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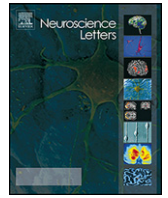
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Review

Voltage-gated sodium channel organization in neurons: Protein interactions and trafficking pathways

Christophe Leterrier^{a,b}, Anna Brachet^{a,b}, Marie-Pierre Fache^{a,b}, Bénédicte Dargent^{a,b,*}

^a INSERM UMR 641, Marseille F-13916, France

^b Université de la Méditerranée, Faculté de Médecine Secteur-Nord, Institut Fédératif de Recherche 11, Marseille F-13916, France

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ABSTRACT

In neurons, voltage-gated sodium (Nav) channels underlie the generation and propagation of the action potential. The proper targeting and concentration of Nav channels at the axon initial segment (AIS) and at the nodes of Ranvier are therefore vital for neuronal function. In AIS and nodes, Nav channels are part of specific supra-molecular complexes that include accessory proteins, adhesion proteins and cytoskeletal adaptors. Multiple approaches, from biochemical characterization of protein–protein interactions to functional studies using mutant mice, have addressed the mechanisms of Nav channel targeting to AIS and nodes. This review summarizes our current knowledge of both the intrinsic determinants and the role of partner proteins in Nav targeting. A few fundamental trafficking mechanisms, such as selective endocytosis and diffusion/retention, have been characterized. However, a lot of exciting questions are still open, such as the mechanism of differentiated Nav subtype localization and targeting, and the possible interplay between electrogenesis properties and Nav concentration at the AIS and the nodes.

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1. Introduction

In neurons, action potentials (APs) are generated at the axonal initial segment (AIS), and their saltatory conduction occurs via the nodes of Ranvier in myelinated axons [15,46]. These processes require a precise distribution of voltage-gated sodium (Nav) channels, which accumulate at high density in these two highly specialized axonal sub-domains. This concentration of Nav channels results from the assembly of supra-molecular complexes composed

of numerous partners, such as adhesion proteins and cytoskeletal adaptors. This review aims to summarize the different partners of Nav channels at the AIS and nodes, and recapitulate their roles in Nav targeting to these compartments. The basic mechanisms of the specific targeting of Nav channels have been unravelled using a combination of biochemical, cellular and functional studies. However, many more questions need to be answered to fully explain Nav targeting and its importance for neuronal transmission in normal and pathological contexts.

2. Structure, function and localization of the Nav channels

Mammalian Nav channels are composed of a highly glycosylated 260 kDa α subunit, the pore forming protein, linked via disulfide bonds to $\beta 2/\beta 4$ subunits and non-covalently with $\beta 1/\beta 3$ subunits

* Corresponding author at: Faculté de Médecine Secteur-Nord, Neurobiologie des Canaux ioniques, Université de la Méditerranée CS80011, Bd Pierre Dramard, 13344 Marseille Cedex 15, France. Tel.: +33 4 91 69 88 59; fax: +33 4 91 09 05 06.

E-mail address: benedicte.dargent@univmed.fr (B. Dargent).

[11]. Nine Nav1 α subunit genes (SCN1A–SCN9A) have been identified in mammals, constituting the Nav1 gene subfamily. The Nav subunits (Nav1.1–9) contain the ion-selective pore and are composed of 4 homologous membrane domains (I–IV) containing 6 transmembrane α helices (S1–S6). S5 and S6 segments from the four domains form the Na⁺ selective pore, and the S4 helix acts as a voltage sensor whose movement within the membrane induces in pore opening [11].

β subunits (β 1–4) are encoded by four different genes (SCN1B–SCN4B). The β subunits share a similar topology, and their structure–function has been reviewed by Patino and Isom in this special issue [63]. β subunits regulate the surface density of Nav1 and modulate the biophysical properties of the channel in complex and opposite ways [67]. In addition, the β subunits exhibit CAM properties, being involved in multiple interactions through either homophilic or heterophilic interactions with members of the L1 CAM family [56], and recognizing molecules in the extracellular matrix (ECM, [80]). Finally, the β 1 subunit promotes neurite outgrowth in cerebellar granule cells (GCs) via a lipid raft mechanism and Fyn kinase signaling [7,8,20].

With the exception of Nav1.4, all Nav1 subunits are expressed in the nervous system. Nav1.1, Nav1.2 and Nav1.6 are abundant in the central nervous system (CNS), whereas Nav1.3 is mostly present during embryonic development. Nav1.7, Nav1.8 and Nav1.9 are predominant in the peripheral nervous system (PNS) [11]. β 1, β 2 and β 4 are the main β subunits expressed in the mammalian brain [63]. Both the expression of the four predominant Nav1 subtypes and the four β subunits are developmentally and spatially regulated in the CNS (for a review see [84]). At the cellular level, Nav1.1 is predominantly found not only in soma and dendrites but also at the AIS and nodes of Ranvier [23,33,90]. Nav1.2 is localized along unmyelinated axons and accumulates at the AIS and in immature nodes [4,5,33,53,90]. Nav1.6 is concentrated in the AIS, nodes of Ranvier and in proximal dendrites in many types of neurons [10,53]. In contrast, little information is available on β subunit localization at the cellular level, although β 2 may be concentrated at the nodes of Ranvier [13] and β 1 has been detected at the AIS in cerebellar GCs [7].

3. Neuronal Nav channels form a supra-molecular complex at the AIS and the nodes of Ranvier

In neurons, Nav channels concentrate at the AIS and nodes of Ranvier as part of well-described multi-molecular complexes [35] (Fig. 1A). In addition to Nav channels, potassium channels Kv7.2/Kv7.3 [21,62] and cell adhesion molecules NF-186 and NrCAM [2,19,24,36] accumulate at the AIS and the nodes of Ranvier where the cytoskeletal adaptor complex ankyrin G (ankG)/ β 4-spectrin is segregated [98] (for reviews see [3,75], Fig. 1B). The AIS and the nodes of Ranvier are also enriched in other proteins, such as protein kinase casein kinase 2 (CK2) which regulates ion channel interaction with ankG [9], SCHIP-1 isoforms [55], FHF2 and FHF4, two members of the fibroblast growth factor homologous factors (FHF), [32]) and members of the NF κ B signaling pathway [66,77]. Lastly, in certain neuronal types, members of the Kv1 voltage-gated potassium channel family are restricted to the distal part of the AIS [39,52].

These supra-molecular complexes at the AIS and nodes are the result of multiple, redundant protein–protein interactions, Nav channels being a core component in this interaction network (Fig. 1C). A key partner of Nav channels is ankG. A well-characterized ankyrin-binding motif is located in the cytoplasmic linker II–III of the pore forming Nav1 protein [26,31,51] (see below). It is possible that β 1, but not β 3, provides an additional link between Nav channel complex and ankG since homophilic β 1 sub-

unit interaction induces ankG recruitment [7,54]. In addition, β 1 subunits interact heterophilically with NF-186 and NF-155, NrCAM [56] and contactin/F3, a CAM protein concentrated in the nodal and paranodal regions of CNS nodes [44]. Nav1 and β 1 subunits associate with the receptor protein tyrosine phosphatase β , a partner of contactin/F3 [69]. The β 2 subunit binds to tenascin-C and tenascin-R [80,93], two ECM molecules that associate with brevican [58]. Finally, studies have shown that several members of the FHF family interact and differentially modulate the functional properties of Nav1 [92]. Furthermore, some of FHF members are enriched at the AIS and influence Nav channel clustering [32,49].

4. Complex Nav expression in the nodes of Ranvier and along the AIS

Initial studies of Nav1 subunit localization in mammalian brain showed that Nav1.2 is preferentially expressed along unmyelinated fibers [33,90] while Nav1.6 is localized at nodes of Ranvier [10]. Boiko et al. [4] confirmed the differential localization of Nav1.2 and Nav1.6 in retinal ganglion cells (RGCs) that form axons with well defined unmyelinated and myelinated segments. During development, a switch between Nav1.2 and Nav1.6 expression occurs in RGCs axons; Nav1.2 is first concentrated at the immature nodes of Ranvier and is replaced by Nav1.6 as the nodes mature [4]. Complemented by others studies [72], Nav1.6 channels have thus been considered predominant in mature nodes of Ranvier (for a review see [84]). However, at present, the situation appears to be more complex. Indeed, Nav1.6 is expressed alone or together with Nav1.1 in CNS and PNS nodes of Ranvier [23]. Moreover, nodes of Ranvier expressing Nav1.1, but not Nav1.6 have been described [23]. The developmental expression of Nav1.1, Nav1.2 and Nav1.6 at the nodes of spinal cord neurons indicates that these three channels can coexist at certain stages of nodal maturation [23]. Finally, Nav1.8, a subtype preferentially expressed in sensory neurons [11], were observed at nodes of Ranvier in physiopathological situations [1,22].

Studies also provide evidence for a complex and cell specific sub-domain organization of Nav1 types along the AIS, with spatially and temporally defined co-expression of Nav1.6, Nav1.1 and Nav1.2. Firstly, Nav1.6 is exclusively and uniformly distributed along the AIS of Purkinje cells [52,85]. Nav1.6 is expressed together with Nav1.1 in the AIS in cortical and cerebellar interneurons, in RGCs and in spinal cord neurons [23,52,60,87]. In these cells, Nav1.1 and Nav1.6 showed a complementary localization along the AIS: Nav1.1 displays a proximo-distal gradient along the AIS, whereas Nav1.6 accumulates in a distal-to-proximal gradient [5,23,86]. In contrast, Nav1.1 and Nav1.6 are uniformly co-distributed along the AIS of main olfactory bulb short axon cells [52]. Nav1.2 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 [38]. Importantly, Nav1.6 accumulation at the distal AIS determines the lowest threshold for AP initiation whereas a proximal Nav1.2 gradient promotes AP back propagation to the soma [38]. Nav1.2 and Nav1.6 are also co-expressed along the AIS of cerebellar GCs [61].

During development, Nav1.2 accumulation precedes Nav1.6 concentration in RGCs and in GCs [5,61]. Mice lacking Nav1.6 (*Scn8a^{medf}* mice) exhibit distinct compensatory change in other Nav1 types. Both Nav1.1 and Nav1.2 compensate the lack of Nav1.6 at the AIS of RGCs while only Nav1.1 replaces Nav1.6 at the AIS of cerebellar Purkinje cells [85], but no compensatory expression between Nav1.6 and Nav1.1 was observed at the AIS in GCs-specific Nav1.6-null mouse [61]. Such a complex organization and regulation of Nav1 types at the AIS is expected to endow neurons with variable excitability properties, which can influence the genera-

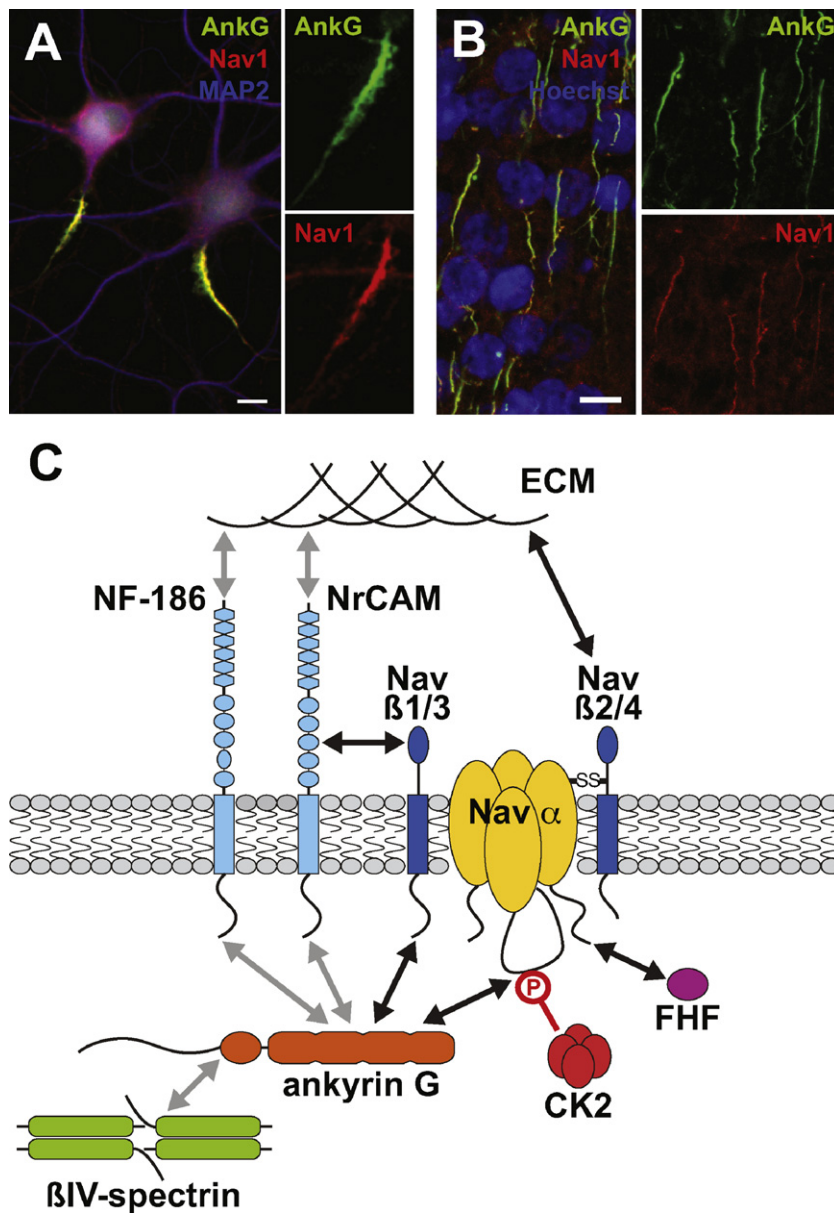


Fig. 1. Nav channel partners at the AIS. (A) Nav channels are highly concentrated at the AIS of hippocampal neurons in culture (red). The AIS is labeled using an anti-ankyrin G antibody (green) and the somatodendritic compartment is labeled using an anti-MAP2 antibody (blue). Scale bar, 10 μ m. (B) Concentration of Nav channels at the AIS of cortical neurons in vivo (red). Nav colocalizes at the AIS with ankG (green). Nuclei are labeled using Hoechst 33258 (blue). Scale bar, 10 μ m. (C) Summary of Nav channel partners at the AIS. β subunits 2 and 4 (dark blue) are covalently linked to the Nav α subunit (yellow) by disulfide bonds. β subunits directly bind adhesion molecules (NrCAM and NF-186 in light blue), possibly ankG (orange) and proteins of the ECM (black). Nav1 subunit directly interacts with ankG (orange), this interaction being regulated by CK2 phosphorylation (red bar and circle). AnkG also binds β IV-spectrin (green) and adhesion molecules. Black arrows symbolize interactions between Nav1 channel and its direct partners; grey arrows represent interaction between Nav1 partners.

tion and the backpropagation of APs [15,38]. Further studies are required for a better understanding of mechanisms accounting for such complex nodal expression and of the respective physiological relevance of each expression pattern.

5. Mechanisms of Nav concentration at the AIS

The AIS is intrinsically specified, being able to assemble in cultured neurons in the absence of glial cells [91,97]. In vivo and in vitro analyses all converge on a crucial role of ankG as the central organizer for AIS assembly and maintenance [36,40,98]. ankG is the first component detected at the AIS by immunohistochemistry [40]. In vivo, mutant mice lacking ankG in Purkinje cells fail to cluster any other AIS component [40,98], and electroporation of

cortical neurons with shRNA against ankG blocks AIS assembly [36]. In cultured hippocampal neurons, knockdown of ankG impedes AIS formation while knockdowns of CAMs, Nav channels or β 4-spectrin have little impact [36]. ankG is an essential organizer of the AIS as Nav, Kv7.2/7.3 channels, CAMs and β 4-spectrin all rely on binding to ankG for their localization [31,51,62,96].

A role for Nav channels in AIS formation and maintenance is, however, not excluded. In cultured motor neurons, Xu and Shrager demonstrated that the decrease of Nav channel expression affects AIS assembly, suggesting that a complementary mechanism might exist in this cell type [94]. In vivo confirmation of the possible role of Nav in AIS assembly may prove difficult, because of the perinatal lethality of the Nav1.2 knockout mouse [64] and the compensation for Nav1.6 loss by Nav1.2 and Nav1.1 in the *Scn8a^{medj}* mouse [85].

The role of Nav β subunits in Nav channel targeting to the neuronal membrane is not fully understood. Tagged β subunits expressed in cultured hippocampal neurons are uniformly distributed [30], indicating that Nav α rather than β subunits may contain information for the sorting and clustering of Nav channel complexes at the AIS. However, cerebellar neurons lacking $\beta 1$ show a decreased concentration of Nav 1.6 at the AIS [7]. Therefore, similarly to $\beta 4$ -spectrin [45,48], β subunits could be necessary for the maintenance of Nav channel complexes at the AIS rather than for their initial concentration at this site.

The key interaction for AIS assembly and function, that is, the binding of Nav channels to ankG, was discovered more than twenty years ago [81]. However, the molecular details of this interaction and its role in Nav channel concentration at the AIS were only recently unravelled [9,26,31,37,51]. To assess whether any of the intracellular loops of Nav1.2 contained sufficient information for its sorting to the AIS, our laboratory developed an approach

based on chimeric protein expression in cultured hippocampal neurons [29]. Using this approach, we showed that the cytoplasmic loop linking the domains II and III (L2) contained a motif responsible for the compartmentalization of Nav channels at the AIS (Fig. 2B and C) [31]. L2 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). This targeting was abolished upon truncation of a sequence of 27 residues within L2 (1102–1128) that was thus called the AIS motif (Fig. 2A) [31]. Another study independently confirmed that the PIALGESD sequence located within the AIS motif directly interacts with the Membrane Binding Domain (MBD) of ankG [51].

ankG is segregated at the AIS, whereas the other neuronal ankyrin, ankyrin B, is located along the distal axon in cultured hippocampal neurons and in myelinated fibers [3]. These axonal ankyrins share a highly conserved MBD, suggesting that an additional mechanism must ensure the specific interaction of Nav

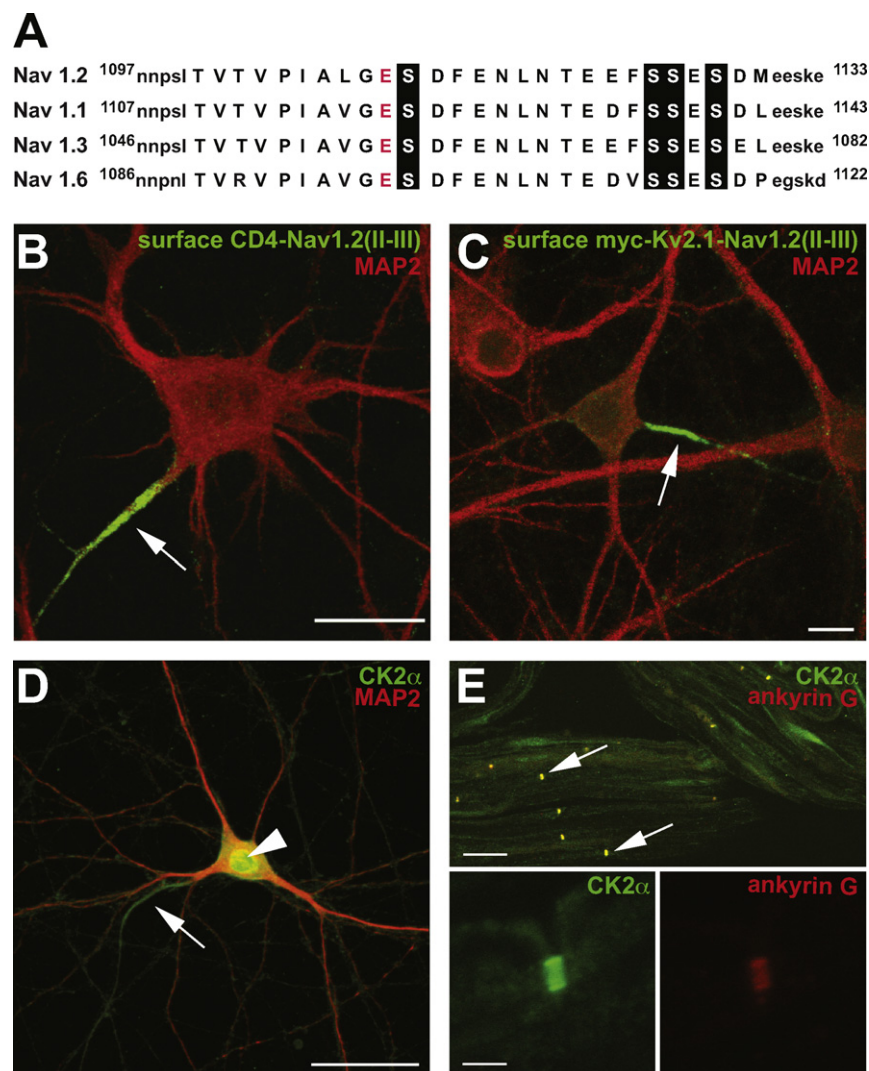


Fig. 2. The ankyrin-binding-motif of Nav 1.2 directs its segregation at the AIS of hippocampal neurons. (A) Sequences of the ankyrin-binding-motif for CNS Nav channels Nav1.1–3 and Nav1.6. Interaction with ankG critically depends on a highly conserved glutamate (E1111 for Nav1.2, in red) and on CK2-phosphorylatable serines (S1112, 1123, 1124 and 1126 for Nav1.2, black boxes). (B) Cell surface distribution of CD4-Nav1.2 (II–III) in transfected hippocampal neurons. Surface CD4-Nav1.2 (II–III) immunolabeled by an anti-CD4 antibody is restricted at the AIS (green). The somatodendritic compartment is identified by MAP2 staining (red). Scale bar, 20 μ m. (C) The ankyrin-binding motif of Nav1.2 is able to target the somatodendritic voltage-gated channel Kv2.1 to the AIS in transfected hippocampal neurons. Surface myc-Kv2.1-Nav1.2 is detected with an anti-myc antibody (green) and the somatodendritic compartment is identified by MAP2 staining. Bar, 10 μ m. Image is reproduced from Bréchet et al. [9] with permission from the Journal of Cell Biology; doi:10.1083/jcb.200805169. (D) and (E) Endogenous CK2 α is concentrated at the AIS in cultured hippocampal neurons (D) and at nodes of Ranvier along the sciatic nerve (E). CK2 α immunoreactivity (green) is expressed at the AIS (arrows) and in the nucleus (arrowheads). Somatodendritic domain is identified by MAP2 (red, D) and ankG labels nodes (red, E). Scale bars, 30 μ m (D); 23.2 μ m (E, top); 3 μ m (E, bottom). Image (E) is reproduced from Bréchet et al. [9] with permission from the Journal of Cell Biology; doi:10.1083/jcb.200805169.

channels with ankG. Recently, Bréchet et al. demonstrated that the AIS motif of neuronal Nav channels is phosphorylated *in vitro* by CK2, resulting in a 1000-fold increase in the binding affinity (from micromolar to nanomolar) for the MBD of ankG [9]. Although CK2 phosphorylation equally enhanced the interaction with the MBD of ankyrin B and G [9], they showed that CK2 appears to be exclusively concentrated at the AIS and nodes along the axon (Fig. 2D and E). This suggests that local concentration of CK2, which phosphorylates Nav channels, enhances their interaction with ankG, and thus contributes to their specific accumulation at the AIS. Now it would be interesting to identify the phosphatase counterbalancing CK2 phosphorylation that could fine-tune this regulation. In addition to its pivotal role in Nav accumulation, ankG modu-

lates the inactivation properties of Na⁺ current in heterologous cells [79], but the electrophysiological consequences of a phosphodependant binding of ankG remains to be explored in neurons. Another unresolved issue is that Nav1.1, Nav1.2 and Nav1.6 bear CK2 phosphorylation sites at their AIS motif, but they are differentially expressed during development at the AIS [5,61], and exhibit distinct subdomain organization along the AIS in defined neuronal types [38,52,87]. Additional mechanisms probably account for this preferential organization. Nevertheless, the discovery of a CK2-dependent Nav–ankG interaction suggests that CK2 can dynamically alter Nav concentration at the AIS. Thus, CK2 could play a role in the activity-driven AIS plasticity that was recently described [34,47].

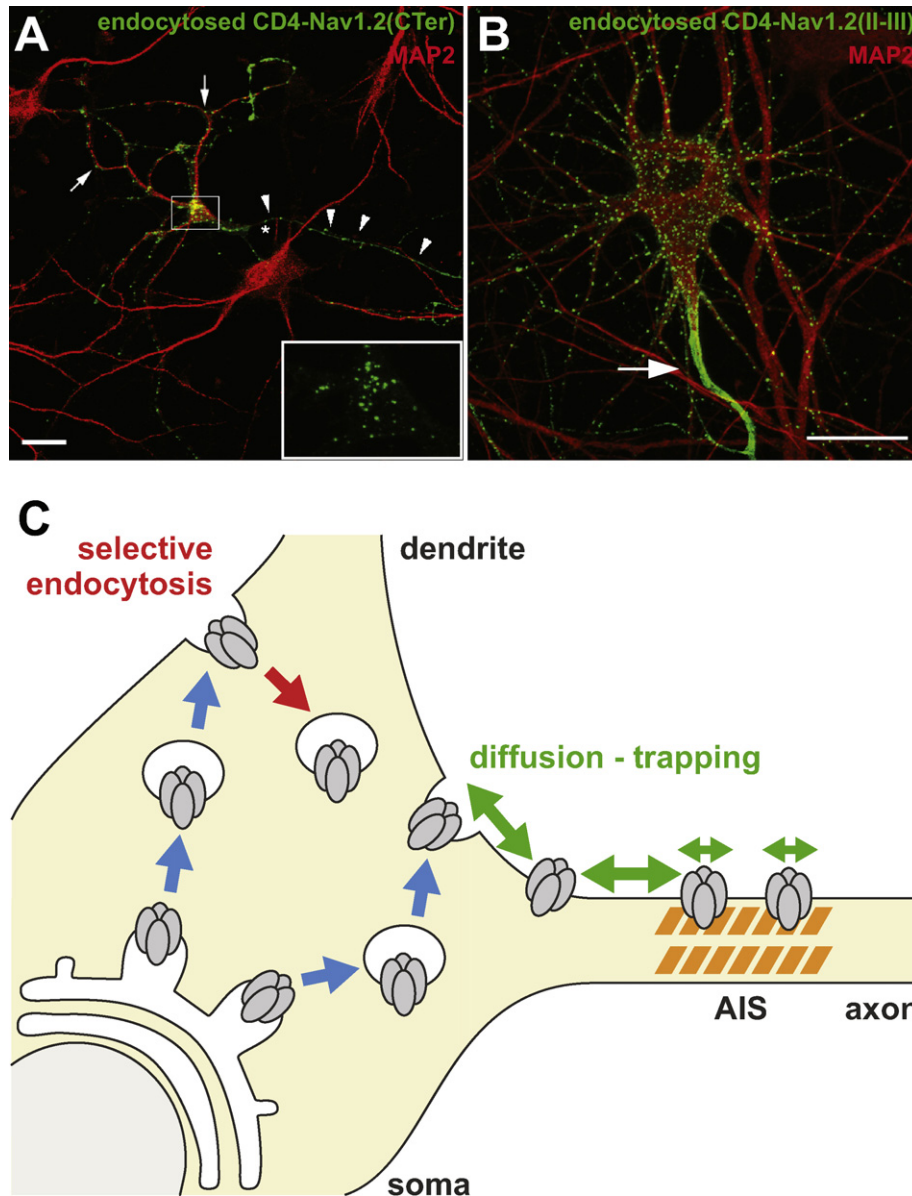


Fig. 3. Trafficking pathways contributing to the compartmentalization of Nav channels. (A) and (B) The C-terminus and cytoplasmic II–III loop of Nav 1.2 are recognized by endocytic pathways. Hippocampal neurons transfected with either CD4-Nav1.2 (CTer) (A) or CD4-Nav1.2 (II–III) constructs (B) were processed for an immuno-endocytosis assay, fixed, permeabilized, stained for CD4 (green) and for MAP2 (red). (A) CD4-Nav1.2 (CTer) containing vesicles (green) are distributed throughout dendrites (arrows), the soma (box) and axon (arrow heads). Some punctate staining appears to be concentrated in the proximal axon (asterisk). Scale bar, 25 μ m. Image is reproduced from Garrido et al. [29] with permission from the EMBO Journal. (B) CD4-Nav1.2 (II–III) containing vesicles are distributed throughout the soma and dendrites identified by MAP2 co-staining. Note the concentration of CD4-Nav1.2 (II–III) at the AIS (arrow). Scale bar, 25 μ m. Image is reproduced from Fache et al. [26] with permission from the Journal of Cell Biology; doi:10.1083/jcb.200312155. (C) A proposed model for Nav1 segregation at the AIS. The neo-synthesized channels are transported and inserted in the somatodendritic compartment (blue arrows). An endocytic mechanism (red arrow) permits the selective elimination of Nav1 in soma and dendrites, whereas channels are trapped by ankG at the AIS after laterally diffusing (green double arrow). These two mechanisms, selective endocytosis and diffusion–trapping, lead to concentration of Nav channels at the AIS.

Beyond the interaction of Nav and ankG at the AIS, two general models may underlie the final compartmentalization of Nav channels at the AIS: (i) the channels could be directly inserted in the AIS plasma membrane from biosynthetic vesicles and/or (ii) the channels could be initially transported and uniformly inserted in the neuronal membrane, subsequently retrieved from the dendrites, soma and the distal axon by endocytosis, while being tethered by ankG at the AIS (elimination/retention model). These two models could coexist and their relative importance may vary with the neuronal developmental stage.

We first showed that endogenous Nav channels could undergo activity-dependent endocytosis in developing neurons [17,18]. Garrido et al. [29] further demonstrated that chimeric proteins bearing the carboxy-terminal tail of Nav1.2 channels were concentrated at the axonal plasma membrane in dissociated hippocampal neurons. This compartmentalization arises from the selective endocytosis of the chimera in the somatodendritic compartment (Fig. 3A), suggesting that Nav channels are addressed to the axon via an elimination/retention model. Finally, the determinants in L2 of Nav1.2 are sufficient to target a chimeric protein to the AIS by an elimination/retention mechanism [26]. Sodium channel L2 actually contains two determinants, the AIS motif responsible for the ankG tethering and an endocytic signal located more distally in the loop. Fache et al. [26] showed that CD4-Nav1.2 is first inserted in the somatodendritic compartment and distal axon, before being endocytosed from these sites and restricted to the AIS (Fig. 3B). In view of these findings, we propose that in developing neurons, Nav channels are first uniformly inserted in the neuronal plasma membrane, including soma and dendrites. Channels would then diffuse laterally until they are endocytosed or trapped by ankG when they reach the AIS (Fig. 3C). Consistent with this scenario, the presence of a conserved binding site for Nedd4 and Nedd4-2 ubiquitin ligases in the C-terminus of several Nav1 subunits could target endocytosed channels to degradative pathways [28]. The key ankyrin-binding and endocytosis motifs are conserved between neuronal Nav subunits (Nav1.1–3 and Nav1.6), and the known localization for these subunits includes AIS and nodes [84]. However, the fact that Nav 1.1 and Nav 1.6 have also been detected in other compartments such as soma, dendrites and synapses [84] suggest that other motifs and/or partner proteins can overcome these mechanisms in some neuron types.

The fact that Nav channels are able to concentrate at the AIS due to their interaction with ankG implies that the AIS is a diffusion trap for these proteins. A decreased diffusion of Nav channels-bound toxins at the axon hillock has been described [59], and confirmed by recent data obtained in our laboratory using single particle tracking of quantum-dot labeled ion channels [6]. Brachet et al. [6] show that the ion channel chimera Kv2.1-Nav1.2 diffuses slowly at the AIS membrane and that this immobilization depends on its ability to bind ankG. Moreover, consistent with a role of phosphorylation increasing the binding affinity of Nav to ankG, inhibition of CK2 enhances Kv2.1-Nav1.2 diffusion and impacts Nav channels accumulation at the AIS. As CK2 can directly modulate ion channel chimera mobility at the AIS, it would be very interesting to test if activity-dependent Nav phosphorylation by CK2 can modulate excitability by altering Nav channels mobility and localization at the AIS.

6. Mechanisms of Nav concentration at the nodes of Ranvier

The molecular architecture of the nodes of Ranvier is strikingly similar to that of the AIS, with a concentration of Nav channels and adhesion proteins tightly anchored to an ankG/ β 4-spectrin submembrane cytoskeleton [75]. However, the presence of myeli-

nating cells (Schwann cells or oligodendrocytes) and their complex relationship with nodal region organization leads to crucial differences in the mechanism of nodes formation and maintenance [82]. Additionally, nodes of Ranvier can be very far from the soma, and trafficking mechanisms allowing for delivery and concentration of Nav channels at such distal locations are yet to be unravelled.

In the PNS, the main steps of node formation have now been delineated [76]. Immunocytochemistry studies have shown that nodes are first formed, followed by delineation of paranodes and juxtaparanodes [57,65]. At the node itself, initial results suggest an extrinsic mechanism initiated by glial cells [14], with recruitment of adhesion proteins occurring before ankG and Nav concentration [50]. Later in vitro studies have detailed the sequence of events leading to node formation. Schwann cells secrete gliomedin, an oligomeric ligand that recruits NF-186 to nascent nodes [25]. NF-186 is then recruits ankG to nodes, which in turn recruits Nav channels [24] and β 4-spectrin [96]. This sequence of events, and the prominent role of NF-186 in node assembly, is confirmed by the general absence of nodes in the PNS of NF-186 knockout mice [78]. Mice deficient for nodal components not directly involved in node assembly such as NrCAM [16] and β 4-spectrin [45,48,95] also exhibit altered nodes, demonstrating the interdependence of every nodal component for long-term nodal maintenance.

The mechanism of node formation in the CNS is much less well understood, in part because of the lack of practical in vitro models. As gliomedin is absent in the CNS, it has been proposed that a redundant network of ECM proteins such as brevican, versican and tenascin-R are responsible for the initiation of NF-186 recruitment [82]. Notably, clustering of Nav channels can be induced in RGC cultures by an unidentified soluble factor released by oligodendrocytes [43]. However, the requirement for NF-186 is not as absolute as in the PNS [99]. Other intriguing results have been reported relative to node formation in the CNS: concentration of paranodal components occurs before nodal ones [68], and ankG clusters can appear before adhesion proteins at the optic nerve nodes [41]. This suggests the presence of complementary or alternative mechanisms directing the assembly of CNS nodes [82].

In contrast to the AIS, this mechanism places ankG and Nav channels as downstream participants in nodes formation. The effect of ankG absence at nodes in vivo is unknown, as the cerebellar ankG knockout model still exhibit ankG labeling at nodes of Ranvier [40]. In myelinating 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 [24]. Although CK2 is concentrated at the nodes of Ranvier in the CNS and PNS [9], whether it directly regulates the ankyrin-Nav interaction at nodes has not been determined yet.

Nav β subunits are also present at the nodes [12,13,42,70], and NF-186 and Nav β subunits can interact in cis [70], suggesting an alternative mechanism for Nav channel recruitment to the nodes through β subunits. In support to this, mice deficient for Nav β 1 show defects in nodal architecture, and both β 1 and β 2 knockout mice exhibit altered nerve conduction characteristic of a reduced Nav channel density [12,13]. However, it is likely that β subunits are involved primarily in Nav cluster maintenance rather than formation, as a NF-186 mutant deficient in ankyrin binding but capable of binding β subunits fails to cluster Nav channels at nodes in vitro [24]. Nav α subunits by themselves probably play a role in the node maintenance, as *Scn8a^{medj}* mice lacking Nav1.6 exhibit elongated, disorganized nodes [71], even if sustained expression of Nav1.2 and Nav1.1 compensate for the lack of Nav1.6 [85,88]. Furthermore, the effect of a knockdown of Nav channels at nodes by shRNA, in vitro or in vivo, is still unknown.

It is likely that nodal membrane proteins are directly inserted into the node, although this has not been demonstrated yet. A critical role of paranodal junctions in limiting subsequent diffu-

sion of nodal membrane proteins, in particular Nav channels, was proposed thirty years ago [73]. Consistent with this hypothesis, different mouse models with altered paranodal junctions have longer and more diluted clusters of Nav channels [72,74,83]. Restriction of nodal components diffusion by paranodes has been proposed as an additional mechanism of Nav channel clustering in the CNS [68,99] and in the PNS [27]. The internode region, where the axonal membrane is apposed to glial cells, has long been thought to be passive with respect to nodal component clustering, but recent studies have shown that it actively directs Nav channels toward the nodes [89]. Direct studies of Nav channel diffusion by live-cell imaging techniques in myelinating cultures would be instrumental to characterize the restriction of diffusion at nodes and the role of paranodes.

Strikingly, paranodal junctions seem to have a prominent role in the differential expression of Nav1.2 and Nav1.6 types at nodes [4,72]. Mice with altered paranodes exhibit incomplete replacement of Nav1.2 by Nav1.6 in PNS nodes [72,83]. The fact that Nav1.2, but not Nav1.6, can cluster to form immature nodes in RGC cultures treated with oligodendrocytes-conditioned medium [42] further suggests that myelinating cells could direct the differential targeting of the two Nav α subunits at the nodes.

7. Conclusion

In conclusion, interactions of Nav channels with their partner proteins, such as ankG, are required for their proper concentration at the AIS and nodes of Ranvier. A number of important questions remain to be answered, like the mechanism of the interplay between neuronal activity and Nav channel concentration [34,47], and the *in vivo* relevance for several assembly and targeting mechanisms demonstrated using *in vitro* models. Hopefully, answering these questions will provide new therapeutic targets for diseases where misexpression and mislocalization of Nav channels cause functional impairments.

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