Chapter 7
Glycans and Carbohydrate-Binding/ Transforming Proteins in Axon Physiology

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

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Abbreviations

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

1 Introduction

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

2   Axon Determination/Early Neuronal Differentiation

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

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

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

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

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

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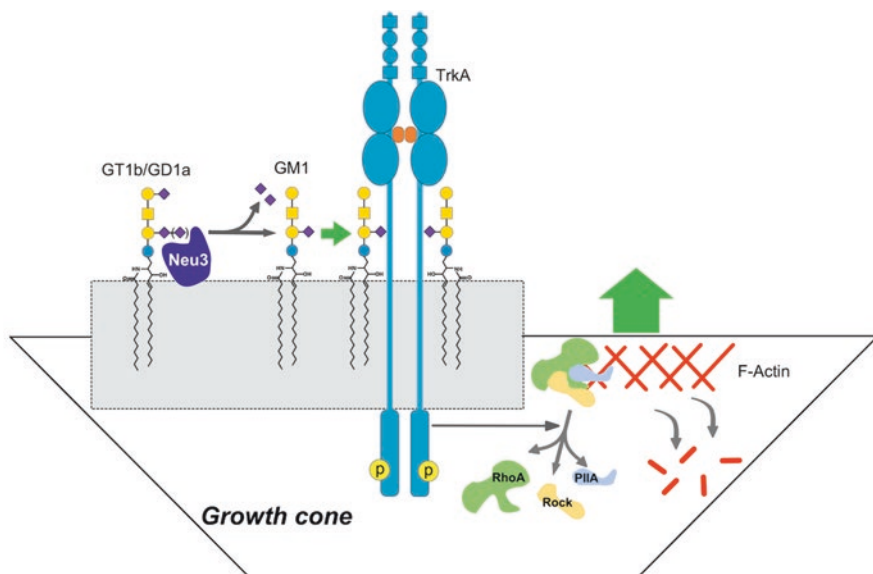

Fig. 7.1 Neu3/GM1-induced axon outgrowth. Membrane neuraminidase Neu3 locally removes neuraminic acid moieties (purple diamonds) from gangliosides GD1a or GT1b to produce GM1, which spatially-gathers neurotrophin receptor TrkA by direct interaction, stimulating signalling for the inactivation of RhoA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

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

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

TrkA displays up to eleven putative N-glycosylation sites and only N-glycosylated TrkA forms bearing fully mature N-glycans can be targeted to the axon membrane (Watson et al. 1999). GM1 specifically binds to the mature form of TrkA, gathering it to transport vesicle membrane rafts for an efficient anterograde transport to the axon plasma membrane (Abad-Rodriguez and Díez-Revuelta 2015; Mutoh et al. 1995; Rabin and Mocchetti 1995). Cells lacking GM1, or expressing deficiently glycosylated TrkA, fail to target the receptor to the membrane precluding any neurotrophic effect (Mutoh et al. 2000; Mutoh et al. 2002). GM1 also interacts with glycosylated α5β1-integrin (Wu et al. 2007) and both molecules are stabilized in the complex by the specific cross-linking with homobivalent dimers of Gal-1. This membrane process stimulates axon growth by triggering the signalling cascade of focal adhesion kinase (FAK)/phospholipase Cγ/phosphoinositide-3 kinase (PI3K), and the opening of TRPC5 channels leading to a transient raise of intracellular Ca^{2+} (Wu et al. 2016).

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

3   Axon Guidance

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

3.1 Axon Guidance by Glycosylated Cell Adhesion Molecules (CAMs)

Neuronal cell adhesion molecule (NCAM) presents a characteristic glycosylation with terminal polysialic acid (PSA), a polymer of α2–8 linked neuraminic acid. These polymers are formed by ST8Sia II and ST8Sia IV polysialyltransferases acting on hybrid, bi- to tetra-antennary complex glycans bearing, preferably, a terminal α2–6-linked neuraminic acid (Bhide and Colley 2016). Supporting the role of PSA-NCAM in guidance, impaired N-glycosylation or polysialylation by enzymatic ablation produces defective development of the mouse olfactory bulb and failed neuronal migration (Hildebrandt et al. 2009; Weinhold et al. 2005), projection errors in the chicken optic fiber layer due to the blockade of axon exit from the retina, and increased fasciculation of chicken spinal ganglia (Monnier et al. 2001). Similarly, impaired PSA synthesis affects granular cell projections (mossy fibers) along the hippocampal *stratum lucidum*. Their axons become misrouted due to over-adhesion to the pyramidal cell layer eliminating the lamination of the supra- and infra-pyramidal mossy fiber bundles (Fig. 7.2) (Koyama and Ikegaya 2018; Weinhold et al. 2005). There is a structural reason that explains these effects. In fact, PSA in NCAM confers numerous negative charges on its structure creating a strong electrostatic repulsion and increasing intercellular space that hampers adhesion (Rutishauser and Landmesser 1996). In all, PSA regulates NCAM-mediated neuronal migration and axonal fasciculation and guidance, becoming essential for the development of the nervous system.

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

Besides the regulation of axon growth and guidance by PSA-induced electrostatic hindrance, other adhesion molecules act through heterophilic interactions with glycosylated molecules on the membranes of adjacent cells. Such is the case of L1CAM that binds in *trans* to the highly glycosylated mucin glycoprotein CD24 carrying α2–3 linked sialic acid and Lewis[x] terminals on its structure. Interestingly, L1CAM/CD24 interaction can exert axon outgrowth stimulation in mouse cerebellar neurons or inhibition in DRG neurons (Kleene 2001). Neuronal L1 interacts in *trans* with α2–3 linked sialic acid in glial CD24 through a siglec homologous sequence, while CD24 Lewis[x] terminals interact with TAG1 and contactin in cerebellar neurons to assemble a neurite-growth stimulating complex. In contrast, TAG1 and contactin in DRG neurons, interacting in *trans* with CD24 Lewis[x] terminals, form complexes by *cis* interactions on neuronal surfaces with Caspr1 and Caspr2, respectively, triggering outgrowth inhibitory signalling (Lieberoth et al. 2009). Noteworthy, contactin binding to Caspr1 is also regulated by contactin glycosylation state. This will be discussed below within the Sect. 4.
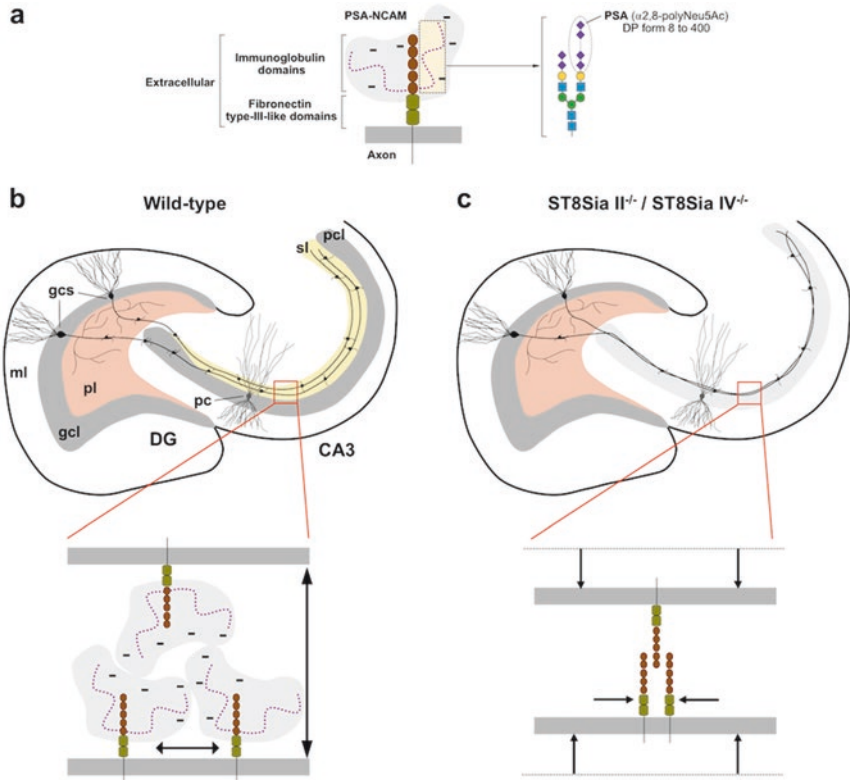
Fig. 7.2 Anomalous lamination of hippocampal mossy fibers in PSA-deficient mice. (**a**) PSA chains are synthesized on 5th and 6th N-glycosylation sites in immunoglobulin (Ig) domain 5 of NCAM, and are formed by repeated α2–8 N-acetylneuraminic acid units (degree of polymerization –DP- from 8 to 400 units). (**b**) Schematic design of wild type mouse dentate gyrus (DG) and hippocampus CA3 zone illustrating the mossy fibers, axons of granule cells (gcs) that project along the *stratum lucidum* (sl) in parallel to the pyramidal cell layer (pcl). These axons send collaterals to contact pyramidal cells (pc), constituting a fully laminated structure (ml, molecular layer; pl, polymorphic layer; gcl, granular cell layer). This laminated structure is partially maintained by PSA through the repulsive field provided by its bulky, polyanionic properties (**b**, bottom panel). Such an electrostatic repulsion regulates cell-cell interspace distance, avoiding the direct interaction of NCAM. (**c**) In PSA-deficient mice lacking the sialyl-transferases that synthesize PSA (ST8Sia II$^{-/-}$ /ST8Sia IV$^{-/-}$) mossy fiber bundling and lamination is severely perturbed and axons become misrouted. The absence of PSA negatively charged "clouds" reduces the distance between axon membranes, and permit the direct interactions of NCAM with itself and other molecules in *cis* or *trans* (**c**, bottom panel) that provoke the over-adhesion among granular axons and to the pyramidal cell layer

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

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

3.2 Glycosaminoglycans in Axon Guidance

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4 Axon/Glia Interactions

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

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

In addition, the myelin-associated glycoprotein (MAG, Siglec-4), that binds gangliosides GD1a and GT1b with high affinity in vitro (Vinson 2001; Vyas et al. 2002;
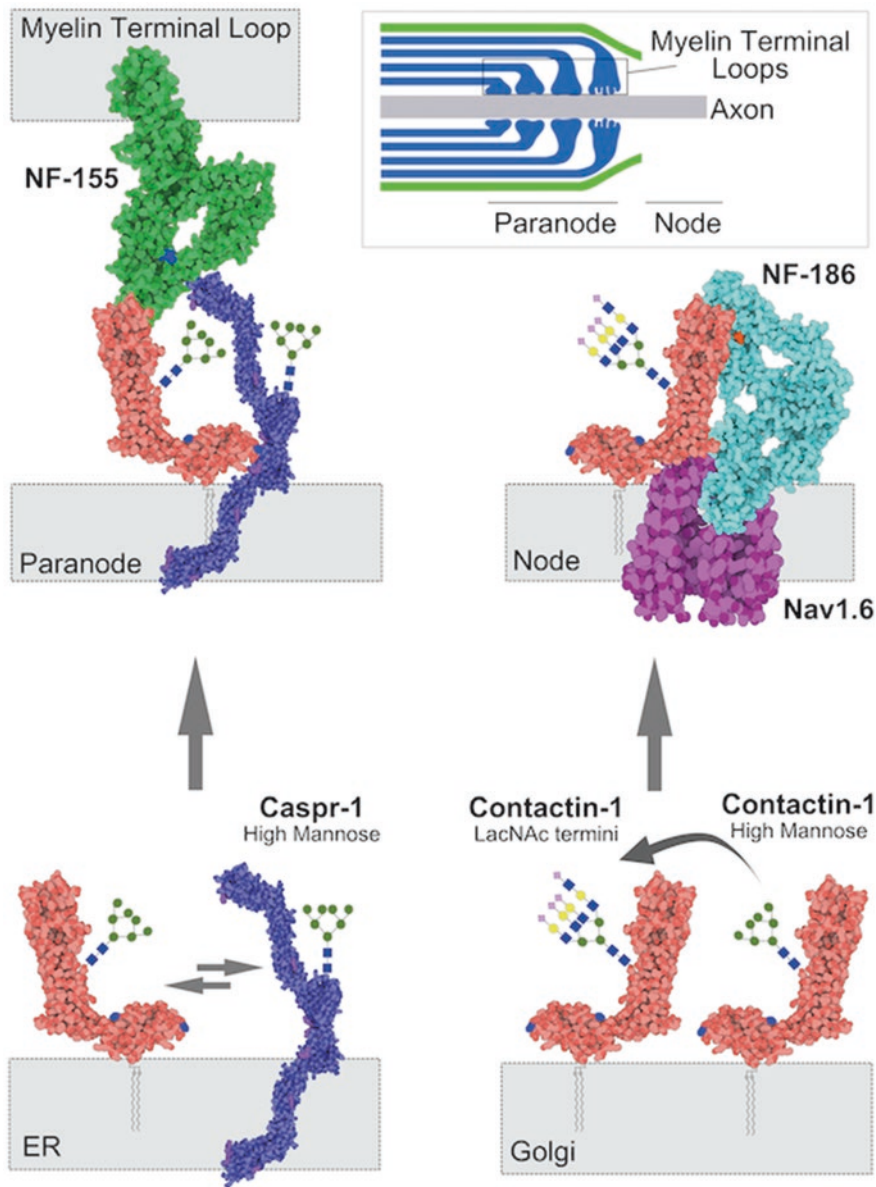
Fig. 7.3 Glycosylation role in the organization of nodes of Ranvier and paranodes. In the left panel it is shown how contactin1 and caspr1 bearing immature N-glycans (high mannose termini) in ER membranes associate in *cis* and target the axon paranodes where they interact with neurofascin-155 (NF-155) of the adjacent myelin terminal loop membrane. Alternatively, as shown in the right panels, contactin1 N-glycans can turn mature by addition of N-acetyl-lactosamine (NAcLac) termini within the Golgi apparatus. This blocks its interaction with caspr1, and targets contactin1 to the nodes of Ranvier, where it interacts with neurofascin-186 (NF-186) and recruits the Nav1.6 sodium channel required for fast nerve impulse conduction. Protein complex structures displayed in this figure are idealized for an intuitive comprehension of the processes. Protein 3D models have been obtained from www.3dproteinimaging.com

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

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

5   Nerve Impulse Generation/Transmission

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

5.1   Voltage-Gated Ion Channels

Voltage-gated ion channels exert important neuronal functions such as the control of cell excitability, generation and propagation of action potentials, and modulation of synaptic transmission. Of particular importance for the correct functioning of the AIS and the nodes of Ranvier are several channels that regulate membrane permeability for sodium, potassium, and calcium ions. Generally, these ion channels and their auxiliary subunits are heavily glycosylated, with some subunits having up to 36% of their mass in the form of glycans (Messner and Catterall 1985; Roberts and Barchi 1987). The post-translational modification of these channels and/or their auxiliary subunits by N-glycan attachment can affect their function through different mechanisms (Fig. 7.4b). N-glycosylation can affect the folding and trafficking of ion channels to the cell surface. Once on the cell surface, N-glycosylation can
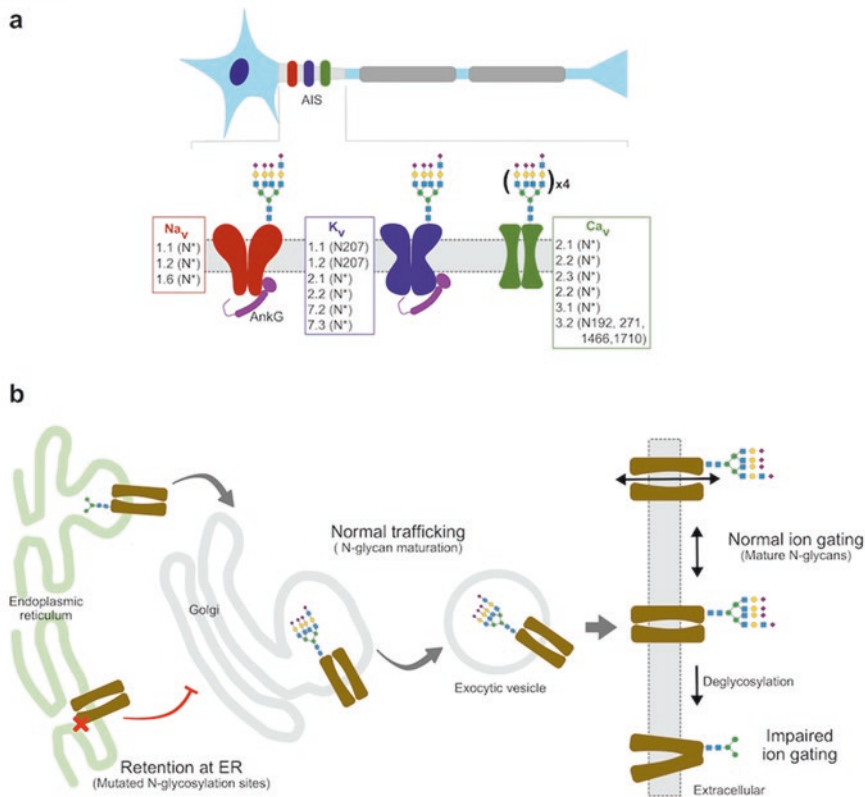
Fig. 7.4 Role of glycosylation in voltage-gated ion channels of the Axon Initial Segment (AIS). (**a**) Schematic representation of ion channel expressed at the AIS. Channels of the three families (sodium, potassium and calcium) have been described in the AIS, all of them present putative N-glycosylation sites, although the presence of N-glycans have been only demonstrated in some of them, denoted as (N + number). Those sites with no current evidence of attached glycans are symbolised as (N*). Updated information on channel glycosylation can be found at https://www.gly-gen.org. (**b**) The post-translational modification of voltage-gated ion channels (generic channel depicted in brown) and/or their auxiliary subunits by N-glycosylation can affect their function through different mechanisms. Lack of N-glycosylation can affect the channel folding as has been proven by directed mutagenesis of the different N-glycosylation sites. This usually induces the retention of non-glycosylayed channels at the ER precluding their trafficking to the cell surface. In contrast, intact channels exit the ER and their N-glycans undergo full maturation before reaching the plasma membrane. Once on the cell surface, mature N-glycosylated channels can perform their normal ion gating function. Nevertheless, N-glycans hydrolysis by glycosidase enzyme activities (deglycosylation) at the membrane can also alter the channel distribution and stability (altered endocytosis and recycling) and impair their ion gating function, thus affecting the transmission of the nerve impulse

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

5.2 Voltage-Gated Sodium Channels

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

comparison to the muscle and cardiac channels, neuronal isoforms (Nav1.2 and Nav1.7) are moderately glycosylated and consequently showed moderate depolarizing shifts upon desialylation (Johnson et al. 2004a). Therefore, the effect of glycosylation on VGSC function is dependent not only on the specific isoform being expressed but also on the cell type in which it is expressed. Consequently, VGSC activity is regulated in a cell-specific manner by glycosylation. As mentioned before, VGSC β subunits are also extensively glycosylated (Yu et al. 2003; Zhou et al. 2012; Cortada et al. 2019). Although fewer studies have explored the role of glycosylation on their function, evidence indicates that β subunit glycosylation can regulate the trafficking of sodium channels to the cell surface. A recent study explored the functional significance of β2 subunit glycosylation on Nav1.5 channel trafficking (Cortada et al. 2019). The β2 subunit was glycosylated at three different sites (N42, N66 and N74). An unglycosylated triple mutant was mostly retained at the endoplasmic reticulum and was defective at promoting cell surface expression of the Nav1.5 α subunit.

5.3 Voltage-Gated Potassium Channels

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

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

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

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

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

5.4   Voltage-Gated Calcium Channels

In excitable cells, voltage-gated calcium channels (VGCCs) elevate intracellular calcium concentrations after membrane depolarization. Particularly in neurons, these channels are the primary mediators of depolarization-induced calcium entry where they regulate the timing and generation of action potential bursts. VGCCs consist of a pore-forming α1 subunit and two auxiliary subunits - a cytoplasmic β subunit and an extracellular α2δ subunit. A great variety of channel subtypes can be co-assembled through the use of ten α1 subunit genes, four β subunit genes, and four α2δ subunit genes. VGCCs are classified in three channel subfamilies (Cav1, Cav2 and Cav3) according to their α1 subunit, or into five classes (L-type: Cav1.1 - Cav1.4, P/Q-type: Cav2.1, N-type: Cav2.2, R-type: Cav2.3, and T-type: Cav3.1–3.3) based on their biophysical and pharmacological properties (Catterall 2011). There is a widespread expression of VGCCs throughout the nervous system. Cav1 channels are present in dendritic spines where calcium entry regulates long-term potentiation (Grover and Teyler 1990; Navakkode et al. 2022). Cav2 channels are present in presynaptic nerve terminals where calcium entry triggers neurotransmitter release (Mochida 2019). Cav3 channels are mainly present in the soma and dendrites of neurons, and they regulate cellular excitability and oscillatory behavior as well as neurotransmitter release (Cheong and Shin 2013; Perez-Reyes 2003; Turner and Zamponi 2014; Weiss et al. 2013; Weiss et al. 2012). Importantly, several VGCCs have been localized at the AIS including Cav2.1, Cav2.2, Cav2.3, and more recently, Cav3.1 and Cav3.2 (Bender and Trussell 2009; Lipkin et al. 2021). Experiments performed on different types of neurons revealed that the activation of these channels at the AIS is essential to the generation and timing of action potential bursts (Bender and Trussell 2009).

Like other voltage-gated ion channels, mature calcium channels are also glycosylated proteins, and their glycosylation regulates the cell surface channel density and their gating properties. In silico analysis of the amino acid sequence of the ten α1 pore-forming subunits reveal canonical N-glycosylation sites (Lazniewska and Weiss 2017). Evidence pointing to the glycosylation of VGCCs have come from studies using lectin labelling to identify glycosylation sites, from biochemical analyses were the molecular mass of the channels change upon glycosidase treatment or N-glycosylation inhibition with tunicamycin, as well as from site-directed mutagenesis studies.

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

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

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

In addition to the pore-forming subunit, the α2δ auxiliary subunit is also a glycosylated protein. It is highly N-glycosylated, with approximately 30 kDa of its mass in the form of glycans (Marais et al. 2001). The first studies concerning the functional significance of the glycosylation of this subunit revealed it has a role in stabilizing its interaction with the pore-forming subunit and for the functional increase in the channel's current amplitude (Gurnett et al. 1996). Site-directed mutagenesis studies identified positions N136 and N184 as functional N-glycosylation sites necessary for enhancement of calcium current amplitudes (Andrade et al. 2009; Sandoval et al. 2004). Glycosylation of the α2δ subunit at position N633 was also identified as necessary for its surface expression and as essential for its role as a regulator of the channel's calcium current (Tétreault et al. 2016). Additional glycosylation sites (N348, N468 and N812) also contribute to the stability of this subunit (Tétreault et al. 2016).

5.5   *O-Linked β-N-Acetylglucosaminylation of AIS Proteins*

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

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

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

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

6   Axon Regeneration

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

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

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

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

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

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

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