PNAS COMMENTARY

Reframing the axon initial segment: Giant ankyrin-G as a modulator of excitability and plasticity in neurodevelopment

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ANK3 is a large, alternatively spliced gene that produces multiple ankyrin-G (AnkG) isoforms with distinct functions in neuronal organization and excitability. Among these are a group termed "giant AnkGs" (gAnkGs), which include a single 7.8 kilobase exon (giant exon) between the sequences encoding the UPA and death domains (Ensembl ID: ENSE00000997921) (1, 2). There has been growing interest in gAnkGs, due to their critical roles within neurons and their implications in neurodevelopmental disorders (NDDs). gAnkG isoforms are known for their roles at the axon initial segment (AIS), nodes of Ranvier, and at GABAergic synapses (3) and have repeatedly been implicated in autism spectrum disorder (ASD), bipolar disorder (BD), epilepsy, and a spectrum of NDDs through genome-wide association studies and rare genetic variant studies (Fig. 1 *A* and *B*) (4–8).

In this issue of PNAS, Li et al. investigated a human missense variant, *ANK3* p.T1861M, identified in children with NDDs presenting with ataxia, seizures, and ASD features. This variant lies within the giant exon, a domain central to AnkG's role in scaffolding proteins to the plasma membrane at the AIS (9). To mechanistically evaluate the pathogenicity of this variant, Li and colleagues employ a mouse knock-in model carrying the homologous variant, T1935M, and investigate its effects at the molecular, cellular, circuit, and behavioral levels.

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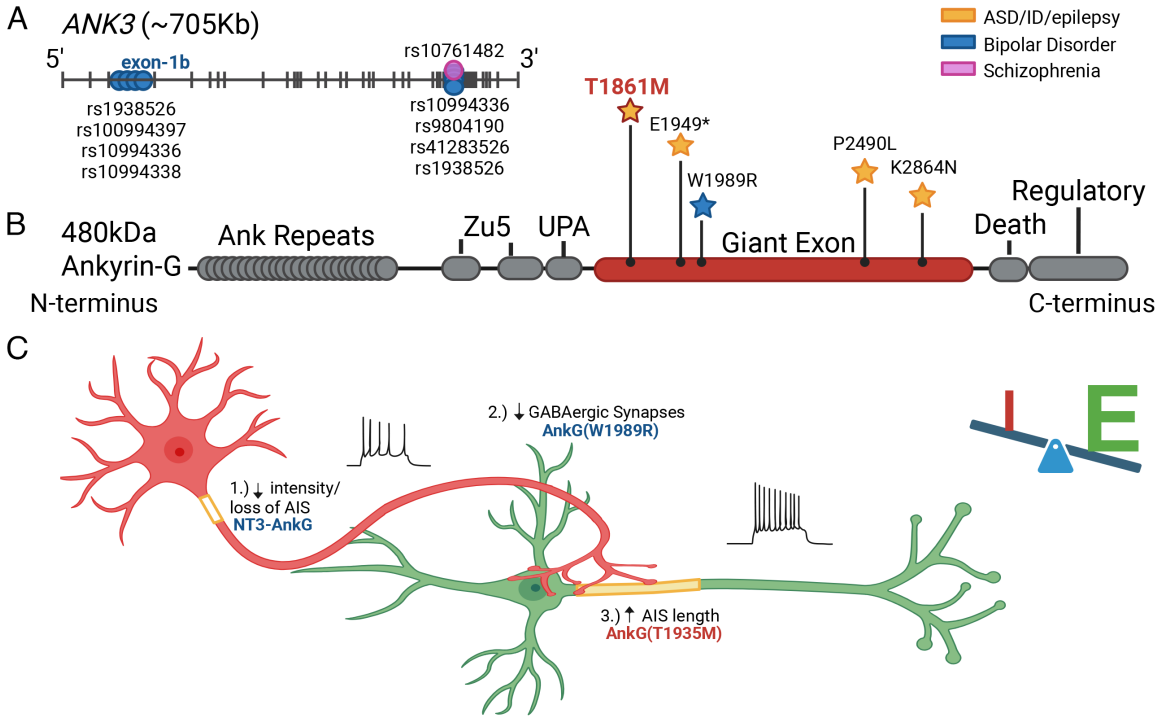

Fig. 1. Giant AnkG mechanisms in NDDs. (*A*) Locations of NDD-related SNPs within the *ANK3* gene. BD-related SNPs (blue circles) and schizophrenia-related SNPs (purple dots) fall within 2 hotspots, the 5′ end upstream of exon-1b and the 5′ end upstream of the giant exon. (*B*) Locations of NDD-related rare variants found in the giant exon in patients with BD (blue star) and ASD (yellow stars). The T1861M variant (red outlined, orange star) studied by Li et al. is in the N-terminal portion of the giant exon (red). (*C*) Schematic of overlapping mechanisms involving gAnkG in relation to NDDs. 1.) *Ank3-1b* deletion leads to a loss of gAnkG and Na_V densities at the AIS of inhibitory PV interneurons (red), reducing PV interneuron excitability. 2.) Introducing the W1989R variant into gAnkG causes reduced GABAergic synapses onto pyramidal neurons (green), leading to pyramidal neuron hyperexcitability. 3.) Li et al. show that the introduction of the T1861M homologous variant, T1935M (red), into gAnkG in mice leads to increased AIS length (yellow) and pyramidal neuron hyperexcitability. All three mechanisms lead to a shift in E/I balance toward hyperexcitability. Figure created with BioRender.

Their major discovery is that this mutation disrupts activity-dependent AIS plasticity, preventing normal developmental AIS shortening, and causing pyramidal neuron hyperexcitability due to abnormally elongated AISs. This defect emerges during a critical developmental window between postnatal days 28 and 60, around the time when AIS maturation fine-tunes neuronal excitability in the cortex (10). These data suggest a novel role for gAnkG in AIS remodeling and plasticity.

AIS elongation was unexpected and contrasts with previously studied *Ank3* models including *Ank3 exon1b* (*Ank3-1b*), *Ank3 exon37* (*exon37*), and *Ank3 W1989R* mouse models. In forebrain, *Ank3-1b*$^{-/-}$ and *exon37*$^{-/-}$ mice have neurons lacking AISs in parvalbumin (PV) interneurons or all neurons, respectively (9, 11). *Ank3-1b*$^{+/-}$ mice show reduced AnkG and voltage-gated sodium channel (Na_V) densities at the AIS of PV interneurons and shortened AIS length with no change to pyramidal neuron AISs (12); *Ank3*$^{W1989R/+}$ knock-in mice also exhibit shortened pyramidal cell AISs (7).

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Li et al. show that the failure of *Ank3 T1935M*-expressing pyramidal cells to remodel their AIS causes excessive accumulation of AIS-enriched Na_Vs and βIV-spectrin, consistent with impaired structural refinement. These cortical pyramidal neurons were hyperexcitable, with depolarized resting membrane potentials, reduced action potential (AP) thresholds, shortened AP half-widths, and increased miniature excitatory postsynaptic current frequency. Interestingly, this cortical hyperexcitability is mirrored in other AnkG models, though by different mechanisms. For example, *Ank3*$^{W1989R/+}$ mouse pyramidal neurons exhibit hyperexcitability characterized by increased AP frequencies and decreased GABAergic innervation (7).

Li et al. also demonstrated that gAnkG-regulated AIS plasticity is not just structural but also functional. To test whether activity-dependent AIS plasticity is also impaired in *Ank3*$^{T1935M/T1935M}$mice, Li et al. used two approaches, KCl-induced chronic depolarization, and chemogenetic activation using designer receptors exclusively activated by designer drugs. In both contexts, wild-type neurons shorten their AIS, while *Ank3*$^{T1935M/T1935M}$ neurons do not. Multielectrode array recordings revealed that mutant neurons also lack the expected increase in firing rate after depolarization. These experiments collectively demonstrate that gAnkG is required for both AIS assembly and to enable its adaptation to developmental and activity-dependent changes.

To look for potential effects of the T1935M mutation on behavior in *Ank3*$^{T1935M/T1935M}$ mice that may be relevant to symptomology in human patients with the *ANK3* T1861M variant, Li et al. performed a battery of behavioral assays. They found that *Ank3*$^{T1935M/T1935M}$ mice have impaired motor function in the rotarod test, social behavior deficits in urine territory marking, and increased repetitive behaviors in the marble burying assay. These results again mirror phenotypes seen in other AnkG models of NDDs (13).

The findings by Li et al. show consistently converging phenotypes with other *ANK3* models (Fig. 1*C*). *Ank3 W1989R* knock-in mice, carrying a rare BD-associated variant, shows reduced inhibitory GABAergic synapses onto pyramidal neurons, decreased inhibitory postsynaptic currents, shortened pyramidal neuron AISs, and elevated action potential firing rates (7) that parallel those seen in *Ank3*$^{T1935M/T1935M}$ neurons. Likewise, the *Ank3-1b* knockout (KO) mice, studied because of BD-associated SNPs in the 5′ regulatory region of *ANK3*, exhibits loss of gAnkG at the AIS of PV interneurons, diminished PV interneuron excitability, network hyperexcitability, ataxia, repetitive behaviors, and seizures (12).

Despite the unique deficits identified among these AnkG models, such as the loss of AIS scaffolding, destabilized GABAergic synapses, or impaired phosphorylation, the phenotypic convergence is intriguing. These AnkG models share features that include disrupted excitability homeostasis, excitation/inhibition (E/I) imbalance, and recapitulation of ASD/BD-related behaviors. This convergence highlights shared pathogenic mechanisms across NDDs and suggests that therapeutic strategies aimed at these common pathways could benefit a variety of conditions.

In the broader context, one of the most compelling implications of Li et al.'s study is how seamlessly it fits into a growing multigene framework for NDDs, one in which disruption of neuronal excitability is a recurring and unifying characteristic. Many high-confidence ASD, epilepsy, and BD genes directly influence AIS structural and functional plasticity, intrinsic excitability, or activity-dependent tuning. For example, *SCN2A* is widely implicated in epilepsy and ASD, and mutations in this gene alter Nav1.2 function and disrupt AIS-mediated excitability (14). *ANK2* has also been implicated in ASD and encodes the ankyrin-B protein which is a homolog of AnkG and scaffolds Na_V1.2 (15). Mutations in *ANK2* have been shown to disrupt axonal branching and synaptic connectivity during development and result in changes to neuronal E/I balance (15).

A similar pattern of mechanistic overlap is evident in *SHANK3* and *SHANK1* models, where disruptions in synaptic scaling and excitability thresholds have been linked to ASD and BD (16). Mutations in the Fragile-X and Rett Syndrome genes, *FMR1* and *MECP2*, also alter intrinsic excitability and disrupt activity-dependent plasticity programs (17, 18). Mutations in *CNTNAP2*, *NRXN1*, and *NLGN3/4* are also found in patients with NDDs and have been shown to affect E/I balance through impaired synaptic maturation (19–21). Although these genes encode diverse proteins including ion channels, scaffolding proteins, transcriptional regulators, and synaptic adhesion molecules, they all play a role maintaining cortical excitability and homeostatic plasticity during development. The data presented by Li et al. solidifies *ANK3*'s place on the growing list of NDD genes and reframes AnkG not simply as an AIS scaffolding molecule, but as a central node in plasticity and excitability homeostasis, whose disruption may tilt neural circuits toward ASD, epilepsy, BD, or schizophrenia depending on the developmental timing, cell type affected, and interacting genetic landscape.

Their data also raise the question of how the T1935M mutation drives the structural and functional changes seen in mice

and how it relates to the human T1961M mutation. Li et al. propose that the threonine-to-methionine substitution abolishes a phosphorylation site critical for gAnkG-dependent AIS remodeling. They propose phosphorylation as a switch regulating AIS maturation: A process that, when disrupted, may contribute to various NDDs. This phosphorylation "switch" could be a new mechanism by which AnkG calibrates neuron excitability during development. Overall, their work establishes a mechanistic connection among distinct *ANK3* variants implicated in NDDs, and it demonstrates convergent circuit-level phenotypes that draw strong parallels with non-AnkG models of NDDs. Most importantly, it identifies a potential therapeutic target wherein restoring activity-dependent AIS remodeling may represent a promising strategy for restoring E/I balance in a broad range of NDDs.

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