COMMENTARY

An unfolding role for ankyrin-G at the axon initial segment

James L. Salzer[a,b,c,1]

The ability of neurons to integrate convergent inputs and generate action potentials, the physiological currency of activity, relies on the axon initial segment (AIS). This specialized segment of the proximal axon is the site of electrogenesis in neurons (1), reflecting its striking enrichment in voltage-gated sodium channels (NaV). The AIS has additional, critical roles in the organization and function of neurons. It is a locus of homeostatic plasticity, i.e., activity-dependent remodeling that serves to dampen swings in activity (2). It provides a barrier to the ingress of somatodendritic components into the axonal compartment, thereby helping to establish neuronal polarity (3, 4). Finally, the AIS receives inhibitory (axo-axonic) synaptic input from Chandelier cells. These GABAergic interneurons can thereby each coordinately regulate the activity of hundreds of pyramidal neurons (5). Given these varied, critical functions, it is not surprising that the AIS is increasingly appreciated as the site of pathology in a number of neurological and psychiatric disorders (6). In PNAS, Yang et al. (7) describe several human mutations in ankyrin-G (AnkG)—the master scaffold of the AIS—that result in neurodevelopmental disorders. Analysis of these mutants indicates they impair a key conformational change in AnkG that is crucial for the assembly/maturation of the AIS, providing important insights into this essential neuronal domain.

The AIS is composed of a macromolecular complex that forms autonomously in the proximal axon. This complex includes the NaV and KCNQ ion channels and members of the L1 family cell adhesion molecules, i.e., neurofascin 186 (NF186), NrCAM, and L1CAM (Fig. 1*A*). All of these proteins bind to AnkG, which itself binds to the C terminus of βIV spectrin to form a submembranous scaffold characteristic of the AIS (8). AnkG is critical for AIS assembly (9). It is also required for proper innervation of the AIS by Chandelier cells by regulating the abundance of L1CAM (10) and for formation of the barrier between the somatodendritic and axonal domains (3, 4). Finally, AnkG by tethering many of these components to the actin/spectrin cytoskeleton coordinates the distinctive microarchitecture of the AIS. Superresolution microscopy indicates the AIS complex is linked to a series of submembranous, circumferential actin rings that extend the length of the axon (11). These rings are arrayed at ~190-nm intervals, spacing dictated by spectrin tetramers that bridge the actin rings (12, 13). Accordingly,

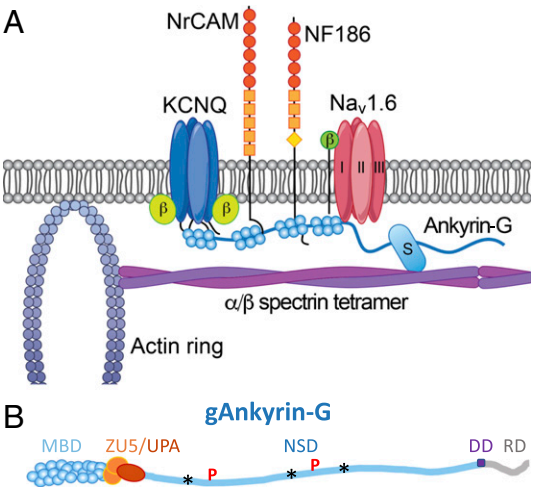

Fig. 1. Organization of the AIS and of Ankyrin-G. (*A*) Schematic of key components of the AIS. These include cell adhesion molecules (NrCAM and NF186) and ion channels (KCNQ and NaV) all bound to ankyrin repeats in the amino terminus. AnkG is, in turn, linked to the spectrin tetramer which is shown associated with an actin ring. Tetramers and the associated actin rings are spaced ~190 nm apart. (*B*) Schematic of the organization of gAnkG in the open conformation based on figure 1 in Yang et al. (7). gAnkG contains a MBD consisting of 24 ankyrin repeats, a ZU5/UPA module that is a canonical spectrin-binding site, an ~2,500-aa NSD, a death domain (DD), and the C-terminal RD. The overall length of gAnkD is just over 4,000 aa and ~150 nm. The approximate location in the NSD of the human mutations Yang et al. (7) describe and the phosphorylation sites they mutated are shown by the 3 asterisks and the 2 red Ps, respectively.

[a]Neuroscience Institute, New York University School of Medicine, New York, NY 10016; [b]Department of Neuroscience and Physiology, New York University School of Medicine, New York, NY 10016; and [c]Department of Neurology, New York University School of Medicine, New York, NY 10016

Author contributions: J.L.S. wrote the paper.

The author declares no conflict of interest.

See companion article on page 19717.

[1]Email: James.Salzer@nyulangone.org.

First published September 9, 2019.

www.pnas.org/cgi/doi/10.1073/pnas.1914002116

AnkG and its various transmembrane partners, e.g., NF186 and NaV, are organized in register (11, 13).

AnkG is 1 of 3 vertebrate ankyrin genes: *ANK1*, *ANK2*, and *ANK3*, corresponding to AnkR, AnkB, and AnkG proteins, respectively. Ankyrins have a conserved role as essential scaffolds that organize diverse proteins into functional microdomains in different cell types (8). Only AnkG is enriched at electrogenic sites in the nervous system, i.e., the AIS and nodes of Ranvier. All ankyrins share a canonical organization that includes a membrane-binding domain (MBD), consisting of 24 ankyrin repeats to which various transmembrane proteins bind, followed by a ZU5/UPA module to which spectrins bind, and an intrinsically disordered C-terminal regulatory domain (RD) (Fig. 1*B*). Ankyrins are further diversified by alternative splicing. Notably, AnkG can incorporate a very large, neurospecific domain (NSD) encoded by a single giant exon resulting in giant AnkG (gAnkG) isoforms that are either 270 or 480 kDa; the latter is the key isoform at the AIS (and nodes) required for ion channel clustering (9). Underscoring its significance, each of the 3 human mutations identified by Yang et al. (7) reside in the NSD.

To elucidate the effects of these mutations on gAnkG function, and on the AIS, Yang et al. (7) expressed the mutant proteins in cultured hippocampal (Hc) neurons, which are frequently used to study AIS assembly in vitro. The Hc neurons were engineered to lack all endogenous AnkG isoforms (by Cre-mediated recombination of a floxed ANK3 gene) to avoid any confounding effects of wild-type (WT) gAnkG. Expression of each of these mutations resulted in gAnkG-positive initial segments that were both aberrantly elongated (~2x) and markedly attenuated in intensity (~50%). All other AIS components were likewise elongated and attenuated commensurate with that of the mutant gAnkGs with the exception of β4 spectrin, which was essentially absent. This latter result suggests loss of β4 spectrin may account for the altered AIS morphology in these gAnkG mutants. In strong support, knockout of β4 spectrin in Hc neurons by Crispr/Cas9 phenocopied the effects of the gAnkG mutants; i.e., it resulted in an extended, attenuated AIS. While reexpression of WT β4 spectrin in these knockout neurons restored the normal AIS phenotype, expression of a mutant β4 spectrin that cannot bind to AnkG did not. Thus, the interaction of gAnkG with β4 spectrin is essential to establish a normal AIS morphology.

These results raise the question of how these human point mutations in the NSD interfere with spectrin binding given the presumptive binding site—the ZU5 domain—is located some 1,000 to 2,000 amino acids (aa) away. Of note, a previously described mutation in the NSD of AnkG, in which a serine phosphorylation site is mutated to an alanine, similarly blocked recruitment of β4 spectrin to the AIS (9). This suggested gAnkG phosphorylation might be an important regulator of spectrin binding in a manner similar to that of the human mutations. Yang et al. (7) thus undertook a detailed and parallel analysis of the effects of gAnkG's phosphorylation on spectrin binding. Mass spectrometry identified 13 phospho-serine or threonine sites in the NSD, many phosphorylated to very high stoichiometries (in some cases 30% or more). They next analyzed the effects of individually rendering 9 of these gAnkG sites nonphosphorylatable by mutating the serines or threonines to alanine. Blocking phosphorylation at 3 of these sites blocked recruitment of endogenous β4 spectrin, aberrantly increasing the length and attenuating the concentration of AIS components.

How does blocking phosphorylation at these various sites, which are scattered over an extended segment of the NSD, block interactions with spectrin and do the various human missense mutations act similarly? gAnkG normally exists in an extended conformation of ~150 nm based on platinum replica EM (9). However, recent structural studies suggest that gAnkG can also adopt a folded "head-to-tail" configuration in which the C-terminal RD interacts with and autoinhibits different MBD sites at the N terminus of gAnkG (14). These considerations suggested mutations in the NSD might result in an aberrant conformation in which the N- and C-terminal regions are in close proximity to likewise preclude β4 spectrin binding.This was indeed corroborated by proximity ligation assays (PLA) (15). Yang et al. (7) used antibodies to the N- and C-terminal domains as probes that would be expected to report a positive PLA signal only when the distance between these domains is less than 40 nm. Strikingly, the PLA signal was much higher in the 3 human mutants and in the 2 nonphosphorylatable mutants, even though gAnkG levels in the AIS were markedly attenuated. Further, early in development, when the AIS is elongated and prior to βIV spectrin recruitment, PLA levels are quite high whereas with AIS maturation, and βIV spectrin recruitment, PLA levels decline substantially. These results strongly suggest that gAnkG transitions from a closed, folded to an open, extended conformation during AIS development and that this transition is regulated by amino acids within the NSD.

In PNAS, Yang et al. describe several human mutations in ankyrin-G (AnkG)—the master scaffold of the AIS—that result in neurodevelopmental disorders.

Taken together, these results indicate the AIS assembles in stages. AnkG initially accumulates in the AIS in a closed conformation to which NaV, NF186, and other components bind to accessible ankyrin repeats in the MBD. Other studies suggest this initial accumulation of AnkG in the AIS results from multiple mechanisms including interactions with microtubule end-binding proteins (16) and the activity of contractile actomyosin (17). After initial assembly, the AIS then matures over a period of days which, as Yang et al. (7) now show, almost certainly results from developmentally regulated phosphorylation of AnkG that drives its transition to an open conformation. This conformational change promotes βIV spectrin binding, driving AIS maturation to the compact (~20 to 40 μm), robust domain characteristic of mature neurons. The human gAnkG mutations Yang et al. (7) describe fail to acquire an extended conformation and the AIS accordingly fails to mature—arresting instead at the stage of initial assembly. The associated neurological impairments of these mutations underscore the importance of AIS maturation for its proper function.

These studies raise a number of compelling questions. These include how the change in the conformation of AnkG is regulated and how it affects β4 spectrin binding. gAnkG's conformational change likely reflects developmentally regulated phosphorylation, although a corresponding change in phosphorylation levels has yet to be demonstrated. The salient kinases and phosphatase are not yet known. It is also unclear how phosphorylation opens up AnkG's conformation given that recent structural studies predict interactions of the MBD with the C terminus and not the NSD (14). Conversely, how do the human mutants preclude this conformational change? Do they do so by interfering with phosphorylation, perhaps unlikely given their distance from the phosphorylation sites? The NSD is intrinsically disordered and elucidating how these phosphorylation sites and mutations affect its folding will be of great interest and likely require structural studies. Identifying

the βIV binding site on AnkG remains to be established—is it the canonical ZU5 site or another site, perhaps in the NSD? This will be important in determining whether it is occluded when AnkG is in the closed conformation.

A key finding is that βIV spectrin drives maturation of the AIS—the mechanisms by which it does so remain to be established. The ΣVI isoform of βIV spectrin, a shorter isoform which lacks the N-terminal actin-binding module, can still drive maturation, suggesting that maturation is independent of spectrin's link to the actin cytoskeleton. One potential candidate to drive maturation is Ca^{2+}/calmodulin-dependent protein kinase II (CaMK2), which is complexed to βIV spectrin (18). Other kinases and phosphatases that regulate interactions between AIS components and its organization have also been described (4, 6). It is unclear whether these or CaMK2 have any role in how spectrin regulates AIS maturation.

As noted, these studies demonstrate that defects of AIS maturation result in substantial neurodevelopmental defects. Several mechanisms seem likely to contribute including alterations in AIS firing rates and in inhibitory tone. Quivering (qv^{3J}) mice, a βIV spectrin hypomorph with a similarly elongated, attenuated AIS, are instructive in this regard. Despite markedly reduced NaV levels, the AISs of qv^{3J} mice still generate action potential but do so with impaired temporal precision, likely contributing to network deficits (19). In addition, Chandelier cell innervation and thus inhibitory control of the elongated, attenuated AISs in these various mutants are expected to be diminished given reduced L1CAM expression that ensues with loss of AnkG or βIV spectrin in the AIS (10). Of note, a mutation in the NSD that impairs interactions of AnkG with the $GABA_A$ receptor-associated protein results in diminished inhibitory tone, pyramidal cell hyperexcitability, and disrupted network synchronization (20). In the future, generation of mice that model these human mutations or block these phosphorylation sites in gAnkG will further clarify the role of the AIS as a nexus of neurodevelopmental disorders.

1 K. J. Bender, L. O. Trussell, The physiology of the axon initial segment. *Annu. Rev. Neurosci.* **35**, 249–265 (2012).
2 R. Adachi, R. Yamada, H. Kuba, Plasticity of the axonal trigger zone. *Neuroscientist* **21**, 255–265 (2015).
3 M. N. Rasband, The axon initial segment and the maintenance of neuronal polarity. *Nat. Rev. Neurosci.* **11**, 552–562 (2010).
4 C. Leterrier, The axon initial segment: An updated viewpoint. *J. Neurosci.* **38**, 2135–2145 (2018).
5 Y. Wang, P. Zhang, D. R. Wyskiel, Chandelier cells in functional and dysfunctional neural circuits. *Front. Neural Circuits* **10**, 33 (2016).
6 C. Y. Huang, M. N. Rasband, Axon initial segments: Structure, function, and disease. *Ann. N. Y. Acad. Sci.* **1420**, 46–61 (2018).
7 R. Yang *et al.*, Neurodevelopmental mutation of giant ankyrin-G disrupts a core mechanism for axon initial segment assembly. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 19717–19726 (2019).
8 V. Bennett, D. N. Lorenzo, An adaptable spectrin/ankyrin-based mechanism for long-range organization of plasma membranes in vertebrate tissues. *Curr. Top. Membr.* **77**, 143–184 (2016).
9 P. M. Jenkins *et al.*, Giant ankyrin-G: A critical innovation in vertebrate evolution of fast and integrated neuronal signaling. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 957–964 (2015).
10 Y. Tai, N. B. Gallo, M. Wang, J. R. Yu, L. Van Aelst, Axo-axonic innervation of neocortical pyramidal neurons by GABAergic chandelier cells requires AnkyrinG-associated L1CAM. *Neuron* **102**, 358–372.e9 (2019).
11 K. Xu, G. Zhong, X. Zhuang, Actin, spectrin, and associated proteins form a periodic cytoskeletal structure in axons. *Science* **339**, 452–456 (2013).
12 G. Zhong *et al.*, Developmental mechanism of the periodic membrane skeleton in axons. *eLife* **3**, 3 (2014).
13 C. Leterrier *et al.*, Nanoscale architecture of the axon initial segment reveals an organized and robust scaffold. *Cell Reports* **13**, 2781–2793 (2015).
14 K. Chen, J. Li, C. Wang, Z. Wei, M. Zhang, Autoinhibition of ankyrin-B/G membrane target bindings by intrinsically disordered segments from the tail regions. *eLife* **6**, 6 (2017).
15 S. Fredriksson *et al.*, Protein detection using proximity-dependent DNA ligation assays. *Nat. Biotechnol.* **20**, 473–477 (2002).
16 A. Fréal *et al.*, Cooperative interactions between 480 kDa Ankyrin-G and EB proteins assemble the axon initial segment. *J. Neurosci.* **36**, 4421–4433 (2016).
17 S. L. Berger *et al.*, Localized myosin II activity regulates assembly and plasticity of the axon initial segment. *Neuron* **97**, 555–570.e6 (2018).
18 T. J. Hund *et al.*, A β(IV)-spectrin/CaMKII signaling complex is essential for membrane excitability in mice. *J. Clin. Invest.* **120**, 3508–3519 (2010).
19 E. Lazarov *et al.*, An axon initial segment is required for temporal precision in action potential encoding by neuronal populations. *Sci. Adv.* **4**, u8621 (2018).
20 A. D. Nelson *et al.*, Ankyrin-G regulates forebrain connectivity and network synchronization via interaction with GABARAP. *Mol. Psychiatry* 10.1038/s41380-018-0308-x (2018).