A hierarchy of ankyrin-spectrin complexes clusters sodium channels at nodes of Ranvier

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The scaffolding protein ankyrin-G is required for Na+ channel clustering at axon initial segments. It is also considered essential for Na+ channel clustering at nodes of Ranvier to facilitate fast and efficient action potential propagation. However, notwithstanding these widely accepted roles, we show here that ankyrin-G is dispensable for nodal Na+ channel clustering in vivo. Unexpectedly, in the absence of ankyrin-G, erythrocyte ankyrin (ankyrin-R) and its binding partner βI spectrin substitute for and rescue nodal Na+ channel clustering. In addition, channel clustering is also rescued after loss of nodal βIV spectrin by βI spectrin and ankyrin-R. In mice lacking both ankyrin-G and ankyrin-R, Na+ channels fail to cluster at nodes. Thus, ankyrin R–βI spectrin protein complexes function as secondary reserve Na+ channel clustering machinery, and two independent ankyrin-spectrin protein complexes exist in myelinated axons to cluster Na+ channels at nodes of Ranvier.

The high density of Na+ channels at nodes of Ranvier is an essential feature of myelinated axons and facilitated the evolution of the complex and efficient vertebrate nervous system1. Nodal Na+ channel clusters confer several important functional advantages including decreased energy and space requirements and increased action potential conduction velocity.

Three overlapping mechanisms work together to cluster Na+ channels at nodes. These include (i) a glia-derived extracellular matrix that interacts with and clusters the axonal cell adhesion molecule neurofascin-186 (NF186), (ii) paranodal axoglial junctions that restrict the location of nodal proteins between adjacent myelin segments and (iii) submembranous cytoskeletal proteins such as βIV spectrin that link the nodal Na+ channel protein complex to the actin-based axonal cytoskeleton2–5. These three mechanisms of channel clustering converge on the scaffolding protein ankyrin-G (AnkG)6. AnkG is highly enriched at nodes of Ranvier, where it interacts directly and simultaneously with Na+ channels, NF186 and βIV spectrin7–9. Reduction in AnkG expression by short hairpin RNA (shRNA) in myelinated dorsal root ganglion–Schwann cell co-cultures was reported to block the clustering of Na+ channels at nodes10. Similarly, loss of AnkG from axon initial segments (AIS) blocks the clustering of these same proteins at the AIS11–13.

Together, these observations have led to the widely accepted model that neuron-glia interactions first gather NF186 in the axonal membrane. Next, clustered NF186 functions as a nucleation site for the assembly of nascent nodes by recruiting and clustering AnkG. Na+ channels are enriched at nodes through their AnkG-binding motif9,14,15. Finally, the entire NF186–AnkG–Na+ channel protein complex is stabilized and linked to the actin cytoskeleton through βIV spectrin. Further support for this sequence of events has come from the analysis of mutant mice lacking paranodal neuron-glia interactions, nodal NF186-binding extracellular matrix proteins, NF186 or βIV spectrin12,13,16. Thus, AnkG’s requirement for nodal Na+ channel clustering at nodes of Ranvier in vivo is broadly accepted17–20.

Here, using AnkG conditional knockout mice, we show that in contrast to the predictions of the widely accepted model, AnkG is dispensable for Na+ channel clustering at nodes of Ranvier. Remarkably, we found a second, nondominant ankyrin-R (AnkR)–βI spectrin protein complex (traditionally thought to function mainly in erythrocytes) that is also found in myelinated axons and can function as a reserve mechanism to rescue Na+ channel clustering. We show that the interactions between NF186, Na+ channels and the two distinct ankyrin-spectrin complexes have different affinities leading to a hierarchy of clustering activities. Finally, by generating mice deficient in both AnkG and AnkR, we prove that ankyrins are required for nodal Na+ channel clustering.

RESULTS
AnkG conditional knockout mice
To investigate the requirement for AnkG to cluster Na+ channels at nodes of Ranvier in vivo, we generated a conditional-null mutant allele in which exons 23 and 24 of the AnkG gene (Ank3) are flanked by loxP sites (Ank3loxF/F mice) and can be excised in the presence of Cre recombinase. To confirm the utility of these mice for our proposed experiments and their loss of AnkG expression, we first crossed these mice with Nestin-Cre (Nes-Cre) mice to eliminate neuronal AnkG. Nes-Cre;Ank3loxF/F mice died at birth and failed to form AISs. Immunostaining of developing cortex at postnatal day 0 (P0) in Ank3loxF/F control mice revealed widespread AIS βIV spectrin and

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AnkG throughout the cortical plate (Fig. 1a,b; AnkG staining not shown). In contrast, the Nes-Cre;Ank3<sup>F/F</sup> mice lacked AISs (Fig. 1c). Na<sup>+</sup> channels were clustered at the AIS of individual cortical neurons in Ank3<sup>F/F</sup> control mice, where they colocalized with AnkG (Fig. 1d,e, arrows, and Supplementary Fig. 1a), but Nes-Cre;Ank3<sup>F/F</sup> mice lacked Na<sup>+</sup> channel clustering in the cortex except in those few neurons that did not undergo recombination (Fig. 1f, arrow). Immunoblots confirmed the nearly complete loss of AnkG protein from brains of Nes-Cre;Ank3<sup>F/F</sup> mice (Fig. 1j). Together, these results showed that AnkG is required...

**Figure 1** AnkG-deficient axons have Na<sup>+</sup> channels clustered at nodes of Ranvier. (a-c) PO Ank3<sup>F/F</sup>, Nes-Cre;Ank3<sup>F/F</sup> and Nes-Cre;Ank3<sup>F/F</sup> mouse brain cortices stained with Hoechst to visualize nuclei (blue) and with antibodies against βIV spectrin (green). Scale bar, 50 μm. (d-f) Axon initial segments (arrows) from PO Ank3<sup>F/F</sup>, Nes-Cre;Ank3<sup>F/F</sup> and Nes-Cre;Ank3<sup>F/F</sup> mouse cortices immunostained using antibodies against neurofascin (NF, red) and Na<sup>+</sup> channels (green). Roots were collected at P14. Scale bar, 10 μm. (g-i) Nodes of Ranvier (arrows) from the indicated genotypes (Supplementary Fig. 9). (j) The percentage of nodes at P14 labeled for Na<sup>+</sup> channels and AnkG in the indicated tissues and genotypes. n = 3 animals per tissue and genotype. Error bars indicate mean ± s.e.m. (k) Immunostaining of P30 Ank3<sup>F/F</sup> and Six3-Cre;Ank3<sup>F/F</sup> mouse optic nerves using antibodies against Caspr (red), AnkG (blue) and Na<sup>+</sup> channels (green). Scale bar, 2 μm.
AnkG is dispensable for nodal Na$^+$ channel clustering

Since Nes-Cre;Ank$^{3/F}$ mice die at birth, and myelination is a postnatal event, they cannot be used to study the formation of nodes of Ranvier. Therefore, to circumvent the perinatal lethality, we used AdvilinCre$^+$ (Avil-Cre) and Six3-Cre mice, which express Cre recombinase from early developmental stages in peripheral sensory neurons and retinal ganglion cells respectively$^{21,22}$, to specifically eliminate AnkG expression in these tissues. We reasoned that more restricted recombination might allow mice to survive past birth and permit the analysis of node assembly in the absence of AnkG. Avil-Cre;Ank$^{3/F}$ mice are also useful because recombination occurs only in sensory neurons, allowing for a direct comparison between dorsal (sensory) and ventral (motor) roots from the same animal. Avil-Cre;Ank$^{3/F}$ and Six3-Cre;Ank$^{3/F}$ mice appeared normal at birth.

To confirm the loss of AnkG from nodes of Ranvier and to determine whether AnkG is required for nodal Na$^+$ channel clustering, we immunostained P14 dorsal roots from Ank$^{3/F}$ and Avil-Cre;Ank$^{3/F}$ mice and ventral roots (control) from Avil-Cre;Ank$^{3/F}$ mice. Whereas the control ventral roots had intense AnkG and Na$^+$ channel immunoreactivity at nodes (Fig. 1g,h, arrows), Avil-Cre;Ank$^{3/F}$ dorsal roots were completely devoid of nodal AnkG (Fig. 1i,k). Unexpectedly, we found normal enrichment of Na$^+$ channels at nodes in AnkG-deficient dorsal roots (Fig. 1i, arrows, k).

To determine whether Avil-Cre;Ank$^{3/F}$ sensory roots were functionally impaired, we measured their conduction velocity at P14. We found no difference between control Ank$^{3/F}$ and AnkG-deficient roots (4.14 ± 0.31 m s$^{-1}$ in control and 4.02 ± 0.39 m s$^{-1}$ in AnkG-deficient roots; n = 11 dorsal roots from 3 mice per genotype; P = 0.79, unpaired two-tailed t-test). We also observed no difference in compound action potential waveform (Supplementary Fig. 1d). Similarly, at P28 Avil-Cre;Ank$^{3/F}$ mice showed no impairment on the rotarod (latency to fall from the rotorod: 163.2 ± 10.7 s and 151.7 ± 15.5 s for Ank$^{3/F}$ and Avil-Cre;Ank$^{3/F}$ mice, respectively; n = 8 mice of each genotype; P = 0.55, unpaired two-tailed t-test), on the wire hang test (Supplementary Fig. 1b) or in thermal nociception (Supplementary Fig. 1c). Much as for Avil-Cre;Ank$^{3/F}$ mice, we found Na$^+$ channel clustering in the optic nerves of Six3-Cre;Ank$^{3/F}$ mice in the absence of AnkG (Fig. 1l). Avil-Cre;Ank$^{3/F}$ and Six3-Cre;Ank$^{3/F}$ mice were fertile and appeared normal, with no signs of impairment or loss of Na$^+$ channel clustering even at 2 years and 18 months of age, respectively (Supplementary Fig. 2a,b and Supplementary Movies 1–4).

Thus, AnkG is dispensable for nodal Na$^+$ channel clustering and axon function in both the PNS and CNS.

Although we found no difference in Na$^+$ channel clustering between control and AnkG-deficient roots at P14, when we examined node formation during early development, we found a significantly lower density of Na$^+$ channels in Avil-Cre;Ank$^{3/F}$ dorsal roots (Supplementary Fig. 1e,f). Thus, although AnkG is dispensable for Na$^+$ channel clustering, the compensatory mechanism that rescues channel clustering is less efficient than the mechanism involving AnkG.

AnkR rescues Na$^+$ channel clustering

We previously showed that the AnkG-binding motif in Na$^+$ channels is both necessary and sufficient for their localization to nodes of Ranvier$^2$. If the results of that study are correct, how can Na$^+$ channels be clustered at nodes without AnkG? Although it is possible that some ankyrin-independent mechanism can also facilitate Na$^+$ channel clustering through this motif, a simpler explanation is that channel clustering is rescued through compensation by another ankyrin that can also interact with Na$^+$ channels at the AnkG-binding motif. Vertebrates have three ankryns: AnkG (encoded by Ank3), AnkB (encoded by Ank2) and AnkR (encoded by Ank1). AnkB has previously been reported to be localized in unmyelinated axons$^{23–25}$ and...
Furthermore, AnkB-deficient mice die shortly after birth owing, in part, to widespread nervous system defects. AnkR, also known as erythrocyte ankyrin, functions in red blood cells to link the Cl⁻–HCO₃⁻ anion exchanger to the submembranous spectrin cytoskeleton. Mutations in AnkR cause hereditary spherocytosis.

To determine whether Na⁺ channel clustering is rescued in Avil-Cre;Ank3f/f sensory neurons by AnkB or AnkR, we performed triple immunostaining for AnkG, AnkB and AnkR. Consistent with previous reports, Ank3f/f control dorsal roots had nodal AnkG (blue) and paranodal AnkB (red), but little or no nodal AnkR immunoreactivity (Fig. 2a, arrows). Remarkably, in AnkG-deficient dorsal roots, we found a dramatic increase in AnkR immunoreactivity (green) at nodes (Fig. 2b, arrows) but no change in AnkB (red). When we immunostained control optic nerve axons using antibodies against AnkG and AnkR, we found robust AnkG immunoreactivity at nodes and paranodes but no nodal AnkR immunoreactivity (Fig. 2c, arrows; paranodal AnkG is oligodendroglial in origin; K.-J.C. and M.N.R., unpublished results). However, when we examined Six3-Cre;Ank3f/f optic nerve axons, we found that nodes deficient in AnkG had robust AnkR immunoreactivity (Fig. 2d, arrows). Thus, AnkR is found at nodes in AnkG-deficient axons and may substitute for AnkG.

AnkR colocalizes with Na⁺ channels at nodes of Ranvier in AnkG-deficient neurons (Supplementary Fig. 3a,b). To confirm that AnkR can substitute for AnkG and interact with Na⁺ channels, we generated GST fusion proteins containing the intracellular loop 2 AnkG-binding motif (GST-Nav II–III) and performed pulldown experiments with AnkG and AnkR; phosphorylation of several key serine residues in GST-Nav II–III by the kinase CK2 significantly enhances its binding affinity for ankyrins. We found that although both AnkG and AnkR can interact with Na⁺ channels, it was necessary to dramatically increase the amount of AnkR input to begin to detect an interaction (Fig. 2e), suggesting that AnkG's preference for binding GST-Nav II–III is much higher than that of AnkR.

The remarkable rescue of Na⁺ channel clustering by AnkR during development led us to ask whether AnkR could also preserve Na⁺ channel clustering after loss of AnkG in adult neurons. To test this possibility, we injected AAV-GFP or AAV-Cre-GFP into the eyes of P21 Ank3f/f mice to transduce retinal ganglion cells (RGCs). Two months later, the axons of transduced RGCs were easily identified by GFP immunoreactivity (Fig. 3). The axons of RGCs transduced with AAV-GFP had normal AnkG (Fig. 3a,g) and normal Na⁺ channel clustering (Fig. 3c,g) and no nodal AnkR immunoreactivity (Fig. 3e,g). We also found that although 86% of nodes along the axons of RGCs transduced with AAV-Cre-GFP had no AnkG (Fig. 3b, arrows, g), 93% had Na⁺ channel clustering (Fig. 3d, arrows, g). Consistent with the preservation of many nodal Na⁺ channel clusters, we found that 75% of nodes along GFP-labeled axons had AnkR immunoreactivity (Fig. 3f, arrows, g). Thus, AnkR also compensates for loss of AnkG in mature, fully myelinated axons and can rescue Na⁺ channel clustering.

**βI spectrin substitutes for βIV spectrin at AnkG-deficient nodes.** (a,b) Immunostaining of P14 dorsal root nodes (arrows) from Ank3f/f (a) and Avil-Cre;Ank3f/f (b) mice using antibodies against Caspr (red), βIV spectrin (blue) and βI spectrin (green). Insets in a and b show additional examples of nodes with βIV spectrin that lack βI spectrin (a, arrowheads) and nodes with βI spectrin that lack βIV spectrin (b, arrow). Scale bar, 10 μm. (c) Quantification of the percentage of nodes in Ank3f/f and Avil-Cre;Ank3f/f mice labeled for βIV spectrin (*P = 0.011, unpaired two-tailed t-test, n = 3 mice for each genotype) or βI spectrin (**P = 0.007, unpaired two-tailed t-test, n = 3 mice for each genotype). Error bars indicate mean ± s.e.m. (d) Co-immunoprecipitation (IP) of AnkG-GFP or GFP-AnkR with spectrin repeats 13–17 of βIV spectrin (Myc-βI SR13–17). DS, depleted supernatant. Fusion proteins were expressed in COS cells. (e) Quantification of the results from co-immunoprecipitation between ankyrins and spectrins. The percent of input spectrin precipitated by the indicated GFP-fusion protein is shown. Error bars indicate mean ± s.e.m. For βI spectrin co-immunoprecipitations, **P = 0.0058 and 0.0035 for comparisons between AnkR and GFP or between AnkR and AnkG, respectively (two-tailed t-test, n = 3 experiments). For βIV co-immunoprecipitations, **P = 0.008 for comparisons between AnkG and GFP or between AnkR and AnkG (two-tailed t-test, n = 3 experiments). (f) Co-immunoprecipitation of AnkG-GFP or GFP-AnkR with spectrin repeats 10–15 of βIV spectrin (Myc-βIV SR10–15) shows βIV spectrin binds preferentially to AnkG. Fusion proteins were expressed in COS cells. (Full-length blots for d,f are provided in Supplementary Fig. 9.)

**βI spectrin replaces βIV spectrin in AnkG-deficient axons.** βIV spectrin is also clustered at nodes of Ranvier (Supplementary Fig. 4a), where it links Na⁺ channels and AnkG to the submembranous...
cytoskeleton. Intriguingly, loss of nodal AnkG is accompanied by a dramatic reduction and, at some nodes, even a complete loss of βIV spectrin (Supplementary Fig. 4b, arrow). Without βIV spectrin, how can nodes be maintained? We wondered whether some β spectrin other than βIV might preferentially partner with AnkR to rescue Na+ channel clustering and stabilization at nodes. In erythrocytes, AnkR’s spectrin binding partner is β spectrin2. Therefore, to determine whether β spectrin also contributes to the rescue of nodes in AnkG-deficient axons, we immunostained Ank3F/F control and Avil-Cre;Ank3F/F mice using antibodies against β spectrin. In control dorsal roots the vast majority of nodes had βIV spectrin and were devoid of any β spectrin (Fig. 4a, inset and arrowheads, c). In contrast, Avil-Cre;Ank3F/F dorsal roots frequently had robust nodal βIV spectrin staining (Fig. 4b, arrows, c); both Avil-Cre;Ank3F/F dorsal roots and Six3-Cre;Ank3F/F optic nerves had β spectrin that colocalized with Na+ channels (Supplementary Fig. 3c,d). Furthermore, there was often a mutually exclusive relationship between βIV spectrin and β spectrin at nodes: nodes with βIV spectrin had no β spectrin (Fig. 4b, arrowheads), and nodes with β spectrin had no βIV spectrin (Fig. 4b, arrows). Thus, Na+ channel clustering in AnkG-deficient axons is not rescued just by substitution of a single protein; instead, the entire AnkG-βIV spectrin protein complex is replaced by AnkR-β spectrin.

To determine whether AnkG and AnkR favor binding of one spectrin over another, we coexpressed GFP-tagged AnkG or AnkR protein complex is replaced by AnkR-β spectrin. We found that in sciatic nerves, AnkR and β spectrin interact much better with AnkG than with AnkR (Fig. 4c,f) and that βIV spectrin preferentially interacts with AnkG rather than AnkR (Fig. 4e,f). AnkR and β spectrin rescue βIV spectrin-deficient axons

Because AnkR and β spectrin substitute for AnkG and βIV spectrin in AnkG-deficient axons, we reasoned that a similar substitution should occur in βIV spectrin-deficient axons. To test this possibility, we used ‘quivering 3J’ mutant mice (Spnb4+3F/F;3J), which have a frameshift mutation resulting in truncated βIV spectrin32 and loss of βIV spectrin from nodes33. Although Spnb4+3F/F;3J mice have progressive ataxia and widened nodes of Ranvier, they still cluster Na+ channels at nodes33. Neither βIV spectrin nor AnkR were detected at control optic nerve nodes of Ranvier (Fig. 5a, c, arrows, e). Remarkably, immunostaining of optic nerves from Spnb4+3F/F;3J mice showed robust nodal βIV spectrin at every node (Fig. 5b, arrows, e) and AnkR immunoreactivity at 68% of nodes (Fig. 5d, arrows, e). These results demonstrated that AnkG and βIV spectrin form the primary ankyrin-spectrin complex at nodes and suggest that loss or disruption of either AnkG or βIV spectrin permits a secondary ankyrin-spectrin complex consisting of AnkR and β spectrin to rescue Na+ channel clustering.

Nodal AnkR and β spectrin derive from pre-existing pools

What is the source of the AnkR and β spectrin that rescues nodal Na+ channel clustering in AnkG- and βIV spectrin-deficient axons? We considered two possibilities: the nodal AnkR and β spectrin could (i) be newly synthesized in response to loss of AnkG or βIV spectrin or (ii) comprise a pre-existing pool of unclustered protein complexes not detectable by immunostaining. To distinguish between these two, we performed RT-qPCR and measured the relative abundances of AnkG, AnkR, βIV spectrin and β spectrin transcripts in Ank3F/F and Avil-Cre;Ank3F/F DRG neurons. Although AnkG transcripts were nearly undetectable in Avil-Cre;Ank3F/F mice, we observed no difference in the expression levels of AnkR, βIV spectrin or β spectrin in response to the absence of AnkG (Fig. 6a), suggesting that transcriptional upregulation does not account for the nodal substitution by AnkR and β spectrin.

Consistent with the idea that AnkR and β spectrin comprise a pre-existing pool of axonal proteins, we found that compared to those in Ank3F/F controls, the levels of both proteins in P0.5 AnkG-deficient brain (Nes-Cre;Ank3F/F; Fig. 6b) and P14, P31 and 4-month-old dorsal root homogenates (Avil-Cre;Ank3F/F; Fig. 6c and Supplementary Fig. 5a) remain unchanged. If AnkR-β spectrin complexes are normally found in the axon and can be clustered at nodes when AnkG is not present, we reasoned that a nascent node, consisting primarily of clustered NF186, might provide a substrate for both AnkG and AnkR binding during early development, resulting in nodes labeled for both AnkG and AnkR. To test this possibility, we immunostained developing sciatic nerve and optic nerve with antibodies against AnkG and AnkR. We found that in sciatic...
Figure 6  AnK and βI spectrin come from a pre-existing pool of proteins. (a) RT-qPCR for AnK, AnK, βI spectrin and βI spectrin. RNA was collected from P31 dorsal root ganglia of AnK/ F/F and Avil-Cre;AnK/ F/F mice. The targets were normalized to P02a, encoding RNA polymerase II polypeptide A, and all samples were then normalized to WT samples. n = 3 mice for each genotype. Error bars indicate mean ± s.e.m. (b) Immunoblot of postnatal day 0.5 brain homogenates from AnK/ F/F, Nes-Cre;AnK/ F/F and Nes-Cre;AnK/ F/F mice using antibodies against AnK, βI spectrin (βI sp) and neurofilament M (NF-M). (c) Immunoblot of P31 dorsal root homogenates from AnK/ F/F, Avil-Cre;AnK/ F/F and Avil-Cre; AnK/ F/F mice using antibodies against AnK, βI spectrin (βI sp) and neurofilament M (NF-M). (d) Quantification of the ratio of nodal AnK fluorescence to nodal AnK fluorescence (gray, n = 2 mice) in wild-type mouse sciatic nerve as a function of age and the percentage of nodes with AnK (black, bottom; n = 2 mice). Quantification of nodal dorsal root node AnK fluorescence intensity normalized to Avil-Cre;AnK/ F/F dorsal root node AnK fluorescence intensity (black, top; n = 2 mice). Error bars indicate the range of the data. (e) Co-immunoprecipitation of Myc-AnK-MBD or Myc-AnK-MBD with HA-NF186 or HA-NF186aFIPQY shows NF186 preferentially binds AnK. (f) Immunostaining of wild-type developing sciatic nerve from P1 to P30 using antibodies against AnK (red) and AnK (green). Nodes are indicated by arrows. Scale bar, 10 μm. (Full-length gels for a,b,c,e are provided in Supplementary Fig. 9.)

nerve AnK could be detected at nearly every node of Ranvier during the first 2 weeks of development (Fig. 6d, arrows, d). However, the nodal AnK was transient and rapidly decreased in both intensity and frequency of nodal staining, such that by P30, 40% of nodes had no detectable AnK, and the remaining nodes had only a very little AnK that was only weakly detected by immunostaining (Fig. 6d). Furthermore, the AnK fluorescence intensity observed in wild-type animals was always much less than the AnK fluorescence intensity observed in Avil-Cre;AnK/ F/F mice (Fig. 6d). Unlike in the PNS, in the CNS AnK was detected at very few forming nodes of Ranvier (Supplementary Fig. 5b). This may reflect differences in the primary mechanisms of node assembly between the PNS and CNS.

Figure 7  AnK-deficient mice have normal PNS and CNS nodes of Ranvier. (a) Immunoblot of AnK+/+ and AnK pale/pale mice using antibodies against AnK and actin. (Full-length gel is provided in Supplementary Fig. 9.) (b) Quantification of the number of nodes of Ranvier labeled for AnK or Na+ channels in control and AnK pale/pale mice. n = 3 mice; error bars indicate mean ± s.e.m. (c) P5 sciatic nerves from AnK+/+ and AnK pale/pale mice immunostained using antibodies against Na+ channels (red), neurofilament (blue) and AnK (green). Nodes formed normally in both genotypes. Scale bar, 10 μm. (d) P22 optic nerves from AnK+/+ and AnK pale/pale mice immunostained using antibodies against Na+ channels (red), AnK (blue) and AnK (green). Nodes formed normally in both genotypes. Scale bar, 10 μm.
Ankyrins are required for nodal Na$^+$ channel clustering

Although both AnkG and AnkR are by themselves dispensable for Na$^+$ channel clustering at nodes of Ranvier, previous studies indicated that the AnkG-binding motif in the II–III linker domain of Na$^+$ channels is both necessary and sufficient for nodal channel clustering\(^9\). To finally prove that ankyrins are required for nodal Na$^+$ channel clustering, we generated mice deficient for both AnkG and AnkR. *Avil-Cre;Ank3\(^{f/f}\);Ank1\(^{f/f}\) mice lack AnkG and AnkR in DRG sensory neurons. These mice usually died at or before P3, although we obtained a few animals that survived to P4–P7. At P3–P7, both Ank3\(^{f/f}\) and *Avil-Cre;Ank3\(^{f/f}\)* mice had Na$^+$ channel clustering at nodes (Fig. 8a–c). In stark contrast, *Avil-Cre;Ank3\(^{f/f}\);Ank1\(^{pale/pale}\) mice had no detectable Na$^+$ channels between neurofascin (NF)-labeled paranodes (Fig. 8a,b). Similarly, in animals that survived from P4–P7, although we found many Caspr-labeled paranodes, some of which had well-defined gaps between them indicating a node (Fig. 8d, arrow), we were unable to detect any Na$^+$ channel clusters between or flanking these Caspr-labeled paranodal junctions. Quantification of the percentage of nodes with Na$^+$ channel clusters showed that animals lacking both AnkG and AnkR had virtually no detectable Na$^+$ channel clusters in their dorsal roots (Fig. 8b). Thus, axonal ankyrins are required for Na$^+$ channel clustering at nodes of Ranvier.

**DISCUSSION**

Nodes of Ranvier are a remarkable example of reciprocal subcellular differentiation. Their evolution has been proposed as a key adaption in the divergence and success of vertebrates\(^3\). Loss or disruption of nodes by disease, autoimmune attack or injury can cause nervous system dysfunction and even death\(^3\). Thus, elucidating how nodes are assembled is essential to understand nervous system function and is an important consideration for any therapeutic strategy aimed at nervous system repair or regeneration.

**A new nodal ankyrin-spectrin protein complex**

Neurons and myelinating glia interact to facilitate the high-density clustering of voltage-gated Na$^+$ channels. Many of the protein-protein interactions contributing to node formation have been described, and...
the model placing AnkG at the nexus of nodal Na⁺ channel clustering is widely accepted. Here, we tested this model in both the PNS and CNS by generating mice with AnkG-deficient dorsal root ganglia and retinal ganglion cells, respectively. Much to our surprise, and in stark contrast to its reported necessity, we found that AnkG is dispensable for nodal Na⁺ channel clustering. Remarkably, we discovered another clustering mechanism that depends on a second ankyrin-spectrin protein complex consisting of AnkR and βI spectrin, both of which were previously thought of as 'erythrocytic' in nature. Furthermore, loss of βIV spectrin also led to replacement of AnkG by AnkR–βI spectrin and preservation of Na⁺ channel clustering (Supplementary Fig. 6a). The rescue of Na⁺ channel clustering and nervous system function in AnkG-deficient neurons by AnkR is a striking example of compensation. The rescue of Na⁺ channel clustering is even more remarkable since compensation is effected not just by a single protein but by a protein complex.

In support of an AnkG-independent clustering mechanism, Zhang et al. investigated the sources and targeting of proteins to nodes of Ranvier. They induced myelination of degeneration-resistant axons separated from neuronal cell bodies and observed a small minority of Na⁺ channel clusters (~20%) at newly formed heminodes that had only weak or undetectable AnkG staining. We suggest that this small population of Na⁺ channel clusters may have accumulated through the actions of AnkR.

Our results are likely generalizable to all myelinated axons, rather than being a tissue- or cell-type-specific phenomenon, since DRG and RGC neurons have different embryonic origins (neural crest and neuroectoderm, respectively). However, Barry et al. recently reported, on the basis of their examination of a constitutive knockout mouse lacking exon 1b of AnkG (ex1b AnkG KO; AnkG has five identified alternative first exons), that AnkG is required for axonal trafficking and nodal clustering of Na⁺ channels; exon 1b–containing AnkG was previously thought to be mainly expressed in cerebellum and Purkinje neurons. We also analyzed ex1b AnkG KO mice, but in contrast to the results of Barry et al. we found robust nodal Na⁺ channel clustering at AnkG-deficient nodes (Supplementary Fig. 7). Despite the loss of AnkG from PNS and CNS axons, we found Na⁺ channels clustered at nodes, indicating that AnkG is required neither for nodal clustering nor for axonal trafficking of Na⁺ channels. We are unable to explain the discrepancy between their results and ours, although the complexity of AnkG's alternative splicing and the expression of exon 1b–containing AnkG in both Purkinje neurons and oligodendrocytes favor the more specific experimental approach used in our study. Nevertheless, since AnkR binds Na⁺ channels and is found in axons, the axonal trafficking of Na⁺ channels may still depend on their interaction with ankcyrin.

A hierarchy of ankyrin-spectrin interactions

The rescue of Na⁺ channel clustering by AnkR–βI spectrin is not a consequence of genetic reprogramming but rather a redistribution of a pre-existing pool of axonal AnkR–βI spectrin. If AnkR–βI spectrin complexes are normally found in mature myelinated axons, why are they not at nodes? Our results indicate that AnkR, AnkG, βI spectrin and βIV spectrin each have different affinities for one another and can interact with Na⁺ channels and NF186; AnkG has the highest affinity for NF186, Na⁺ channels and βIV spectrin, while AnkR has a higher affinity for βI spectrin. This hierarchy of ankyrin-spectrin interactions and preference for specific binding partners immediately suggests a simple model that can explain the steady-state molecular composition of nodes, the transient detection of AnkR–βI spectrin at nodes during early development, and the rescue of Na⁺ channel clustering in AnkG- or βIV spectrin–deficient axons (Supplementary Fig. 6b). We propose that during early development neuron-glia interactions first cluster NF186, resulting in a local excess of NF186 relative to AnkG and AnkR, allowing both to bind to NF186. Na⁺ channels are then recruited to developing nodes by binding to either AnkG or AnkR. Over time, turnover of AnkR and the higher affinity of AnkG for NF186 and Na⁺ channels results in the displacement and gradual loss of AnkR from nodes. The interaction between AnkG and βIV spectrin further stabilizes the NF186–AnkG–Na⁺ channel complex, resulting in a mature node that includes AnkG and βIV spectrin rather than AnkR and βI spectrin. Although we transiently detected AnkR at developing PNS nodes, we only rarely detected AnkR at developing CNS nodes. This discrepancy may reflect differences between the PNS and CNS in the mechanisms responsible for the initial clustering of NF186 (refs. 2, 4) or differences in the temporal expression of ankcyrin between the PNS and CNS.

In AnkG-deficient axons, AnkR does not have to compete with AnkG for NF186 or Na⁺ channels, and thus it can effectively bind these molecules despite its lower binding efficiency compared to AnkG. We speculate that the reduced density of Na⁺ channels seen in AnkG-deficient sensory axons during early development reflects this lower binding efficiency (Supplementary Fig. 1e,f). Finally, in AnkG-deficient axons the NF186-AnkR-Na⁺ channel complex is stabilized in the membrane by AnkR's preferential binding to βI spectrin. In βIV spectrin–deficient axons, although AnkG is expressed, we propose that spectrin-actin interactions stabilize the NF186–ANKyar–Na⁺ channel complex; without βIV spectrin, the balance of stable complexes is shifted toward NF186–AnkR–Na⁺ channel complexes.

If AnkR–βI spectrin can compensate for the loss of AnkG–βIV spectrin, why are AISs not rescued in the Nes-Cre;Ank3F/F mice? Ankyrins are enriched at nodes of Ranvier by interacting with NF186. Thus, nodal clustering of ankcyrins depends on extrinsic interactions between NF186 and myelinating glia. In contrast, AIS clustering of AnkG does not depend on any extrinsic factors; rather, AnkG is restricted to the proximal axon by an intra-axonal cytoskeletal boundary consisting of ankcyrin-B, αI spectrin and βIV spectrin. Interestingly, AnkR is not subject to this intra-axonal cytoskeletal boundary and instead is diffusely distributed throughout neurons (T.S.-Y.H. and M.N.R., unpublished results). Immunostaining of dorsal root and retinal ganglion cells in Avil-Cre;Ank3F/F and Six3-Cre;Ank3F/F mice, respectively, shows no enrichment for Na⁺ channels or AnkR in an AIS like structure (Supplementary Fig. 8). Future studies comparing AnkG and AnkR may reveal key domains and/or interactions that are necessary for AIS clustering.

What is the AnkR–βI spectrin protein complex doing in myelinated axons and the nervous system? Although previous studies have reported AnkR in the brain, little is known about its function. AnkR is not required for normal node formation since Ankypale/pale mice showed no defect in PNS or CNS node formation (Fig. 7c,d). Another AnkR mutant mouse (the nb/nb mouse) with normoblastosis and hemolytic anemia also shows reduced levels of AnkR but can survive for many months. Interestingly, nb/nb mice have cerebellar Purkinje cell degeneration, suggesting AnkR may play important roles in the nervous system besides the node of Ranvier. Furthermore, a few case reports describe patients with hereditary spherocytosis who also show neurological symptoms including spastic paraplegia and cerebellar dysfunction.

Ankyrins are required for nodal Na⁺ channel clustering

AnkG was first reported at nodes, and suggested to be responsible for Na⁺ channel clustering, nearly two decades ago. Proof that ankcyrin
are required for nodal Na+ channel clustering has not been forthcoming owing to the lack of appropriate animal models and reagents. Using full-length wild-type and mutant Na+ channel expression constructs, and the ankyrin-binding fragment of Na+ channels, we showed the AnkG-binding motif of Na+ channels is both necessary and sufficient for their localization to nodes. However, the results presented here show that AnkG is dispensable. Instead, the rescue of channel clustering by AnkR in AnkG-deficient neurons indicates that the motif in the Na+ channel is more correctly described as an ankyrin-binding motif.

In conclusion, we provide here evidence that a hierarchy of ankyrin-spectrin interactions exists in myelinated axons to cluster nodal Na+ channels. Finally, the analysis of mice deficient for both AnkG and AnkR in dorsal sensory neurons proves that ankyrins are required for Na+ channel clustering at nodes of Ranvier.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.N.R. and T.S.-Y.H. conceived the project, designed the experiments and wrote the manuscript. D.R.Z. performed the electrophysiology experiments and analyzed the data. M.N.R. performed intravitreal injections of AAV. T.S.-Y.H. performed all other experiments and analyzed the data. K.-J.C., M.X., E.C.C., M.C.S. and V.B. provided crucial reagents, mice and support.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. AnkG conditional knockout mice were generated by cell-type-specific Cre-mediated deletion of the Ank3 gene. The targeting construct was designed and Ank3loxPlox mice were generated as previously described4. Exon 1b AnkG KO mice have been described21. AnkR mutant mice (Ank1palepale, C57BL/6) and Nes-Cre transgenic mice (C57BL/6) were obtained from the Jackson Laboratory (009157 and 003771, respectively). Advillin-Cre (Adv-Cre) mice were provided by W. H. Klein (University of Texas MD Anderson Cancer Center, Houston, TX). Adv-CreAnk3F/i;Ank1palepale were generated by crossing Adv-Cre;Ank3F+/i; Ank1palepale with Ank3F/i;Ank1palepale mice. βIV spectrin mutant mice (Spinb−/−) were described previously22. Both male and female mice were used in our studies. All mouse work and procedures were approved by the Animal Care and Use Committee at Baylor College of Medicine and were performed in accordance with the NIH guide for the humane care and use of animals.

Antibodies. Mouse monoclonal pan Na+ channel (pan-Nav) antibody was described previously60. Other mouse monoclonal antibodies used were sourced as follows: AnkG (N106/36, UC Davis/NIH NeuroMab facility, 1:250), AnkB (N105/17 and N105/13, UC Davis/NIH NeuroMab facility, 1:200), Caspr (K65/35, UC Davis/NIH NeuroMab facility, 1:200), GST (N100/13, UC Davis/NIH NeuroMab facility, 1:500), GFP (N86/38, UC Davis/NIH NeuroMab facility, 2 μg per IP reaction), actin (C4, EMD Millipore, 1:2,000), Myc (9E10, Sigma, 1:2,000) and hemagglutinin (HA) (16B12, Covance, 1:1,000). Rabbit polyclonal antibodies against GFP and neurofilament M were purchased from Life Technologies (cat. no. A1112, 1:1,000) and EMD Millipore (cat. no. AB1987, 1:6,000), respectively. Rabbit antibodies against AnkG were generated against the N-terminal domain (1:200). Rabbit anti-AnkR antibodies were made by immunizing rabbits with His-tagged full-length human AnkR protein and then performing affinity purification from rabbit serum (1:400 for immunostaining; 1:2,000 for immunoblotting). Rabbit anti-βⅢ spectrin was raised against and affinity purified against the unique C terminus of βⅢ2 spectrin (1:400 for immunostaining; 1:1,500 for immunoblotting). Rabbit anti-βⅢ spectrin antibodies (1:400) were previously described97, and chicken anti-βⅣ spectrin (1:100) was generated using the same peptide; both antibodies were purified by affinity chromatography against the immunizing peptide. The chicken antibody to neurofascin was purchased from R&D Systems (cat. no. AF3235, 1:400). The goat anti-AnkK antibody was generated against AnkK’s C-terminal domain (1:2,000). The rat anti-GFP antibody was purchased from Nacalai USA (cat. no. 04040-84, 1:1,000). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories and Life Technologies. All antibody dilutions used here should be considered examples, and researchers are encouraged to validate dilutions for themselves.

DNA constructs. The full-length rat 270-kDa AnkG-GFP has been described40. To construct Myc-tagged AnkG membrane binding domain (Myc-AnkG MBD) the DNA fragment encoding 1–846 amino acids (aa) of AnkG was PCR amplified and inserted into the EcoRI-XhoI sites of pCS3+MT vector. The primers for amplifying AnkG MBD are forward, TCCGAATTCAATGGCTCATGCCGCCTCCCAG, and reverse, GAACTTGTGGCCGTTTAC, and replacing the insert between GGAGCCATCTTCGTTGAAC and forward, ACTGTCAGAAAGGACAAGG, with the DNA fragment encoding 1–846 amino acids (aa) of AnkG was PCR amplified and inserted into the EcoRI-XhoI sites of pCS3+MT vector. The primers for amplifying SR1–17 are forward, TCCGAATTCAATGCCCTATTCTGTGG, and reverse, GCCTCGAGGTGAGCCGGGCGTCCTCAC. GST-Nav II–III intracellular loop, was inserted into BamHI+SalI sites of pGEX4T-1. Myc-tagged spectrin repeat 10–15 (SR10–15) of βIV spectrin was described previously60. To construct Myc-tagged β spectrin ankyrin-binding domain, the DNA fragment encoding SR13–17 of β spectrin was PCR amplified from the construct GST-β1 spectrin SR13–17 (a gift from S. Cunha, University of Texas Health Science Center) and introduced into the EcoRI-XbaI sites of the pCS3+MT vector. The primers for amplifying SR13–17 are forward, TCCGAATTCACTCCTATGCTATCCGC, and reverse, GCTCTAGAGCGACTGACTCCCAGGAAC. GST-Nav II–III was generated by PCR amplification using the rat NavL2 cDNA clone48 as template and the primers GAAGGATCCGCAAACTGGGTCCGACCAGTACG (forward) and GAA GTGCACTAATCTGAGCCATTTTCTTCAGTCC (reverse). The PCR product, corresponding to residues 989–1203, representing the entire domain II–III intracellular loop, was inserted into BamHI+SalI sites of pGEX4T-1.

Immunofluorescence and image analysis. Nervous system tissues were dissected and fixed in 4% paraformaldehyde for 30 min (optic nerves, sciatic nerves and nerve roots) to 1 h (brains) on ice and subsequently immersed in 20% sucrose overnight at 4 °C. Immunostaining and image processing were performed as previously described25.

Immunoblotting. Mouse forebrains and dorsal roots were dissected, frozen on dry ice and homogenized in ice-cold homogenization buffer (0.32 M sucrose, 5 mM sodium phosphate, pH 7.2, 1 mM sodium fluoride, 1 mM sodium orthovanadate and protease inhibitors). For dorsal roots, 10–15 roots per animal were pooled for each preparation and homogenized in 100 μl homogenization buffer. The homogenates were centrifuged at 700g for 10 min at 4 °C to remove nuclei and debris. For brains, the supernatant was further centrifuged at 27,200g for 90 min at 4 °C, and the pellet was resuspended in homogenization buffer (3 ml/gram of brain). Protein concentrations were measured using a Bradford assay (Bio-Rad). The samples were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with the appropriate antibodies.

In vitro phosphorylation and GST-pulldown assay. GST fusion proteins were expressed in Escherichia coli strain BL21 (DE3) and induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). GST fusion proteins were isolated by lysis in 0.5% Triton X-100/PBS/Complete protease inhibitor (Roche). The cells were sonicated, and proteins isolated from the cleared lysate and affinity-purified with glutathione-Sepharose 4B (GE Healthcare). The protein was dialyzed against 1 L 1× PBS using a 10-kDa-MWCO dialysis cassette (Pierce). The protein concentration was estimated from SDS gels after colloidal blue staining (Invitrogen) against a standard curve generated using bovine serum albumin, snap-frozen in liquid nitrogen and stored at −80 °C. Phosphorylation reactions were performed by incubating purified GST fusion proteins (2 μg) with 1× CKII buffer containing 200 μM ATP and 10 units of CKII (NEB, Inc) for 1 h at 30 °C. For controls, protein samples were treated identically, except CKII was omitted. In vitro-phosphorylated GST proteins were then coated on glutathione-Sepharose 4B (GE Healthcare) by 2 h of incubation at 4 °C. At the meantime, COS7 cells transfected with full-length AnkG or AnkR were solubilized in lysis buffer49 for 1 h at 4 °C, and then the lysates were centrifuged at 13,000g for 15 min at 4 °C. The soluble materials were incubated overnight with GST protein–coated beads. The beads were washed 5 times with lysis buffer and then eluted with 30 μl SDS sample buffer at 100 °C for 5 min. The samples were analyzed by immunoblotting with anti-AnkG, anti-AnkR, or anti-GST antibody.

Co-immunoprecipitation. For co-immunoprecipitation, Myc-β1 spectrin SR13–17 or Myc-βIV spectrin SR10–15 was co-transfected with AnkG-GFP, GFP-AnkR, or GFP in COS cells. Cells were lysed with lysis buffer for 1 h at 4 °C and then centrifuged at 13,000g for 15 min at 4 °C. Protein A agarose beads (Thermo Scientific) were washed 5 times with lysis buffer, the beads were eluted with 20 μl of 2× reducing sample buffer at 100 °C for 5 min. The samples were analyzed by immunoblotting using Myc or GFP antibody. For co-immunoprecipitaiting NF186 and ankryin membrane binding domains (MBD), HA-NF186 was co-transfected with Myc-AnkG MBD or Myc-AnkR MBD in HEK293 cells. The samples were immunoprecipitated with mouse anti-HA antibody and immunoblotted with Myc antibody.
Electrophysiology. Compound action potential (CAP) recording in P14 dorsal roots was performed as described⁵⁰.

Behavior analysis. P28 Ank3⁴⁶/F and Avil-Cre, Ank3⁴⁶/F mice were subjected to accelerating rotarod, wire hang and hot-plate tests as previously described⁵⁰.

AAV viruses and intravitreal injection. AAV2-Cre-GFP or AAV2-GFP (Vector Biolabs) was injected into the eyes of P21 Ank3⁴⁶/F mice using a pulled glass needle. Both Cre and GFP were driven by their own CMV promoters. The optic nerves were dissected 2 months after virus injection and then immunostained.

Reverse transcription—quantitative polymerase chain reaction (RT-qPCR). Total RNA from the dorsal root ganglia of 1 month old mice was extracted using RNeasy Plus Mini kit (Qiagen). No DNase treatment was performed since gDNA Eliminator spin columns were used, all the primer pairs span at least one exon-exon junction and negative controls without the reverse transcriptase were included (see below). The RNA integrity was confirmed by ~2:1 intensity ratios of 28S and 18S rRNAs as sharp bands on agarose gels. cDNA was synthesized with SuperScript III First-Strand Synthesis System (Life Technologies) using random hexamers and 0.21–0.48 µg of total RNA in 20 µl of reactions. Real-time PCR was carried out using Power SYBR Green PCR master mix (Life Technologies) in 25 µl of reactions including each primer at 400 nM and 2 µl of the fourfold-diluted RT reactions on a StepOnePlus Real-Time PCR System (Life Technologies); 95 °C 10 min; 40 cycles of 95 °C 15 s and 60 °C 45 s. The primers used are (5′-3′): AnkG (Ank3:: forward, GTGAATGGGAACACAGCTC; reverse, TCATCGTTTCTGGGACATTC; 178-bp amplicon), AnkR (Ank1:: forward, CCTTCGATACAGCATTCTCAG; reverse, GGAGTTAGGGTTTTCTACACG; 142-bp amplicon), and Polr2a (Polr2a:: forward, GAATAAGGAGTGGCTGGAG; reverse, CTCATTCTGCAGGGTCTG; 149-bp amplicon), βII spectrin (Spbbn4:: forward, GAATAAGGAGTGGCTGGAG; reverse, GATCTCACCTAATTTCTTGCG; 100-bp amplicon), βI spectrin (Spth:: forward, CCAAGCCAAGCTCAAAAC; reverse, CTCTCTGTGAGGTTCTG; 149-bp amplicon), and Polr2α (Polr2a:: forward, CATCAAGAGAGTGCAGTTCG; reverse, CCATTAGTCCCCAGTTTG; 125-bp amplicon). The efficiency of all the primer pairs was validated on six points of fourfold serial dilutions of adult mouse brain cDNAs and confirmed to be between 93% and 101%. The specificity of the RT reactions without the reverse transcriptase were also carried out and included for qPCR. Either no amplification was observed or the Cq values were all higher than those derived from the RT with the reverse transcriptase by more than 5. Polr2α was chosen as the internal control in view of the previous study⁵¹ and the tests we performed. We observed consistently close Cq values across samples in each comparison group (the s.d. of Cq values among different samples is 0.25–0.30). The targets were normalized to Polr2α, and all samples were then normalized to WT samples (ΔΔCq method).

Statistical analyses. Unpaired, two-tailed Student’s t-test was used for statistical analysis unless otherwise indicated. Data were collected and processed randomly and were analyzed using Microsoft Excel and GraphPad Prism. All error bars are ±s.e.m. unless otherwise indicated. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported previously². Except for electrophysiological recording, data collection and analysis were not performed blind to the conditions of the experiments. Data distribution was assumed to be normal but was not formally tested.

A Supplementary Methods Checklist is available online in the supplementary information.

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