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REVIEW ARTICLE

The clustering of voltage-gated sodium channels in various excitable membranes

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Abstract

In excitable membranes, the clustering of voltage-gated sodium channels (VGSC) serves to enhance excitability at critical sites. The two most profoundly studied sites of channel clustering are the axon initial segment, where action potentials are generated and the node of Ranvier, where action potentials propagate along myelinated axons. The clustering of VGSC is found, however, in other highly excitable sites such as axonal terminals, postsynaptic membranes of dendrites and muscle fibers, and pre-myelinated axons. In this review, different examples of axonal as well as non-axonal clustering of VGSC are discussed and the underlying mechanisms are compared. Whether the clustering of channels is intrinsically or extrinsically induced, it depends on the submembranous actin-based cytoskeleton that organizes these highly specialized membrane microdomains through specific adaptor proteins.

KEYWORDS

axoglial adhesion molecules, axon initial segment, cytoskeleton, node of Ranvier, sodium channels

1 | INTRODUCTION

In vertebrates, the clustering of voltage-gated sodium channels (VGSC) at specific membrane domains is at the basis of fast neuronal conduction. The two major sites where VGSCs are found at high densities are the axon initial segment (AIS) where action potentials (APs) are generated and the node of Ranvier (NOR) where APs propagate by saltatory conduction in myelinated axons. In neurons, the clustering of VGSCs in the plasma membrane depends on interactions with the actin-spectrin submembranous cytoskeleton through ankyrins. Sodium channels acquired ankyrin-binding anchor motif already 50 million years prior to the evolution of myelin. That is, the AIS predated the NOR which, as myelin evolved, utilized similar intracellular mechanisms for its assembly (Hill et al., 2008). In agreement with this evolutionary timeline, the formation of NOR depends on axon-glia contact, while the AIS is determined intrinsically (Eshed-Eisenbach & Peles, 2013; Zhang & Rasband, 2016). Apart from the AIS and NOR, clustered VGSCs are found at sites where enhanced excitability is required, such as axonal terminals, postsynaptic membranes and pre-myelinated axons. In this review, different examples of axonal as well as non-axonal clustering of VGSC are discussed and the underlying mechanisms are compared. Although most research effort has expectedly focused on the AIS and NOR, evidence points to a common universal model with site-specific modifications.

2 | THE FAMILY OF VGSC

Neuronal excitably critically depends on VGSC that mediate inward sodium currents and are responsible for the depolarization phase of the AP. VGSC consist of a pore-forming α subunit (Nav1.1–Nav1.9) associated with one or more auxiliary β

subunits (β1–β4) (Goldin et al., 2000) that modulate the surface expression and kinetics of the channels (Namadurai et al., 2015). Nav1.1 is mostly somatodendritic in principal neurons, but can be found at the AIS, where it is localized very close to the soma and at NOR in a few neuronal cell types including motor neurons (Dumenieu, Oule, Kreutz, & Lopez-Rojas, 2017). Nav1.3 is expressed in axons of DRG neurons and is upregulated after injury (Lindia, Kohler, Martin, & Abbadie, 2005). Nav1.4 is a skeletal muscle isoform and Nav1.5 is expressed in cardiomyocytes. Nav1.7, Nav1.8 and Nav1.9 (and the axonal Nav1.6) are expressed in various subtypes of DRGs sensory neurons, including nociceptive fibers (Bao, 2015; Cummins, Sheets, & Waxman, 2007), hence although less studied compared to CNS-expressed isoforms, they are targets for local anesthetic and analgesic drug-related research. Nav1.2 and Nav1.6 are the major axonal channels. They are clustered at high density at the AIS, where APs are generated and at NOR (Nelson & Jenkins, 2017). Nav1.2 is also uniformly distributed along the axon. In addition, it is transiently expressed in AIS and NOR during development and is replaced by Nav1.6 (Boiko et al., 2001). In some mature AIS, Nav1.2 can be detected but not colocalized with Nav1.6 expression (Hu et al., 2009).

3 | CHANNEL CLUSTERING: PHYSIOLOGICAL CONSEQUENCES

VGSC are critical for AP generation and propagation. Therefore, neuronal excitability is controlled by the localization of sodium channels, their subunit composition and density (Heine, Ciuraszkiewicz, Voigt, Heck, & Bikbaev, 2016). Voltage-gated potassium channels also greatly affect different aspects of neuronal excitability as a key part of the AP machinery (Heine et al., 2016). In this review, we will focus on the general phenomenon of VGSC clustering only.

AIS is the site of AP initiation. It is a 20–60 μm long specialized membrane domain located at the proximal region of the axon and contains a high density of VGSC (Huang & Rasband, 2018). APs are generated at the distal end of the AIS, where the density of the low-threshold Nav1.6 is highest (Hu et al., 2009). The excitability of the AIS is controlled by the density of voltage-gated sodium and potassium channels, their types and their precise location (Huang & Rasband, 2018). It has been reported that the AIS length and localization are dynamic and change upon alterations in activity or under pathological conditions. For example, visual deprivation blocks the changes in AIS length that were detected in cortical neurons (ranging from an average of 22.75 to 37.59 μm) during the critical period of visual system development (Gutzmann et al., 2014). In general, input-deprived neurons become more excitable, while hyperpolarized neurons become less excitable and this occurs by alterations in the size and localization of the AIS (Huang & Rasband, 2018).

In unmyelinated axons, conduction velocity depends on the axon diameter as it is inversely correlated with the axial resistance of the axon (Castelfranco & Hartline, 2015). In myelinated axons there are additional parameters that control the speed of conduction. The myelin sheath, a multilamellar structure synthesized by myelinating glia, provides high electric insulation and decreased membrane capacitance to axons. It is organized in segments termed internodes, spaced by gaps in which VGSC are clustered at high densities (Peles & Salzer, 2000). This specialized form of axon-glial interaction enables fast and efficient nerve conduction as APs only occur at nodes of Ranvier, while the electrical signals propagate fast and with a minimum energy usage along the myelinated internodes. It is believed that by enabling faster nerve conduction without the need to increase axonal caliber, myelin allowed the evolution of large vertebrates (Zalc, Goujet, & Colman, 2008). In addition to axon caliber, conduction velocity in myelinated axons depends on the myelin sheath thickness (which is correlated with the axon caliber), the internodal length, the intactness of the paranodal seal, and the size of the NOR and the density of VGSC it contains (Arancibia-Carcamo & Attwell, 2014; Freeman, Desmazieres, Fricker, Lubetzki, & Sol-Foulon, 2016). An increase in the nodal length would result in increased nodal capacitance and thus slower conduction (Waxman, Pappas, & Bennett, 1972). Indeed, it was recently reported that in the rat optic nerve and cortical axons, the nodal length varies considerably (ranging from an average of 0.43 to 3.72 μm in the cortex), mostly between axons (Arancibia-Carcamo et al., 2017). The authors suggest that axon-specific adjustment of NOR length may be another means of regulating conduction velocity in myelinated CNS axons. In all excitable sites in neurons, the type of sodium channels that is expressed or locally clustered, has a critical significance too. For example, the major sodium channel expressed in AIS and NOR is Nav1.6 that has low threshold, slow inactivation and fast recovery from inactivation (Herzog, Cummins, Ghassemi, Dib-Hajj, & Waxman, 2003; Zhou & Goldin, 2004).

The AIS and NOR are the major and most studied sites where sodium channels cluster at high densities; however, some VGSC isoforms are also accumulated in the somatodendritic compartment (Wu, Ivanova, Cui, Lu, & Pan, 2011) and synaptic locations (Hossain, Antic, Yang, Rasband, & Morest, 2005), in un-myelinated axons (Pristera, Baker, & Okuse, 2012), as well as in non-neuronal excitable cells (Bailey, Stocksley, Buckel, Young, & Slater, 2003). In these sites, based on physiological experiments and mathematical modeling, the clustering of channels increases membrane excitability. Computer models revealed that clusters at a specific size ("magic size") can enhance weak signals in small membrane patches (Shuai & Jung, 2006). In dendrites, it enhances the responsiveness of the membrane to weak stimuli (Hanson, Smith, & Jaeger, 2004); it supports AP propagation with a smaller amount of

channels in unmyelinated axons (Johnston, Dyer, Castellucci, & Dunn, 1996) and allows for a micro-saltatory conduction in un-myelinated C-fibers due to the dense clustering of sodium channels at nodal-like domains (Pristera et al., 2012). These examples and others will be further discussed below.

4 | VGSC CLUSTERING AT AIS AND NOR

4.1 | The pivotal role of ankyrin G

Nav1.6 is the major component of both AIS and NOR (Boiko et al., 2001, 2003; Dumenieu et al., 2017). In these sites, sodium channels are anchored to the actin-spectrin submembranous cytoskeleton through ankyrin G (Chang & Rasband, 2013). All sodium channel isoforms possess a 9-amino acid long ankyrin-binding domain that targets them to the AIS and NOR (Garrido et al., 2003). It has been shown that in cultured hippocampal neurons, Nav1.2 interacts with ankyrin G already during its synthesis in the ER and that ankyrin G mediates its transport to the membrane via KiF5B/kinesin-1 (Barry et al., 2014). The interaction of sodium channels with ankyrin G is enhanced by a casein kinase 2 (CK2)-mediated phosphorylation of their ankyrin-binding domain (Brechet et al., 2008). It has recently been shown that FGF13 promotes the endocytosis of Nav1.2 from the somatodendritic membrane, whereas FGF14 stabilizes them at the AIS in a CK2-dependent manner (Pablo, Wang, Presby, & Pitt, 2016). Thus, targeted protein transport and endocytosis from the cell soma and dendrites promote axonal localization of Nav1.2, and likely of Nav1.6. But what are the mechanisms that cluster axonal sodium channels specifically at the AIS and the NOR? The key factor is again ankyrin G, that through binding to βIV spectrin provides a scaffold that recruits all the AIS and NOR proteins and anchors them to the cytoskeleton. Anchorage of membrane proteins to the submembranous cytoskeleton not only prevents their lateral diffusion but also stabilizes their membrane expression by inhibiting endocytosis (Fache et al., 2004). Ankyrin G is thus the master organizer of both domains (Nelson & Jenkins, 2017). In its absence, NOR but not AIS can still form by ankyrin R -βI spectrin that are normally not expressed in this membrane domain (Ho et al., 2014).

4.2 | Clustering at the NOR versus AIS

The clustering of sodium channels in both AIS and NOR thus depends on the clustering of ankyrin G in these domains. However, an important difference between these two domains is that, while the clustering of ankyrin G at the forming AIS is determined intrinsically by the neuron, NOR form in association with the myelinating glial cells (Eshed-Eisenbach & Peles, 2013). For the AIS to assemble, a membrane barrier that restricts lateral diffusion of membrane proteins has to form right distally to the prospective site. This barrier, that dictates the exact localization of the AIS, is formed by ankyrin B, αII spectrin and βII spectrin that are completely segregated from ankyrin G and βIV spectrin, similarly to the nodal-paranodal membrane organization along myelinated axons (Galiano et al., 2012). The assembly of NOR in myelinated axons is more complex and is controlled by several mechanisms that operate together to ensure the correct formation of nodes. In cases where one of them fails, nodes would usually still form due to compensation by the additional mechanisms (Feinberg et al., 2010; Susuki et al., 2013). These mechanisms similarly include the formation of a membrane diffusion barrier, but in this case, the barrier is positioned by an external cue, namely the paranodal axo-glial junction that forms between the edge of each glial loop of myelin and the apposing axolemma. In the paranodal axolemma, a cell adhesion complex consisting of Caspr and contactin is linked to an αII spectrin and βII spectrin-dependent cytoskeleton by protein 4.1B (Ogawa et al., 2006), thus creating a diffusion barrier similar to the intra-axonal barrier that participates in the formation of AIS. Indeed, it was shown that both 4.1B and βII spectrin are necessary for the formation of a functional diffusion barrier at the paranodal axolemma (Brivio, Faivre-Sarrailh, Peles, Sherman, & Brophy, 2017; Zhang, Susuki, Zollinger, Dupree, & Rasband, 2013). A second glial-dependent mechanism for NOR formation requires the axonal cell adhesion molecule Neurofascin (the 186 isoforms, Nfasc186). This Ig domain-containing cell adhesion molecule (CAM) interacts with ankyrin G and also binds the β1 and β3 sodium channel subunits directly, although the latter cannot mediate channel clustering, which requires ankyrins (Ho et al., 2014).

4.3 | Clustering of Nfasc186 in PNS versus CNS NOR

Nfasc186 is clustered at the newly formed NOR by a complex of ECM and adhesion molecules, primarily gliomedin and NrCAM at the PNS, that are secreted by the myelinating glia (i.e., Schwann cells in PNS). Gliomedin and NrCAM interact with NF186 and induce its clustering owing to the self-oligomerization ability of gliomedin (Eshed, Feinberg, Carey, & Peles, 2007; Maertens et al., 2007). In addition, dystroglycan and its ligand perlecan are also secreted by Schwann cells at the developing node and enhance gliomedin-Nfasc186-mediated sodium channel clustering (Colombelli et al., 2015). Unlike Schwann cells in the PNS, oligodendrocytes in the CNS do not contact the nodal axolemma. In addition, and in contrast to the PNS, CNS NOR form after the paranodal junction had been established and thus CNS node formation relies primarily on the flanking paranodal membrane diffusion barrier (Eshed-Eisenbach & Peles, 2013; Susuki et al., 2013). A second mechanism involving the nodal glial-derived ECM

of the CNS (includes the proteoglycans brevican, versican, neurocan and phosphacan in addition to tenascin-R, BRal1), which directly interacts with axonal CAMs at the nodes (Nfasc186, contactin-1 and the β-subunit of sodium channel), is involved in long-term stabilization rather than the initial assembly of the CNS NOR (Chang & Rasband, 2013; Susuki et al., 2013).

In summary, the accumulation and stabilization of sodium channels at both the AIS and NOR depend on anchorage to the submembrane cytoskeleton through ankyrin G and on adjacent membrane barriers. In addition, NOR but not AIS, depends on axonal cell adhesion molecules that interact with glial ECM to localize and stabilize the node. It is not surprising then, that AIS are highly dynamic structures affected by the electrical activity of the cell, while the NOR are more stable structures under physiological conditions (Huang & Rasband, 2018).

5 | MORE SITES OF VGSC CLUSTERING

5.1 | The neuro-muscular junction

In the mammalian neuro-muscular junction (NMJ), it was initially suggested that VGSC are clustered by the dystrophin-associated protein complex (DAPC) similarly to acetylcholine receptors (AChR), as the muscle isoform, Nav1.4, unlike the neuronal isoforms, contains a PDZ-binding domain that can bind to syntrophin (an adaptor dystrophin-associated protein) (Gee et al., 1998). DAPC, much like the spectrin-ankyrin complex, connects membrane proteins to the submembrane cytoskeleton, and links these proteins to the ECM via dystroglycan. As shown for AChR, VGSC were copurified with syntrophin and dystrophin from the extracts of skeletal and cardiac muscles (Gee et al., 1998). However, it was later found that the muscular channels are actually segregated from AChR at the postsynaptic sarcolemma. It has been shown that VGSC were colocalized with β-spectrin, ankyrin G and dystrophin in the depth of the postsynaptic folds (i.e., the troughs), while AChRs, rapsyn and utrophin were localized to the crests of the postsynaptic folds. However, α-and β-dystroglycan were found to localize at both sites (Bailey et al., 2003; Colledge & Froehner, 1998). These observations precluded the model according to which both sodium channels and AChR accumulate at the NMJ by binding the PDZ domain of syntrophin. In the rat NMJ, sodium channel clusters form approximately 2 weeks after the clustering of AChRs (Lupa, Krzemien, Schaller, & Caldwell, 1993). Nevertheless, there was evidence that agrin, a neuronal proteoglycan that clusters AchR on the crests, can stimulate VGSC clustering in cultured rat muscle fibers (Sharp & Caldwell, 1996), which again links between the dystrophin complex (i.e., agrin binds via dystroglycan) and these channels. However, the possible involvement of MUSK, which mediates agrin-induced AChR clustering, is still an open question. Bailey et al. followed the process of initial segregation between AChR and VGSC during NMJ development (Bailey et al., 2003). They found that the segregation begins before the synaptic folds had formed. During this process, sodium channels were always co-labeled with ankyrin G and never with syntrophin that exclusively co-distributed with AChRs. The authors thus concluded that ankyrin G, and not syntrophin, is likely to mediate sodium channel clustering at the NMJ and suggested that AChR-sodium channel segregation results from the physical exclusion of sodium channels and ankyrin G from the region of nerve-muscle contact rather than by a process of active clustering (Bailey et al., 2003). This model strikingly resembles the segregation between nodal (ankyrin G-βIV spectrin-sodium channels) and paranodal (Caspr-contactin, βII spectrin, 4.1B) components at the NOR. It would be interesting to test whether the same cytoskeletal elements that segregate nodal from paranodal proteins in myelinated nerves also play a role in the segregation of proteins from the crests and troughs of the NMJ and whether a membrane barrier exists at the nerve-muscle contact sites at the crests. In summary, unlike the AIS and NOR, the mechanisms by which VGSC cluster at the NMJ are still unclear although evidence points to a similar ankyrin G-based mechanism supported by an adjacent membrane barrier. Deciphering these mechanisms will require the analysis of VGSC expression at the NMJ of the different KO mice as was extensively done for both AIS and NOR. Without such analyses, the involvement of the agrin-dystroglycan-syntrophin system in VGSC clustering at the NMJ cannot be ruled out. Interestingly, it was suggested that the sodium channels at the NMJ are linked to the cytoskeleton through ankyrin-spectrin and to the ECM through syntrophin-dystrophin-dystroglycan-agrin (Kordeli, 2000). Notably, at the peripheral NOR, perlecan which is a heparan sulfate proteoglycan and a dystroglycan ligand-like agrin, enhances the clustering of sodium channels by binding to gliomedin-Nfasc186 complex (Colombelli et al., 2015). Whatever scenario is proven correct, the NMJ and AIS/NOR seem to utilize similar mechanisms for the clustering of sodium channels (Figure 1). Interestingly, at the NMJ, the neuron is the provider rather than the receiver of the signal that induces channel clustering.

5.2 | Cardiomyocytes

In adult cardiomyocytes, VGSC segregate into defined subdomains. Nav1.5 has been described to distribute into two separate pools with distinct functional properties, one at the intercalated disc, where Nav1.5 interacts with ankyrin-G, synapse-associated protein 97, and plakophilin 2 and a "lateral membranes" pool, where Nav1.5 associates with

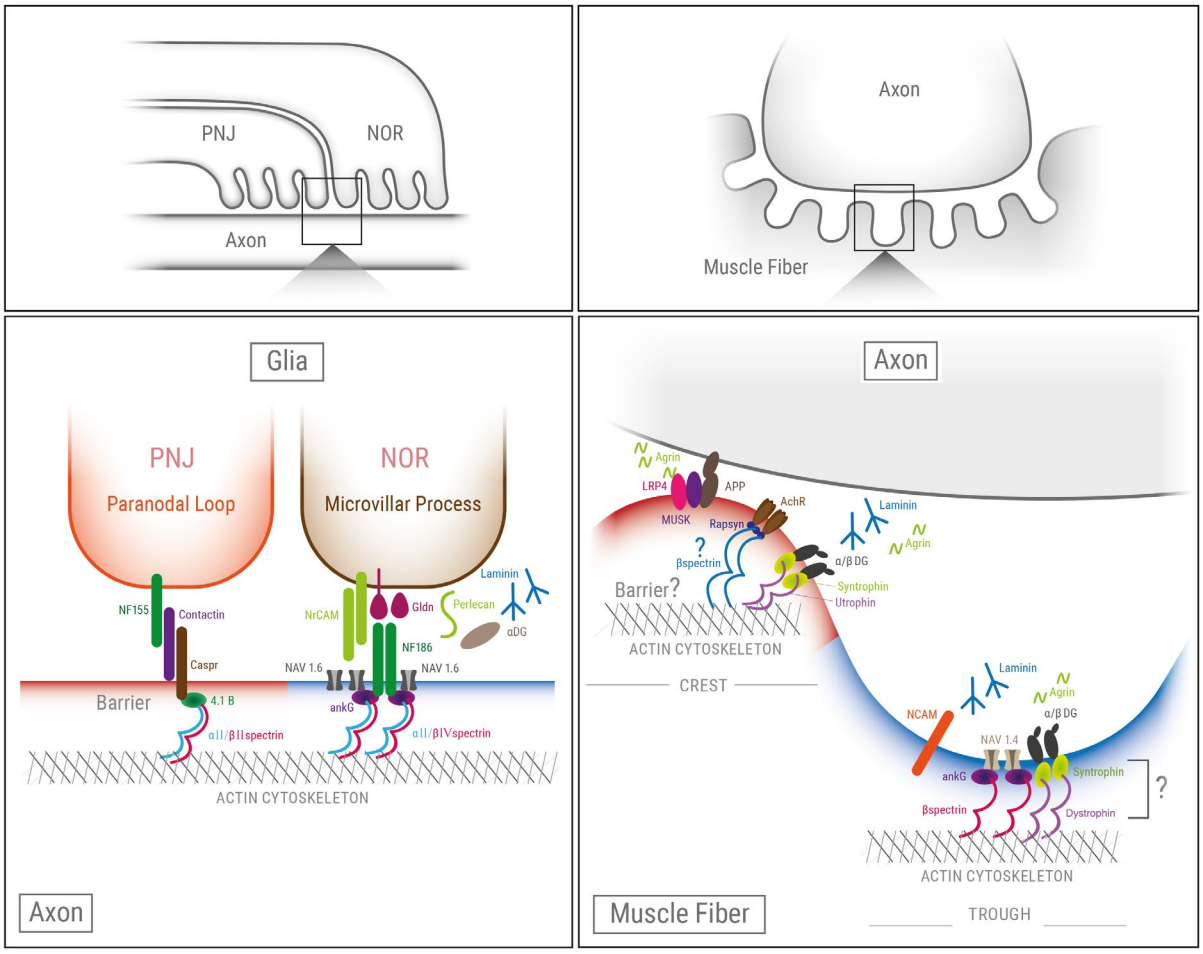

FIGURE 1 A scheme describing the molecular mechanisms of voltage-gated sodium channel clustering in the axonal membrane of the peripheral NOR (left) and postsynaptic folds of the sarcolemma at the NMJ (right). The microdomains where the clustering occurs are marked in blue and the domain in which a membrane diffusion barrier exists (PNJ) or may exist (crests) is marked in red

the syntrophin–dystrophin complex through a PDZ-binding motif (Lin et al., 2011; Petitprez et al., 2011; Sato et al., 2011). Indeed, truncation of the PDZ-binding motif in Nav1.5 resulted in the reduction of only the lateral membrane Nav1.5 channels (Shy et al., 2014). It appears that both adaptor proteins syntrophin and ankyrin G are required for the normal surface expression of cardiac Nav1.5 (Gavillet et al., 2006; Lowe et al., 2008). Accordingly, both the ankyrin-binding and PDZ-binding domains of Nav1.5 have been associated with Brugada syndrome which involves the loss of cardiac VGSC function (Mohler et al., 2004; Shy et al., 2014). Using a new technique named "super resolution scanning patch clamp," Bhargava and colleagues demonstrated that specifically in the lateral membranes of cardiomyocytes, functional sodium channels form clusters of various densities, the largest reside in the crest region of the sarcomere. Between these clusters they found areas with no electric activity (Bhargava et al., 2013). The function of these clusters is still to be studied. This is a unique example of channel clustering independent of ankyrin G and possibly the clustering occurs through DAPC as was initially suggested for the channels at the NMJ.

5.3 | Cochlea

In the cochlea, the hearing organ at the inner ear, Nav1.6 and 1.2 are clustered in a mutually discrete fashion at the cochlear afferents and efferent, respectively (Hossain et al., 2005). In cochlear afferents, that is, type I and type II cochlear ganglion cells, Nav1.6 was found to be clustered at the recepto-neural segments which are the axonal terminals that contact the inner (type I) and outer (type II) hair cells. In addition, Hossain et al. identified the accumulation of Nav1.6 in two AIS emanating of each type II ganglion cell body, that is, in both the central and peripheral axons of these unmyelinated neurons (Hossain et al., 2005). The authors conclude that the sites of VGSC clustering are the spike generators of hearing and suggest that in type II ganglion cells all three sites of high VGSC density they identified are critical for AP initiation and propagation.

Interestingly they showed that in contrast to afferent cochlear innervation, the efferent axons were not stained for Nav1.6. In these neurons, Nav1.2 was found to be at a high density in the axonal endings. Analysis of quivering mice, which carry mutations in βIV spectrin and have a hearing loss, revealed that Nav1.6 was not clustered at initial segments and in the recepto-neural segment, indicating that the clustering at this domain also depends on the ankyrin G-βIV spectrin submembrane cytoskeleton (Hossain et al., 2005). Not all axonal terminals of the auditory system contain VGSC clusters. In the calyx of Held located in the auditory brainstem, axon terminals are devoid of sodium channels. In pathological situations, as occurs in the dysmyelinated axons of the mutant rat LES, sodium channels invade the terminals, suggesting that myelination restricts sodium channels localization to the last NOR thereby regulates the excitability of the axon terminal (Berret, Kim, Lee, Kushmerick, & Kim, 2016).

5.4 | Pre-myelinated axons

In the mouse, both CNS and PNS myelination occurs during early postnatal life. At this period the axons undergoing myelination switch from continuous to the saltatory mode of conduction. It has recently been suggested that prior to myelination, VGSC clustering occurs on specific populations of pre-myelinated neurons (Freeman et al., 2015). Freeman et al. showed that in hippocampal GABAergic neurons, sodium channel clusters, colocalized with Nfasc186 and AnkG, appear before myelination. They could induce the clustering by adding oligodendrocyte-conditioned medium, as shown two decades ago in cultured retinal ganglion cells (Kaplan et al., 1997). They showed that the medium could induce the clustering of Nav1.1 and 1.2 but not of 1.6, in an ankyrin G-dependent but NF186-independent manner. This clustering could increase the speed of conduction in cultured neurons. In a recent follow-up article from the same group, the identity of the glial signal was revealed. Using mass spectrometry analysis on active protein fractions isolated from the glial-conditioned medium, the authors identified a complex of proteins namely, contactin and ECM proteins RPTP/Phosphacan or Tenascin-R to be sufficient to induce nodal protein clusters on hippocampal GABAergic neurons (Dubessy et al., 2019). They also showed that in addition to NF186, NrCAM was dispensable for this clustering as well (whether these CAMs compensate for each other is yet to be determined). This finding makes these early clusters the first example of glial-induced axonal clustering that is not mediated by axonal Nfasc186. This is a very unexpected result as Nfasc186 can interact with contactin in *trans* (Gollan, Salomon, Salzer, & Peles, 2003). The authors suggest that the contactin may interact with another ankyrin G-binding CAM on the axolemma directly or indirectly through the proteoglycans it is complex with. (Dubessy et al., 2019). Another possibility is that the contactin complex binds directly to the sodium channel β subunit (O'Malley & Isom, 2015). The ability of the soluble contactin/proteoglycan complex to cluster Nav1.2 and not Nav1.6 could be explained by the higher availability of the former in young axonal processes, as Nav1.2 but not 1.6 contains a sequence that promotes clearance from the somatodendritic compartment (Garrido et al., 2001). In the PNS, nodal assembly is initiated by gliomedin secreted from Schwann cells. Nevertheless, sodium channels are clustered only at the edges of the growing myelin segment and only after the Schwann cell had fully differentiated. This spatiotemporal regulation on node formation that prevents the formation of premature clusters in the PNS is achieved by proteolytic enzymes that cleave and negatively regulate the channel clustering activity of gliomedin (Eshed-Eisenbach and Peles, unpublished data).

5.5 | C-fibers

The distribution of VGSC along C-fibers, which are nociceptive peripheral axons, has been described as largely uniform along the axon under normal conditions, while the abnormal clustering of channels after injury was associated with neuropathic pain (Omana-Zapata, Khabbaz, Hunter, Clarke, & Bley, 1997). Interestingly, it was found that in C-fibers isolated from rat DRGs, Nav1.8 was normally localized in clusters where it was associated with lipid rafts. Moreover, it was shown that the disruption of lipid rafts led to a shift of NaV1.8 into the non-raft portion of the membrane and this redistribution correlated with impaired neuronal excitability. Although the authors do not mention that removal of glial cells from these DRG cultures was done, one can assume that indeed the culture was mostly glia-free and thus this raft-associated clustering is an intrinsic property of the neurons. Clustered localization of Nav1.8 was also observed in rat sciatic nerve (Pristera et al., 2012). Interestingly, lipid raft-associated clustering may be a unique example of actin cytoskeleton-independent clustering, as actin-spectrin membrane microdomains are functionally and structurally distinct from lipid rafts (Bennett & Lorenzo, 2016). Nevertheless, Neishabouri et al. were later able to show that these 0.1–0.3 μm long clusters that resemble nodes of Ranvier, permitted micro-saltatory conduction in those thin axons (Neishabouri & Faisal, 2014). By modeling biophysically realistic unmyelinated axons with sodium channels conventionally distributed or clustered with lipid rafts, they found that APs were reliably conducted in a micro-saltatory fashion along with lipid rafts. It would be interesting to test whether this unique mode of conduction is truly a characteristic of unmyelinated peripheral axons in-vivo. Intriguingly, in Zebrafish, numerous ectopic VGSC clusters form in the absence of Schwann cells or in the presence of Schwann cells that fail to differentiate. These clusters

were shown to be ankyrin G but not Nfasc186-dependent and are thus probably a result of axon-intrinsic mechanisms (Voas, Glenn, Raphael, & Talbot, 2009). The authors concluded that myelinating Schwann cells inhibit axon-intrinsic clustering thus allowing it to occur only in the AIS. As C-fibers are not associated with myelinating SC, the mechanisms could be similar. In addition to the hypomyelinated fish, researchers have found that in Aplysia californica, a sea slug that lacks myelin, VGSCs are not homogeneously distributed along axons, but are rather present in distinct clusters (Johnston et al., 1996). Another example for local accumulations of VGSC in unmyelinated axons comes from the human dental pulp, where sodium channels were found to accumulate on axonal processes that were unmyelinated or even not associated with Schwann cells. Such clusters may have implications for dental pulp pain (Henry, Luo, & Levinson, 2012).

5.6 | Retina

The inner plexiform layer (IPL) of the retina contains multiple cell processes and synapses belonging to bipolar (input), amacrine, glial and ganglion (output) cells. Van Wart et al. first found that Nav channels, ankyrin-G, and neurofascin are clustered in a subset of processes in IPL of the rat retina. They noted that in the retina, clusters that contained Nav1.1 channels were replaced by Nav1.2 early in development, at a time when the switch from Nav1.2 to Nav1.6 occurred in the PNS (Van Wart, Boiko, Trimmer, & Matthews, 2005). These sodium channel clusters at the IPL were later identified by Wu et al. as a unique AIS-like domain in the axonless AII amacrine cell (Wu et al., 2011). Using adeno-associated virus carrying the sodium channel ankyrin-binding motif fused to membranous GFP they showed that this protein is targeted to AII dendritic processes where it colocalized with ankyrin G and Nfasc. Moreover, they showed that the expression of the above fusion protein disrupted the clustering of endogenous Nav1.1 in the AII process, which abolished the ability of AII amacrine cells to generate spiking. This striking result shows that the specialized AIS-like domains in dendrites can function as spike generators similarly to the conventional AIS. Another example of VGSC clustering in dendrites was described in the mouse globus pallidus, where dendritic channels exhibited specific clustering at sites of excitatory synaptic inputs. They show that these dendrites mediate synaptic amplification allowing dendrites to respond to weaker stimuli (Hanson et al., 2004).

6 | PATHOLOGICAL FORMS OF VGSC CLUSTERING

In general, most demyelinating and dysmyelinating diseases involve the disruption of the axonal membrane domains at and around the NOR, resulting in diffuse expression of their components which impairs axonal conduction (Freeman et al., 2016). Interestingly, in some pathologic conditions affecting both PNS and CNS, disease-induced ectopic clustering of sodium channels has been described. In conditions of chronic demyelination of peripheral nerves or diseases that involve defected PNS myelination, large clusters of amorphous sodium channels appear on demyelinated axons (Levinson, Luo, & Henry, 2012). Similarly, it was shown that in human neuromas, which are masses of regenerating axons embedded within the peripheral nerve trunk, there is the abnormal accumulation of sodium channels within many axons, especially within the axonal tips. Such changes have also been described at the site of nerve injury in an animal model for neuropathic pain (Henry, Freking, Johnson, & Levinson, 2007). It is believed that these accumulations of sodium channels may cause axonal hyperexcitability resulting in abnormal sensory phenomena (pain and paresthesias), which characterizes peripheral nerve injury symptoms (England et al., 1996). Moreover, it was shown that in demyelinated nerves, isoforms other than Nav1.6 are expressed along the demyelinated axons and in nodes (Luo, Perry, Levinson, & Henry, 2008). The authors suggested that demyelination induces the expression of the non-nodal isoforms, just as developmental myelination reduces their expression (Levinson et al., 2012). In the CNS, the clustering of sodium channels has also been observed in PLP-negative remyelinated axons within lesions in multiple sclerosis (MS) patients (Coman et al., 2006). In these lesions, unlike PNS lesions, such clusters are believed to accelerate conduction velocity before remyelination and thus participate in the functional recovery of the lesion (Freeman et al., 2016). Hence, due to the high contents of nociceptive fibers in peripheral nerves, ectopic VGSC clustering at sites of peripheral nerve damage are considered harmful (and are therefore known therapeutic targets), whereas such clusters in CNS lesions are thought to promote recovery.

7 | SUMMARY

VGSC control neuronal excitability. The two major sites of neuronal excitability and concomitantly of sodium channel clustering, are the AIS and NOR. Nevertheless, sodium channels clusters exist at other axonal, non-axonal or even non-neuronal excitable membranes (summarized in Table 1). Physiologically, the clustering of sodium channels results in increased responsiveness of the membrane to electric stimuli and accelerated conduction and thus has critical functional impacts. In terms of clustering mechanisms, neuronal clustering depends on anchorage to the ankyrin-spectrin cytoskeleton and a flanking or neighboring membrane diffusion barriers,

TABLE 1 VGSC clustering at various excitable membranes and their underlying mechanisms

Site of clustering	Reference	VGSC isoform	Clustering mechanism
AIS	Nelson and Jenkins (2017) Huang and Rasband (2018) Zhang and Rasband (2016)	Nav1.2 Nav1.1 Nav1.6	Neuronal intrinsic: 1. CS: Ankyrin-spectrin 2. Diffusion barrier: Intra-axonal
NOR	Nelson and Jenkins (2017) Eshed-Eisenbach and Peles (2013)	Nav1.2 Nav1.6	Glial induced: 1. CS: Ankyrin-spectrin 2. Diffusion barrier: Paranodal 3. CAM-ECM: Nfasc186 gliomedin/NrCAM/dystroglyacn-perlecan
Hippocampal GABAergic neurons	Freeman et al. (2015) Dubessy et al. (2019)	Nav1.1, 1.2	Glial induced: 1. CS: Ankyrin-spectrin 2. CAM-ECM: Soluble glial contactin-proteoglycan complex with an unknown axonal CAM (Nfasc186-independent)
Cochlear ganglion cell terminals	Hossain et al. (2005)	Nav1.6	Induced by hair cell-axon contact CS: Ankyrin-spectrin
Cochlear efferent fibers	Hossain et al. (2005)	Nav1.2	Unknown
Cardiomyocyte lateral membrane	Bhargava et al. (2013)	Nav1.5	CS: Dystrophin-syntrophin (DAPC)
Unmyelinated C-fibers (rat)	Pristera et al. (2012)	Nav1.8	Intrinsic, unknown, associated with lipid rafts
Schwann cell-ablated peripheral axons (Zebra fish)	Voas et al. (2009)	Unknown	Neuronal intrinsic: 1. CS: Ankyrin-spectrin (inhibited by myelinating glia)
Unmyelinated dental pulp axons (human)	Henry et al. (2012)	Unknown	Probably intrinsic
Amacrine dendrites In inner plexiform layer of retina	Van Wart et al. (2005) Wu et al. (2011)	Early Nav1.2 switches to Nav1.1	CS: Ankyrin-spectrin
NMJ	Gee et al. (1998) Sharp and Caldwell (1996) Bailey et al. (2003)	Nav1.4	Induced by synaptic contact, may involve: 1. CS: Ankyrin-spectrin *2. CS: Dystrophin-syntrophin (DAPC)* *3. Membrane barrier at axon-muscle contact sites* *4. ECM: Agrin*
Injured axons and neuromas	Levinson et al. (2012)	Nav1.6, 1.7, 1.8	Unknown

Note: CS cytoskeleton, CAM cell adhesion molecules and ECM extracellular matrix. Light blue, orange and white backgrounds refer to intrinsically and extrinsically-induced or unknown clustering, respectively. Mechanisms that await further experimental evidence are labeled in italics.

while the clustering in the heart and skeletal muscle probably depends on both ankyrin-spectrin and DAPC-associated submembranous cytoskeletons. There is evidence for the existence of a membrane barrier segregating membrane proteins in the postsynaptic membrane of the NMJ. Interestingly, while the axon plays opposite roles in each case, there is a striking similarity between the glial-induced VGSC clustering at the PNS NOR and the axon-induced VGSC clustering at the postsynaptic sarcolemma of the NMJ. Compared to the extensively studied mechanisms of VGSC clustering along the neuronal axolemma, the mechanisms controlling VGSC clustering at the sarcolemma and in cardiomyocytes are much less clear. The various (fish and mice) cytoskeletal, CAM and ECM mutants that were used to study AIS and NOR formation could be excellent tools to study the clustering of VGSC channels at the NMJ.

In summary, the clustering of VGSC as means to enhance excitability had been adopted at specific membrane domains throughout evolution. The clustering of channels is based on two important features of the submembranous actin-spectrin cytoskeleton: 1. Formation of membrane diffusion barriers by extensive linkage to the plasma membrane via adaptor proteins such as the ankyrins, 4.1 proteins and syntrophin. 2. Stabilization of membrane proteins by limiting their lateral diffusion and inhibiting their endocytosis. At the AIS and at other sites where clustering is determined intrinsically, the mechanisms of clustering relies only on the barrier and stabilization functions of the actin-spectrin

cytoskeleton. However, at sites where clustering is induced by external factors such as cell-cell contact (NOR) or secreted proteins (hippocampal GABAergic neurons), additional mechanisms involving cell adhesion molecules and ECM components determine the exact localization and timing of sodium channels cluster formation. In conclusion, although featuring different cytoskeletal adaptor proteins, adhesion molecules and ECM proteins, the clustering of sodium channels in most sites described so far, share common mechanisms.

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