Physical and functional convergence of the autism risk genes Scn2a and Ank2 in neocortical pyramidal cell dendrites

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ABSTRACT

Dysfunction in sodium channels and their ankyrin scaffolding partners have both been implicated in neurodevelopmental disorders, including autism spectrum disorder (ASD). In particular, the genes SCN2A, which encodes the sodium channel NaV1.2, and ANK2, which encodes ankyrin-B, have strong ASD association. Recent studies indicate that ASD-associated haploinsufficiency in Scn2a impairs dendritic excitability and synaptic function in neocortical pyramidal cells, but how NaV1.2 is anchored within dendritic regions is unknown. Here, we show that ankyrin-B is essential for scaffolding NaV1.2 to the dendritic membrane of mouse neocortical neurons, and that haploinsufficiency of Ank2 phenocopies intrinsic dendritic excitability and synaptic deficits observed in Scn2a+/- conditions. Thus, these results establish a direct, convergent link between two major ASD risk genes and reinforce an emerging framework suggesting that neocortical pyramidal cell dendritic dysfunction can be etiological to neurodevelopmental disorder pathophysiology.

INTRODUCTION

A decade of gene discovery has identified hundreds of genes whose dysfunction is associated with autism spectrum disorder (ASD) (lossifov et al., 2014; Neale et al., 2012; O’Roak et al., 2012; Sanders et al., 2015, 2012; Satterstrom et al., 2020). A key challenge remains to translate these findings into an understanding of pathophysiology at the cellular and circuit level. Loss-of-function in SCN2A, which encodes the neuronal sodium channel NaV1.2, has the strongest evidence of ASD association based on exome sequencing (Ben-Shalom et al., 2017; Fu et al., 2021; Satterstrom et al., 2020; Spratt et al., 2019). Given this critical role in ASD etiology, alterations in cellular function due to SCN2A loss may illuminate common causes of dysfunction shared with other ASD-associated genes.

A novel role for NaV1.2 was identified recently in neocortical pyramidal cells, a cell class whose dysfunction is implicated in ASD (Satterstrom et al., 2020; Willsey et al., 2013). In contrast to the well-characterized roles for sodium channels (NaV) in axonal action potential (AP) electrogensis and propagation, NaV1.2 was found to be critical for dendritic excitability, with ASD-associated Scn2a haploinsufficiency impairing postsynaptic features of synaptic function and plasticity (Spratt et al., 2019). This dendritic NaV1.2 localization is presumably controlled by ankyrins, which are a family of intracellular scaffolding proteins that link ion channels to the underlying actin cytoskeleton (Bennett and Lorenzo, 2016; Lemaitre et al., 2003; Nelson and Jenkins, 2017). Ankyrin-NaV interactions have been studied extensively in excitable axonal compartments, where NaV’s are anchored by ankyrin-G (ANK3) (Jenkins and Bennett, 2001; Jenkins et al., 2015; Pan et al., 2006; Zhou et al., 1998), but how NaV’s are scaffolded to dendritic domains to regulate postsynaptic excitability is unknown.

Insight into this question may come from ASD gene discovery, where another ankyrin family member, ankyrin-B (ANK2), has strong evidence of ASD association (Fu et al., 2021; Satterstrom et al., 2020). Interestingly, immunostaining for ankyrin-G and ankyrin-B in cultured neurons indicates that they occupy largely non-overlapping domains, with ankyrin-G enriched in the axon initial segment (AIS) and nodes of Ranvier, and ankyrin-B enriched in other regions, including dendrites (Lorenzo et al., 2014). Thus, ankyrin-B is well-positioned to scaffold dendritic NaV1.2 channels. In this way, loss-of-function in either SCN2A or ANK2 could impair dendritic excitability, either directly through reduced NaV density or function or indirectly by reduced NaV scaffolding, respectively. This would implicate dendritic excitability as a convergent feature disrupted in ASD.

Here, we paired cellular and molecular biology with electrophysiology and two-photon imaging to demonstrate that the protein products of these two ASD risk genes, SCN2A and ANK2, interact in neocortical pyramidal cell dendrites to mutually regulate dendritic excitability. Using a novel epitope-tagged NaV1.2, we found that NaV1.2 co-localizes with ankyrin-B in the dendrites of mature neocortical neurons. Removal of ankyrin-B eliminated NaV1.2 dendritic localization. Furthermore, dendritic ankyrin-B loss was not compensated for by other ankyrin family members, indicating that ankyrin-B has a unique scaffolding role in this neuronal compartment. Ex vivo studies revealed that Ank2 haploinsufficiency results in intrinsic and synaptic dendritic deficits that closely phenocopy those observed in Scn2a heterozygous neurons. Thus, these findings suggest that deficits in dendritic excitability may be a common point of convergence in ASD, with direct convergence between two high-risk genes SCN2A and ANK2.
mature neocortical neuron dendrites clamp recordings from HEK293 cells transfected with either wild type (WT) characterize ASD-associated variants (Ben-Shalom et al., 2017). Voltage-was created by cloning a codon-optimized compartments, we first generated a cDNA encoding full-length NaV1.2 across development in all neuronal To determine whether NaV1.2 is enriched in the AIS of neocortical pyramidal cells in early development (Boiko et al., 2003; Gazina et al., 2015; O’Brien and Meisler, 2013; van Wart et al., 2007) (Figure 1A). Consistent with staining of native channels, NaV1.2-3xFLAG was similarly restricted to the AIS at day 7 (DIV7) in cultured neocortical neurons, where it colocalized with ankyrin-G (Figure 1C). Later in development, NaV1.2 is largely displaced from the AIS and instead increases in density throughout somatodendritic domains (Hu et al., 2009; Lorincz and Nusser, 2010; Spratt et al., 2019, 2021; Zhang et al., 2021) (Figure 1A). Consistent with this shift in NaV1.2 subcellular localization, NaV1.2-3xFLAG was visualized at high levels throughout dendrites at DIV21 (Figure 1C). Dendritic NaV1.2 was not co-localized with ankyrin-G (ANK3) (Figure 1C), suggesting that NaV1.2 or NaV1.2-3xFLAG indicated that the introduction of this epitope tag did not alter channel biophysics (Figure S1A) or its ability to interact with β1 subunits (Figure S1B), suggesting that this approach is a viable way to visualize NaV1.2 without altering its function. Immunostaining and electrophysiological measurements indicate that NaV1.2 is enriched in the AIS of neocortical pyramidal cells in early development (Boiko et al., 2003; Gazina et al., 2015; O’Brien and Meisler, 2013; van Wart et al., 2007) (Figure 1A). Consistent with staining of native channels, NaV1.2-3xFLAG was similarly restricted to the AIS at day 7 (DIV7) in cultured neocortical neurons, where it colocalized with ankyrin-G (Figure 1C). 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another ankyrin may be important for NaV1.2 scaffolding in dendritic regions. Based on genetic and co-expression data related to ASD (Fu et al., 2021; Satterstrom et al., 2020; Willsey et al., 2013) (Figure 1B), we hypothesized that this dendritic scaffold is ankyrin-B (ANK2). To compare the subcellular localization patterns of ankyrin-B and NaV1.2 across development, we transfected cultured neocortical neurons with NaV1.2-3xFLAG-ires-eGFP and immunostained with antibodies against ankyrin-B at DIV7 and DIV21. At DIV7, ankyrin-B was predominantly localized to the distal axon (Figure 1D). At DIV21, however, ankyrin-B was enriched along the membrane of dendritic shafts, colocalizing with NaV1.2 (Figure 1D). Importantly, there was no correlation between dendritic NaV1.2-3xFLAG and plasmid expression levels, inferred from GFP fluorescence intensity, suggesting that NaV1.2 expression is tightly regulated and that its dendritic localization is not an off-target effect of overexpression (Figure 1E). These data indicate ankyrin-B is well-positioned to scaffold NaV1.2 to the dendritic membrane of mature neocortical pyramidal neurons.

Ankyrin-B localizes NaV1.2 to the dendritic membrane of mature neocortical neurons

To determine whether ankyrin-B directly scaffolds NaV1.2 to the dendritic membrane, we performed knockout-and-rescue experiments in cultured neocortical neurons generated from Ank2<sup>fl/fl</sup> mice, which contain loxP sites flanking exon 24 of the Ank2 gene (Roberts et al., 2019). In wild type (WT) conditions, NaV1.2-3xFLAG was highly enriched in the dendritic membrane with endogenous ankyrin-B in DIV21 neurons (Figure 2A). Co-transfection of NaV1.2-3xFLAG-ires-eGFP with Cre-2A-BFP (blue fluorescent protein) in Ank2<sup>fl/fl</sup> neurons resulted in the complete absence of ankyrin-B and a corresponding loss of dendritic NaV1.2 (Figure 2A). Simultaneous knockout of endogenous ankyrin-B via Cre-2A-BFP and rescue with the canonical wild-type ankyrin-B restored NaV1.2 to the dendritic membrane (Figure 2A).

Figure 2: 220 kDa ankyrin-B scaffolds NaV1.2 to the dendritic membrane

A. Top left: Confocal images of DIV21 WT or total Ank2-null cultured neocortical neurons co-transfected with NaV1.2-3xFLAG-IRES-eGFP or Cre-2A-BFP and rescued with WT or FF/QQ mutant 220 kDa ankyrin-B. Cells were immunostained with antibodies against ankyrin-B (white), FLAG (magenta), and GFP (green). Top right: zoomed images of dendrite labeled with yellow box. Bottom left: Quantification of mean fluorescence intensity of NaV1.2-3xFLAG in dendrites. Circles represent individual neurons. (WT: 0.44 ± 0.06, n = 18 cells; Ank2<sup>FF/QQ</sup>: 0.12 ± 0.02, n = 16 cells; Ank2<sup>+</sup> + WT 220 kDa AnkB: 0.54 ± 0.1, n = 16 cells; Ank2<sup>FF/QQ</sup> + 220 kDa AnkB: 0.54 ± 0.01, n = 16 cells; Ank2<sup>FF/QQ</sup> + FF/QQ 220 kDa AnkB: 0.15 ± 0.02, n = 15 cells). WT vs. Ank2<sup>FF/QQ</sup> was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.
Ankyrin-B is highly homologous with ankyrin-G, especially throughout the ankyrin repeat domain, which contains the canonical NaV binding site (Cai and Zhang, 2006). Therefore, we examined whether ankyrin-B requires this same sequence to localize NaV1.2. We generated a double mutant (F1310Q, F164Q) in the ankyrin repeats of ankyrin-B, which has been shown previously to reduce binding affinity between ankynrs and NaV1.2 by more than 40-fold (Wang et al., 2014). Knockout-and-rescue with the FF/QQ mutant ankyrin-B failed to scaffold NaV1.2, demonstrating the importance of this site for the proper localization of NaV1.2 to adult neocortical dendrites (Figure 2A). Importantly, NaV1.2 and western blot of IP lysates probed with antibodies to ankyrin-B from P60-P75 mice. Black arrows highlight bands of ankyrin-B or NaV1.2. Non-immune IgG used as a negative control. Note: 440 kDa ankyrin-B band consistently runs anomalously high as reported previously (Jenkins et al., 2015).

Figure 3: Ankyrin-B directly interacts with NaV1.2 in adult mouse brain

A. Schematic of NaV1.2 highlighting the ankyrin-binding motif (purple) located within the intracellular loop between domains II and III (cyan). Core nine amino acids (D9 motif) within the II-III loop are essential for ankyrin binding.

B. Left: Representative images of proximity ligation assay (PLA) signal (cyan) between anti-HA and anti-ankyrin-B antibodies from HEK293 cells transfected with ankyrin-B-GFP (green) and HA-tagged NaV1.2 II-III loop (left) or the HA-tagged D9 mutant loop (right). Right: Quantification of PLA signal (a.u.) between ankyrin-B and WT NaV1.2 II-III loop versus ankyrin-B and D9 NaV1.2 II-III loop. (WT: 11.6 ± 1.5, n = 26 cells; D9: 1.4 ± 0.2, n = 24 cells). ***p < 0.0001. Mann-Whitney test.

C. Co-immunoprecipitation (co-IP) of ankyrin-B-GFP with WT NaV1.2 II-III loop or D9 NaV1.2 II-III loop. Western blots were probed with antibodies against anti-GFP (to label ankyrin-B-GFP) and anti-HA (to label NaV1.2 II-III loop-HA). Non-immune IgG used as a negative control.

D. Left: Confocal images of cultured neocortical neurons transfected with WT NaV1.2-3xFLAG-IRES-eGFP or D9 NaV1.2-3xFLAG-IRES-eGFP. Cells were immunostained with anti-GFP (purple) and anti-FLAG (magenta) antibodies. Right: Quantification of mean fluorescence intensity (a.u.) of NaV1.2-3xFLAG in the AIS (top) and dendrites (bottom). AIS: (WT: 1445 ± 233.5, n = 5 cells; D9: 170.6 ± 15.2, n = 4 cells). *p = 0.016. Mann-Whitney test. Dendrites: (WT: 620 ± 71.0, n = 5 cells; D9: 46.1 ± 9.7, n = 4 cells). *p = 0.016. Mann-Whitney test.

E. Left: IP of endogenous ankyrin-B and western blot of IP lysates probed with antibodies to ankyrin-B or endogenous NaV1.2 from P60-P75 mice. Right: IP of endogenous NaV1.2 and western blot of IP lysates probed with antibodies to NaV1.2 or ankyrin-B from P60-P75 mice. Black arrows highlight bands of ankyrin-B or NaV1.2. Non-immune IgG used as a negative control. Note: 440 kDa ankyrin-B band consistently runs anomalously high as reported previously (Jenkins et al., 2015).

Ankyrin-B directly interacts with NaV1.2 in adult mouse brain

We next evaluated the molecular basis underlying the interaction between ankyrin-B and dendritic NaV1.2. Due to their large size, it is difficult to study direct protein–protein interactions between full-length ankynrs and ion channels (Garrido et al., 2003; Lemalilet et al., 2003). Therefore,
we evaluated binding by ankyrin-B-GFP and an epitope-tagged fragment of Na$_{1.2}$ that contains a highly conserved core nine amino acid motif necessary for ankyrin binding (Na$_{1.2}$ II-III loop-HA) (Lemailliet al., 2003) (Figure 3A). We co-transfected these constructs into HEK293 cells and performed a proximity ligation assay (PLA), which reports whether ankyrin-B and the Na$_{1.2}$ II-III loop are associated within 10 nanometers of one another. Using PLA, we found that full-length ankyrin-B readily complexes with the Na$_{1.2}$ II-III loop in HEK293 cells (Figure 3B). To assess direct binding between ankyrin-B and Na$_{1.2}$, we then transfected HEK293 cells with a mutant Na$_{1.2}$ II-III loop (termed Δ9-mutant Na$_{1.2}$) from which we excised the core 9 amino acid ankyrin-binding motif. Expression of the Δ9-mutant Na$_{1.2}$ completely abolished PLA signal, indicating the importance of this motif for binding between ankyrin-B and Na$_{1.2}$ (Figure 3B). Of note, deletion of the Δ9 sequence in the Na$_{1.2}$-3xFLAG construct did not affect channel biophysical properties when compared to WT Na$_{1.2}$ recordings in HEK cells (Figure S1C). We further validated their interaction by successfully immunoprecipitating ankyrin-B-GFP and Na$_{1.2}$ II-III loop from HEK293 cells (Figure 3C). Again, we failed to detect any Δ9-mutant Na$_{1.2}$ following immunoprecipitation of ankyrin-B (Figure 3C).

Next we tested whether ankyrin-B localized full-length Na$_{1.2}$ to pyramidal cell dendrites if these channels lacked the ankyrin-binding motif. Cultured neurons were transfected with a plasmid encoding either WT Na$_{1.2}$-3xFLAG-IRE5-GFP or Δ9-mutant Na$_{1.2}$-3xFLAG-IRE5-GFP. At DIV21, WT Na$_{1.2}$-3xFLAG properly localized to the dendrites as well as the AIS; however, the Δ9-mutant Na$_{1.2}$-3xFLAG-IRE5-GFP, which is unable to interact with ankyrin-B, failed to localize to the dendrites (Figure 3D). Since excising the nine amino acid motif prevents all ankrys from binding, Na$_{1.2}$-3xFLAG clustering at the AIS was also lost (Figure 3D). Additionally, these data with the Δ9-mutant Na$_{1.2}$ are consistent with those obtained with the FF/QQ mutant ankyrin-B, further confirming that the dendritic localization of Na$_{1.2}$-3xFLAG is not an artifact of overexpression.

The experiments above demonstrate that ankyrin-B is in complex with Na$_{1.2}$ in pyramidal neuron dendrites when each protein is overexpressed in cultured neocortical neurons. To determine if this interaction occurs with endogenous ankyrin-B and Na$_{1.2}$ in native adult neocortex, we immunoprecipitated ankyrin-B using antibodies that detect both the 220 kDa and 440 kDa isoforms. Western blot and immunoblotting with antibodies against Na$_{1.2}$ revealed ankyrin-B is in complex with Na$_{1.2}$ in pyramidal neurons.
the adult neocortex (Figure 3E and S2). Since ankyrin-B is expressed in almost every cell-type within the neocortex (Smith and Penzes, 2018), we wanted to confirm their interaction in pyramidal cell dendrites, where Na\textsuperscript{+} predominantly resides, by immunoprecipitating Na\textsubscript{V}\textsubscript{1.2} -3xFLAG labeling in cultured Ank2\textsubscript{+/-} neurons compared to WT and Ank2\textsubscript{+/-} cells. While interleaved experiments in (Spratt et al., 2019, 2021). While we hypothesized that Ank2 loss would impair measures of dendritic excitability, we wanted to determine whether it would also affect somatic excitability.

To test how Ank2 haploinsufficiency affects neuronal AP electrogenesis and propagation, we recorded AP waveform properties from layer 5 (LS) thick-tufted (pyramidal tract) neurons in acute slices of P43-75 Ank2\textsubscript{+/-}:CaMKII\textsubscript{Cre} mice, which express Cre in all neocortical pyramidal neurons after -P16 (Spratt et al., 2019; Xu et al., 2000) (Figure 4A-E and S4A). This allowed for the study of Ank2 haploinsufficiency in mature cells, a period with a previously defined ASD-associated role for Scn2a in pyramidal cell dendrites (Spratt et al., 2019). Western blots of neocortical lysates generated from adult Ank2\textsubscript{+/-}:CaMKII\textsubscript{Cre} and WT littermates confirmed a significant reduction in the 440 kDa and 220 kDa isoforms of ankyrin-B, without any change in Na\textsubscript{V}\textsubscript{1.2} or ankyrin-G expression levels (Figure S3). While interleaved experiments in Scn2a\textsubscript{+/-} cells revealed expected reductions in peak somatic dV/dt, Ank2\textsuperscript{+/-} cells were not different than WT (Figure 4D-E). In addition, we observed no difference in the FI curves of Ank2\textsuperscript{+/-} neurons compared to WT and Scn2a\textsuperscript{+/-} neurons, or in measures of AIS excitability (threshold and AIS-associated peak dV/dt) (Figure 4B-E).

These empirical data suggest that loss of Ank2 does not affect somatic or axonal excitability. This result contrasts with previous results in Scn2a\textsuperscript{+/-} neurons and compartmental models where Ank2 haploinsufficiency results in similar reductions in Na\textsubscript{V}\textsubscript{1.2} density throughout the somatodendritic domain (Figure 4F). Instead, models indicate that peak dV/dt is dependent exclusively on somatic Na\textsubscript{V}\textsubscript{1.2} density and is insensitive to changes in dendritic channel density (Figure 4G), and therefore suggests that Na\textsubscript{V}\textsubscript{1.2} densities are at WT levels in Ank2\textsuperscript{-/-} cells in the soma. This motivated us to re-examine Nax1.2-3xFLAG labeling in cultured Ank2\textsuperscript{-/-} neurons, focusing on somatic regions. Indeed, Na\textsubscript{V}\textsubscript{1.2} expression was sensitive to changes in somatic ankyrin-B loss in both heterozygous or homozygous knockout conditions. While somatic recordings described above suggest that somatic Na\textsubscript{V}\textsubscript{1.2} density is not affected by Ank2 haploinsufficiency, they cannot inform on changes in dendritic excitability, which modeling suggests would still be impaired in Ank2\textsuperscript{-/-} conditions (Figure S4B). To evaluate the effects of ankyrin-B loss on dendritic Na\textsubscript{V}\textsubscript{1.2} channel density and excitability, we examined dendritic Na\textsubscript{V}\textsubscript{1.2} function directly with AP-evoked Na\textsuperscript{+} imaging using the sodium indicator ING-2 (500 μM), which is sensitive to relatively large changes in sodium concentration (K\textsubscript{Na} = 20 mM) but has higher signal-to-noise than other sodium indicators (Bömör et al., 2021, Filipis and Canepari, 2021; Lipkin et al. 2021). In WT neurons, trains of APs (40 APs at 100 Hz) evoked detectable sodium transients within the first 125 μm of the apical dendrite (Figure 5A). These transients were largest proximal to the soma and fell off with increasing distance from the soma. At 25 μm from the soma, Na\textsuperscript{+} transient amplitudes were comparable in WT and Ank2\textsuperscript{-/-} neurons. In Scn2a\textsuperscript{-/-} neurons, Na\textsuperscript{+} influx was reduced by 50%, consistent with haploinsufficiency of Na\textsubscript{V}\textsubscript{1.2} density. Farther from the soma, however, transients in Ank2\textsuperscript{-/-} neurons became significantly smaller and exhibited a 40% reduction in Na\textsuperscript{+} influx compared to the WT average (Figure 5A-B). This stark change in Na\textsuperscript{+} influx between 25 and 50 microns from the soma corresponds well to the distribution of endogenous ankyrin-G, which can extend into the first tens of microns of dendrite in cultured neurons (Figure 2C). Thus, these data are most consistent with a model where ankyrin-G is capable of scaffolding Na\textsubscript{V}\textsubscript{1.2} in the soma and proximal dendrite in Ank2 haploinsufficiency conditions, but that ankyrin-B is solely responsible for Na\textsubscript{V}\textsubscript{1.2} scaffolding in more distal dendritic domains.
Ca²⁺ transients were markedly reduced in the neurons, including distal dendritic tuft branches (Figure 6A). By contrast, previous observations, bursts of APs (a set of 5 spike doublets at 100 Hz) have not been reported to interact with ankyrin-B (Choi et al., 2019; Kline et al., 2018). Converge to regulate dendritic, but not somatic, intrinsic excitability. (Figure S5 with no difference in branch number or length compared to WT neurons note, dendritic arborization was unaltered in adult L5 neurons (Spratt et al., 2019) (Figure S6). Importantly, this dendritic Ca influx is mediated largely by arbors of layer 5 prefrontal pyramidal cells (Gulledge and Stuart, 2003; with reliable engagement of voltage-dependent calcium channels throughout dendritic haploinsufficiency affects excitability in more distal dendritic compartments, be imaged only within 150 microns of the soma. To understand how due to the sensitivity of sodium indicators, dendritic Na⁺ influx could be imaged only within 150 microns of the soma. To understand how Ank2 haploinsufficiency affects excitability in more distal dendritic compartments, we took advantage of the fact that bursts of backpropagating APs (bAPs) reliably engage voltage-dependent calcium channels throughout dendritic arbors of layer 5 prefrontal pyramidal cells (Gulledge and Stuart, 2003; Larkum et al., 1999; Short et al., 2017; Spratt et al., 2019; Stuart and Häusser, 2001). Importantly, this dendritic Ca influx is mediated largely by Ca²⁺ and Ca³⁺ channels, which, in contrast to Ca₂⁺, Ca³⁺, and Ca²⁺, 2 channels, have not been reported to interact with ankyrin-B (Choi et al., 2019; Kline et al., 2014; McKay et al., 2006; Pérez-García et al., 2013). Consistent with previous observations, bursts of APs (a set of 5 spike doublets at 100 Hz) evoked robust calcium transients throughout the apical dendrite of WT neurons, including distal dendritic tuft branches (Figure 6A). By contrast, Ca²⁺ transients were markedly reduced in the Ank2⁻/⁻ neurons, mirroring observations made in Scn2a⁻/⁻ neurons (Spratt et al., 2019) (Figure 6A). Note, dendritic arborization was unaltered in adult L5 Ank2⁻/⁻ neurons, with no difference in branch number or length compared to WT neurons (Figure S5). Taken together, these data demonstrate that Ank2 and Scn2a converge to regulate dendritic, but not somatic, intrinsic excitability.

**Ank2 heterozygous mice demonstrate impaired excitatory synaptic function**

In Scn2a⁻/⁻ cells, impaired dendritic excitability weakens postsynaptic aspects of excitatory synaptic transmission by reducing the relative number of functionally mature, AMPA-containing synapses (Spratt et al., 2019). We hypothesized that attenuated bAP in Ank2⁻/⁻ dendrites would result in similar synaptic deficits as observed in Scn2a⁻/⁻ mice. To test this, we evaluated pre- and postsynaptic components of synaptic function in Ank2⁻/⁻ mice injected with a Cre-expression adeno-associated virus (AAV-EF1α-Cre-mCherry, injections at P30, experiments at P52-60) (Figure 6B). Whole-cell voltage-clamp recordings of miniature excitatory and inhibitory mEPSCs recorded from P54-P60 WT (black) and Ank2⁻/⁻-Cre-mCherry-positive (green) neurons. Dotted line indicates when NMDA component was calculated (50 μs after stimulation onset). Bottom: Quantification of AMPA:NMDA ratio. (WT: 3.9 ± 0.6, n = 7 cells; Ank2⁻/⁻-Cre: 2.2 ± 0.2, n = 8 cells) **p = 0.0093. Mann-Whitney test. 50 ms – (WT: 1.9 ± 0.1, n = 13 cells; Ank2⁻/⁻-Cre: 1.5 ± 0.09, n = 11 cells) *p = 0.03. Mann-Whitney test. 100 ms – (WT: 2.0 ± 0.1, n = 11 cells; Ank2⁻/⁻-Cre: 1.3 ± 0.08, n = 12 cells) *p = 0.0317. Mann-Whitney test.
Inversely proportional to release probability, was also altered, an effect not observed in Scn2a−/− conditions. Interestingly, we observed a decrease, rather than an increase, in PPR, suggesting that release probability had increased despite an overall reduction in mEPSC frequency (Figure 6E). This suggests that ankyrin-B has differential roles in dendritic and axonal compartments and that impaired dendritic excitability results in an increased proportion of AMPA-lacking synapses and a reduction in mEPSC frequency. This large effect of silent synapses may mask the expected increases in mEPSC frequency due to increased release probability.

To test this, we isolated the postsynaptic contributions of ankyrin-B by injecting a dilute AAV-EF1α-Cre-mCherry virus into the mPFC of Ank2+/- mice at P30 (Figure 7A). In these conditions, cell-autonomous effects of Ank2 haploinsufficiency in dendrites could be assessed by patching one of the few mCherry positive neurons that largely receive input from largely mCherry-negative (e.g., WT) inputs (Figure 7B). In these conditions, we found a significant reduction in AMPA/NMDA ratio in Ank2−/− neurons with no change in PPR (Figure 7C-D). Taken together, these results indicate that, postsynaptically, ankyrin-B is critical for scaffolding of dendritic Na⁺s and that Ank2 haploinsufficiency phenocopies dendritic deficits observed in Scn2a−/− neurons. Additional functions for Ank2 are present in axons, where its loss increases release probability. Thus, in neocortex, Ank2 haploinsufficiency converges with Scn2a haploinsufficiency in pyramidal cell dendrites, but has points of divergence in axons and the soma.

DISCUSSION:
Ankyrin-B variants have long been known to contribute to cardiac dysfunction through their function in scaffolding a range of membrane pumps, ion exchangers, and receptors (Mohler et al., 2003). Here, we provide evidence that ankyrin-B (ANK2) functions as the primary scaffold for Na⁺,1,2 (SCN2A) in the dendrites of neocortical pyramidal neurons. Haploinsufficiency of Ank2 in prefrontal neocortical neurons caused dendritic excitability and synaptic deficits, due to reduced Na⁺ channel density within the dendritic membrane, which phenocopies Scn2a haploinsufficiency conditions. These findings establish a direct, convergent mechanism between two major ASD-associated genes and add to a growing body of literature demonstrating dysfunction in dendritic excitability in ASD (Brager and Johnston, 2014; Brandalise et al., 2022; Johnston et al., 2016; Nelson and Bender, 2021).

Subcellular patterning of ankyrins
While ion channels and ankyrin scaffold interactions have been studied extensively in axonal domains (Bender and Trussell, 2012; Huang and Rasband, 2018; Kole and Stuart, 2012; Leterrier, 2018; Nelson and Jenkins, 2017), the mechanisms governing dendritic localization of Na⁺s have received far less attention. Data here support a model where ankyrin-G and ankyrin-B have largely distinct roles in Na⁺ scaffolding, with ankyrin-G localized to excitatory parts of the axon (e.g., AIS and nodes of Ranvier), and ankyrin-B localized to other regions, including dendrites. We show that ankyrin-B is critical for scaffolding Na⁺,1,2 in this domain, and does so along the dendritic shaft membrane. Consistent with this, complete knockout of ankyrin-B in cultured neurons eliminates dendritic Na⁺,1,2 immunostaining (Figure 1), and Ank2−/− conditions decrease AP-evoked dendritic Na⁺ influx by ~50% (Figure 5). Interestingly, ankyrin-G is known to localize within spines, but does so in support of synapse function rather than Na⁺ scaffolding (Smith et al., 2014). Thus, these data are consistent with the hypothesis that dendritic sodium channels are scaffolded exclusively by ankyrin-B, and that heterozygous or homozygous loss of ankyrin-B cannot be compensated for by other ankyrins.

Intriguingly, one place where ankyrin-B and ankyrin-G overlap, and appear to serve compensatory Na⁺ scaffolding roles, is at the somatic membrane. Here, we observed a disconnect between Ank2−/− and Scn2a−/− conditions, as Ank2−/− neurons did not show a decrease in peak AP velocity, which is one hallmark of Scn2a−/− neurons (Figure 4) (Spratt et al., 2019, 2021). This indicates that somatic Na⁺s can be scaffolded at the soma at WT levels in Ank2−/− conditions, either by ankyrin-G, or by preferential recruitment of extant ankyrin-B to the soma over other compartments. In support of the former, we found that Na⁺,1,2-3xFLAG constructs were still present at the soma in Ank2−/− cultured neurons, despite being absent from dendrites (Figure 4). Methods to label each ankyrin, their splice variants, and Na⁺ subtypes scaffolded to each of these compartments in neurons will be useful to unravel these complexities.

Taken together, these findings suggest that both ankyrins can scaffold somatic Na⁺s, but why they fail to compensate for one another outside the soma remains unclear. One explanation may lie in how ankyrin-G and ankyrin-B are themselves localized to different neuronal compartments. Previous studies have shown that ankyrin-G requires the post-translational modification S-palmitoylation for its membrane association at the AIS (He et al., 2012). S-palmitoylation is a process mediated by a family of 23 palmitoyl acyl transferases (zDHHC PATs) that covalently adds a 16-carbon fatty acid chain to a conserved cysteine 70 (C70) that resides within the ankyrin repeats (Globa and Bamji, 2017). Two of these PATs, zDHHC5 and zDHHC8, are known to palmitoylate ankyrin-G at the AIS (He et al., 2012), but whether ankyrin-B is palmitoylated in the dendrites has not been determined. Alternatively, ankyrin localization to discrete domains may be driven by autoinhibition mechanisms from unstructured C-terminal ankyrin.

Figure 7: Cell-autonomous Ank2 haploinsufficiency results in postsynaptic excitatory synaptic function
A. Left: Neurons were sparsely transduced by injecting a diluted AAVs-EF1α-Cre-mCherry virus in mPFC of P30 Ank2+/− neurons. Right: 2PLSM single optical sections of mCherry fluorescence (red) overlaid with scanning DIC image (grayscale) showing Ef1a-Cre-mCherry expression in a subset of Ank2+/− L5 pyramidal neurons.
B. Schematic demonstrating Ank2+/−-Cre-mCherry neuron targeted for whole-cell recording (red). Presynaptic Cre-neurons are WT (gray).
C. Top: AMPA/NMDA ratio of evoked EPSCs from P52-73 WT (black) and Ank2+/−-Cre-mCherry (green) neurons. Bottom: Summary of AMPA/NMDA ratio. WT: (3.3 ± 0.2, n = 10 cells; Ank2+/−-Cre: 2.5 ± 0.3, n = 9 cells) *p = 0.02. Mann-Whitney test.
D. Top: Paired-pulse ratio of evoked excitatory inputs at 25, 50, and 100 ms intervals in P52-73 WT (black) and Ank2+/−-Cre-mCherry (green) neurons. Bottom: PPR grouped by inter-stimulus interval. 25 ms – (WT: 1.8 ± 0.1, n = 10 cells; Ank2+/−-Cre: 1.9 ± 0.2, n = 10 cells) * p > 0.99. Mann-Whitney test. 50 ms – (WT: 1.9 ± 0.1, n = 11 cells; Ank2+/−-Cre: 1.9 ± 0.1, n = 11 cells) * p = 0.7. Mann-Whitney test. 100 ms – (WT: 1.6 ± 0.9, n = 10 cells; Ank2+/−-Cre: 1.6 ± 0.07, n = 10 cells) * p = 0.6. Mann-Whitney test.

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domains, which have been shown to play an important role in restricting ankyrin activity throughout the cell (Chen et al., 2017).

In addition to differential localization of ankyrin family members, individual splice variants of ankyrin-G and ankyrin-B have unique localization patterns and functions. Alternative splicing of Ank2 gives rise to two main isoforms of ankyrin-B in the brain: a canonical 220 kDa isoform and a larger 440 kDa splice variant, which contains a single 6.4-kb neuron-specific exon within the middle of the gene (Chan et al., 1993; Kunimoto, 1995; Kunimoto et al., 1991). Here, we found that the 220 kDa ankyrin-B is expressed throughout dendrites, whereas 440 kDa isoform was dominant in distal axons (Figure 2). Using conditional Ank2 alleles, we removed Ank2 from prefrontal pyramidal cells to isolate cell-autonomous, postsynaptic roles of the 220 kDa isoform, and found that it is capable of scaffolding NaV1.2 through direct interaction. This impaired dendritic excitability and excitatory synaptic function in ways that converged with those observed in Scn2a+/− mice (Scn2a+/− mice result in comparable impairments in AP-evoked Na+ transients, AMPA:NMDA ratio, and mEPSC frequency (Aru et al., 2020; Branco and Häusser, 2010; Branco et al., 2010; Gidon and Johnston, 1995; Shah, 2014), or changes in excitation that promote dendritic non-linear events or inhibition that limits such activity (Gidon and Segev, 2012; Megias et al., 2001; Ujfalussy et al., 2015; Wilson et al., 2012; Zhang et al., 2013). These effects can be overt, with ASD-associated variants directly affecting genes encoding dendritic or synaptic proteins in question, or covert, with ASD-associated variants instead affecting gene regulatory elements that in turn alter protein expression (reviewed in Nelson and Bender, 2021).

NaV1.2 is critical for dendritic excitability throughout life. Conditionally-induced heterozygosity of Scn2a late in development results in identical impairments in dendritic and synaptic function as observed in constitutive Scn2a heterozygotes (Spratt et al., 2019). Excitatory synapses appear similar to those found in immature neurons, with relatively small spine heads and low AMPA:NMDA receptor ratios, suggesting that synapses may be maintained in an immature, pre-critical period state (Spratt et al., 2019). Indeed, restoration of near WT levels of Scn2a, either via Cre-induced genetic rescue or CRISPR activator-based upregulation of the residual, functional allele in Scn2a heterozygotes, restores dendritic excitability, synapse morphology, and synapse function to WT levels (Tamura et al., 2022). This suggests that restoration of Ank2 function would have similar effects to Scn2a, at least in dendritic regions where it actively scaffolds NaV1.2. ANK2 gene are too large for traditional gene therapy approaches; however, several other approaches are maturing for gene regulation in neurodevelopmental disorders, with marked progress for a number of genetic conditions (Colasante et al., 2020; Derbis et al., 2021; Han et al., 2020; Tamura et al., 2022; Ure et al., 2016; Weuring et al., 2021; Wolter et al., 2020). In addition, a better understanding of the unique roles of different ankyrin proteoforms in scaffolding and function of various binding partners (Gidon and Segev, 2012; Ujfalussy et al., 2015; Wilson et al., 2012; Zhang et al., 2013) provide insight into methods that allow ankyrins to better compensate for one another in dendritic compartments. Overall, these establish a framework that both Scn2a and Ank2 models are forms of channelopathies contributing to ASD (Plaček, 2015), motivating future research on potential convergent impairments in channel and dendritic function associated with other ASD/ID risk genes.

METHODS:

CONSTRUCTS, ANTIBODIES, AND EXPERIMENTAL MODELS:

Human Na1,2-3xFLAG IRES eGFP was generated by Genscript (Piscataway, NJ) by the addition of a short linker (AAARG) and a triple-FLAG epitope (DYKDDDDK) to the carboxy terminus of codon-optimized human Na1,2 IRES eGFP (Ben-Shalom et al., 2017). Δ9 Na1,2-3xFLAG IRES eGFP was created by Genscript by deletion of the necessary nine amino acid core ankyrin-binding motif, described previously (Lemailliet et al., 2003). The HA-tagged Na1,2 II-III loops (wild-type and Δ9) were cloned by creating the coding sequence corresponding to amino acids 991-1211 of human Na1.2 into pENTR D-TOPO by polymerase chain reaction. The loops were shuttled into pCSF107mT-GATEWAY-3-3HA (gift from Todd Stucken, Addgene plasmid # 67616) using Gateway LR clonase, according to manufacturer’s instructions (Thermo Fisher). Na1.2-V5-2A-DsRed was a generous gift from Dr. Lori Isom (University of Michigan) (Bouza et al., 2021). 220 kDa ankyrin-B GFP was previously described (Mehler et al., 2002). 440 kDa ankyrin-B-GFP was created by subcloning the additional giant exon from 440 kDa ankyrin-B-Halo (Yang et al., 2019) into 220 kDa ankyrin-B-GFP using BsiZ171 and SacII sites. 220 kDa ankyrin-B F131Q/I164Q (Wang et al., 2014) was created by
Genscript by site-directed mutagenesis. TagBFP and Cre-2A-TagBFP were previously described (Tseng et al., 2015). All plasmids were sequenced across the entire coding sequence by Sanger sequencing prior to use in experiments.

Lab-generated antibodies to ankyrin-B and ankyrin-G were described previously, including rabbit anti-ANK2-G C-terminus (Kizhatil et al., 2007), goat anti-ankyrin-G C-terminus (He et al., 2014), rabbit anti-270/480 kDa ankyrin-G (Jenkins et al., 2015), rabbit anti-480kDa ankyrin-G (Jenkins et al., 2015), rabbit anti-ankyrin-B C-terminus (Ayalon et al., 2008), sheep anti-ankyrin-B C-terminus. Specificity of all lab-generated antibodies are confirmed using respective null mouse tissue. In addition, antibodies are tested for ankyrin cross-reactivity by both immunocytochemistry and western blotting in HEK293 cells expressing 220 kDa ankyrin-B-GFP or 190 kDa ankyrin-G-GFP. Commercial antibodies used in these studies include rabbit anti-nav1.1 (Abcam, ab51613), mouse anti-FLAG-M2 (Sigma, F8578), rabbit anti-anti-GFP (clone e62, Cell Signaling Technologies), rabbit anti-HA epitope (clone C29F4, Cell Signaling Technologies), and guinea pig anti-MAP2 (Systicrpath, 188-004).

C57BL/6J mice were obtained from Jackson Laboratories (stock #000664). The Ank2 exon 24a in vivo mouse line was a gift from Dr. Peter Mohler (The Ohio State University) (Robert et al., 2019). Scn2a mice were provided by Drs. E. Glasscock and M. Montal (Mishra et al., 2017; Planelles-Cases et al., 2000).

CO-IMMUNOPRECIPITATION:

Whole brain was dissected from C57Bl/6J adult mice (P60-75). Each brain was homogenized in 2 ml of reaction buffer (0.3 M sucrose, 10 mM Phosphate, 2 mM EDTA; pH 7.4), mixed with phosphatase inhibitor and protease inhibitor, 500 µl of 20 mM DSP (Lomant’s Reagent) was added to the sample and incubated for 2 hours on ice, after which the crosslinking reaction was quenched by adding 1x Tris to a final concentration of 50 mM and incubated on ice for 15 minutes. Samples were lysed by mixing with lysis buffer (30 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% IGEPAI, 0.5% Sodium Deoxycholate; pH 6.8) and sonication 20 times at 1-second-long pulses, followed by ultracentrifugation at 100k g for 30 minutes. Solubilized proteins were then subjected to immunoprecipitation using magnetic beads bound to antibodies (Bio-Rad SureBeads Protein A; rabbit ankyrin-B 1:250, rabbit Nav1.2 1:900, rabbit IgG 1:250). Lysate samples were rotated with the bead mixture overnight at 4°C. Beads were collected the next day, washed 3 times with lysis buffer, and mixed 1:1 with 5x PAGE buffer (5% SDS, 25% sucrose, 50 mM Tris; pH 9, 0.5 mM EDTA) and heated to 68°C for 10 minutes.

WESTERN BLOT:

Samples were separated on a 3.5-17% gradient gel in 1x Tris buffer, pH 7.4 (40 mM Tris, 20 mM NaOAc, and 2mM NaEDTA) with 0.2% SDS. Transfer to nitrocellulose membrane was performed overnight at 300 mA at 4°C in 0.5x Tris buffer with 0.1% SDS. Membranes were blocked with 5% Bovine Serum Albumin (BSA) in TBS at room temperature for 1 hour and incubated in primary antibodies (rabbit ankyrin-B 1:1000; rabbit Nav1.2 1:500; mouse a-tubulin 1:1000) diluted in 5% BSA in TBS-T overnight at 4°C. Membranes were washed 3x for 10 minutes with TBS-T and incubated for 1 hr at room temperature with LiCor fluorescent secondaries (1:15,000) in 5% BSA in TBS-T. Membranes were then washed 3x for 10 minutes in TBS-T, 3x for 5 minutes in ddH₂O before being imaged on LiCor Odyssey Clx imager.

PROXIMITY LIGATION ASSAY:

HEK293 cells were obtained from the American Type Culture Collection and maintained in a humidified environment at 37 °C with 5% CO₂. Cells were cultured in DMEM (Invitrogen #11995) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. 100,000 cells were cultured in DMEM (Invitrogen #11995) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. 100,000 cells were cultured in DMEM (Invitrogen #11995) with 10% fetal bovine serum, 2% CO₂, 1.5 CaCl₂, 1.5 MgCl₂, 100 mM Hepes, then triturated gently through a glass pipette with a fire-polished tip. The dissociated neurons were then plated on 35mm MatTek dishes, precoated with poly-D-lysine and laminin, in 0.5ml of Neurobasal-A medium containing 10% FBS, B27 supplement, 2 mM glutamate, and penicillin/streptomycin. On day in vitro 1 (DIV), the neurons were washed with Neurobasal-A medium and fed with growth media (2.5mL of fresh Neurobasal-A medium containing 1% FBS, B27, glutamine, penicillin/streptomycin, and 2.5 µg/mL ARAc). On DIV3, plasmsids were introduced into neonatal neurons through lipofectamine 2000-mediated transfection. In one tube, 500 ng of each plasmid was added to 200 µL of Neurobasal-A, and in another tube, lipofectamine 2000 (3 µL 1 µg plasmid) was added to 200 µL of Neurobasal-A. The two tubes were then mixed and incubated at room temperature for 15 min. The neuronal growth media was then removed from the dishes and saved, and transfection media was added to the tubes. Cells were incubated in transfection media for 1 hr at 37°C. The transfection media was aspirated, cells were washed once with warm Neurobasal-A, and growth media was added back to plates. The cells were maintained in culture until 7 DIV or 21 DIV and fixed for immunofluorescence as described below.

Dissociated neonatal neurons were fixed for 15 minutes at room temperature with 4% paraformaldehyde, followed by permeabilization with 0.2% Triton in 1X PBS pH7.4 for 10 minutes at room temperature. They were then blocked with blocking buffer (5% BSA, 0.2% Tween 20 in 1X PBS pH7.4) at room temperature for 30 minutes. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. The next day, cells were washed at room temperature three times for 15 minutes with PBS containing 0.2% Tween 20. Then the cells were incubated with secondary antibodies diluted in blocking buffer for one hour at room temperature. The cells were washed at room temperature three times for 15 minutes with PBS containing 0.2% Tween 20 and then mounted with ProLong Gold antifade reagent before imaging with confocal microscopy as described below.

CONFOCAL MICROSCOPE:

Samples were imaged on a Zeiss LSM 880 with Airyscan using a 63x 1.4 Plan-Apochromat objective and excitation was accomplished using 405-, 488-, 561-, and 633-nm lasers. Each experiment was repeated at least three independent times. Measurements were taken using Fiji software (Schindelin et al., 2012). Laser power and imaging parameters were kept constant for each immunocytochemistry condition.

IN VITRO CELL ELECTROPHYSIOLOGY:

Voltage-clamp recordings were performed at room temperature in standard whole-cell configuration, using Axopatch 700B amplifier and pClamp (version 10, Axon Instruments, Foster City, CA) and a Digitizer 1440A digitizer (Molecular Devices). Sodium current was recorded in the presence of external recording solution containing in mM: 120 NaCl, 4 KCl, 1 MgCl₂, 1.5 CaCl₂, 10 HEPES, 45 Glucose and 30 Sucrose (pH 7.35 with CsOH; osmolality was 300–305 mOsm). For the 81 subunit co-transfection experiments, the external sodium concentration was reduced to 60mM. Fire-polished patch pipettes obtained from borosilicate glass capillary (WPI) which resistance was between 1.5-3.5 MΩ, were filled with an internal solution containing in mM: NaCl, 105 Cs-A, 10 CsCl, 10 EGTA, 10 HEPES, (pH 7.2 with H₂SO₄). To determine sodium current amplitude and voltage dependence of activation, currents were evoked by depolarization to 250 ms to different potentials,(from −120 to 30 mV on 5 or 10 mV steps) from holding potential of −80 mV and a hyperpolarizing −120mV, 250 ms pre-pulse. Voltage-dependence of inactivation was determined by applying a 50 ms test pulse of 0 mV after the 250 ms pulses used for voltage dependence of activation. Series resistance was compensated no more than 40%–65% when needed, and leak subtraction was performed by application of a standard P/4 protocol. Signals were low-pass filtered at 10 kHz, and data were sampled at 40 kHz online. Current densities were determined by dividing current amplitude by the cell capacitance (Cm) measured by pClamp software. Normalized conductance and inactivation curves were generated as previously described (Patino et al., 2017).
COMPARTMENTAL MODELING:
A pyramidal cell compartmental model was implemented in the NEURON environment (v7.7) based on the Blue Brain Project thick-tufted layer 5b pyramidal cell (TTPC1) model used in our previous study (Ben-Shalom et al., 2017; Markram et al., 2015; Ramaswamy and Markram, 2015; Spratt et al., 2021). The TTPC1 model was adjusted to include an AIS, and the original Na channels in the TTPC1 model were replaced with Na1,2 and Na1,6 channels in compartments with densities as previously shown (Spratt et al., 2021). For phase plane comparisons, the first AP was evoked with a stimulus of 500 pA intensity (25 ms duration) in each model configuration. Threshold was defined as the membrane potential when dV/dt exceeds 15 V/s. For AP backpropagation, a single AP was evoked with a 1.2 nA, 8 ms step current applied to the somatic membrane. In model conditions with only Na1,2 contributing Na conductance in the distal apical dendrite, Na1,6 was replaced with Na1,2 after ~30 microns from soma and total Na1,2 conductance was increased by a factor of 1.9 to match total conductance levels in the mixed Na1,2/Na1,6 model (since Na1,6 voltage dependence is more hyperpolarized). Voltage was recorded from the soma, shaft of the apical dendrite (460 µm from soma), and branch of the apical tuft (975 µm from soma). Conductance densities for sodium channels in different compartments across models were as in Table 1: 1.2 and Na1,6 in WT conditions in all compartments

<table>
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<tr>
<th>Compartment</th>
<th>Conductance (mho/cm2)</th>
<th>Model with both Na1,2 and Na1,6 in distal dendrite</th>
<th>Model with Na1,2 alone in distal dendrite</th>
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<td>(peak)</td>
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EX VIVO ELECTROPHYSIOLOGY:
All experiments were performed in accordance with guidelines set by the University of California Animal Care and Use Committee. Mice aged P5-75 were anesthetized under isoflurane. Brains were dissected and placed in 4 °C cutting solution consisting of (in mM) 87 NaCl, 25 NaHCO3, 25 glucose, 75 sucrose, 2.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2, and 7 MgCl2, and bubbled with 5% CO2/95% O2. Coronal slices 250 µm-thick were obtained that included the medial prefrontal cortex. Slices were then incubated in a holding chamber with sucrose solution for 30 mins at 33 °C, then placed at room temperature until recording. Recording solution consisted of (in mM): 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 25 NaHCO3, 1.25 NaH2PO4, 25 glucose, bubbled with 5% CO2/95% O2. Osmolarity of the recording solution was adjusted to approximately 310 mOsm. All recordings were performed at 32-34 °C.

Neurons were identified using differential interference contrast (DIC) optics for conventional visual-guided whole-cell recording, or with two-photon-guided imaging of AAV-EF1α-Cre-mCherry fluorescence overlaid on a scanning DIC image of the slice. Patch electrodes were pulled from Schott 8250 glass (3-4 MΩ tip resistance). For current-clamp recordings, patch electrodes were filled with a K-glucuronate-based internal solution that contained (in mM): 113 K-Glucuronate, 9 HEPES, 4.5 MgCl2, 0.1 EGTA, 14 Tris-2-phosphocreatine, 4 Na2-ATP, 0.3 Tris-GTP; 290 mOSM, pH: 7.2-7.25. For Ca2+ imaging, EGTA was replaced with 250 µM Fluo-5F and 20 µM Alexa 594. For voltage-clamp recordings, a CsCl-based internal solution was used that contained (in mM): 110 CsMeSO4, 40 HEPES, 1 KCl, 4 NaCl, 4 Mg-ATP, 10 Na-phosphocreatine, 0.4 Na-GTP, 5 OX-314, and 0.1 EGTA; ~290 mOsm, pH 7.22.

Electrophysiological recordings were collected with a Multiclamp 700B amplifier (Molecular Devices) and a custom data acquisition program in Igor Pro software (Wavemetrics). Current-clamp recordings of action potential waveform were acquired at 50 kHz and filtered at 20 kHz. Pipette capacitance was compensated by 50% of the fast capacitance measure under gigahorn seal conditions in voltage-clamp prior to establishing a whole-cell configuration, and the bridge was balanced. Voltage-clamp experiments were acquired at 10-20 kHz and filtered at 3-10 kHz. Pipette capacitance was completely compensated, and series resistance was compensated 50%. All data were corrected for measured junction potentials of 12 and 11 mV in K-glucuronate and Cs-based internals, respectively. Data inclusion was based on previously established metrics (Bender and Trussell, 2009; Bender et al., 2010; Spratt et al., 2019), and includes measures for recording stability and cell health [e.g., stable series resistance >180 MΩ, membrane potential (Vm), and input resistance (Rin) with less than 15% change over data collection epochs]. All recordings were made using a quartz electrode holder (Sutter Instrument) to minimize electrode drift within the slice.

All acute slice recordings were made from layer 5b thick-tufted pyramidal tract (PT) neurons in the medial prefrontal cortex. In current-clamp, layer 5b neurons were characterized as those that exhibited a voltage rebound more depolarizing that Vth in response to a strong hyperpolarizing current (~400 pA, 120 ms) that peaked within 90 ms of current offset and depolarizing (300 ms, 200-300 pA) square current pulses from a holding potential of ~80 mV (Clarkson et al., 2017). AP threshold, AIS dV/dt and peak dV/dt were determined from the first AP evoked by a step current (300 ms duration; 200-300 pA) delivered to the somatic pipette within the first 2 minutes of establishing the whole-cell recording configuration. AP threshold was defined as the Vm when dV/dt first exceeded 15 V/s. AIS peak dV/dt was defined at the saddle point between two positive inflection points in the second voltage derivative that occur during the depolarizing phase of the AP.

Miniature inhibitory and excitatory postsynaptic currents (mEPSCs, mIPSCs) were acquired in voltage-clamp configuration at ~80 mV and 0 mV, respectively, in the presence of 10 µM R-CPP and 400 nM TTX. Events were analyzed using a deconvolution-based event detection algorithm within IgorPro (Pernia-Andrade et al., 2012). Detectable events were identified using a noise threshold of 3.5x with a minimum amplitude of 2 pA and a 2 ms inter-event interval. Events were subsequently manually screened to confirm appropriate event detection. Event detection code is available at https://benderlab.ucsf.edu/resources. Cumulative probability distribution of mEPSCs and mIPSCs event intervals were generated per cell and then averaged. Distributions were compared using the Kolmogorov-Smirnov test. A confidence interval of 95% (P < 0.05) was required for values to be considered statistically significant. In experiments measuring AMPA:NMDA ratio and paired-pulse ratio (PPR), EPSCs were evoked using a bipolar glass theta electrode placed in layer 5b ~200 µm lateral from the recording neuron. AMPA: NMDA ratio was initially measured at ~80 mV to assess the AMPA contribution and then at +30 mV to evaluate the NMDA-mediated component in the presence of 25 µM picrotoxin. AMPA was defined as the peak inward current at -80 mV and NMDA as the outward current 50 ms after stimulus onset at +30 mV. PPR was acquired at ~80 mV in the presence of 10 µM R-CPP and 25 µM picrotoxin. mEPSCs, mIPSCs, and AMPA:NMDA ratio were collected from Ank2+/- mice injected with AAV-EF1α-Cre-mCherry and wild type littermates. Mice were anesthetized with isoflurane and positioned in a stereotaxic apparatus. 500 nL volumes of AAV-EF1α-Cre-mCherry (UNC vector core) were injected into the mPFC of Ank2+/- mice (n = 3) with a 1:1.7:0.35 dilution of dextran (Dil) to -2.6). Experiments were conducted four-week post-injection. Paired-pulse ratio (PPR) was recorded from Ank2+/-:CaMKIIα-Cre mice and wild type littermates. For AMPA:NMDA and PPR experiments in spare cre-expressing animals, AAV-EF1α-Cre-mCherry was diluted 1:3 in saline, then injected into the mPFC of Ank2+/- mice.

TWO-PHOTON IMAGING:
Two-photon laser scanning microscopy (2PLSM) was performed as previously described (Bender and Trussell, 2009). A Coherent Ultra II was tuned to 810 nm for calcium imaging, ING-2 sodium imaging, and morphology experiments. Epi- and transfluorescence signals were captured either through a 40x, 0.8 NA objective for calcium imaging or a 60X, 1.0 NA objective for ING-2 imaging paired with a 1.4 NA oil immersion condenser (Olympus). For calcium imaging, fluorescence was split into red and green channels using dichroic mirrors and band-pass filters (575 DCXR, ET525/70 m-2p, ET620/60 m-2p, Chroma). Green fluorescence (Fluo-5F) was captured with 10770-40 photomultiplier tubes selected for high quantum efficiency and low dark counts (PMTs, Hamamatsu). Red fluorescence (Alexa 594) was captured with R9110 PMTs. Data were collected in linescan mode (2-2.4). Cumulative probability distribution of (Fluo-5F) was captured with 10770-40 photomultiplier tubes selected for high quantum efficiency and low dark counts (PMTs, Hamamatsu). Red fluorescence (Alexa 594) was captured with R9110 PMTs. Data were collected in linescan mode (2-2.4). Cumulative probability distribution of
REFERENCES:


Chen, K., Li, J., Wang, C., Wei, Z., and Zhang, M. (2017). Ultrafast Sodium Imaging of the Axon Initial Segment of Neurons in Mouse Brain Slices. BioRxiv 2020.05.31.494205; this version posted June 1, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY-NC-ND 4.0 International license.