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Current Opinion in

Neurobiology

The making of a node: a co-production of neurons and glia

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Nodes of Ranvier are specialized axonal domains formed in response to a glial signal. Recent research advances have revealed that both CNS and PNS nodes form by several overlapping molecular mechanisms. However, the precise nature of these mechanisms and the hierarchy existing between them is considerably different in CNS versus PNS nodes. Namely, the Schwann cells of the PNS, which directly contact the nodal axolemma, secrete proteins that cluster axonal components at the edges of the growing myelin segment. In contrast, the formation of CNS nodes, which are not contacted by the myelinating glia, is surprisingly similar to the assembly of the axon initial segment and depends largely on axonal diffusion barriers.

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Current Opinion in Neurobiology 2013, **23**:1049–1056

This review comes from a themed issue on **Development of neurons and glia**

Edited by **Samuel Pfaff** and **Shai Shaham**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 5th July 2013

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<http://dx.doi.org/10.1016/j.conb.2013.06.003>

Introduction

Myelin is the vertebrates' solution for fast and energetically efficient axonal conduction. Action potentials generated at the axon initial segment (AIS) are passively transmitted under the myelin sheath and regenerate in the periodic gaps in the myelin called nodes of Ranvier (NOR). Thus, for the myelinated nerve to function, voltage gated sodium and potassium channels (Nav and Kv) must be specifically localized to AIS and NOR where action potentials take place. In terms of neuronal conduction, the physiological consequences of having myelin without nodes would probably be more detrimental than not having myelin at all. It is thus not surprising that several mechanisms have evolved to facilitate and ensure the correct assembly and stabilization of these highly important axonal domains. In this review we aim to provide the reader with an up-to-date model for node formation while emphasizing PNS–CNS differences as well as comparing the formation of the node to that of its evolutionary 'ancestor', the AIS.

For any specialized membrane domain to form, proteins must first be transported to the cell membrane then

tethered to a particular site on the membrane where they assemble together by specific protein–protein interactions. Finally, the newly formed protein complex is stabilized by restricting its lateral diffusion. Each of these steps can be achieved by different possible mechanisms. The major components of nodes and initial segments are voltage gated ion channels. All other components, namely cell adhesion molecules (CAMs), extracellular matrix (ECM) proteins and cytoskeletal proteins serve to cluster and stabilize the channels in these crucial axonal domains.

Trafficking of nodal components

Targeting proteins to specific membrane domains is achieved either by selective transport, or random transport followed by specific retention in the correct domain or removal of mislocalized proteins. Retention can be accomplished by anchoring to large molecular scaffolds or by restricting lateral diffusion [1,2]. The ion channels of the AIS and nodes are initially transported to all parts of the neuron and then anchored and immobilized by ankyrin G (ankG). Like numerous other axonal proteins, the channels are endocytosed from the somatodendritic membrane [3] via two endocytosis signals they possess [4]. Interestingly, ankG itself may be subjected to a retention/exclusion targeting mechanism as it is apparently excluded from the submembranous cytoskeleton of the distal axon [5^{**}]. Once the AIS is formed, the high density of membrane proteins and their anchorage to the cytoskeleton creates a diffusion barrier that immobilizes all AIS components, including small lipid molecules, regardless of their binding to the cytoskeleton [6,7^{*}].

It was recently shown that during PNS node formation, CAMs are initially assembled from a cell surface pool, whereas the accumulation of Nav and ankG requires vesicular transport from the cell soma [8^{**}]. These results are in line with current models for node formation in which, according to the removal–retention model, Nav channels targeting to the axolemma is ankG-dependent, whereas gliomedin-induced CAM clustering requires a mobile surface pool of CAMs. The surface expression of Nav channels may also depend on the $\beta 1$ and $\beta 2$ auxiliary subunits [9]. These ankyrin-binding proteins enhance the surface expression of the Nav pore forming alpha subunit [10], and their deficiency results in reduced levels of Nav channels at the optic nerve NOR [11]. Moreover, $\beta 2$ and $\beta 4$ possibly associate with the alpha subunit before its transport to the axolemma, since their AIS and nodal localizations depend on a single extracellular disulfide bond with the alpha subunit [12,13].

Assembly

The scaffold protein ankG was first described as a master regulator for AIS assembly 15 years ago when Bennet and colleagues showed that cerebellar Purkinje cells lack AIS in the absence of ankG [14]. Almost a decade later, ankG was shown to be crucial for NOR formation [15] and remains the only protein whose correct localization is consistently both necessary and sufficient for AIS and NOR assembly [16]. This is not surprising as ankG can bind and cluster most if not all of the known axonal proteins that these domains contain (Table 1), namely Nav and Kv channels, the CAMs neurofascin 186 isoform (NF186) and NrCAM, as well as β IV spectrin, an actin binding scaffold protein. β IV spectrin thus connects the ankG-organized AIS and nodal protein complexes to the submembranous actin cytoskeleton.

For nodes and AIS to form, ankG has to be tethered to the membrane at specific sites. The specific locations are determined by the neurons alone (AIS) or by the

myelinating glial cell, which is the oligodendrocyte in the CNS and the Schwann cell in the PNS. One major difference between CNS and PNS nodes is that PNS nodes are contacted by microvillar processes sent from the Schwann cells that myelinate the flanking internodes. In the CNS, however, the myelinating cells themselves do not contact the nodes which are to some degree contacted by astrocyte processes [17]. The second major difference is the extracellular proteins, mostly ECM, that interact with the nodal axolemma (Table 1). These differences are reflected in the way in which nodes form in the CNS and PNS tissues, as depicted in Figure 1.

PNS nodes

During PNS myelination, heminodal clustering of Nav channels, that is, their accumulation at the edges of a growing myelin segment, depends on the interaction of axonal NF186 with glial gliomedin and NrCAM [18,19**]. Gliomedin incorporates into the nodal ECM where it creates high avidity CAM-binding multimolecular complexes that drive the accumulation of NF186 in the underlying axolemma [20]. AnkG is recruited to NF186 complexes, followed by β IV spectrin and Nav [15,18,21,22]. A recent paper by Zhang *et al.* provides an elegant support for this model showing that initial accumulation of NF186 at heminodes as well as its internodal clearance requires its ectodomain, while its stabilization in mature nodes requires its cytoplasmic ankG binding domain. This is in contrast to the AIS where the ankG binding domain is sufficient for early clustering of NF186 [8**].

Flanking the nodes are the paranodal junctions (PNJ) that form between the terminal loops of the glia and the axon. These junctions form after the formation of nodes in the PNS [23]. Nevertheless the PNJ themselves, serving as diffusion barriers that limit the lateral diffusion of membrane proteins, can drive the assembly of PNS nodes in the absence of a functioning heminodal clustering mechanism (e.g. in gliomedin or NrCAM null mice). Consequently, only in the absence of both heminodal and paranodal mechanisms, nodes would not form. Thus in the PNS clustering of nodal components is induced by a glial signal at the heminodes. A second mechanism, which depends on intact paranodal junctions, ensures nodal assembly [19**].

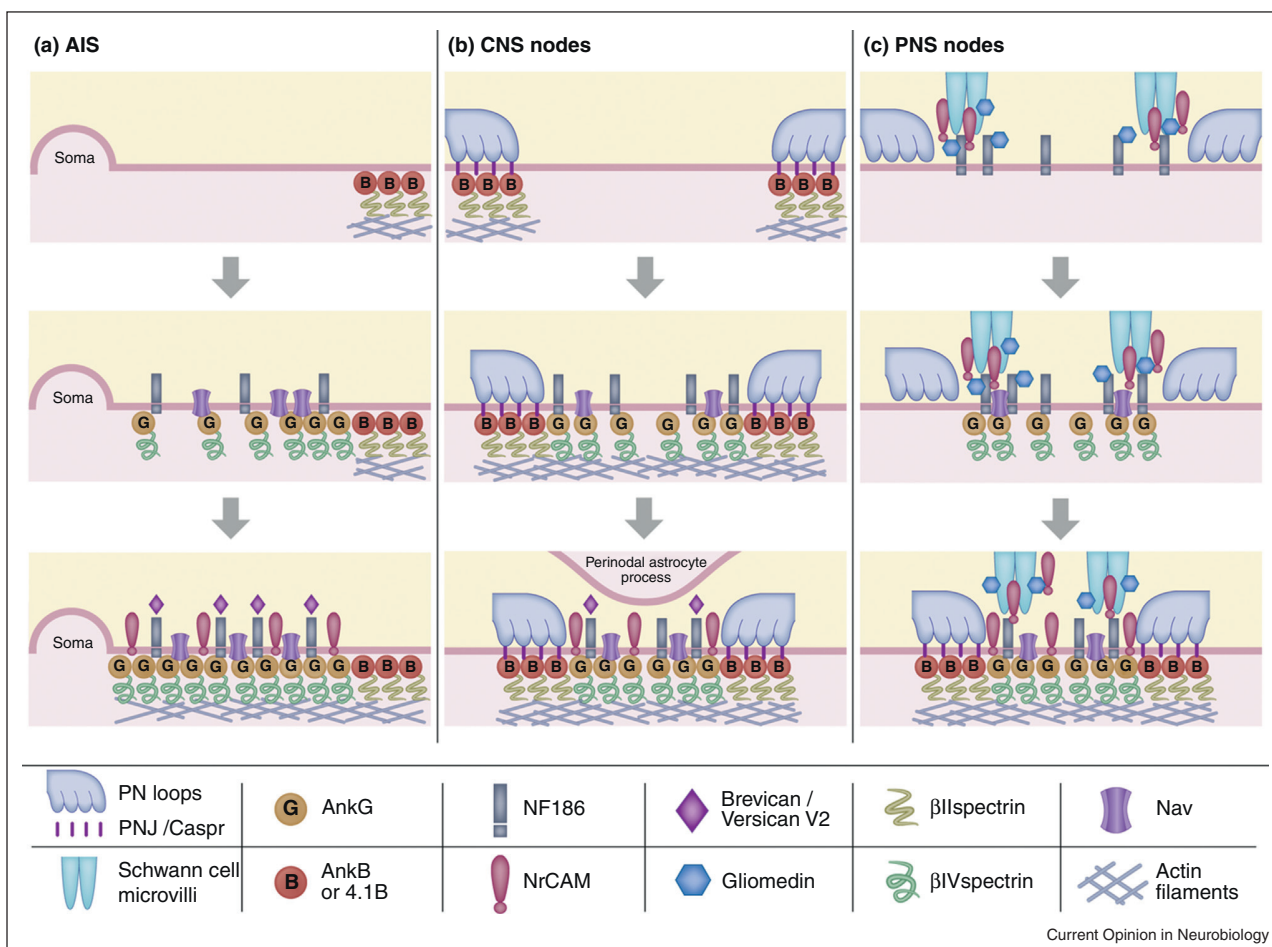
CNS nodes

A different sequence of events is observed in the developing CNS node: the PNJ appear first [24], followed by ankG which in turn recruits the nodal CAMs and Nav [25]. Therefore, The primary inductive signal does not appear at the nascent nodes themselves (which lack oligodendrocyte contact), but at the PNJ driving the accumulation of ankG in the adjacent nodal axolemma. In a recent paper Suzuki *et al.* show that similarly to the PNS, CNS nodes form by several mechanisms [26**].

Table 1

Molecular components of nodes and AIS			
	AIS	CNS NOR	PNS NOR
Cell adhesion			
NF186 [59]	+	+	+
NrCAM [59]	+	+	+
Navb1-4 [9]	+	+	+
Caspr2 [9,60]	+	–	–
TAG1 [61]	+	–	–
ADAM22 [62]	+	–	–
Dystroglycan [63]	–	–	+
Ion channels			
Nav1 [16,64]	+	+	+
KCNQ2,3 [65,66]	+	+	+
Kv1 [60,61]	+	–	–
Kv3.1b [67]	–	+	+
ECM			
Gliomedin [18]	–	–	+
Syndecan-3,4 [68]	–	–	+
Collagen XXVII [69]	–	–	+
Perlecan [70]	–	–	+
Collagen V [71]	–	–	+
Brevican [39,72]	+	+	–
Versican V2 [73,74]	+	+	–
Versican V1 [71]	–	–	+
Neurocan [39,75]	–/+	+	–
Brall [54*,73]	nd	+	–
Phosphacan [72]	nd	+	–
Tenascin-R [72,76]	nd	+	–
Hyaluronan [73,76]	+	+	–
NG2 [77,78]	nd	+	+
Scaffolds			
AnkG-scaffold [79]	+	+	+
β IV spectrin [79]	+	+	+
PSD93 [61]	+	–	–
Signaling			
IQCC-SCHIP-1 [80]	+	+	+
CK2 [30**,31]	+	+	+
FHFs [81–83]	+	+	+

Figure 1



Current models for the formation of nodes and AIS. **(a,b)** AIS and CNS nodes. The assembly of both the AIS and CNS nodes starts by the formation of a diffusion barrier marked by ankB and 4.1B. For nodes, this occurs at the base of the formed paranodal junction. In the adjacent AIS or nodal axolemma, accumulation of ankG results in the clustering of Nav and of NF186. ECM is the last to appear, and together with NF186, is probably required for the stabilization of AIS and nodal complexes. An astrocyte process may contact CNS nodes late in development, although the exact timing is still to be determined. **(c)** PNS nodes. The assembly of PNS nodes is induced by glial gliomedin and NrCAM that cluster NF186 on the axolemma, resulting in the accumulation of ankG and Nav. Linkage of the forming complex to the submembranous actin cytoskeleton likely occurs earlier in CNS nodes formation [26**] and later in AIS and PNS nodes [7*,26**]. See Table 1 for additional components not described here.

This paper focuses on three elements known to be involved in node formation: The PNJ, NF186-binding ECM proteins, and the spectrin-based cytoskeleton. By generating mice lacking different pairs of nodal elements, as well as testing the function of mutant NF186 constructs, they unraveled the existence of a hierarchy between the different mechanisms that control node formation. They concluded that the PNJ play the primary role during CNS node formation, while the ECM components, which as they show are the last to appear in the node, play a role in stabilizing rather than assembling the nodes. In addition they show that linkage to the actin-spectrin cytoskeleton contributes to CNS nodal assembly. This is in contrast to the PNS, as mice lacking both paranodal and spectrin-based mechanisms had normal

PNS nodes [26**]. These results demonstrate that while ECM-based interactions play a primary role in PNS node formation, in the CNS, paranodal and cytoskeletal (rather than ECM-based) mechanisms collaborate to assemble nodes. Notably, in both PNS and CNS, removing the primary assembly mechanisms would not result in nodal loss because of the compensation by the other mechanisms [19**,26**,27]. Thus, the crucial importance of the NOR for neural function has prompted the evolution of a fail-safe system for assembling nodes, using multiple mechanisms with mutual backup capacities.

AnkG-based mechanisms

Nav1 channels contain an ankyrin binding motif, that binds both ankB, expressed along distal axons, and ankG

[28]. This sequence was shown to be sufficient for targeting proteins to AIS [28,29] and NOR [30**]. Phosphorylation by the protein kinase CK2 dramatically increases the affinity of this site specifically to ankG [31]. AIS localization of CK2 and ankG are mutually dependent and are both necessary for microtubule stabilization at AIS [32], which in turn is necessary for the preservation of AIS integrity and neuronal polarity [33]. A CK2-ankG mutual dependence makes it hard to conclude whether CK2 is truly necessary for Nav-ankG association in this domain. However, using mutants of full length Nav1.6 alpha subunit, Gasser *et al.* showed that while Nav1.6 interaction with ankG is crucial for its AIS and NOR localization, its phosphorylation by CK2 is not critical but serves to facilitate channel clustering at these domains [30**]. They attribute the apparent discrepancy with previous publications to the different reporter protein that were used. In this paper, the use of the full length alpha subunit rather than a kv2.1/Nav1.2 chimera also revealed that Nav-ankG interactions are necessary and sufficient for Nav clustering at both AIS and NOR, as no other protein-protein interaction can substitute for the loss of ankG binding [30**]. Interestingly, ankG can associate to the MDCK cell membrane independently of its binding partners E-cadherin and β II spectrin. This membrane association depends on ankG's palmitoylation at Cys-70, which was shown to be crucial for its function as the AIS organizer [34*]. As a C70A mutant could localize to AIS in WT but not in ankG-deficient neurons, this may indicate that ankG initially accumulates in the forming AIS via its Cys-70 palmitate modification [34*].

NF186

Owing to its capacity to bind both ankG [35] and ECM proteins [20,26**], NF186 plays a key role during node formation in both CNS and PNS [36–38]. In contrast, it is mostly involved in stabilization rather than formation of the AIS [39,40,41*]. In the PNS, secretion of glial gliomedin and NrCAM at the nodal gap initiates node assembly [18]. Different binding sites for gliomedin and NrCAM on NF186 probably allow the multimolecular glial complexes to cluster axonal NF186 at heminodes rapidly and efficiently [42].

Can the PNJ-induced mechanism for node formation compensate for NF186 deficiency or is it NF186-dependent as well? The fact that NF186 accumulates at mature nodes in both PNS [19**] and CNS [26**] ECM mutants implies that NF186 may be involved in the PNJ-mediated node formation. Using neuron-specific ablation of neurofascin (as the glial isoform 155 is necessary for PNJ formation) it was concluded that NF186 is necessary for both CNS and PNS node formation [38]. On the other hand, using a NF155 transgene that recovers PNJ in neurofascin-null animals, it was shown that NF186 was necessary for PNS but not for CNS node formation

[36,37]. Finally, Feinberg *et al.* showed that in DRG myelinating cultures, NF186 is not necessary for node formation when the PNJ is intact. The apparent discrepancies between these papers most probably arise from the different models that were studied. In Ref. [38], the neuronal specific ablation of neurofascin was only partial. As a result, nodal counting was based on N186-negative nodes, which could result in a bias toward the less stable node population. The finding that in DRG cultures, representing an early developmental stage, NF186 is dispensable, could suggest that NF186 is crucial for early stabilization of the node and not for its assembly, two processes that are hard to distinguish.

The paranodal junction

How do the PNJ drive the accumulation of ankG in the developing node? The role of the transverse bands, intercellular densities at the paranodal junction, is unknown. Recently it was shown that in contrast to previous hypotheses, this structure does not create an extracellular seal [43]. However, using electron tomography on thin sections of mouse corpus callosum, Nans *et al.* identified an extensive network of filamentous linkers in the paranodal axoplasm. These linkers interconnected the three cytoskeletal systems of the axon to one another as well as to the transverse bands, making the PNJ a center of cytoskeletal connections. In addition they revealed that various membranous organelles, including transport vesicles, were tethered to the PNJ by short filaments. These observations support a role for the PNJ as a diffusion barrier fencing the NOR, and suggest an additional role in targeted trafficking of proteins to the nodes [44*]. Additional support for a role for cytoskeletal connections in the paranodes comes from two different papers showing that the association of caspr and the actin-spectrin cytoskeleton via protein 4.1B is necessary for an efficient paranodal membrane barrier [45,46].

In addition to their role in creating diffusion barriers, cytoskeletal structures may promote node formation in various other ways. One such mechanism has recently been introduced for AIS formation by Galiano *et al.* Searching for cellular cues that localize ankG at the forming AIS, these authors found that in young unmyelinated neurons, a submembranous cytoskeleton consisting of ankB, α II-spectrin, and β II-spectrin defines an intra-axonal boundary that precedes and localizes ankG at the developing AIS [5**]. Moreover 4.1B, another spectrin binding protein, was shown to be involved in the segregation between the AIS and the first myelin segment [47]. These cytoskeletal components are also present in PNJ [48]. It would thus be interesting to test whether the paranodal localization of these proteins precedes nodal ankG. If this were the case, then CNS nodes would form very similarly to the AIS, differing only in the initiating signal, that is, an axoglial contact versus a cell-autonomous process.

Maintenance

AnkG appears to play a major role not only in the assembly but also in the maintenance of AIS and nodes. Knock down of ankG in fully polarized neurons resulted in AIS disintegration, as well as loss of neuronal polarity [49]. In the AIS, NF186 and NrCAM accumulate late and depend on ankG for their localization [50]. Although both CAMs are probably dispensable for the assembly of AIS and CNS nodes [37,39], NF186 is critical for the maintenance of the AIS and PNS nodes [8^{**},41^{*}], but not for the maintenance of CNS nodes [41^{*}]. The authors also show that the NF186 turnover rate is much slower at the node than at AIS, which suggests that nodes are much less plastic than the AIS, that emerges as a highly plastic domain [51,52]. Interestingly, the authors note that the stabilization of the AIS requires NF186 as a linker between extracellular proteins (e.g. brevican) and the axolemma, similarly to its role at the NOR.

In the CNS, perinodal ECM components accumulate after the nodes had assembled, and thus probably play a stabilizing role [26^{**}]. This highly variable and redundant ECM always contains Bral1 which is a brain specific link protein that stabilize the binding of lecticans and hyaluronic acid [53]. In Bral1 KO mice, changes in the nodal ECM (e.g. brevican, versican V2 and hyaluronan are missing), do not result in changes in Nav channel clustering at the node. However, Bral1 mice exhibit slower salutatory conduction [54^{*}], probably since the Bral1-associated ECM serves as an extracellular ion pool that facilitates nodal action potentials. Thus the nodal ECM, at least in the CNS, is crucial for maintaining functional nodes. These results also emphasize the importance of conducting physiological tests when dealing with nodal mutants, as there is often much discrepancy between histological and physiological observations. Although the mechanisms operating to stabilize PNS nodes are still under investigation, it seems that they constitute very stable domains similarly to their CNS counterparts [8^{**}]. Given the analogy between CNS and PNS nodes, it is likely that additional 'late-arriving' ECM components that bind NF186 are involved in stabilization and maintenance of PNS nodes.

Concluding remarks

The picture emerging from recent studies indicate that the AIS and CNS nodes probably form by similar mechanisms (Figure 1). In both cases, accumulation of ankG is induced by an intra axonal barrier formed autonomously or by axoglial contact, respectively. The extremely long and large caliber axons of the PNS probably require a more rapid and robust nodal assembly, thus in the PNS, ankG-Nav complex formation is catalyzed by a gliomedin-NrCAM glial signal that binds axonal NF186. Interestingly, in both CNS nodes and AIS, although not crucial for their formation, NF186-ECM interactions are essential for the stabilization and functionality of these

domains. It is thus plausible that these NF186-binding ECM proteins are subjected a spatial and/or temporal regulation, possibly by matrix proteinases.

Node formation requires the cooperation between neurons and glia for the formation of diffusion barriers as well as clustering of membrane proteins in a specific time and place. It is thus tempting to speculate that these two intimately connected cells may communicate on additional levels. Vesicle exchange between cells was shown to be a means of sharing proteins, microRNAs, mRNAs and ribosomes between neighboring cells [55]. Moreover, transfer of ribosomes from Schwann cells to injured axons has been recently described [56–58], raising the possibility that the formation and maintenance of remote peripheral nodes may be aided by supplies from the ensheathing Schwann cells.

Acknowledgements

We would like to thank Dr. Matt Rasband for helpful discussions and Ms. Genia Brodsky for the graphics. The author's laboratory is supported by the NIH (NS50220), the Israel Science Foundation, and the Adelson Medical Research Foundation.

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