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REVIEW

The role of protein *N*-glycosylation in neural transmission

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Recent studies have explored the function of *N*-linked glycosylation in the nervous system, demonstrating essential roles of carbohydrate structures in neural development. The function of *N*-glycans in neural physiology remains less understood; however, increasing evidence indicates that *N*-glycans can play specific modulatory roles controlling neural transmission and excitability of neural circuits. These roles are mediated via effects on synaptic proteins involved in neurotransmitter release, transporters that regulate nerotransmitter concentrations, neurotransmitter receptors, as well as via regulation of proteins that control excitability and response to milieu stimuli, such as voltage-gated ion channels and transient receptor potential channels, respectively. Sialylated *N*-glycan structures are among the most potent modulators of cell excitability, exerting prominent effects on voltage gated Na^+ and K^+ channels. This modulation appears to be underlain by complex molecular mechanisms involving electrostatic effects, as well as interaction modes based on more specific steric effects and interactions with lectins and other molecules. Data also indicate that particular features of *N*-glycans, such as their location on a protein and structural characteristics, can be specifically associated with the effect of glycosylation. These features and their functional implications can vary between different cell types, which highlight the importance of in vivo analyses of glycan functions. Experimental challenges are associated with the overwhelming complexity of the nervous system and glycosylation pathways in vertebrates, and thus model organisms like *Drosophila* should help elucidate evolutionarily conserved mechanisms underlying glycan functions. Recent studies supported this notion and shed light on functions of several glycosylation genes involved in the regulation of the nervous system.

Keywords: Drosophila / glycosylation / ion channels / neural transmission / sialylation

Introduction

Protein *N*-glycosylation, one of the most abundant and well-studied types of protein glycosylation, has been found in all three domains of life: Eukarya, Bacteria and Archaea (Abu-Qarn et al. 2008; Stanley et al. 2009; Moremen et al. 2012). The majority of all secretory pathway glycoproteins in the human organism appear to be *N*-glycosylated (Apweiler et al. 1999). The genetic or pharmacological inhibition of *N*-glycosylation does not preclude cell viability in mammalian tissue cultures (Gottlieb et al. 1975; Stanley et al. 1975). At the same time, the abrogation of biosynthesis of hybrid and complex *N*-glycans causes embryonic lethality in mice (Ioffe and Stanley 1994; Metzler et al. 1994), which indicates the essential requirement of *N*-glycosylation for cell communications within mammalian organisms. General functions of *N*-linked glycans inside the secretory pathway have been extensively studied, including prominent roles in protein folding, quality control and trafficking (Helenius and Aebi 2001). At the same time, *N*-linked glycosylation also plays a variety of important roles outside of the cell. These roles are significantly less understood, because they are commonly pleiotropic, complex and not directly amenable to ex vivo analyses. Moreover, *N*-glycans of a protein can show significant heterogeneity at the cellular level, and their biosynthesis is intimately linked to metabolism, representing a dynamic physiological read-out of a metabolic state of the cell (Dennis et al. 2009). Many of the extracellular functions of *N*-glycans are underlain by mechanisms of interactions with lectins, proteins that bind specific glycan structures (Varki et al. 2009). Lectin interactions play prominent roles in cell adhesion and signaling, and they can sculpt the molecular landscape of cell surfaces (Sharon 2007; Dennis et al. 2009). Molecular functions of *N*-glycans can also include the stabilization of functional protein conformations and steric interactions protecting from proteolysis (Wittwer and Howard 1990, reviewed in Wormald and Dwek 1999). Several novel paradigms have started to emerge for functions of *N*-glycans in the nervous system. With the goal of focusing on some interesting findings that shed light on these paradigms, while not intending to provide a comprehensive account of data accumulated in the field, we will discuss the neural functions of *N*-glycosylation in the current review.

N-Glycans in the nervous system development

Studies of human congenital disorders of glycosylation unveiled that inborn defects of *N*-glycosylation almost inevitably result in prominent neurological abnormalities (reviewed in Freeze et al. 2012), which highlights the crucial role of this

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posttranslational protein modification in the nervous system. The genetic inactivation of glycosyltransferase genes in mice has been an invaluable tool for elucidating in vivo functions of glycan structures (Lowe and Marth 2003). Thus, brain-targeted inactivation of β-1,6-*N*-acetylglucosaminyltransferase (GlcNAcT)-I, the enzyme required for the biosynthesis of hybrid and complex *N*-linked carbohydrates, was found to result in severe neurological phenotypes, including locomotor abnormalities, tremors and paralysis (Ye and Marth 2004). While the complexity of mammalian glycosylation and its pleiotropic effects on development and physiology provide serious obstacles for studying mutants with defects in core structures, the knockouts affecting more specialized and certain terminal structures proved to be more amenable for analyses. They demonstrated the involvement of some *N*-linked modifications in specific regulatory events. For instance, genetic deletions of ST8Sia II and ST8Sia IV polysialyltransferases modifying *N*-glycans of neural cell adhesion molecule (NCAM) with polysialic acid (PSA) unveiled the remarkable role of PSA in the nervous system (Weinhold et al. 2005; Angata et al. 2007; Hildebrandt et al. 2009). PSA, a unique long homopolymer of α2,8-linked sialic acid (Sia) residues, is present on *N*-glycans of NCAM and regulates brain development, neurite outgrowth and targeting, while also affecting synaptic plasticity, learning and memory (the structure and function of NCAM-PSA has been discussed extensively in several reviews, e.g. Muhlenhoff et al. 1998; Rutishauser 2008; Muhlenhoff et al. 2009; Colley 2010). Interestingly, the severe malformation of major axonal tracts in the brain, progressive hydrocephalus and early postnatal lethality associated with PSA deficiency are caused by the gain-of-function effect of NCAM lacking PSA, and the genetic removal of NCAM rescues these gross defects in PSA-deficient mice (Weinhold et al. 2005). Another striking example of a specialized *N*-glycan structure affecting neural development is represented by poly-*N*-acetyllactosamine (poly-LacNAc) oligosaccharides synthesized by β3GnT2. Genetic deletion of the β3GnT2 glycosyltransferase in mice leads to multiple abnormalities of the olfactory system, including prominent axonal guidance defects. This phenotype appears to result primarily from hypoglycosylation of adenylyl cyclase 3, a multi-pass membrane glycoprotein highly expressed in olfactory sensory neurons, which leads to the loss of cyclase's enzymatic activity that generates cAMP, a key signaling molecule in olfactory axon targeting (Henion et al. 2011). These examples probably represent just a tip of the iceberg of many other crucial roles of *N*-glycans in the nervous system that are waiting to be discovered. The panoply of glycoproteins, including cell surface and extracellular matrix components involved in cell signaling and adhesion, is known to regulate neural development and physiology (Kleene and Schachner 2004; Dityatev et al. 2010). In some cases, functions of these glycoproteins were found to be affected by *N*-glycosylation in biological contexts outside of the nervous system. For example, laminin 332 and α3β1 integrins were shown to be regulated by bisecting and β1, 6-branching *N*-acetylglucosamine (GlcNAc) modifications that modulated cell adhesion and contributed to increased cell motility (Zhao et al. 2006; Kariya et al. 2008). Another striking example of a carbohydrate modification that can affect different molecular interactions is α2,6-sialylation that was found to control the receptor protein tyrosine phosphatase CD45 and β1 integrins via modifying their conformation and interactions with functionally important partners (Amano et al. 2003; Woodard-Grice et al. 2008). Specific role of α2,6-linked Sia's in negative regulation of galectin binding has emerged as a paradigm that is likely pertinent to many cellular and molecular contexts (reviewed in Zhuo and Bellis 2011). These and other examples suggest that similar, yet unknown, glycan-dependent regulatory processes may operate in the nervous system. Recent studies have also shed light on several novel mechanisms implicating *N*-glycosylation in the modulation of neural transmission, which we will be discussed in more detail below.

Glycans in synaptic transmission

Recent genetic analysis of congenital myasthenic syndromes, a group of hereditary abnormalities of synaptic transmission at neuromuscular junctions (NMJs; e.g., OMIM 608931, Engel 2012), unveiled a novel genetic lesion associated with mutations in GFPT1, the glutamine-fructose-6-phosphate transaminase 1. GFPT1 mediates the first, rate-limiting step of the hexosamine pathway required for the biosynthesis of uridine diphospho-*N*-acetylglucosamine (UDP-GlcNAc), a key substrate for glycosylation pathways (Senderek et al. 2011); hence, these data highlight that glycosylation in general is an essential prerequisite for synaptic functions. The pertinence of synaptic glycosylation has been discussed in many studies (see reviews Kleene and Schachner 2004; Dityatev et al. 2010; Dani and Broadie 2012). However, the molecular and cellular mechanisms underlying the effects of glycans on synaptic transmission is complex and still poorly understood. The role of protein *N*-glycosylation at the synapse is multifaceted since it controls the function of a number of key players of synaptic processes. For instance, *N*-linked glycosylation was found to be crucial for the function of the synaptic vesicle protein 2 (SV2) that is ubiquitously present at vertebrate synapses. The importance of SV2 has been revealed by gene-targeting experiments that demonstrated that the deletion of two out of three existing SV2 isoforms resulted in postnatal lethality in mice due to severe seizures. No brain developmental abnormalities were found in these SV2 mutants, which indicated that this protein has a dedicated role in synaptic physiology (Janz et al. 1999). Experiments suggested that SV2 determines a novel maturation step of primed synaptic vesicles and enhances their responsiveness to Ca^{2+}. Moreover, all SV2 isoforms are *N*-glycosylated at multiple sites within their intravesicular loop. The removal of all three glycosylation sites present on the most ubiquitous SV2a isoform abolishes its synaptic targeting along with its function (Chang and Sudhof 2009), suggesting that *N*-glycans are required for proper folding and trafficking of SV2 within the neuron. A more recent study of single *N*-glycosylation site mutants indicated that individual glycans are partially dispensable and their function is redundant for SV2a sorting to synaptic vesicles (Kwon and Chapman 2012). Similar analyses of two other major synaptic vesicle glycoproteins revealed that the role of glycosylation in their sorting to synaptic vesicles ranges from being dispensable (synaptotagmin 1) to essential (synaptophysin) (Kwon and Chapman 2012). This highlights a common theme in glycobiology: glycans can play highly individualized roles tailored for a particular glycoprotein and its specific function.

Ligand-gated channel proteins can function as synaptic neurotransmitter receptors that mediate communications among

neurons or between neurons and muscles. These receptors usually carry several *N*-linked carbohydrate chains. Substantial evidence uncovering the biological importance of these carbohydrate modifications has started to accumulate. Thus, the effect of *N*-glycans on the function of nicotinic acetylcholine receptors (nAChRs) has been revealed by several studies. These receptors represent 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 of NMJs, while also affecting diverse brain functions, such as learning, memory and processing of sensory information (Miwa et al. 2011). A number of studies suggested that glycosylation plays a role in channel gating and desensitization (Gehle et al. 1997; Chen et al. 1998). *N*-glycans of *Torpedo* nAChRs receptors have been implicated in receptor modulation, as receptors with mutated glycosylation sites have abnormal desensitization (the rate of current decay) and conductances (Nishizaki 2003). Interestingly, concanavalin A (ConA) can mimic this effect of mutations in vitro when pharmacologically included in the assays with wild-type receptors. ConA is a lectin that binds *N*-linked glycans, while showing a preference for oligomannosidic structures, and thus its effect on wild-type nAChRs suggested that these glycans may function as a modulating "lid" at the channel pore (Nishizaki 2003). More recent research on structure–function relationship of nAChR glycosylation indicated that carbohydrate modifications can be important for the surface expression and cholinergic agonist-dependent gating of nAChRs, but not for the binding affinity for the agonists or the stability of folded receptors (da Costa et al. 2005; Dellisanti et al. 2007).

The modulatory effect of ConA was also demonstrated for ionotropic glutamate receptors (iGluRs). iGluRs mediate fast transmission at the majority of excitatory synaptic connections within the mammalian nervous system, while also playing important roles in the regulation of synaptic plasticity and extrasynaptic modulation of neurons (reviewed in Traynelis et al. 2010). These receptors represent tetrameric complexes that function as ligand-gated ion channels and encompass large subfamilies of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and *N*-methyl-D-aspartate (NMDA) receptors (Traynelis et al. 2010). ConA exerts a pronounced effect on kainate receptors by inhibiting their desensitization (Partin et al. 1993; Everts, Villmann et al. 1997; Everts, Petroski et al. 1999). Most iGluRs appear to be modified by *N*-glycosylation, while having consensus glycosylation sites within the amino-terminal domain (ATD) involved in the receptor assembly and modulation, as well as in the ligand-binding domain (LBD) (Partin et al. 1993; Everts, Villmann et al. 1997, Everts, Petroski et al. 1999; Mah et al. 2005). The in vivo utilization of these sites has not been well characterized, but it was suggested that ConA interacts with ATD-attached *N*-glycans and affects conformational changes of the receptors (Everts, Villmann et al. 1997; Everts, Petroski et al. 1999; Fay and Bowie 2006). Other lectins, such as wheat germ agglutinin, soybean agglutinin and succinyl-ConA, can also potentiate kainate receptors (Thio et al. 1993; Yue et al. 1995). The effect of heterologous lectins in vitro suggests that glycans may play a specialized role in iGluR modulation (Everts et al. 1997; Nanao et al. 2005) mediated by interactions with some endogenous, yet unidentified lectins. However, currently, there is no direct evidence to support this scenario. Nevertheless, the appealing hypothesis that lectins recognizing *N*-glycans can be involved in the regulation of neurophysiology is consistent with several studies that shed light on lectin functions in the nervous system. Thus, experiments demonstrated the involvement of Galectin 1 in the degeneration of neuronal processes and the inhibition of neurotoxicity by affecting the expression of the NR1 NMDA receptor (Lekishvili et al. 2006; Plachta et al. 2007). Although the exact mechanisms of these functions remain to be elucidated, they appear to depend on galectin carbohydrate-binding activity and require galectin dimerization. More recently, Galectin 1 was implicated in the regulation of circadian rhythms in mice, which also points to its role in neural physiology (Casiraghi et al. 2010). Narp, a member of pentraxin family of putative endogenous lectins with structural similarity to wheat germ agglutinin, localizes to excitatory synapses and specifically clusters AMPA-type glutamate receptors (O'Brien et al. 1999). Finally, studies in *Drosophila* indicated that Mind-the-Gap (MTG), a putative fly lectin, is required for synaptic localization of iGluRs (Rushton et al. 2012).

While glycans can influence maximal currents and desensitization of AMPA and kainite receptors (Hollmann et al. 1994; Everts et al. 1997), *N*-glycosylation appears to be not generally required for the function of these iGluRs, and glycans do not significantly affect the synthesis, transport or subunit assembly of AMPA or kainate receptors (Sumikawa et al. 1988; Everts et al. 1997; Gill et al. 2009). Crystallization experiments indicated that glycans are not directly involved in ligand binding and subunit association of kainate receptors (Armstrong et al. 1998; Nanao et al. 2005). Unlike AMPA and kainate iGluRs, the NMDA-type receptors can be dramatically downregulated by inhibition of *N*-glycosylation. This effect is primarily due to the specific reduction in NR1 subunits (Everts et al. 1997). Although the exact mechanism of this defect remains largely unknown, data suggested a possible requirement for glycans in the folding or trafficking of these subunits.

Interestingly, a specialized structure that can be present on *N*-glycans, the HNK-1 glycoepitope, was shown to regulate the AMPA-type receptor subunit GluR2. HNK-1 is represented by a sulfated glucuronic acid linked to GlcNAc on the nonreducing termini of oligosaccharides (HSO_3–3GlcAβ1–3Galβ1–4GlcNAc) and its expression is enriched in the nervous system. Mouse knockouts of enzymes involved in the biosynthesis of this structure (glucuronyltransferase GlcAT-P, sulfotransferase HNK-1^{ST} and β4-galactosyltransferase-2) showed impairment of neural plasticity, learning and memory, suggesting the role of HNK-1 in synaptic functions (Senn et al. 2002; Yamamoto et al. 2002; Yoshihara et al. 2009). Experiments with GluR2 expressed in cultured hippocampal neurons and heterologous cells demonstrated that HNK-1 epitope downregulates receptor endocytosis and promotes GluR2 stability on neuronal membranes, probably via enhancing GluR2 interactions with *N*-cadherin (Morita et al. 2009).

Neurotransmitter transporters of the solute carrier 6 (SLC6) family represent another example of synaptic glycoproteins that play essential roles in synaptic transmission (Kristensen et al. 2011). They include ion-coupled plasma membrane cotransporters that control the synaptic cleft concentration of key

neurotransmitters such as dopamine, GABA, glycine, norepinephrine and serotonin. These transporters share a similar structure including 12 transmembrane domains and a large *N*-glycosylated extracellular loop between transmembrane domains III and IV. The glycosylation of this loop is important for transporter functions, since removal of glycan chains by mutations, pharmacological inhibition of glycosylation or glycosidase treatment can dramatically reduce transporter activity at the cell surface (Melikian et al. 1996; Martinez-Maza et al. 2001; Li et al. 2004; Hahn et al. 2005). These and other experiments demonstrated that the most prominent effect of glycans on these transporters is due to the requirement of proper *N*-glycosylation for trafficking and cell surface retention, while glycans usually do not significantly influence ligand binding and catalytic activity of the transporters (Tate and Blakely 1994; Olivares et al. 1995; Nguyen and Amara 1996). Importantly, the structure of glycan chains can also affect the function of these transporters. Complex sialylated glycans were found to play a role in the functional oligomerization of serotonin transporter (SERT). Sia modifications can potentiate hetero-oligomeric interactions of SERT with the cytoskeletal protein myosin IIA and stimulate serotonin uptake, presumably due to their effect on transporter homo-oligomerization and the accessibility of domains involved in hetero-oligomeric interactions (Ozaslan et al. 2003). Sia was also found to influence the function of GAT1, the major GABA transporter in the brain. Deficient sialylation of GAT1 was found to slow down the kinetics of GABA transport cycle and decrease the apparent affinity for extracellular Na^+ (Cai et al. 2005; Hu et al. 2011). Notably, several single-nucleotide polymorphisms (SNPs) affecting *N*-glycosylation and potentially associated with a disease condition have been identified in transporter genes in human populations. Thus, several SNPs that significantly decrease the glycosylation and activity of human norepinephrine transporter were found to be potentially associated with defects of synaptic transmission at postganglionic sympathetic nerve terminals implicated in cardiovascular abnormalities (Hahn et al. 2005). A SNP in the *SLC6A4* gene encoding serotonin transporter was found to create an ectopic glycosylation site (K201N) that increases the glycosylation of hSERT along with its expression and activity. By analogy to another polymorphism that similarly affects the expression of serotonin transporter and was phenotypically characterized, it was suggested that the K201N mutation can potentially influence personality traits and psychiatric disease susceptibility (Rasmussen et al. 2009).

It is important to keep in mind that many experiments with neurotransmitter receptors and transporters have been performed in various types of heterologous cells (such as insect cells (Tate and Blakely 1994), frog oocytes (Everts et al. 1997; Gehle et al. 1997; Nishizaki 2003), mammalian tissue cultures (Dellisanti et al. 2007; Gurba et al. 2012)) that could execute glycosylation and glycan-dependent regulation differently from each other and from neural cells in vivo. Moreover, glycosylation of multisubunit receptor complexes could also depend on a particular combination of expressed receptor subunits (e.g., as in the case of $GABA_A$ receptors that can have differently glycosylated β2 subunits, depending on co-expression of other subunits (Gurba et al. 2012)). Therefore, a caution should be exercised when interpreting data from cell culture experiments and relating them to in vivo mechanisms. Nevertheless, taken together, experimental data clearly indicate that glycosylation can significantly influence the function of neurotransmitter receptors and transporters. This influence can be dissimilar for distinct neurotransmitter systems, and even within one family of closely related glycoproteins, such as iGluRs, glycosylation can engage various modulatory mechanisms resulting in different functional outcomes and depending on particular structures of carbohydrate chains. These specific effects of *N*-glycans add an additional dimension to the regulatory space of processes that control synaptic transmission and plasticity.

N-glycosylation regulates ion channels in vertebrate neurons

N-glycosylation was found to be an important modulator of ion channels in neural cells. Usually, glycans modulate channels via two mechanisms: by affecting their cell surface expression and by influencing biophysical properties of channel proteins. The first mechanism depends on the effect on protein folding, stability and subcellular trafficking, in particular exocytosis and internalization. The effect on cell surface expression has been demonstrated for several types of neuronal channels, including acid-sensing channels (e.g., acid-sensing ion channel (ASIC) 1a 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)). Many channel proteins have glycans attached to their pore loops, a protein region frequently influencing channel gating, which promotes the effect of glycans on channel's biophysical characteristics. Thus, the function of TRPM8, a member of a large family of transient receptor potential (TRP) ion channels playing essential roles in sensory physiology, is modulated by pore loop glycans that cause a 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). Since its glycosylation affects the temperature threshold of channel activation, it was proposed that TRPM8 glycans represent critical molecular determinants that establish cold sensitivity in primary sensory neurons (Pertusa et al. 2012). Interestingly, the membrane localization of TRPM8 appears to be largely unaffected by glycosylation (Pertusa et al. 2012). While glycans change the biophysical properties of several other TRP channels (TRPC3, TRPC6 and TRPV1 (Dietrich et al. 2003; Wirkner et al. 2005)), glycosylation can also control TRP channels by influencing their expression and subcellular localization (TRPV4 and TRPV5 (Chang, et al. 2005; Xu et al. 2006)). These different mechanisms appear to be not mutually exclusive and could operate at the same time, while one of them could dominate, depending on particular molecular and cellular contexts.

A fascinating glycan-dependent regulation has recently been described for the renal epithelial Ca^{2+} channel TRPV5. This channel can be efficiently internalized, and its retention on the cell surface is an essential process that regulates channel function. Although this regulation is not yet fully understood, it is most likely mediated by converging effects of several mechanisms. One of them was found to rely on *N*-glycan-dependent stabilization of the channel by the hormone Klotho. Klotho is a

glycosidase that appears to modify channel glycans, which in turn facilitates channel interactions with galectin and potentiates cell surface retention (Cha et al. 2008; Chang et al. 2005; Leunissen et al. 2013). Yet another regulation engages sialylation and appears to work independently from *N*-glycosylation by promoting lipid-raft–mediated internalization of the channel (Leunissen et al. 2013). It would be interesting to investigate if similar mechanisms operate in the nervous system to control channels involved in neural transmission.

In addition to direct effects mediated by channel glycans, *N*-glycosylation can exert its effect indirectly, in molecular non-autonomous manner, via regulating glycoproteins that control channel functions, e.g. auxiliary subunits promoting membrane localization of a channel (Cotella et al. 2010). This extra layer of regulation, however, remains largely unexplored.

Sialylated *N*-glycans regulate voltage-gated ion channels and membrane excitability

Glycans can directly influence cell excitability in vertebrates by affecting the function of different members of a large superfamily of voltage-gated ion channels, including Na^+, K^+ and Ca^{2+} channel (e.g., Recio-Pinto et al. 1990; Zhang et al. 1999; Bennett 2002; Gong et al. 2002; Watanabe, Wang et al. 2003; Watanabe, Zhu et al. 2007; Johnson et al. 2004; Schwetz et al. 2010; Weiss et al. 2013). The glycosylation of mammalian voltage-gated Na^+ and K^+ channels has been shown to be developmentally and spatially regulated in the heart and the nervous system, suggesting that glycans are involved in the control of cellular excitability to fulfill different demands of particular cells and developmental stages (Castillo et al. 1997; Tyrrell et al. 2001; Schwalbe et al. 2008; Montpetit et al. 2009). Many studies have been specifically focused on sialylated glycans because of their negative charge and potential electrostatic effect on channel gating (reviewed in Ednie and Bennett 2012). Vertebrate voltage-gated Na^+ channels are especially heavily glycosylated and sialylated: glycans can comprise up to 30% of their molecular mass, with Sia representing almost 50% of these glycan chains (Miller et al. 1983; Elmer et al. 1985; Messner and Catterall 1985; James and Agnew 1987; Roberts and Barchi 1987).

Electrophysiological analyses of cultured cells and in vitro experiments revealed that sialylation can significantly influence the conductance properties of Na^+ channels (Recio-Pinto et al. 1990; Bennett et al. 1997; Zhang et al. 1999; Cronin et al. 2005). The effect of Sia is isoform- and subunit-specific, and it significantly varies for different channel proteins (Bennett 2002; Johnson et al. 2004; Johnson and Bennett 2006). This effect has been usually attributed to a large negative charge due to numerous Sia residues attached to vertebrate channel glycans. Interestingly, vertebrate voltage-gated Na^+ channels represent one of only few types of glycoproteins shown to be modified with PSA (James and Agnew 1987; Zuber et al. 1992). It is estimated that these channels can carry more than 100 Sia residues, most of them being in a form of PSA (Miller et al. 1983; James and Agnew 1987; Zuber et al. 1992). The role of PSA in channel modulation was confirmed by experiments with ST8Sia II polysialyltransferase mutant mouse cardiomyocites that showed defects in excitability and gating of voltage-gated Na^+ channels (Montpetit et al. 2009). However, several lines of evidence indicate that the effect of sialylation is not always associated with PSA and a significant charge accumulated in the vicinity of channel pore, suggesting that Sia can have a more specific effect on channel functions. For instance, endo-N treatment that specifically removes PSA did not significantly influence evoked synaptic transmission and action potentials in the rat brain (Muller et al. 1996). Similarly, cell culture experiments indicated that cardiac sodium channel could be affected by some "functional" Sia residues rather than by the total charge of sialylation (Stocker and Bennett 2006). Moreover, experiments in mutant isogenic Chinese hamster ovary (CHO) cell lines with different defects in sialylation found that the effects of PSA and non-PSA sialylation on the function of alpha-$Na_V1.4$ channel were distinct. The complete loss of sialylation in the 6B2 clone due to a mutation in the Golgi CMP-Sia transporter resulted in opposite shifts of voltage-dependent activation and steady-state inactivation of the channel, when compared with the consequence of a PSA defect due to genetic inactivation of the polysialyltransferase ST8SiaIV in the 2A10 clone, while only the loss of Sia had a significant effect on recovery from fast inactivation (Ahrens et al. 2011). Finally, metabolic introduction of unnatural Sia's with *N*-acetyl groups changed to *N*-pentanoyl and *N*-propanoyl structures was found to have an effect on conductance properties of the Kv3.1 voltage-gated K^+ channel, suggesting that Sia residues can modulate channels through specific steric interactions (Hall et al. 2011).

While many studies using transgenic approaches in various heterologous cultured cells have demonstrated the involvement of sialylation in the regulation of voltage-gated channels, the structure of glycosylation and its functional implication can significantly vary between different cell types, and between cultured cells and in vivo. At the same time, experiments that address the function of Sia's in vivo remain scarce. Biological importance of sialylation of voltage-gated Na^+ channels has been most clearly shown in the context of cardiac functions using mouse genetic models and cardiomyocyte cultures. These experiments indicated that deficient sialylation of channels can be implicated in cardiac abnormalities and heart failure (Ufret-Vincenty, Baro, Lederer, et al. 2001; Ufret-Vincenty, Baro, Santana, et al. 2001; Stocker and Bennett 2006; Montpetit et al. 2009). Treatments with glycosidases that remove sialylated glycans and the inhibition of endogenous neuraminidase, an enzyme that trims Sia's from carbohydrate chains, demonstrated that sialylation influences network excitability and seizure threshold in kindling epilepsy models and suggested that Sia's modulate voltage-gated Na^+ channels in brain neurons (Tyrrell et al. 2001; Isaev, Isaeva et al. 2007; Isaeva, Lushnikova et al. 2010; Isaev, Zhao et al. 2011). While these experiments revealed that sialylation can significantly potentiate the excitability of neural networks, whether this effect is due to the sialylation of voltage-gated channels or some other molecules remains unknown. It is challenging to address this question in vertebrates because of the complexity of the nervous system, intricacies of glycosylation pathways, as well as the ubiquity of sialylation affecting panoply of glycoconjugates. With its power of genetic approaches, a spectrum of well-established neurobiological approaches, and simplified glycosylation pathways, *Drosophila* has recently emerged as promising model for elucidating conserved genetic and molecular mechanisms of neural glycosylation.

N-glycosylation regulates the nervous system of *Drosophila*

The repertoire of *N*-glycans in insects is different from that in vertebrate species. Detailed analyses of *Drosophila* glycome revealed that paucimannose and high mannose structures dominate the spectrum of *N*-glycosylation, while the complex and hybrid-type oligosaccharides, the glycans corresponding to more processed mature structures prevalent in vertebrates cells, represent only 12% of the total *N*-glycan profile (Aoki et al. 2007). These minor glycan species, however, appear to play prominent roles in the nervous system, suggesting conservation

Table I. Examples of effects of *N*-glycosylation on glycoproteins involved in neural physiology

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	(Gehle et al. 1997; Chen et al. 1998; 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	(Partin et al. 1993; Thio et al. 1993; Yue et al. 1995; Everts et al. 1997, 1999) (Senn et al. 2002; Yamamoto et al. 2002; Morita et al. 2009; 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 its affinity for Na^+.	(Tate and Blakely 1994; Olivares et al. 1995; Melikian et al. 1996; Nguyen and Amara 1996; Martinez-Maza et al. 2001; Ozaslan et al. 2003; Li et al. 2004; Cai et al. 2005; Hahn et al. 2005; Rasmussen et al. 2009; Hu et al. 2011; Kristensen et al. 2011)
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	(Kadurin et al. 2008; Jing et al. 2012)
TRP channels (transient receptor potential ion channels)	Playing essential roles in sensory physiology	Affects the temperature threshold of TRPM8 activation in response to cold. Affect biophysical properties of TRPC3, TRPC6 and TRPV1. Affect expression and subcellular localization of TRPV4 and TRPV5.	(Dietrich et al. 2003; Chang et al. 2005; Wirkner et al. 2005; Xu et al. 2006; Pertusa et al. 2012)
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	(Thornhill et al. 1996; Freeman et al. 2000; Gong et al. 2002; Watanabe et al. 2003, 2004; Sutachan et al. 2005; Watanabe et al. 2007; Johnson and Bennett 2008; Noma et al. 2009; Zhu et al. 2009; Schwetz et al. 2010; Hall et al. 2011; 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)	(Recio-Pinto et al. 1990; Bennett et al. 1997; Zhang et al. 1999; Johnson et al. 2004; Cronin et al. 2005; Stocker and Bennett 2006; Repnikova et al. 2010)

of their functions in evolution (Schachter 2010). The importance of these glycans has become evident from a number of studies that analyzed neurological phenotypes of mutants affecting *N*-glycosylation pathway. Thus, genetic inactivation of *N*-acetylglucosaminyltransferase I, a key enzyme in the production of mature *N*-glycan structures, was found to result in neurological abnormalities in fruit flies, including locomotion phenotypes, shortened life span and developmental defect of the mushroom bodies, a specialized brain structure involved in memory formation (Sarkar et al. 2006; Sarkar et al. 2010). Mutations of *fused lobes*, a gene encoding Golgi β-*N*-acetylglucosaminidase that promotes the biosynthesis of paucimannose structures at the expense of hybrid and complex *N*-glycans, result in similar mushroom body defects (the mushroom body *lobes* become *fused*) and cause cell-lethal phenotype in mosaic clones of olfactory projection neurons (Boquet et al. 2000; Leonard et al. 2006; Sekine et al. 2013). Downregulation of *sugar-free frosting*, a gene encoding a *Drosophila* homolog of SAD kinase regulating secretory flux through the Golgi, increases *N*-glycan complexity and leads to NMJ defects in larvae and locomotor abnormalities in adult flies (Baas et al. 2011). Meigo, a putative nucleotide sugar transporter, affects *N*-glycosylation of ephrin and regulates dendrite and axonal targeting in the olfactory system (Sekine et al. 2013). Mutations in β1,4-*N*-acetylgalactosaminyltransferases A (β4GalNAcTA), a glycosyltransferase potentially involved in the biosynthesis of complex and hybrid *N*-glycans, including sialylated structures, result in prominent neurological phenotypes (Haines and Irvine 2005; Haines and Stewart 2007; Nakamura et al. 2012). All these examples illustrate that there are important requirements for a proper amount and structural diversity of *N*-glycans in the nervous system. These studies also highlight an intriguing possibility that many genes involved the *N*-glycosylation pathway may be associated with conserved mechanisms regulating the nervous system.

Sialylated *N*-glycans control neural excitability in *Drosophila*

Although sialylated glycans represent a minuscule fraction of *Drosophila N*-glycome (<0.1% of total *N*-glycan profile), they are unambiguously detected by multidimensional mass spectrometry in embryos and adult heads (Aoki et al. 2007; Koles et al. 2007) and play important roles in neural regulation (see below). Unlike vertebrate species that have multiple sialyltransferase enzymes, *Drosophila* possesses only one sialyltransferase, DSiaT, which facilitates the analysis of sialylation function in vivo (Koles et al. 2009). DSiaT modifies *N*-glycans with α*2,6*-linked Sia's and shows close evolutionary relationship to ST6Gal family of mammalian sialyltransferases (Koles et al. 2004; Repnikova et al. 2010). The expression of sialylation genes is dynamic and largely restricted to a subset of CNS neurons throughout development, indicating that sialylation undergoes a tight cell-specific and temporal regulation (Koles et al. 2009; Repnikova et al. 2010; Islam et al. 2013), which partially explains the low amount of sialylated glycans in the *Drosophila* CNS (Koles et al. 2007).

Targeted mutagenesis that inactivated the sialylation pathway has revealed that sialylated *N*-glycans have a specific role in the regulation of the nervous system. *DSiaT* mutations result in a significantly reduced life span, locomotion abnormalities and

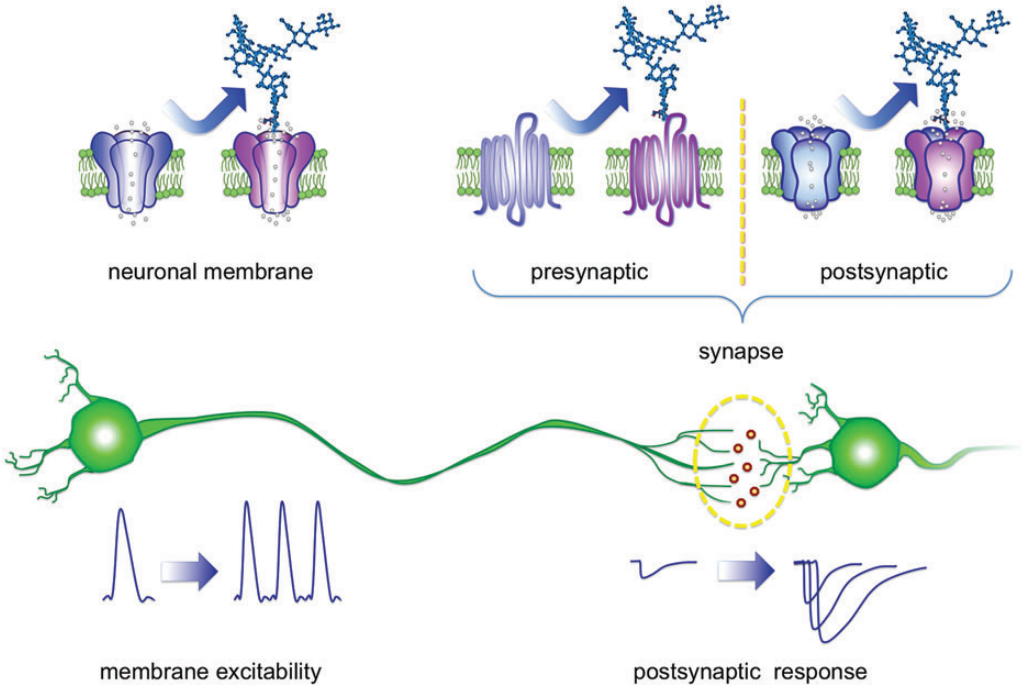

Fig. 1. *N*-glycosylation can regulate neural transmission by modulating voltage-gated ion channels that generate action potential on neuronal membranes, and by affecting synaptic transmission via impact on the function of synaptic vesicle proteins and postsynaptic neurotransmitter receptors. *N*-glycosylation is sketched as a single generic *N*-glycan (not to scale), while the number of modifications sites can be different for distinct proteins, and particular glycan structures can significantly vary and include some specific modifications, such as polysialylation and the HNK-1 epitopes (not shown).

temperature-sensitive (TS) paralysis. *DSiaT* mutants have defects of larval NMJs, and electrophysiological analyses indicated that DSiaT regulates neuronal excitability and affects the function of Paralytic (Para), a major *Drosophila* voltage-gated Na^+ channel (Repnikova et al. 2010). Similar phenotypes result from mutations in the *CMP-sialic acid synthetase* gene (*CSAS*) encoding an enzyme that produces activated sugar donor, CMP-Sia, for DSiaT-mediated sialylation (Islam et al. 2013). Notably, the paralysis phenotype of *CSAS* mutants can be ameliorated by an extra copy of *para*, which suggests that sialylation may control the amount of voltage-gated channels available for neural transmission (Islam et al. 2013). Moreover, genetic interactions between *DSiaT* and *β4GalNAcTA* indicated that Sia's may function by masking the interaction of LacNAc terminal structures of *N*-glycans with some endogenous lectins (Nakamura et al. 2012). These interesting hypotheses await further detailed investigation. Taken together, the data from *Drosophila* experiments shed light on a novel, nervous system-specific function of α2,6-sialylated *N*-glycans as essential modulators of neural transmission. This function probably represents one of the most ancient roles of Sia's in metazoan organisms. It is tempting to speculate that this role is evolutionarily conserved between flies and mammals. This intriguing possibility remains to be further investigated.

Concluding remarks

Functional modalities of *N*-glycosylation include effects on glycoproteins' folding, stability, trafficking, interactions with other molecules, such as recognition of specific sugar structures by lectins, as well as electrostatic and steric effects on protein dynamics and conformation. In the nervous system, *N*-linked glycans influence a spectrum of biological processes by mechanisms that largely depend on particular molecular and cellular contexts. Many major players of neural transmission are modified with *N*-glycans, and their effect on these proteins' functions can range from a modulation to the obligatory requirement (Table I). This wide range of possible effects appears to be well suited to the regulation of neural processes, providing a basis for achieving a whole additional continuum of states of neural transmission (Figure 1). Moreover, these effects can potentially provide an important link between neural transmission and metabolism. The modulation of neural excitability and synaptic transmission by *N*-glycans is predicted to influence essential neural functions, including plasticity and memory formation. This poses several central questions, such as what are the molecular, cellular and genetic mechanisms that underlie these roles of glycans in vivo? What are the pathobiological functions of these glycans in neurological diseases? What are the roles of lectins in mediating these glycan functions? The progress in this fundamentally important but challenging and underexplored area should be facilitated by combining in vitro and in vivo approaches, including experiments using genetically tractable model organisms.

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Abbreviations

β4GalNAcTA, β1,4-*N*-acetylgalactosaminyltransferase A; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ASIC, acid-sensing ion channel; ConA, concanavalin A; CSAS, CMP-sialic acid synthetase; DSiaT, *Drosophila* sialyltransferase; GABA, γ-aminobutyric acid; GlcNAcT, β-1,6-*N*-acetylglucosaminyltransferase; NMJ, neuromuscular junction; iGluR, ionotropic glutamate receptor; LacNAc, *N*-acetyllactosamine; nAChR, nicotinic acetylcholine receptor; NCAM, neural cell adhesion molecule; NMDA, *N*-methyl-D-aspartate; Para, paralytic; PSA, polysialic acid; Sia, sialic acid(s); SV2, synaptic vesicle protein 2; TRP, transient receptor potential; UDP-GlcNAc, uridine diphospho-*N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

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