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### Review

# Determinants of voltage-gated sodium channel clustering in neurons

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### ABSTRACT

In mammalian neurons, the generation and propagation of the action potential result from the presence of dense clusters of voltage-gated sodium channels (Nav) at the axonal initial segment (AIS) and nodes of Ranvier. In these two structures, the assembly of specific supra-molecular complexes composed of numerous partners, such as cytoskeletal scaffold proteins and signaling proteins ensures the high concentration of Nav channels. Understanding how neurons regulate the expression and discrete localization of Nav channels is critical to understanding the diversity of normal neuronal function as well as neuronal dysfunction caused by defects in these processes. Here, we review the mechanisms establishing the clustering of Nav channels at the AIS and in the node and discuss how the alterations of Nav channel clustering can lead to certain pathophysiologicals.

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### Contents

1. Introduction .....	00
1.1. Molecular structure and organization of neuronal Nav channels .....	00
1.2. Nav channel neuronal distribution: not so simple! .....	00
2. How do Nav channels cluster at the AIS and the node of Ranvier? .....	00
2.1. Mechanisms regulating Nav channel clusters at the AIS .....	00
2.1.1. Regulation of ankyrinG-Nav1 channel interaction .....	00
2.1.2. Mechanism of Nav channel segregation .....	00
2.2. Mechanisms regulating Nav channel clusters at the nodes of Ranvier .....	00
2.2.1. Regulation of nodal ankyrinG-Nav channel interaction .....	00
2.2.2. Mechanism of nodal Nav1 channel segregation .....	00
2.3. Future directions .....	00
3. Role of Nav channel clustering in neuropathology .....	00
4. Conclusions .....	00
References .....	00

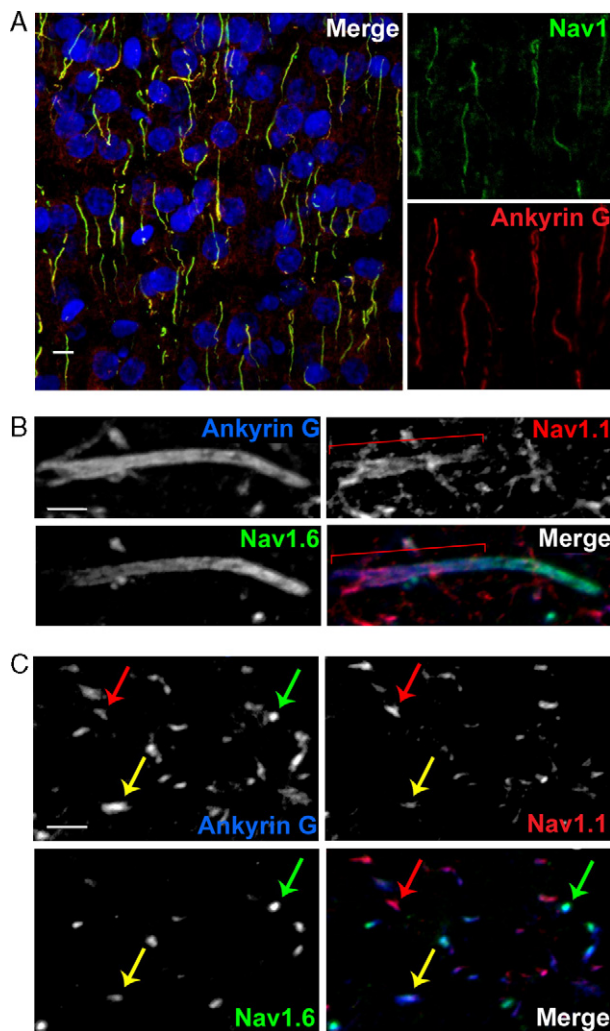
### 1. Introduction

The intrinsic electrical properties and the synaptic input–output relationships of neurons are governed by the action of a wide repertoire of ion channels. The localization of specific populations of ion channels with distinct functional properties at discrete sites in neurons dramatically impacts excitability and synaptic transmission. In mammalian neurons, dense clusters of voltage-gated

sodium channels (Nav) at the axonal initial segment (AIS) and nodes of Ranvier underlie action potential generation and fast propagation [1,2] (Fig. 1). Nav clustering depends on the formation of supra-molecular complexes linked to the actin/spectrin cytoskeleton via the scaffold protein ankyrin G (ankG). Recently, much effort has focused on determining the molecular identity of these native neuronal Nav channel complexes, their cellular and subcellular distributions, as well as the mechanisms determining their discrete axonal subcellular distributions, as a crucial step in understanding their contribution to specific aspects of neuronal function. Here, we review progress made by recent studies aimed at determining the basic mechanisms of the specific targeting of Nav channels to the AIS and the node of Ranvier. We also discuss the currently asked questions and the implications for neuronal physiology.

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**Fig. 1.** *In vivo* localization of Nav channels. (A) *In vivo* concentration of Nav channels at the AIS of cortical neurons (green). Nav colocalizes at the AIS with ankG (red). Nuclei are labeled using Hoechst 33258 (blue). Scale bar, 10  $\mu\text{m}$ . (B) Triple immunostaining of ankG, Nav1.1 and Nav1.6 in the adult lumbar spinal cord grey matter. Nav1.1 expression displayed a proximo-distal gradient, with a higher expression level in the AIS region proximal to the soma. Scale bar, 5  $\mu\text{m}$ . (C) *In vivo* concentration of Nav1.1 and Nav1.6 in the nodes of Ranvier of the adult spinal cord white matter. Nav1.1 and Nav1.6 colocalized with ankG at the nodes. Three populations of nodes were identified: expressing Nav1.1 only (red arrows), Nav1.6 only (green arrows) or both (yellow arrows). Scale bar, 5  $\mu\text{m}$ . (B) and (C) panels were reproduced with permission from Molecular and Cellular Neuroscience; doi:10.1016/j.mcn.2008.06.008.

### 1.1. Molecular structure and organization of neuronal Nav channels

The first family of Nav channels contains all the classical voltage dependent sodium channels [3]. The outlying second family, termed Nax, contains channels whose sequences share some similarity to Nav1 sequences. In the mammalian genome, at least eight of the nine Nav1  $\alpha$  subunit genes (SCN1A (Nav1.1); SCN2A (Nav1.2); SCN3A (Nav1.3); SCN4A (Nav1.4); SCN5A (Nav1.5); SCN8A (Nav1.6); SCN9A (Nav1.7); SCN10A (Nav1.8); SCN11A (Nav1.9)) are expressed in the nervous system, the exception being the muscle-specific Nav1.4. Nav1.1, 1.2 and Nav1.6 are abundant in the central nervous system (CNS), while Nav1.3 is mostly present during embryonic development. Nav1.7, Nav1.8, and Nav1.9 are predominant in the peripheral nervous system (PNS) [12]. Nav channels consist of a highly processed  $\alpha$  subunit, which is approximately 260 kDa, associated with auxiliary  $\beta$  subunits (~30 kDa)

through either covalent or non-covalent linkage [4]. The  $\alpha$  subunits are organized into four homologous domains (I–IV), each of which contains six transmembrane  $\alpha$  helices (S1–S6) and an additional pore loop located between the S5 and S6 segments. The pore loops line the outer, narrow entry to the pore, whereas the S5 and S6 segments line the inner, wider exit from the pore. The pore-forming  $\alpha$  subunit is sufficient for functional expression, but the kinetics and voltage dependence of channel gating are modulated by the  $\beta$  subunits. These auxiliary subunits are involved in channel localization and interaction with cell adhesion molecules (CAMs), extracellular matrix (ECM), and intracellular cytoskeleton [5,6]. The role of  $\beta$  subunits in neuronal Nav channel clustering will not be discussed in this review, for more details about their multiple roles see [7].

### 1.2. Nav channel neuronal distribution: not so simple!

Recent studies provide compelling evidences for a complex cell specific sub-domain organization of Nav1 subunits, with a spatially and temporally defined expression. In mature CNS and PNS nodes, Nav1.6 channels can be expressed either alone or with Nav1.1, while some nodes of Ranvier have been found expressing only Nav1.1 [8–10]. In some pathophysiological situations, Nav1.8, a subtype preferentially expressed in sensory neurons [3], can also be observed at nodes [11,12]. In addition, developmental changes occur at the nodes, where Nav1 subunits can all coexist at certain stages of maturation (e.g. nodes of the spinal cord [8]) (Fig. 1C). In mature retinal ganglion cell (RGC) axons, which consist of distinct unmyelinated and myelinated zones, Nav1.2 is found uniformly throughout unmyelinated regions, whereas Nav1.6 is specifically targeted to the nodes [13]. During the development of the RGCs, Nav1.2 is expressed first and clustered at the immature nodes of Ranvier, but as the myelination proceeds, Nav1.6 progressively replaces Nav1.2 at nodes [13].

In the same line, a cell-specific subcellular distribution of Nav1 subunits occurs at the AIS (Fig. 1). Nav1.6 can be exclusively and uniformly distributed along the AIS of Purkinje cells [14,15], or co-expressed with Nav1.1 at the AIS of cortical and cerebellar interneurons, in RGCs and in spinal cord neurons [8,14,16,17]. Moreover in these latter cells, Nav1.1 and Nav1.6 showed a complementary localization along the AIS: Nav1.1 subunits are found highly clustered at the proximal edge of the AIS, while Nav1.6 subunits exhibit an increase in density towards the proximal end of the AIS (Fig. 1B) [8,18,19]. In contrast, Nav1.1 and Nav1.6 channels are uniformly co-distributed along the AIS of main olfactory bulb short axon cells [14]. With regards to Nav1.2, it is co-expressed with Nav1.6 in layer 5 pyramidal cells of the neocortex with a complementary gradient of Nav1.2–Nav1.6 concentration along the AIS [20]. Nav1.2 and Nav1.6 are also co-expressed along the AIS of cerebellar granular cells (GCs) [21]. During development, Nav1.2 accumulation at the AIS precedes Nav1.6 in RGCs and in GCs [19,21]. It is important to note that these complex organizations of Nav1 channels at the AIS have a tremendous impact on the electrical properties of the cell. For example, in cortical pyramidal neurons, Nav1.6 accumulation at the distal AIS, determines the lowest threshold for AP initiation [20]. In contrast, a higher concentration of Nav1.2 channels in the proximal AIS promotes AP back propagation into the somadendritic compartment [20]. To complicate matters further, a recent study revealed, using a highly sensitive electron microscopic immunogold technique, the presence of the Nav1.6 subunit in the proximal and distal dendrites of hippocampal CA1 pyramidal cells. In these cells, Nav1.6 subunits have a lower density than that found in the AIS (by a factor of 35–80), and exhibit a gradual decrease in density along the proximodistal axis of the dendritic tree without being expressed in dendritic spines [22]. Consequently,

further studies are required for a better understanding of the mechanisms accounting for such complex expressions and of the respective physiological relevance of each expression pattern.

## 2. How do Nav channels cluster at the AIS and the node of Ranvier?

The AIS and the node of Ranvier have a common molecular organization, due to multiple protein–protein interactions where Nav channels are a core component of this interaction network [23]. Both sub-domains contain mainly ion channels (Nav, Kv7, and Kv1 only at the AIS) [14,24–26], CAMs (Neurofascin 186 (NF-186) and neuronal cell adhesion molecule (NrcAM)) [27–30], cytoskeletal adaptor complex like ankG and  $\beta$ IV-spectrin [31–33], and ECM (brevican and versican). More recently, new proteins present at the AIS and the nodes have been identified: protein kinase CK2 [34], SCHIP-1 isoforms [35], FHF2 and FHF4 (two members of the fibroblast growth factor homologous factors (FHF)) [36] and members of the NF $\kappa$ B signaling pathway [37,38]. Despite the molecular similarities between these two neuronal domains, there is one major difference between the AIS and the node of Ranvier: clustering of proteins at nodes requires the influence of myelinating glia (i.e. extrinsic factors), but recruitment of these same proteins to the AIS does not (i.e. intrinsic factors) [23].

### 2.1. Mechanisms regulating Nav channel clusters at the AIS

The AIS is intrinsically specified, being able to assemble in cultured neurons in the absence of glial cells [39–41]. *In vivo* and *in vitro* analysis all demonstrate a crucial role of ankG as the master organizer for AIS assembly and maintenance [30,33,42]. AnkG is the first component localized at the AIS [42], and through its membrane-binding domain (MBD) (ankyrin repeat domain), specifically clusters Nav [43,44], Kv7.2/7.3 channels [25] and CAMs [45], as well as the scaffolding protein  $\beta$ IV-spectrin through its spectrin domain [46]. In fact, mice lacking ankG in Purkinje cells fail to cluster any other AIS components [33,42], and electroporation of cortical neurons with shRNA against ankG blocks AIS assembly [30]. In cultured hippocampal neurons, knockdown of ankG impedes AIS formation, while knockdowns of CAMs, Nav channels or  $\beta$ IV-spectrin have little impact [30]. A role for Nav channels in AIS formation and maintenance is, however, not excluded. In cultured motor neurons, the decrease of Nav channel expression affects AIS assembly, suggesting that a complementary mechanism might exist in this cell type [47]. However, the *in vivo* confirmation of the role of Nav channel in AIS assembly may prove difficult, because of the perinatal lethality of the Nav1.2 knockout mouse [48] and the compensation of Nav1.6 disappearance by Nav1.2 and Nav1.1 in the medj mouse [15].

#### 2.1.1. Regulation of ankyrinG-Nav1 channel interaction

AnkG has been known to link Nav channels to the underlying AIS cytoskeleton, but the molecular details of this interaction and its role in Nav channel concentration at the AIS were only recently unraveled [34,43,44,49,50]. The use of chimeric protein expression in cultured hippocampal neurons showed that the cytoplasmic loop linking domains II and III was responsible for the AIS concentration of Nav channels [43]. This II–III loop was sufficient to either segregate a chimera of the CD4 protein (CD4-Nav1.2) at the AIS, or redirect the somatodendritic potassium channel Kv2.1 (Kv2.1–Nav1.2) to the AIS. Two independent studies revealed that the AIS targeting was abolished upon truncation of a sequence of 27 residues within the Nav1.2 II–III loop (a.a. 1102–1128) [43], and that the PIALGSD sequence located within these 27 residues

directly interacted with the MBD of ankG [44]. However, this 27-residue sequence, called ank-binding domain, is not only specific to ankG, but it also allows binding to ankyrin-B (ankB), which shares the canonical MBD. AnkB is located along the distal axon in cultured hippocampal neurons and in myelinated fibers [31]. Thus, this suggests the existence of an additional mechanism ensuring the specific interaction of Nav channels with ankG at the AIS. Recent findings showed that specific residues within the ank-binding domain are phosphorylated by the protein kinase CK2, allowing for a high-affinity recognition of ankG [34]. *In vitro* assays demonstrated that the CK2-mediated phosphorylation of the ank-binding domain of neuronal Nav channels resulted in a 1000-fold increase in binding affinity for the MBD of ankG (from micromolar to nanomolar). Although, in this study CK2-mediated phosphorylation equally enhanced the affinities for either the MBD of ankG or ankB, the authors showed that CK2 was highly concentrated at the AIS (Fig. 2A and B) and that inhibition of CK2 activity disrupted the AIS localization of Nav channels [34]. This implies that the local concentration of CK2 enhances the interaction of Nav channels with AnkG via their phosphorylations, contributing to their specific accumulation at the AIS. However, the mechanisms regulating the AIS targeting and timing of CK2 as well as the phosphatase counterbalancing of CK2 phosphorylation are still unknown. AnkG also modulates the inactivation properties of Na<sup>+</sup> current in heterologous cells [51]. However, the electrophysiological consequences of a phosphodependant binding of ankG remain to be explored in neurons. Finally, it is important to note that the ank-binding domain is highly conserved among Nav channels and that some neuron-specific subtypes of Nav channel can be present in the somatodendritic domain. Therefore, it is likely that some Nav channels bear an additional determinant that overrides the Ank-binding domain.

#### 2.1.2. Mechanism of Nav channel segregation

Some important questions concerning the targeting of Nav channels to the AIS still remain: are the Nav channels directly inserted into the AIS plasma membrane from biosynthetic vesicles? Or, are the channels initially transported and inserted in the whole neuronal membrane, before being subsequently retrieved from the dendrites, soma and the distal axon by endocytosis? Are these two models mutually exclusive or co-existing at some stage of the neuronal development? Studies have shown that in developing neurons, endogenous Nav channels can undergo activity-dependent endocytosis [52,53]. In fact, Nav subunits possess two endocytosis signals located in the C-terminal tail [54] and in the proximal region of the II–III loop [50]. CD4-chimeric proteins bearing the C-terminal tail of Nav channels are localized to the axonal plasma membrane in dissociated hippocampal neurons. This compartmentalization arises from the selective endocytosis of the chimera in the somatodendritic compartment [54]. In a similar fashion, the same reporter bearing the Nav1.2 II–III loop is first inserted in the somatodendritic compartment and distal axon, before being endocytosed from these sites and restricted to the AIS [50]. One model arising from these findings is that in developing neurons, an elimination/retention mechanism accounts for Nav channel compartmentalization in axons. Nav channels are first sorted indiscriminately to the whole neuronal plasma membrane. These channels would then diffuse laterally until they are endocytosed or trapped by ankG via their ank-binding domain when they reach the AIS (Fig. 2C). It is also possible that the two endocytosis signals lead to differential regulations of endocytosis in the somatodendritic compartment versus the axon. In addition, the presence of a conserved binding site for Nedd4 and Nedd4-2 ubiquitin ligases in the C-terminus of several Nav1 subunits suggests that endocytosed channels are targeted to degradative pathways [55].



2.2. Mechanisms regulating Nav channel clusters at the nodes of Ranvier

The mechanisms of node formation and maintenance are quite complex due to the presence of myelinating cells: Schwann cells for the PNS and oligodendrocytes for the CNS [56,57]. In the PNS, the nodes are first formed, followed by paranodes and juxtaparanodes [58–60]. *In vitro* studies showed that Schwann cells secrete gliomedin, an oligomeric ligand that recruits first the adhesion protein NF-186 to nascent nodes [57]. NF-186 is then able to recruit ankG to nodes, which in turn recruits Nav channels [29] and  $\beta$ IV-spectrin [46]. This sequence of events, and the prominent role of NF-186 in PNS node assembly, is confirmed by the general absence of nodes observed in NF-186 knockout mice [61]. Moreover, mice deficient for nodal components not directly involved in node assembly, such as NrCAM [62] and  $\beta$ IV-spectrin [45,63,64], also exhibit altered nodes, demonstrating the interdependence of nodal component for long-term nodal maintenance.

The mechanisms of node formation in the CNS are not as understood as in the PNS, mainly because of the lack of *in vitro* models. As gliomedin is absent in the CNS, it has been proposed that a redundant network of ECM proteins play a role in the initiation of NF-186 recruitment [56], and that the clustering of Nav channels is induced in RGC cultures by an unidentified soluble factor released by oligodendrocytes [21]. In addition, in contrast to the PNS, the concentration of paranodal components occurs before nodal ones [65], and ankG clusters can appear before adhesion proteins at the optic nerve nodes [66]. This suggests the presence of complementary or alternative mechanisms directing the assembly of CNS nodes

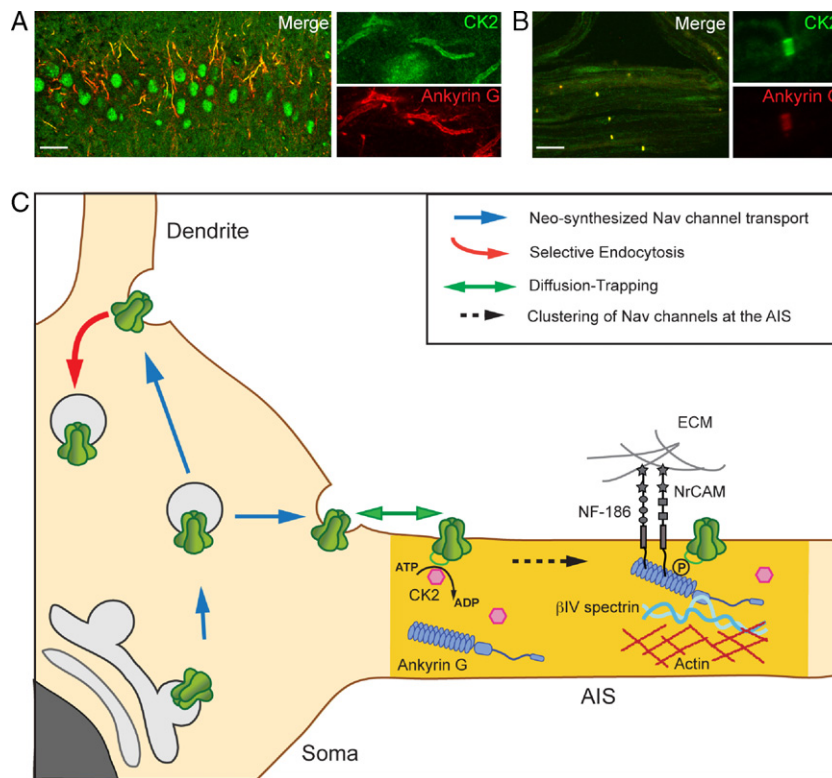
[56], see below), where the requirement for NF-186 is not crucial [67].

2.2.1. Regulation of nodal ankyrinG-Nav channel interaction

All the mechanisms described above, place ankG and Nav channels as downstream participants in node formation. The effect of ankG absence at nodes *in vivo* is unknown, as the current cerebellar ankG knockout mouse model still exhibits ankG labeling at nodes of Ranvier [42]. In myelinated DRG cultures however, knockdown of ankG in neurons abolishes node formation, including NF-186 clustering, indicating a central role for ankG in maintaining the integrity of the nodal complex [29]. Although CK2 is concentrated at the nodes of Ranvier in the CNS and PNS [34], its role in directly regulating the ankG-Nav interaction has not yet been determined.

2.2.2. Mechanism of nodal Nav1 channel segregation

The trafficking mechanisms allowing for delivery and concentration of Nav channels in the nodes of Ranvier have yet to be unraveled. It is likely that nodal membrane proteins are directly inserted into the node where they are confined because of the presence of paranodes and their junctions [65,67–70]. Paranodal junctions limit subsequent diffusion of nodal membrane proteins, in particular, Nav channels [71]. Several mouse models with altered paranodal junctions have longer and more diluted clusters of Nav channels [68–70]. Strikingly, paranodal junctions also have a prominent role in the differential expression of Nav1.2 and Nav1.6 subunits at nodes [13,68], and mice with altered paranodes exhibit incomplete replacement of Nav1.2 by Nav1.6 in PNS nodes [68,70]. The internode region, where the axonal membrane is apposed to



**Fig. 2.** *In vivo* localization of CK2 and simplified steps of Nav channel segregation at the AIS. (A) *In vivo* concentration of CK2  $\alpha$  at the AIS of CA1 hippocampal neurons (green). CK2 colocalizes at the AIS with ankG (red). Scale bar, 26.8  $\mu$ m. (B) *In vivo* concentration of CK2  $\alpha$  at the node of Ranvier of teased fibers of sciatic nerve (green). CK2 colocalizes at the AIS with AnkG (red). Scale bar, 23.2  $\mu$ m. (A) and (B) panels were reproduced with permission from the Journal of Cell biology; doi:10.1083/jcb.200805169. (C) The neo-synthesized channels are transported and inserted in the somatodendritic compartment (blue arrows). An endocytic mechanism (red arrow) permits the selective elimination of Nav channels in soma and dendrites, whereas channels are trapped by ankG at the AIS after laterally diffusing and being phosphorylated at their ankyrin-binding domain by CK2 (green double arrow). These two mechanisms, selective endocytosis and diffusion-trapping, lead to the clustering of Nav channels at the AIS where they will remain firmly anchored via the actin-spectrin-ankG based cytoskeleton and the adhesion molecules (NF-186 and NrCAM)-ECM network.

glial cells, has recently been implicated in Nav channel clustering as it actively forces Nav channels toward the nodes [72]. Finally, the fact that Nav1.2, but not Nav1.6, can cluster to form immature nodes in RGC cultures treated with oligodendrocyte-conditioned medium [73] further suggests that myelinating cells could direct the differential targeting of the two Nav subunits at the nodes.

### 2.3. Future directions

The key ankyrin-binding and endocytosis motifs are conserved between neuronal Nav subunits (Nav1.1–3 and Nav1.6). However, these subunits are present in other compartments such as soma, dendrites and synapses with a lower density than that found in the AIS and the nodes [9,22]. In the same line, all the Nav1.1, Nav1.2 and Nav1.6 subunits bear the same set of CK2 phosphorylation sites in their ank-binding domain, but they are differentially expressed during development, and exhibit distinct cell type-dependent subdomain organizations along the AIS [14,17,20]. Therefore, this suggests the presence of additional mechanisms, which can overcome all the mechanisms described above in a cell type-dependent manner. Different motifs and/or binding-partners, not identified yet, could underlie these preferential sub-cellular and cell-type dependent organizations. Intercellular and intracellular signaling, transcription factors, and micro-RNAs could also control the developmental regulations.

Finally, recent studies have discovered that the AIS can be surprisingly plastic in its responses to ongoing levels and patterns of electrical activity [74,75] as well as to alterations in neuronal health [76]. This plasticity results in different changes in terms of length, position and composition of the AIS. For instance, in avian auditory neurons, the length of the AIS, defined by the distribution of Nav channels and ankG, increases according to the levels of presynaptic activity [75]. However, the mechanisms underlying such modifications of local Nav channel densities and/or distributions are poorly understood. It is likely that CK2 could play a role in the activity-driven AIS plasticity.

### 3. Role of Nav channel clustering in neuropathology

It is important to understand the mechanisms directing Nav channel targeting to the AIS and nodes, as improper localization or expression of Nav channels has been implicated in a number of pathological conditions linked to altered conduction and electrogenic properties of neurons [77]. Strikingly, defects in Nav channel function can lead to either neurological hyperexcitability [78] or conduction failure/axonal degeneration [79] depending on the pathological context. Mutations in Nav1.1, and to a lesser extent Nav1.2, have been detected in several epileptic syndromes, namely Generalized Epilepsy with Febrile Seizures Plus (GEFS+), Severe Myoclonic Epilepsy of Infancy (SMEI) and Benign Familial Neonatal Infantile Seizures (BFNIS). Nav1.1 mutants seem to be mostly loss-of-function, primarily affecting inhibitory interneurons where Nav1.1 is highly expressed [8,14]. The resulting defect in interneuron excitability is likely to explain the global hyperexcitability and the seizure phenotype associated with these mutations in mouse models and epileptic patients [80]. In contrast, some Nav1.2 mutations have been associated with impaired trafficking of channels to the plasma membrane, but it is not clear how a Nav1.2 loss of function can lead to hyperexcitability.

Beyond the pathological consequences of mutations in Nav channels, perturbations of Nav expression and localization has been implicated in demyelinating diseases such as multiple sclerosis [81]. Nav channel concentration at nodes is lost during demyelination, and Nav channels diffuse all along demyelinating segments. Not only Nav1.6, but also Nav1.2 is often detected in these axonal segments, suggesting that new channels are expressed

and inserted along the unmyelinated membrane. The consequence of this mislocalization can be beneficial, as this low concentration of Nav channels can restore conduction along demyelinated segments. However, it has been suggested that expression of Nav1.6, which exhibits a significant persistent current, plays a role in axonal degeneration: the resultant Nav loading in axons is responsible for activation of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and subsequent calcium-mediated axonal damage [82]. Moreover, a misexpression of non-CNS channels also occurs in neurons during MS, as shown for Purkinje cells expressing Nav1.8 in MS patients and mouse models of MS [83].

### 4. Conclusions

It is now clear that Nav channel densities and discrete localizations are dynamically regulated, as was illustrated by the recent characterization of the kinase CK2 [34]. However, to answer to all the questions posed above, it will be absolutely necessary to make a great effort in developing new model systems and to combine them with advanced electrophysiological, imaging and molecular methods. These new approaches will surely reveal the intricate set of mechanisms that allows for a fine-tuning of neuronal excitability and plasticity in normal and pathological conditions.

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