2004; Dityatev et al. 2010). Intriguingly, the functions of a number of these glycoproteins are affected by N-glycosylation outside of the nervous system. Thus, *β1,6*-branching GlcNAc modifications were found to modulate cell adhesion and cell motility by affecting the functions of laminin 332 and α3β1 integrins (Zhao et al. 2006; Kariya et al. 2008). Another example of a carbohydrate structure that can markedly affect molecular interactions is *α2,6*-sialylation. It was found to regulate the functions of α4β1 integrins and receptor protein tyrosine phosphatase CD45 by modifying their conformation or interactions with functionally important partners in immune system cells (Amano et al. 2003; Woodard-Grice et al. 2008). Laminins, integrins, and receptor protein tyrosine phosphatases also function in the nervous system, affecting neuronal migration, axonal growth and myelination, neuromuscular junction

development, neuronal survival, etc. (Wang et al. 2009; Barros et al. 2010; Tan et al. 2011), and glycosylation of these proteins may be implicated in these neural functions. Several studies demonstrated that *α2,6*-linked sialic acids play an important role as negative regulators of galectin binding, which revealed a paradigm that is expected to be pertinent in many cellular and molecular contexts (reviewed in (Zhuo and Bellis 2011)). These and other examples suggest that similar glycan-dependent regulatory mechanisms may operate in the nervous system, and they need to be explored.

Several novel mechanisms implicating N-glycosylation in the modulation of neural transmission have been recently elucidated, and are discussed in more detail below.

17.3 N-Glycans in Neural Physiology

17.3.1 Glycans in Synaptic Transmission

Recent research revealed a connection between mutations in the gene encoding glutamine-fructose-6-phosphate transaminase 1 (GFPT1) and a group of congenital myasthenic syndromes (CMS, e.g. OMIM 608931) characterized by hereditary defects in synaptic transmission at neuromuscular junctions (Engel 2012). GFPT1 mediates the first, rate-limiting step in the synthesis of hexosamine needed for glycan biosynthesis (Senderek et al. 2011). The importance of protein glycosylation for different aspects of synaptic transmission was unveiled by a number of studies (see reviews (Kleene and Schachner 2004; Dityatev et al. 2010; Dani and Broadie 2012)). However, the molecular and cellular bases for the effects seen on synaptic transmission are complex and not well understood. Combined, these observations indicate that glycosylation in general is required for normal synaptic functions.

N-Glycosylation controls the function of many key players in synaptic processes and its effect on synaptic physiology is multifaceted. For instance, the function of synaptic vesicle protein 2 (SV2), ubiquitously present at vertebrate synapses, was found to depend on its N-glycosylation. Targeted gene inactivation in mice demonstrated the importance of SV2 for neural transmission as the deletion of two out of three existing SV2 isoforms resulted in postnatal lethality due to severe seizures. Notably, no developmental defects were found in the brains of these mutants, which indicated that SV2 proteins function mainly in synaptic physiology (Janz et al. 1999). It was suggested that they mediate a novel maturation step of primed synaptic vesicles, which potentiates responsiveness of synaptic vesicles to Ca^{2+} regulation. All SV2 isoforms have multiple N-linked glycan chains attached to their intravesicular loop. The most ubiquitous SV2 isoform, SV2a, has three glycosylation sites, and the removal of all of them inhibits the synaptic targeting of SV2a along with its function (Chang and Sudhof 2009). These results suggest that N-glycans are required for proper folding and trafficking of SV2 within the neuron.

More recent analyses of SV2 mutants lacking individual glycosylation sites indicated that single N-glycans are partially dispensable and their function is redundant for the proper sorting of SV2a to synaptic vesicles (Kwon and Chapman 2012). Similar approaches were used to examine the role of N-glycosylation in the regulation of two other major glycoproteins of synaptic vesicles, synaptotagmin 1 and synaptophysin. It was found that the role of glycosylation in glycoprotein sorting to synaptic vesicles can range from dispensable (synaptotagmin 1) to essential (synaptophysin) (Kwon and Chapman 2012). These results illustrated that glycans can play highly individualized regulatory roles that are tailored for a particular glycoprotein and its specific function in the nervous system.

Another type of prominent players in synaptic transmission is represented by neurotransmitter receptors that function as ligand-gated channel proteins and mediate communication among neurons within the nervous system, or between neurons and muscles at neuromuscular junctions. Neurotransmitter receptors are commonly glycosylated, having several N-glycans attached to their extracellular domains. Substantial evidence indicating the functional importance of these carbohydrate modifications has started to emerge. Thus, a number of studies have demonstrated that N-linked carbohydrate chains are involved in the function of nicotinic acetylcholine receptors (nAChRs). nAChR proteins correspond to founding members of the pentameric ligand-gated super family of ion channels, that also includes serotonin, γ-aminobutyric acid (GABA), and glycine receptors (Chen 2010). nAChRs regulate postsynaptic responses at neuromuscular junctions and a variety of synaptic connections in the brain. These receptors are implicated in diverse neural functions, including the processing of sensory information and learning and memory (Miwa et al. 2011). Results of studies on the involvement of N-glycosylation in the function of nAChRs indicate that glycosylation affects functional properties of the receptors. It was proposed that N-glycans can promote the local folding of some functional protein domains, without influencing interactions between receptor subunits or their cell surface expression (Gehle et al. 1997; Chen et al. 1998). Using *Torpedo* nAChRs as a model system, experiments revealed that N-glycosylation is implicated in receptor modulation, as receptors with mutated glycosylation sites have abnormal conductance and desensitization (the rate of current decay) (Nishizaki 2003). Interestingly, the pharmacological application of concanavalin A (ConA) to in vitro assays of wild type receptors mimicked the effect of mutations affecting N-glycosylation. ConA is a lectin that binds N-linked glycans, and thus its effect on nAChRs was interpreted as evidence that the glycans may function as a modulating "lid" at the channel pore, and the lack of sugar chains or the inhibition of its movement by lectin binding caused the decreased rate of desensitization (Nishizaki 2003). More recent experiments indicated that carbohydrate modifications of nAChRs can influence their surface expression and cholinergic agonist-dependent gating. However, glycosylation does not change their binding affinity for the agonists or the stability of folded receptors (Dacosta et al. 2005; Dellisanti et al. 2007).

The role of N-glycosylation of ionotropic glutamate receptors (iGluRs) was also analyzed. iGluRs mediate fast transmission at the majority of excitatory synapses within the mammalian nervous system, and they play essential roles in synaptic

plasticity and extrasynaptic modulation of neurons (reviewed in (Traynelis et al. 2010)). These receptors form tetrameric complexes that function as ligand-gated ion channels. iGluRs encompass large subfamilies of AMPA, kainate, and NMDA receptors (Traynelis et al. 2010). The majority of these receptors appear to be N-glycosylated, with consensus glycosylation sites in their amino-terminal domains involved in receptor assembly and modulation, as well as in their ligand-binding domains (Partin et al. 1993; Everts et al. 1997; Everts et al. 1999; Mah et al. 2005). While the presence of glycans at these sites has not been well characterized, N-glycans were found to affect desensitization of AMPA and kainite receptors (Hollmann et al. 1994; Everts et al. 1997). At the same time, N-glycosylation is not generally required for iGluR function since the synthesis, transport, and subunit assembly of functional receptors on the plasma membrane are not significantly affected by the lack of glycosylation (Sumikawa et al. 1988; Everts et al. 1997; Gill et al. 2009). In agreement with these data, crystallization studies of kainate receptors showed that N-linked sugar chains are not directly involved in ligand binding and subunit association of iGluRs (Armstrong et al. 1998; Nanao et al. 2005). In contrast to the AMPA and kainite receptors, functional expression of NMDA-type receptors was found to be dramatically downregulated by inhibition of N-glycosylation. This effect was shown to be associated with a specific reduction in expression of the NR1 subunit, suggesting that glycans are required for its folding and/or trafficking (Everts et al. 1997).

Some specialized carbohydrate structures that can be present on N-linked glycans were found to be involved in the functional modulation of glycoproteins participating in neural transmission. These structures include HNK-1 and sialic acid (discussed below). The HNK-1 glycoepitope (initially discovered on *h*uman *n*atural *k*iller cells (Abo and Balch 1981)) was shown to be involved in regulation of the AMPA-type receptor subunit GluR2. The HNK-1 epitope can be also present on some glycolipids. Using these glycolipids, two research groups independently demonstrated that the HNK-1 epitope represents a sulfated glucuronic acid linked to *N*-acetyllactosamine on the nonreducing termini of oligosaccharides (HSO3–3GlcAβ1–3Galβ1–4GlcNAc) (Chou et al. 1986; Ariga et al. 1987). The expression of HNK-1 is highly enriched in the nervous system. Genetic inactivation of enzymes responsible for the biosynthesis of this epitope (glucuronyltransferase GlcAT-P, sulfotransferase HNK-1 ST, and β4-galactosyltransferase-2) lead to neurological phenotypes in mice, including reduced long-term potentiation in hippocampal CA1 synapses, electrophysiological abnormalities of hippocampal interneurons, defects in neural plasticity, and learning and memory, which suggest that HNK-1 is important for synaptic functions (Senn et al. 2002; Yamamoto et al. 2002; Gurevicius et al. 2007; Yoshihara et al. 2009). It was found that the HNK-1 epitope downregulates endocytosis of the AMPA glutamate receptor subunit GluR2 and stabilizes its expression on neuronal plasma membranes. Moreover, the presence of HNK-1 promotes the interaction between GluR2 and *N*-cadherin, which probably regulates the stability of GluR2 on the cell surface at synaptic connections (Morita et al. 2009). The HNK-1 epitope is present on a number of glycoproteins involved in intracellular adhesion, cell migration, and synaptic plasticity (reviewed in (Kleene and

Schachner 2004; Yanagisawa and Yu 2007)). More recently this epitope was found to be expressed on a tenascin-C spliced variant and involved in the regulation of mouse neural stem cells (Yagi et al. 2010).

Synaptic transmission can be significantly influenced by the function of neurotransmitter transporters, synaptic proteins essential for control of the concentration of neurotransmitters in the synaptic cleft. The SLC6 (solute carrier) family of membrane proteins includes a subfamily of transporters that mediate the translocation of neurotransmitters across the plasma membrane by coupling it to the cotransport of Na^+ and Cl^- (reviewed in (Kristensen et al. 2011)). The members of this subfamily include the transporters for serotonin (5-hydroxytryptamine, or 5-HT), dopamine, norepinephrine, GABA, and glycine. All these transport proteins appear to be N-glycosylated at the large extracellular loop 2 region, suggesting that this modification is functionally important. While removal of this glycosylation by mutagenesis or glycosidase treatment reduces the number of transporters at the cell surface, it usually does not have a strong effect on ligand binding and transporter activity. This reduction in transporter amount was attributed to a decrease in protein stability or a disruption in trafficking of nonglycosylated transporters to the plasma membrane (Tate and Blakely 1994; Olivares et al. 1995; Melikian et al. 1996; Nguyen and Amara 1996; Martinez-Maza et al. 2001; Li et al. 2004; Kristensen et al. 2011). N-Glycosylation of GAT1, the predominant GABA transporter in the brain, was found to promote both, the stability of the protein and its trafficking to the cell surface. Moreover, N-glycans were found to be important for GABA-uptake activity of the transporter, with sialic acids appearing to play an essential part in this regulation as the absence of sialylation slowed down the kinetics of the GABA transport cycle and reduced the apparent affinity of GAT1 for extracellular Na^+ (Cai et al. 2005; Hu et al. 2011). Interestingly, a nonsynonymous single nucleotide polymorphism (SNP) in the human *SLC6A4* gene encoding the serotonin transporter (hSERT) creates an ectopic glycosylation site (K201N) that was found to enhance glycosylation of hSERT with a concomitant increase in the level of transporter expression and activity. Although it is not yet known whether this SNP is associated with a neurological phenotype, by analogy to a well-studied polymorphism that also changes the expression level of hSERT, it was suggested that the K201N allele may affect personality traits and psychiatric disease susceptibility (Rasmussen et al. 2009).

17.3.2 N-Glycosylation Regulates Ion Channels in Vertebrate Neurons

N-Glycosylation can be an important modulator of ion channels in the nervous system. In general, glycans can regulate channels via at least three different mechanisms: (1) by promoting their folding and trafficking to the cell surface, (2) by affecting their stability and distribution on the cell surface (e.g., via regulating protein endocytosis and/or recycling at the plasma membrane), and (3) by changing

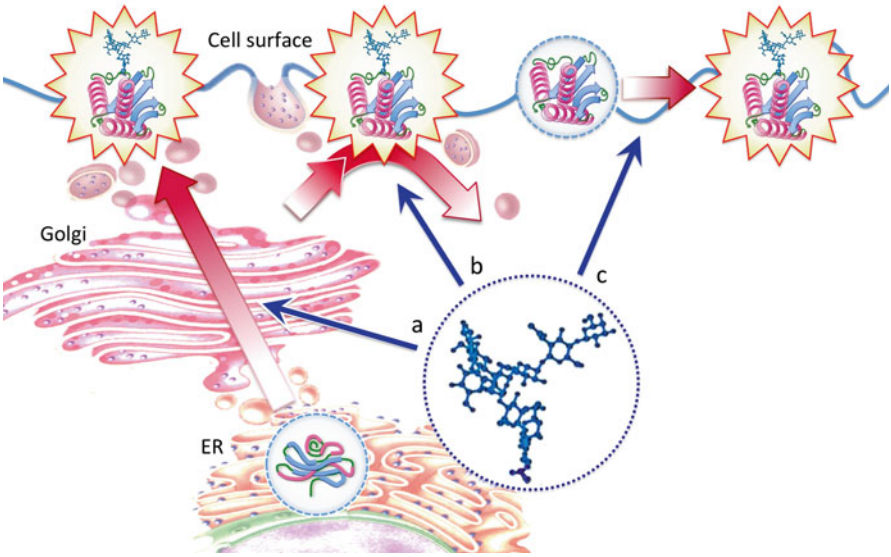

Fig. 17.1 Main effects of protein N-glycosylation. N-glycans can potentiate glycoprotein functions by facilitating protein folding and trafficking to the cell surface (**a**), promoting protein stability on the cell surface via regulation of protein uptake and recycling to the plasma membrane (**b**), and by enhancing protein activity via changing protein biophysical properties (**c**). N-Glycosylation is sketched as a single generic N-glycan (not to scale). The number of glycans can be different for distinct proteins, while glycan structures can vary and have different effects on protein functions

their molecular properties and thus potentiating channel functions (e.g., affecting biophysical characteristics and/or functional interactions with other molecules) (Fig. 17.1). Outcomes of the first two mechanisms impinge on the control of the number of channels on the cell surface. The effect of N-glycosylation on channel cell surface expression was demonstrated for several types of neuronal channels, including acid-sensing channels (e.g., ASIC1a and 1b (Kadurin et al. 2008; Jing et al. 2012)) and voltage-gated ion channels (e.g., potassium channels Kv1.3, Kv1.4, and HERG (Gong et al. 2002; Watanabe et al. 2004; Zhu et al. 2012), and calcium channels Cav3.2 (Weiss et al. 2013)). The effect on biophysical properties is frequently mediated by glycans attached to channel pore loops that can influence channel gating. Channel pore N-glycan modifications can effectively modulate the function of the TRPM8 channel, a member of a large family of transient receptor potential (TRP) ion channels playing essential roles in sensory physiology. TRPM8 glycosylation was found to cause a marked shift in the voltage dependence of channel activation (Pertusa et al. 2012). TRPM8 is expressed in sensory neurons that respond to cold (Mckemy et al. 2002; Peier et al. 2002). The N-linked glycans affect the temperature threshold of TRPM8 activation, and therefore they can function as critical molecular determinants that establish cold sensitivity in primary sensory neurons (Pertusa et al. 2012). Notably, the membrane localization of channels in this case appears to be unaffected by glycosylation and therefore the effect of

glycans is concentrated on the regulation of channel biophysical properties (Pertusa et al. 2012). Similarly, glycosylation was found to be an essential factor for agonist-mediated regulation of TRPV1 (TRP Vanilloid Type 1), a nonspecific cation channel that functions as a key sensor of pain-sensing nerve fibers. A nonglycosylated mutant TRPV1 (N604T) was shown to be properly expressed on the plasma membrane; however, it did not undergo sustained regulation by capsaicin and had substantially altered desensitization properties (Veldhuis et al. 2012). While glycans affect the biophysical properties of several TRP channels (e.g., TRPC3 and TRPC6 (Dietrich et al. 2003; Wirkner et al. 2005)), glycosylation can also promote the function of TRP channels by regulating their expression and subcellular localization and thus influencing the number of available functional channels (TRPV4 and TRPV5 (Chang et al. 2005; Xu et al. 2006)). These different mechanisms mediated by N-glycosylation do not appear to be mutually exclusive. They could operate at the same time, while one of them could become more prominent, depending on particular molecular and cellular contexts.

17.3.3 N-Glycans and Interactions with Lectins

Exogenous lectins that interact with N-linked glycan structures were found to have a strong modulatory effect on some neurotransmitter receptors in pharmacological assays. As mentioned above, ConA can bind to N-glycans of nAChRs and influence desensitization of wild-type receptors in a way that mimics the effect of mutations that eliminate N-glycosylation sites (Nishizaki 2003). The modulatory effect of ConA was also demonstrated for iGluR subfamilies of AMPA, kainate, and NMDA receptors (Traynelis et al. 2010). ConA exerts a pronounced effect on kainate receptors by inhibiting their desensitization (Partin et al. 1993; Everts et al. 1997, 1999). Experiments indicate that ConA can interact with N-glycans attached to the amino-terminal domain of iGluRs and affect receptor conformational changes. This action appears to depend on the conformational state of the channel, since agonist-induced desensitization prior to ConA application eliminates the effect (Everts et al. 1997, 1999; Fay and Bowie 2006). Some other lectins, such as wheat germ agglutinin, soybean agglutinin, and succinyl-ConA, were also shown to potentiate kainate receptors (Thio et al. 1993; Yue et al. 1995). The in vitro effects of these lectins suggested that glycans may play a specialized role in the modulation of receptors in vivo (Everts et al. 1997; Nanao et al. 2005), while some endogenous, yet unknown lectins can potentially bind to these glycans and regulate the function of neurotransmitter receptors. A related mechanism of lectin-dependent regulation has recently been described for the Ca^{2+} TRPV5 channel in renal epithelial cells. Retention of TRPV5 on the cell surface is an essential regulatory process in the control of channel function. This regulation is mediated by Klotho, a humoral factor with glycosidase activity that appears to directly modify channel glycans, which in turn potentiates interactions with galectin and facilitates cell surface retention of the channel (Chang et al. 2005; Cha et al. 2008; Leunissen et al. 2013). However, the

regulation of TRPV5 is not fully understood, and it is likely mediated by the converging effects of several mechanisms, also including sialylation that appears to work in parallel to promote lipid raft-mediated internalization of the channel (Leunissen et al. 2013). It will be important to investigate whether lectin-mediated regulation can also operate in the nervous system to regulate channels involved in neural transmission.

17.3.4 N-Glycans in Regulation of Voltage-Gated Ion Channels and Membrane Excitability

A large group of voltage-gated ion channels represents principal regulators of cell excitability. Glycosylation can affect cell excitability of neurons by modulating the function of various members of this channel superfamily, including channels that regulate membrane permeability for Na^+, K^+, and Ca^{2+} ions (e.g., (Recio-Pinto et al. 1990; Zhang et al. 1999; Bennett 2002; Gong et al. 2002; Watanabe et al. 2003; Johnson et al. 2004; Watanabe et al. 2007; Schwetz et al. 2010; Weiss et al. 2013)). In mammals, N-glycosylation of voltage-gated Na^+ and K^+ channels was found to be regulated developmentally and in a cell-specific manner in the heart and the nervous system, suggesting that glycans participate in setting the distinct levels of excitability required in different cells and at various developmental stages (Castillo et al. 1997; Tyrrell et al. 2001; Schwalbe et al. 2008; Montpetit et al. 2009).

In addition to the direct effects of channel glycans, N-glycosylation can influence ion channels indirectly, in a molecule nonautonomous manner, by regulating other glycoproteins that control channel functions. For example, glycosylation of auxiliary subunits that interact with channels can promote cell surface localization and modify channel biophysical properties (Johnson et al. 2004; Cotella et al. 2010). The nonautonomous effect of N-glycans can be potentially pertinent for regulation of many channels; however, this possibility remains largely unexplored.

Numerous studies of channel glycosylation have concentrated on sialylated glycans (reviewed in (Ednie and Bennett 2012)). Sialylated carbohydrate chains are negatively charged and can participate in electrostatic interactions with ions and other charged groups located on the cell surface, thus potentially affecting channel functions. Vertebrate voltage-gated Na^+ channels are heavily decorated with sialylated structures. It was estimated that up to 30 % of Na^+ channel molecular mass is represented by carbohydrate chains, with sialic acids (Sia) comprising nearly 50 % of channel glycans (Miller et al. 1983; Elmer et al. 1985; Messner and Catterall 1985; James and Agnew 1987; Roberts and Barchi 1987). Electrophysiological assays indicated that sialylated glycans can markedly affect the gating properties of Na^+ channels (Recio-Pinto et al. 1990; Bennett et al. 1997; Zhang et al. 1999; Cronin et al. 2005). This effect varies significantly for different channels, and it can also be isoform- and subunit-specific (Bennett 2002; Johnson et al. 2004; Johnson and Bennett 2006).

The role of sialylation in the modulation of vertebrate voltage-gated Na^+ channels has been generally explained by the electrostatic effect of the large negative charge

provided by the numerous Sia residues present in the vicinity of the channel pore. Remarkably, more than 100 Sia residues can be attached to a channel protein, with the majority of them being incorporated as PSA structures (Miller et al. 1983; James and Agnew 1987; Zuber et al. 1992). The specific role of PSA in the regulation of voltage-gated Na^+ channels was uncovered by analyses of mouse mutant cardiomyocytes that had genetically inactivated ST8Sia II polysialyltransferase, an enzyme involved in PSA biosynthesis. The ST8Sia II deficiency was found to cause defects in cell excitability and channel gating, including abnormal action potentials with a significantly broader waveform and a delayed peak, considerable depolarizing shifts of gating curves, and compromised fast inactivation of channels (Montpetit et al. 2009).

While the effect of PSA was confirmed by several studies, a line of evidence suggested that sialylation can also affect voltage-gated channels via mechanisms that cannot be attributed to PSA or the significant charge of numerous Sia residues attached to channel glycans. These data indicated that Sia can play a more specific role in the modulation of channel functions. Thus, electrophysiological analyses of the cardiac sodium channel in cell culture revealed that its function can be affected by some "functional" Sia residues rather than by the total charge of channel sialylation (Stocker and Bennett 2006). Furthermore, experiments with rat hippocampal organotypic slice cultures suggested that PSA does not always influence the function of voltage-gated Na^+ channels, since treatment with Endo-N sialidase, a glycosidase that specifically removes PSA, was found to have no apparent impact on intracellularly recorded action potentials and evoked synaptic transmission (Muller et al. 1996). Additionally, the effects of PSA and non-PSA Sia residues on the function of α-$Na_V1.4$ channels were found to be distinct when they were analyzed using mutant Chinese hamster ovary (CHO) cell lines with defects in sialylation or polysialylation pathways. The loss of Sia and PSA in these mutant cells results in opposite shifts of voltage-dependent activation and steady-state inactivation of α-$Na_V1.4$, while only the loss of Sia has a significant effect on recovery from fast inactivation (Ahrens et al. 2011). Finally, unnatural Sia residues with *N*-acetyl groups changed to *N*-pentanoyl or *N*-propanoyl structures, when introduced metabolically, were found to have a notable effect on conductance properties of the Kv3.1 voltage-gated K+ channel. Collectively, these data suggest that sialylation can modulate channels through specific steric effects, in addition to its role in electrostatic interactions (Hall et al. 2011).

It is worth noting that most studies on the role of glycosylation in the regulation of ion channels and synaptic glycoproteins have been performed in vitro or in cell culture using transgenic approaches in various types of heterologous cells (for example, using frog oocytes (Everts et al. 1997; Gehle et al. 1997; Nishizaki 2003), different mammalian cell cultures (Bennett 2002; Dellisanti et al. 2007; Watanabe et al. 2007; Hu et al. 2011; Gurba et al. 2012), or in vitro reconstituted lipid membranes (Recio-Pinto et al. 1990; Castillo et al. 2003; Cronin et al. 2005)). It is important to keep in mind that the structure of glycosylation and its functional implications can vary significantly between different cell types, and between cultured cells and neural cells in vivo. Furthermore, the glycosylation of multisubunit protein complexes could also depend on a particular combination of subunits expressed by the cell (e.g., the glycosylation of $GABA_A$ β3 subunits can be affected by the coexpression of

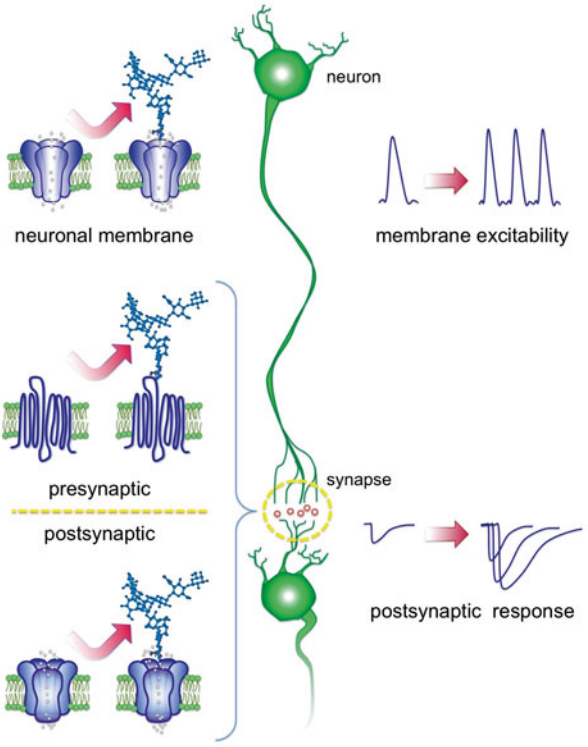

Fig. 17.2 N-Glycosylation can affect neural transmission by modulating voltage-gated ion channels that generate action potentials and determine neuronal excitability, and by influencing synaptic transmission via impact on the function of synaptic proteins, such as synaptic vesicle proteins and neurotransmitter receptors. N-Glycosylation is sketched as a generic N-glycan. Glycans can also include some specific modifications, such as polysialylation and the HNK-1 epitopes (not shown). Modified from (Scott and Panin 2014)

other receptor subunits (Gurba et al. 2012)). Therefore, it is important to exercise caution when interpreting data from in vitro and cell culture experiments in terms of mechanisms that operate in vivo. Nevertheless, taken together, experimental data clearly indicate that glycosylation can substantially influence the function of glycoproteins playing key roles in neural transmission (Fig. 17.2). This influence can be dissimilar for distinct types of factors regulating the nervous system. Moreover, even within the same family of related proteins (e.g., iGluRs) glycosylation can underlie distinct modulatory mechanisms that can also depend on the structure and location of carbohydrate chains. These effects of N-glycosylation potentially create an extra layer of regulatory processes that control neural physiology.

17.3.5 In Vivo Functions of Sialylated N-glycans

Studies that investigate the function of sialic acids in vivo remain relatively scarce. The biological importance of sialylation of voltage-gated Na^+ channels was most unambiguously demonstrated in the context of cardiac functions. Analyses of cardiomyocytes with defective channel sialylation (using mouse genetic models with diminished sialylation or glycosidase-treated rat cardiomyocytes) suggested that

abnormal channel sialylation can result in cardiac excitability phenotypes and heart failure (Ufret-Vincenty et al. 2001; Stocker and Bennett 2006; Montpetit et al. 2009). Murine models were also used to examine the role of channel sialylation in the nervous system. These experiments analyzed neural excitability after treatment with glycosidases to remove sialylated glycans, as well as upon inhibition of endogenous neuraminidases that trim sialic acids from carbohydrate chains in vivo. It was found that glycoprotein sialylation can significantly affect the excitability of neural networks and influence seizure threshold in kindling epilepsy models. These studies suggested that sialic acids can effectively modulate voltage-gated Na^+ channels in brain neurons (Tyrrell et al. 2001; Isaev et al. 2007, 2011; Isaeva et al. 2010).

Recent analyses of the mouse model of Angelman syndrome revealed the possibility that an abnormal sialylation of cell surface proteins plays a key role in the etiology of the syndrome (Condon et al. 2013). This neurological genetic disorder is caused by the maternal loss of the E3 ubiquitin ligase Ube3a and is associated with motor dysfunction, mental retardation, speech impairment, seizures, and a high prevalence of autism (Williams et al. 2006). Loss of Ube3a causes defects in synaptic development and function, including a deficit in experience-dependent synaptic plasticity and decreased plasma membrane localization of AMPA receptors at excitatory synapses (Jiang et al. 1998; Dindot et al. 2008; Yashiro et al. 2009; Greer et al. 2010). Intriguingly, the Ube3a defect also causes a dramatic reduction of glycoprotein sialylation due to the structural and homeostatic disruption of the Golgi apparatus, which indicated that the deficiency of glycoprotein sialylation likely underlies the pathobiological mechanism of Angelman syndrome (Condon et al. 2013).

Although a number of in vivo experiments indicate that glycoprotein sialylation can significantly influence the excitability of neural networks, it remains unknown whether this effect is primarily due to the sialylation of voltage-gated channels or some other glycoproteins. It is challenging to address this question in vertebrates because of the complexity of the nervous system, intricacies of glycosylation pathways, potential functional redundancy of glycosylation genes, as well as the ubiquity of sialylation that affects a panoply of glycoconjugates in the majority of vertebrate cells. With its power of genetic approaches, a spectrum of well-established neurobiological approaches, and simplified glycosylation pathways, *Drosophila* has recently emerged as a promising model for elucidating conserved genetic and molecular mechanisms of neural glycosylation.

17.4 N-Glycosylation Regulates the Nervous System of *Drosophila*

17.4.1 Drosophila *Mutations Affecting N-Glycosylation*

Recent glycoproteomic approaches characterized in detail the totality of *Drosophila* N-glycosylated proteins, identifying more than 450 glycoproteins expressed in the head (Koles et al. 2007; Vandenborre et al. 2010; Baycin-Hizal et al. 2011).

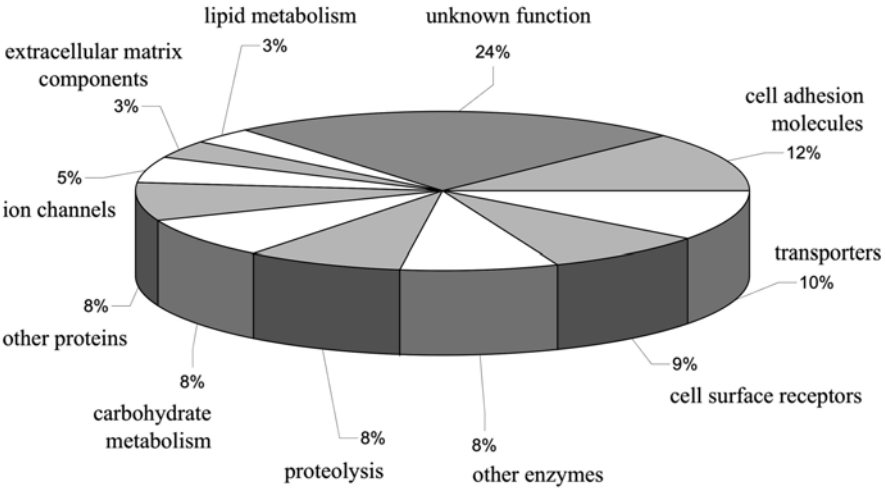

Fig. 17.3 Distribution of the different protein classes among N-glycosylated proteins identified in *Drosophila* head by glycoproteomics approaches. Figure adapted with permission from Koles et al. (2007)

These proteins comprise ion channels, transporters, cell surface receptors, cell adhesion molecules, molecules involved in proteolysis and carbohydrate metabolism, and some other protein families, including a large proportion of proteins with unknown functions (Fig. 17.3) (Koles et al. 2007; Baycin-Hizal et al. 2011). The repertoire of N-glycan structures in *Drosophila* is different from that in mammalian organisms. Detailed mass spectrometry analyses of the *Drosophila* N-glycome revealed that paucimannose and high mannose structures dominate the spectrum of N-glycosylation (Aoki et al. 2007; Koles et al. 2007). In contrast to mammalian N-glycans that are represented by abundant complex structures (Antonopoulos et al. 2011), complex and hybrid-type oligosaccharides that correspond to more processed mature structures represent only 12 % of the total *Drosophila* N-glycan profile (Aoki et al. 2007). Nevertheless, these minor glycan species play prominent roles in the nervous system, suggesting that their functions are evolutionarily conserved (Schachter 2010). The importance of these glycans for the nervous system was revealed in a number of studies that analyzed mutants with defects in the N-glycosylation pathway. Thus, genetic inactivation of the *MGAT1* gene that encodes GlcNAcT I, a key enzyme in the production of processed N-glycan structures, was found to result in severe neurological phenotypes, including locomotor abnormalities, significantly decreased life span, and the "fused lobes" phenotype, a developmental defect affecting a specialized brain structure involved in memory formation, the mushroom bodies (Sarkar et al. 2006, 2010). *MGAT1* mutants have prominent synaptic defects, including overgrowth of neuromuscular junctions and abnormal synaptic vesicle cycling. *MGAT1* mutant synapses have disrupted extracellular synaptomatrix and the accumulation of Mind the gap, a lectin-like extracellular matrix protein of the synaptic cleft. They also show a decreased expression of

several key markers of functional synaptic morphology, such as Bruchpilot, a presynaptic active zone protein, and GLURIIB, a postsynaptic iGluR subunit B (Parkinson et al. 2013). Mutations of *fused lobes* cause cell-lethal phenotype in mosaic clones of olfactory projection neurons and result in mushroom body defects similar to those found in *MGAT1* mutants (the mushroom body *lobes* become *fused*) (Boquet et al. 2000; Sekine et al. 2013). *Fused lobes* encodes Golgi β-N-acetylglucosaminidase that inhibits the biosynthesis of hybrid and complex N-glycans and concomitantly promotes the production of paucimannose structures (Leonard et al. 2006). Downregulation of *sugar-free frosting,* a gene encoding a *Drosophila* homolog of SAD kinase that regulates secretory flux through the Golgi, inhibits synthesis of the HRP glycoepitope (α3-linked core fucose) and increases the amount of hybrid and complex N-glycan structures. *Sugar-free frosting* mutations lead to neuromuscular junction defects in larvae and locomotor abnormalities in adult flies (Baas et al. 2011). Meigo, a putative nucleotide sugar transporter, appears to specifically regulate the targeting of neurite projections in the olfactory system by affecting N-glycosylation of ephrin (Sekine et al. 2013). Deficiency of β1,4-N-acetylgalactosaminyltransferase A (β4GalNAcTA), a glycosyltransferase potentially involved in the biosynthesis of complex and hybrid N-glycans, results in prominent neurological phenotypes, including defects of locomotion, reduction in the number of synaptic boutons at neuromuscular junctions and decreased frequency of spontaneous release of neurotransmitters (Haines and Irvine 2005; Haines and Stewart 2007; Nakamura et al. 2012). Taken together, these examples highlight the notion that protein N-glycosylation plays important and specific functions in the *Drosophila* nervous system, and that these functions require the structural diversity of N-glycans. These data also indicate an intriguing possibility that many genes involved in the N-glycosylation pathway could be associated with evolutionarily conserved mechanisms that regulate the nervous system in a wide range of animals, from arthropods to mammals.

17.4.2 Sialylated N-Glycans Control Neural Excitability in Drosophila

Sialylated glycans represent less than 0.1 % of the total glycan profile of the *Drosophila* N-glycome. As a result, they can only be unambiguously detected and analyzed by the most sensitive glycomic approaches, such as multidimensional mass spectrometry (Aoki et al. 2007; Koles et al. 2007). Despite the fact that sialylation is so scarce, it has a prominent function in the nervous system of *Drosophila*, which was revealed by analysis of mutant phenotypes of the *Drosophila sialyltransferase* (*DSiaT*) and *CMP-sialic acid synthetase* (*CSAS*) genes that play key roles in the sialylation pathway (Koles et al. 2009). Unlike mammalian organisms that have 20 different sialyltransferases, *Drosophila* possesses only one sialyltransferase, DSiaT, which significantly simplifies the in vivo analysis of sialylation functions (Koles et al. 2009). DSiaT shows a close evolutionary relationship to the

ST6Gal family of mammalian sialyltransferases; it modifies glycoproteins by attaching α*2,6*-linked sialic acids to LacNAc termini of N-glycans (Koles et al. 2004; Repnikova et al. 2010). The expression of DSiaT is dynamic and largely restricted to subsets of fully differentiated CNS neurons during development and in adult flies, which indicates that the pattern of sialylation is tightly controlled in a cell-specific and developmentally regulated manner (Koles et al. 2009; Repnikova et al. 2010; Islam et al. 2013). The expression of CSAS, an enzyme generating the CMP-sialic acid sugar donor for sialylation, is similarly restricted, which can partially explain the low overall amount of sialylated glycans present in *Drosophila* (Koles et al. 2007; Repnikova et al. 2010; Islam et al. 2013), even when DSiaT was ectopically expressed throughout the CNS (North et al. 2006).

Genetic inactivation of the sialylation pathway in vivo revealed that sialylated N-glycans play a prominent and specific role in the regulation of the nervous system. Targeted deletion of *DSiaT* results in a significantly shortened life span, locomotion abnormalities, and temperature-sensitive paralysis phenotype. *DSiaT* mutant larvae have structural and physiological defects in their neuromuscular junction synaptic connections. Electrophysiological assays of *DSiaT* mutants indicated that DSiaT activity is required for normal neuronal excitability and specifically affects the function of Para, the main voltage-gated Na^+ channel in *Drosophila* (Repnikova et al. 2010). Similar phenotypes result from *CSAS* mutations that are predicted to also block the sialylation pathway (Islam et al. 2013). Interestingly, the paralysis phenotype of *CSAS* mutants can be significantly ameliorated by an extra gene copy of *para*, which suggests that sialylation potentially controls the number of functional voltage-gated channels on the cell surface (Islam et al. 2013). Moreover, the genetic interactions between *DSiaT* and *β4GalNAcTA* indicated that sialic acids may function as masking residues hindering the recognition of LacNAc termini of glycans by some endogenous lectins (Nakamura et al. 2012). While further experiments are required to test these intriguing hypotheses, taken together, these results reveal an important novel, nervous system-specific function for α*2,6*-sialylated N-glycans in the regulation of neural transmission. It is tempting to speculate that this regulatory role corresponds to one of the most ancient evolutionarily conserved functions of sialylation in metazoan organisms. This intriguing hypothesis requires further investigation.

17.5 Conclusions

N-Glycosylation can affect glycoproteins by a number of mechanisms, e.g., by facilitating protein folding and stability, supporting trafficking, participating in interactions with other molecules, including lectins, as well as by mediating electrostatic and steric effects on protein dynamics and conformation. In the nervous system, many key players of neural transmission bear N-linked carbohydrate modifications. The roles of these modifications usually depend on molecular and cellular contexts and can vary from nonessential effects to obligatory requirements for protein functions (Table 17.1). This broad range of possible effects is expected

Table 17.1 Examples of effects of N-glycosylation on glycoproteins involved in neural physiology. Modified from (Scott and Panin 2014)

Glycoprotein	Function in the nervous system	Role of N-glycosylation	References
SV2 (synaptic vesicle protein 2)	Major synaptic vesicle protein, controls maturation step of primed synaptic vesicles	Required for proper folding and trafficking to synapses	(Chang and Sudhof 2009; Kwon and Chapman 2012)
Synaptophysin	Major synaptic vesicle protein, regulates the kinetics of synaptic vesicle endocytosis	Required for synaptic localization	(Kwon and Chapman 2012)
Nicotinic acetylcholine receptors (nAChRs)	Ligand-gated cation channels, regulate postsynaptic responses to neurotransmitter acetylcholine, regulates diverse brain functions	Regulates desensitization and channel gating	(Chen et al. 1998; Gehle et al. 1997; Nishizaki 2003)
Ionotropic glutamate receptors (iGluRs)	Ligand-gated ion channels, regulate fast transmission at the majority of excitatory synapses	Affects maximal currents and desensitization of AMPA and kainite receptors Required for folding or trafficking of NMDA receptors *HNK-1* structure downregulates endocytosis of AMPA GluR2 subunit and promotes receptors' stability on neuronal membranes	(Everts et al. 1999; Everts et al. 1997; Partin et al. 1993; Thio et al. 1993; Yue et al. 1995) (Morita et al. 2009; Senn et al. 2002; Yamamoto et al. 2002; Yoshihara et al. 2009)
Neurotransmitter transporters	Major determinants of synaptic signaling, mediate uptake of neurotransmitters and regulate synaptic concentration of neurotransmitters	Promotes protein stability and trafficking, increases the number of transporters at the cell surface Sialylated glycans can affect the kinetics of GABA transporter activity and affinity for Na^+	(Hu et al. 2011; Kristensen et al. 2011; Li et al. 2004; Martinez-Maza et al. 2001; Melikian et al. 1996; Nguyen and Amara 1996; Olivares et al. 1995; Tate and Blakely 1994)
Acid-sensing channels (ASICs)	Acidosis-activated cation channels. Play roles in pain, neurological and psychiatric diseases, potential mechanosensory function in sensory neurons	Effect on cell surface expression of ASIC1a and ASIC1b	(Jing et al. 2012; Kadurin et al. 2008)
TRP channels (transient receptor potential ion channels)	Playing essential roles in sensory physiology	Affects the temperature threshold of TRPM8 activation in response to cold. Affects biophysical properties of TRPC3, TRPC6 and TRPV1. Affects expression and subcellular localization of TRPV4 and TRPV5.	(Chang et al. 2005; Dietrich et al. 2003; Pertusa et al. 2012; Wirkner et al. 2005; Xu et al. 2006)

Voltage-gated ion channels	Control cell excitability, generate action potentials, affect neural transmission		
Potassium channels		Affects cell surface expression and stability of Kv11.1, Kv1.3, Kv12.2, and Kv1.4. Affects gating of Kv1.1, Kv1.5, Kv12.2, I_{sK} Affects trafficking and gating of Kv1.2 Affects simulated action potentials for Kv1.1 and Kv1.2 *Sialylation:* Affects gating of Kv1.1, Kv1.5, Kv3.1 Gating of *Drosophila* Shaker channel expressed heterologously in mammalian cells is affected by N-glycans and sialylation	(Freeman et al. 2000; Gong et al. 2002; Hall et al. 2011; Johnson and Bennett 2008; Noma et al. 2009; Schwetz et al. 2010; Sutachan et al. 2005; Thornhill et al. 1996; Watanabe et al. 2003; Watanabe et al. 2004; Watanabe et al. 2007; Zhu et al. 2009; Zhu et al. 2012)
Calcium channels		Controls activity and cell surface expression of Cav3.2, affects glucose-dependent potentiation	(Weiss et al. 2013)
Sodium channels		Affects gating of Nav1.4 and Nav1.5, Alters steady-state inactivation of Nav1.9 *Sialylation:* Affects gating of Nav1.4, and Nav1.5, electroplax channel. Sialylation of Nav beta(2) subunit affects gating of Nav1.5. Affects functional properties of *Drosophila* Nav Para (unknown if the effect is direct)	(Bennett et al. 1997; Cronin et al. 2005; Johnson et al. 2004; Recio-Pinto et al. 1990; Repnikova et al. 2010; Stocker and Bennett 2006; Zhang et al. 1999)

to create a full gamut of states of neural transmission that can be controlled by glycosylation pathways (Fig. 17.2). Collectively, these data suggest that N-glycans can function in vivo as potent regulators of synaptic transmission and excitability of neural circuits, while also providing an important link between neural transmission and metabolism. These data also pose a number of outstanding questions about molecular, cellular, and genetic mechanisms that can underlie the glycan-mediated neural regulation in vivo, as well as a potential involvement of neural N-glycosylation in the pathobiology of neurological disorders. Obtaining answers to these challenging but fundamentally important questions is expected to require a combination of in vitro and in vivo approaches, and should be facilitated by studies using genetically tractable model organisms with simplified glycosylation pathways and a decreased complexity of the nervous system.

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

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