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

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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. 1A). 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, spaced dictated by spectrin tetramers that bridge the actin rings (12, 13). Accordingly,
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. 1B). 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 endogenous gAnkG 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

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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\(^{2+}\)) mice, a βIV spectrin hypomorph with a similarly elongated, attenuated AIS, are instructive in this regard. Despite markedly reduced Na\(^{+}\) levels, the AISs of qv\(^{2+}\) 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 NSF 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.

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