Effects of chronic lithium treatment on neuronal excitability and GABAergic transmission in an *Ank3* mutant mouse model

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ABSTRACT

Bipolar disorder (BD) is a common psychiatric disease that can lead to psychosocial disability, decreased quality of life, and high risk for suicide. Genome-wide association studies have shown that the *ANK3* gene is a significant risk factor for BD, but the mechanisms involved in BD pathophysiology are not yet fully understood. Previous work has shown that ankyrin-G, the protein encoded by *ANK3*, stabilizes inhibitory synapses in vivo through its interaction with the $GABA_A$ receptor-associated protein (GABARAP). We generated a mouse model with a missense p.W1989R mutation in *Ank3*, that abolishes the interaction between ankyrin-G and GABARAP, which leads to reduced inhibitory signaling in the somatosensory cortex and increased pyramidal cell excitability. Humans with the same mutation exhibit BD symptoms, which can be attenuated with lithium therapy. In this study, we describe that chronic treatment of *Ank3* p.W1989R mice with lithium normalizes neuronal excitability in cortical pyramidal neurons and increases inhibitory GABAergic postsynaptic currents. The same outcome in inhibitory transmission was observed when mice were treated with the GSK-3β inhibitor Tideglusib. These results suggest that lithium treatment modulates the excitability of pyramidal neurons in the cerebral cortex by increasing GABAergic neurotransmission, likely via GSK-3 inhibition. In addition to the importance of these findings regarding *ANK3* variants as a risk factor for BD development, this study may have significant implications for treating other psychiatric disorders associated with alterations in inhibitory signaling, such as schizophrenia, autism spectrum disorder, and major depressive disorder.

INTRODUCTION

Bipolar disorder (BD) is a psychiatric illness with a global prevalence estimated to be 1-4% [1,2], characterized by cycles of mania and mild or severe depression, and associated with progressive degenerative functional impairment [3-5]. Patients with BD frequently experience lingering symptoms that affect their mood, psychosocial abilities, and quality of life [6,7], likely contributing to a lower life expectancy [8-10]. The cause of BD is most likely a combination of genetic, neurochemical, and environmental factors, with heritability estimates as high as 80% [11,12]. However, few genes have been consistently linked in large-scale genomic studies [13].

According to genome-wide association studies, the *ANK3* gene is a significant risk locus associated with BD [14-21]. *ANK3* gene encodes ankyrin-G, a scaffolding protein that organizes excitable membrane domains [22,23]. In neurons, ankyrin-G is necessary for the assembly, formation, and organization of the axon initial segment (AIS) and nodes of Ranvier [23,24], which are necessary for action potential (AP) initiation and propagation, respectively [25,26]. Ankyrin-G also stabilizes GABAergic synapses at the AIS and soma of pyramidal neurons by interacting with GABARAP to reduce $GABA_A$ receptor endocytosis, a mechanism that contributes to the regulation of neuronal excitability [27,28]. Experimental models with deficits in ankyrin-G expression or *ANK3*-specific genetic variants found in patients have been developed to elucidate the relationship between *ANK3* and BD. Ablation of *Ank3* in forebrain pyramidal neurons or deletion of *Ank3*-exon1b, which is expressed in the cerebellum and in forebrain inhibitory neurons, causes increased anxiety and mania-like behavior [29,30]. We previously reported that the *Ank3* p.W1989R mouse, which harbors a tryptophan (W) to arginine (R) missense mutation that was found in a family diagnosed with BD, exhibits decreased forebrain gamma oscillations hyperexcitable pyramidal neurons, and reduced GABAergic neurotransmission [28]. Such regulation is significant since the GABAergic system is important for maintaining homeostasis of neuronal circuits and proper brain function. Studies in humans suggest that altered GABAergic function underlies neuronal deficits across multiple psychiatric disorders, including BD, disrupting the excitatory/inhibitory balance (E/I) [31-36]. Other evidence indicates that dysfunction in GABAergic networks may contribute to the psychopathological symptoms observed in patients with BD [37]. This previous evidence suggests that ankyrin-G deficiencies, deficits in GABAergic transmission, and the pathophysiology of BD are likely closely related.

Lithium has been found to be an effective therapy for mania, reducing suicide and overall mortality rates [38]. Despite its limited therapeutic spectrum and potential side effects [39] lithium is still the first-line medication for the prevention of BD symptoms [40,41]. Although lithium has been used extensively for more than 60 years as a medication, its mechanism of action and the reasons why it fails in a majority of patients with BD are poorly understood [42,43]. Since lithium treatment improves anxiety- and mania-like behavior in *Ank3* mutant mouse models [29,30], the objective of this study was to determine how lithium therapy impacts the neuronal physiology in the *Ank3* p.W1989R mice [28].

Chronic lithium treatment in *Ank3* p.W1989R mice resulted in an increase in inhibitory GABAergic postsynaptic currents, reduction of hyperexcitability of pyramidal neurons, and an elongation of the AIS. Since GSK-3 blockade has been proposed as one of the main therapeutic targets of lithium [44-47], we compared the effect of blocking GSK-3β with its specific inhibitor Tideglusib [48-51]. Interestingly, the chronic treatment with Tideglusib likewise increases the inhibitory postsynaptic currents, suggesting that lithium-dependent rescue of GABAergic signaling is due, at least in part, to inhibition of GSK-3β. In addition to providing important information about the mechanism of action of lithium and Tideglusib as therapy, these results advance our understanding of the pathophysiology and treatment of BD and other psychiatric diseases related to loss of inhibitory transmission.

RESULTS

Chronic lithium therapy increases the frequency of inhibitory postsynaptic currents from presynaptic interneurons in *Ank3* p.W1989R mice.

To explore if lithium treatment modifies inhibitory neurotransmission in *Ank3* p.W1989R mice, we performed whole-cell patch-clamp recordings in pyramidal neurons in WT, *Ank3* p.W1989R, and *Ank3* p.W1989R mice treated with lithium for 19-21 days. *Ank3* p.W1989R littermates given lithium

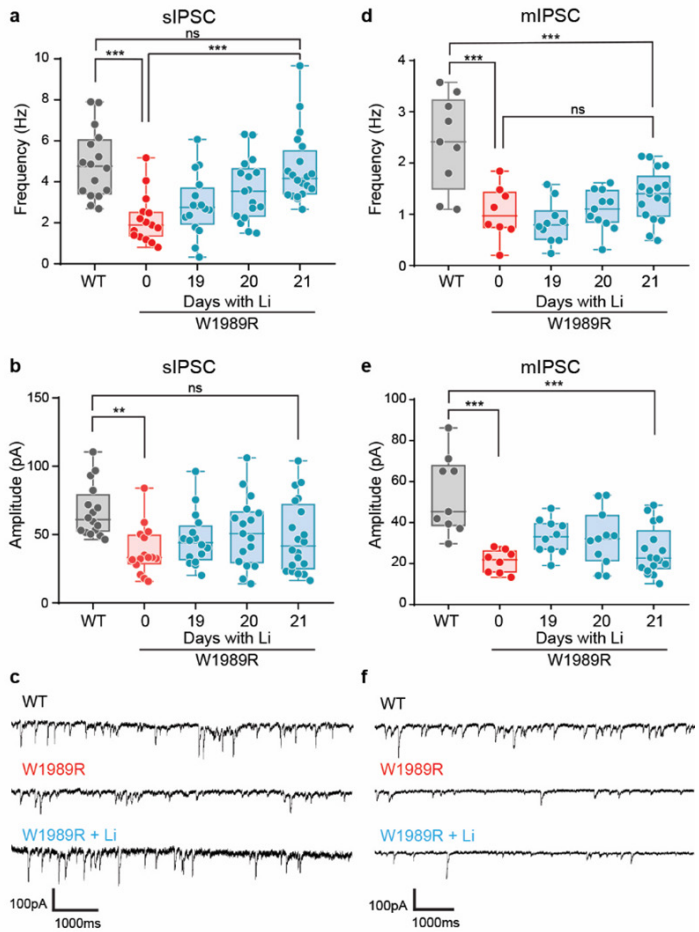

Figure 1. Chronic lithium treatment increases IPSC frequency, while not affecting current amplitude, in *Ank3* p.W1989R mice.
(a) Quantification of sIPSC and frequency (Hz) and (b) amplitude (pA) from cortical neurons from brain slices of WT (dark gray circles), *Ank3* p.W1989R (red circles), and *Ank3* p.W1989R after chronic lithium treatment 19-21 days (blue circles). Kruskal-Wallis, Dunn's multiple comparisons test ***P < 0.01 and P= ns (WT: n=16; p.W1989R: n = 15; p.W1989R/Day 19 + Li: n = 16; p.W1989R/Day 20 + Li: n = 17 and p.W1989R/Day 21 + Li n = 20). (c) Representative traces of sIPSC plots (a) and (b). (d) Quantification of mIPSC frequency (Hz) and amplitude (e) of WT (dark gray circles), p.W1989R (red circles), and *Ank3* p.W1989R after chronic lithium treatment 19-21 days (blue circles). Ordinary one-way ANOVA, Tukey's multiple comparisons tests: ***P < 0.001, and P = ns. (WT: n = 9; p.W1989R: n = 8; p.W1989R/Day 19 + Li: n = 10; p.W1989R/Day 20 + Li: n = 11 and p.W1989R/Day 21 + Li n = 17). (f) Representative traces of mIPSC in (d) and (e).

reached plasma lithium levels of: 1.07±0.039 mmol/L after treatment, concentrations similar to those reached in patients under lithium therapy [30,52]. Lithium-treated *Ank3* p.W1989R mice showed higher spontaneous inhibitory postsynaptic current (sIPSC) frequency compared to vehicle-treated *Ank3* p.W1989R mice (Fig. 1a). Lithium therapy did not impact sIPSC amplitude, compared to *Ank3* p.W1989R mice without treatment (Fig. 1b and C). To evaluate AP-independent GABAergic currents, we measured the miniature inhibitory postsynaptic currents (mIPSCs). The frequency and amplitude of the mIPSCs in *Ank3* p.W1989R and *Ank3* p.W1989R after lithium treatment mice were lower than in WT littermates (Fig. 1d-f), and no significant differences were observed in mIPSC amplitude or frequency in *Ank3* p.W1989R mice after lithium treatment compared with vehicle (Fig. 1d-e). WT mice under treatment did not exhibit any significant variations in the amplitude or frequency of IPSCs (Fig.S1). Furthermore, there was no significant difference in the frequency of inhibitory postsynaptic events between the WT and *Ank3* p.W1989R animals that received lithium treatment (Fig.S2). Also, we did not observe any sex-related differences in inhibitory transmission following lithium therapy (Fig.S3). These findings suggest that chronic lithium therapy increases GABAergic neurotransmission *Ank3* p.W1989R mice, while not affecting the amplitude of postsynaptic currents.

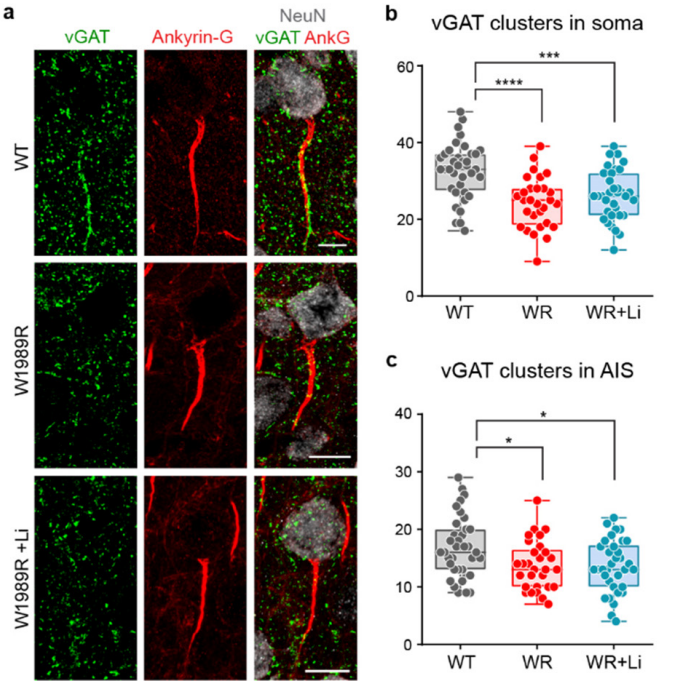

Figure 2. Lithium treatment does not increase the soma or AIS GABAergic synapse clusters in *Ank3* p.W1989R mice.
(a) Representative images of GABAergic synapses on pyramidal neurons in layer II/III somatosensory cortex of wild type (WT), *Ank3* p.W1989R (WR), and *Ank3* p.W1989R mice following lithium treatment (WR+Li). Coronal brain sections immunostained with a presynaptic GABAergic marker vGAT (green), total ankyrin-G (red), and NeuN (white). Scale bars: 10 µm. (b) Quantification of the total number of vGAT-positive clusters per soma above a set intensity threshold from WT (black), WR (red), and WR+Li (blue). (c) Quantification of AIS vGAT-positive clusters from WT (black), WR (red), and WR+Li (blue). One-way ANOVA: ****P < 0.0001, ***P = 0.0004 and P = ns (WT: n=37; WR: n=29; WR+Li: n=34).

Lithium therapy in *Ank3* p.W1989R mice does not affect the density of vGAT puncta on the pyramidal neuron soma or AIS.

Parvalbumin-positive GABAergic neurons synapse onto to the soma and AIS of pyramidal neurons [53,54]. Previously we showed that GABA synaptic clusters are decreased on the somatodendritic domain and AIS of cortical pyramidal neurons in *Ank3* p.W1989R mice [28]. To determine the effect that lithium therapy has on the number of GABAergic synapses, we carried out immunohistochemistry experiments on coronal brain sections taken from adult WT, *Ank3* p.W1989R, and lithium-treated *Ank3* p.W1989R mice. We used vesicular GABA transporter (vGAT) as a presynaptic indicator of GABAergic synapses onto the AIS and soma of pyramidal neurons, identified by ankyrin-G and NeuN labeling, respectively (Fig. 2). As reported previously, *Ank3* p.W1989R mice exhibited a reduction in the number of GABAergic synapse clusters on the somatodendritic domain and AIS of cortical pyramidal neurons compared to WT [28]. Lithium treatment failed to reverse the deficit in inhibitory synapse density on the AIS and soma (Fig. 2a-c). These results imply that lithium treatment does not involve changes in GABAergic synaptic density.

Tideglusib administration increases GABAergic transmission in *Ank3* p.W1989R mice.

Inhibition of GSK-3 is a key physiological mechanism driving lithium's therapeutic effects [55]. Tideglusib, an approved human CNS-permeant GSK-3β inhibitor [48-51] enhances mouse hippocampal development and memory with just 20 days of treatment. [48]. Tideglusib treatment for 20 days in *Ank3* p.W989R mice elevated sIPSC and mIPSC frequencies in layer II/III pyramidal neurons compared to vehicle-treated controls. (Fig. 4 a, d). By contrast, IPSC amplitudes remained unchanged between the two groups (Fig. 4b, c, e, and f). This shows Tideglusib, akin to lithium, boosts presynaptic GABAergic neurotransmission.

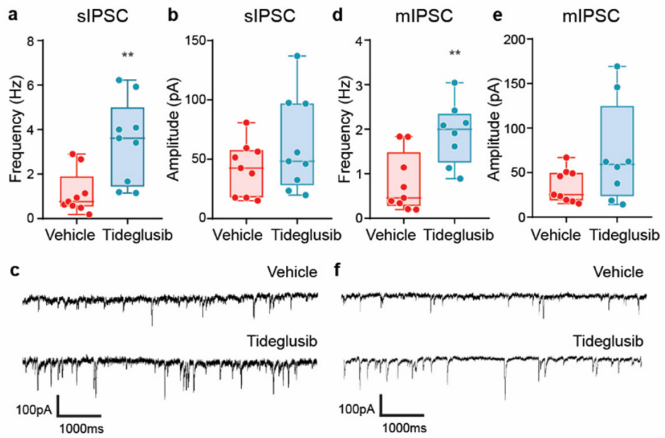

Figure 3. Chronic Tideglusib treatment increases sIPSC and mIPSC frequency while not affecting current amplitude in *Ank3* p.W1989R mice.
(a) Quantification of sIPSC frequency (Hz) and (b) amplitude (pA) from cortical neurons from brain slices of *Ank3* p.W1989R (red circles), and *Ank3* p.W1989R with Tideglusib treatment (blue circles). Unpaired t-test **P < 0.005 (W1989R + vehicle: n = 9; andp.W1989R + Tideglusib n = 9). (c) Representative traces of sIPSC in (a) and (b). (d) Quantification of mIPSC frequency (Hz) and (e) amplitude (pA) from cortical neurons from brain slices of *Ank3* p.W1989R (red circles), and *Ank3* p.W1989R chronic Tideglusib treatment (blue circles). Unpaired t-test **P < 0.005 (W1989R + vehicle: n = 9; andp.W1989R + Tideglusib n = 9) (f) Representative traces of mIPSC plots (d) and (e).

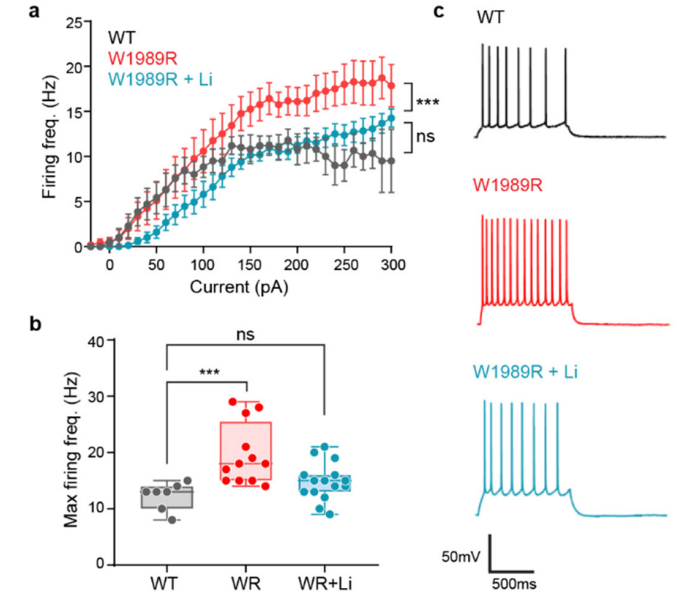

Figure 4. Pyramidal neuron firing frequency is restored in *Ank3* p.W1989R mice following chronic lithium treatment.
(a) Average firing rate (Hz) vs. current injection (pA) from evoked APs in cortical neurons from WT (dark gray circles), *Ank3* p.W1989R (red circles), and *Ank3* p.W1989R after lithium treatment (blue circles). Two-way ANOVA, Tukey's multiple comparisons tests: WT vs.p.W1989R ***P < 0.001, WT vs.p.W1989R + Lithium P = ns, and p.W1989R vs. p.W1989R + Lithium ***P < 0.001. (b) Maximum firing frequency (Hz) graph of pyramidal neurons from WT (dark gray circles), *Ank3* p.W1989R (red circles), and *Ank3* p.W1989R after lithium treatment (blue circles). Kruskal-Wallis, Dunn's multiple comparisons tests: WT vs. p.W1989R ***P < 0.001, WT vs. p.W1989R + Lithium P= ns, p.W1989R + Lithium vs p.W1989R P < 0.05 (WT: n=7; p.W1989R: n=12; p.W1989R + Li: n=15). (c) APs generated by current injection in WT (dark gray), *Ank3* p.W1989R (red), and *Ank3* p.W1989R after lithium

Acute exposure to lithium does not affect inhibitory postsynaptic currents in *Ank3* p.W1989R pyramidal neurons.

Prior research indicates lithium alters CNS neurotransmission in both chronic and acute treatment [56-59]. These findings led us to examine acute lithium's impact on brain slice GABAergic neurotransmission. Electrophysiological recordings of sIPSCs were conducted with a therapeutic 0.8 mmol/L lithium concentration acutely applied in the perfusion bath. However, there was no observed effect on sIPSC frequency or amplitude during the 8-minute acute lithium application, as shown in

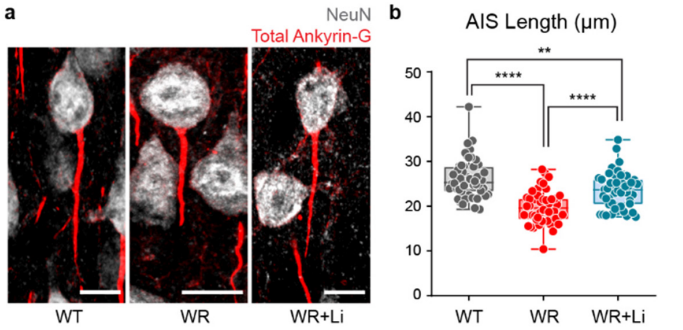

Figure 5. Lithium treatment increases the AIS length in *Ank3* p.W1989R mice.
(a) Representative images of pyramidal neuron AIS length from layer II/III somatosensory cortex of wild type (WT), *Ank3* p.W1989R (WR), and *Ank3* p.W1989R following lithium treatment (WR+Li). Immunostaining for total ankyrin-G (red) and NeuN (white). Scale bars: 10 µm. (b) Quantification of AIS length between WT (black), WR (red), and WR+Li (blue). One-way ANOVA: ****P < 0.0001, ****P < 0.0001 and **P = 0.0033 (WT: n=55; WR: n=48; WR+Li: n=48).

Figure S4a-b, when compared to the control condition. These findings show that acute lithium administration in at a physiological concentration does not affect presynaptic inhibitory transmission.

Chronic lithium treatment restores pyramidal cell firing frequency deficits in *Ank3* p. p.W1989R mice.

To assess lithium's effect on pyramidal neuron excitability in *Ank3* p.W1989R mice, we compared the firing rates of evoked action potentials in somatosensory cortical slices from WT and *Ank3* p.W1989R mice. In line with our prior findings [28], *Ank3* p.W1989R mice displayed significantly increased AP firing frequencies in response to escalating current injections when compared to WT mice (Fig. 4a). Furthermore, the maximum firing rate of *Ank3* p.W1989R neurons was significantly higher compared to WT littermates (Fig. 4b). Lithium-treated *Ank3* p.W1989R mice showed attenuation in the maximum firing frequency in pyramidal neurons compared to *Ank3* p.W1989R untreated mice (Fig. 4b and c). The neuron firing rate at different current injections, as well as the maximum firing frequency, were not significantly different between *Ank3* p.W1989R lithium-treated mice and WT (Fig. 4b-c). Cell membrane and AP properties were not affected after treatment (Fig.S5). However, the threshold of the first AP generated was significantly more depolarized in lithium-treated *Ank3* p.W1989R mice compared with WT mice, but not when compared with their untreated counterparts. These data demonstrated that neuronal excitability in layer II/III pyramidal neurons of *Ank3* p.W1989R mice is reduced by chronic lithium therapy.

Chronic lithium treatment partially restores AIS length deficits in *Ank3* p.W1989R mice.

The morphology and position of the AIS, is modified in response to changes in the strength and frequency of neuronal signals in order to maintain homeostasis and stabilize network activity [60]. We previously reported shortening of the AIS in *Ank3* p.W1989R, likely resulting from homeostatic compensation [28]. Using immunostaining assays for the scaffolding protein ankyrin-G, we measured the AIS length of layer II/III cortical neurons in coronal brain sections in WT, *Ank3* p.W1989R, and *Ank3* p.W1989R lithium-treated mice. Interestingly, the length of AIS in the *Ank3* p.W1989R variant significantly increased after lithium therapy; nevertheless, it remained shorter than the WT (Fig. 5 a-b).

Lithium treatment does not affect the individual AP properties in pyramidal neurons from *Ank3* p.W1989R mice.

To assess lithium's impact on single APs in pyramidal neurons, we analyzed the electrophysiological membrane properties of somatosensory cortex layer II/III neurons in the three experimental groups. No significant differences were found between WT lithium-treated and untreated *Ank3* p.W1989R littermates in single AP characteristics, including threshold, amplitude, half-width, membrane resistance, depolarization, and repolarization velocities (Fig. S5a). Lithium also had no significant impact on the resting membrane potential or rheobase in *Ank3* p.W1989R pyramidal neurons (Fig. S5b). These results indicate that lithium treatment does not alter membrane properties affecting individual APs in *Ank3* p.W1989R pyramidal neurons.

DISCUSSION

Numerous studies have consistently revealed that *ANK3*, the gene associated with bipolar disorder (BD), poses a high genetic risk for this condition [14-21]. Despite its limitations and potential side effects, lithium stands as a primary choice for preventing and managing BD symptom [38,39]. Just as lithium has proven its efficacy in treating BD [38-41], it has also shown effectiveness in reducing anxiety-like behaviors in experimental models lacking ankyrin-G [25,61,62]. These findings help us to understand the role of ankyrin-G in the development of psychiatric diseases and shed light on the therapeutic mechanisms of lithium, which are still being elucidated. Here we report that several neurophysiological deficits in *Ank3* p.W1989R mice are reversed by lithium monotherapy. The *Ank3* p.W1989R mouse suffers from a deficit in inhibitory synaptic density, as well as a reduction in GABA-dependent postsynaptic currents and an increase in the excitability of pyramidal neurons [28]. After chronic lithium treatment, we observed plasma concentrations in mice that were comparable to those typically seen in patients undergoing lithium therapy [30,52]. This treatment increases GABAergic neurotransmission, as is also seen with treatment using the selective GSK-3β inhibitor Tideglusib [48-51], suggesting that inhibitory neurotransmission is restored by lithium therapy through the same signaling pathway. Interestingly, following lithium therapy, an elongation of the AIS length in cortical neurons was observed in *Ank3* p.W1989R mice compared to untreated *Ank3* p.W1989R mice [65]. This compensatory neuronal plastic adjustment is consistent with the decrease in pyramidal neuron excitability observed after lithium therapy in *Ank3* p.W1989R mice. Such results align with reports where chronic treatment with lithium reverses hyperexcitability in neurons derived from patients who also responded to lithium treatment [63].

GABAergic interneurons influence the excitability of pyramidal neurons by synapsing onto their soma and AIS [53,54]. Deficits in GABAergic signaling can harm cerebral cortical neurons, impacting cortical function and neuronal excitability [31-35,64], while reductions in GABAergic markers in BD suggest that GABA dysfunction is a broader susceptibility factor for illness [28,65,66]. In *Ank3* p.W1989R mice, forebrain microcircuits exhibit an imbalance due to inadequate GABAergic signaling, and a patient with the same mutation reported fewer symptoms following lithium therapy [28]. The recovery of inhibitory postsynaptic currents by lithium treatment could be associated with an increase in presynaptic GABA release or the restoration of the number of GABAergic terminals. However, no change in the number of presynaptic vGAT clusters was observed in *Ank3* p.W1989R mice, suggesting that the restoration of inhibitory synaptic density is not occurring. Prior reports have shown that lithium treatment increases GABA levels in CNS in several experimental contexts [67-71]. Notably, lithium treatment increased sIPSC frequency in *Ank3* p.W1989R mice without affecting inhibitory current amplitude, indicating a restoration of GABAergic neurotransmission through elevated inhibitory presynaptic activity. There was no significant effect on mIPSC frequency or amplitude, aligning with the lack of impact on GABAergic terminal density. These findings suggest that lithium therapy in *Ank3* p.W1989R mice primarily boosts spontaneous presynaptic GABA release, without altering postsynaptic inhibitory receptors. These results support previous findings where lithium therapy did not affect $GABA_A$ receptors in the cortex [72,73]. Consequently, changes induced by lithium therapy appear closely tied to neurotransmitter release levels, a concept proposed previously [59,73].

The modulation of neuronal excitability, neurotransmitter release, and synaptic plasticity is influenced by the enzymatic activity of GSK-3 [55,74,75], which can be inhibited by lithium [44-47,76-83]. Interestingly, lithium treatment has been compared to other GSK-3 inhibitors, such as BRD0320 reestablishing homeostatic plasticity in neocortical pyramidal neurons [83], and CHIR99021 rescuing dendrite and spine density defects [84]. Here, we demonstrate that direct GSK-3β inhibition by chronic Tideglusib treatment increased GABAergic transmission comparable to lithium treatment. Similarly, inhibition of GSK-3 activity restores GABAergic signaling in corticolimbic interneurons [85,86]. Based on this evidence, treatment with lithium and Tideglusib appears to correct the deficiency in GABAergic neurotransmission by inhibiting GSK-3β and enhancing the inhibitory signaling.

Lithium treatment has varied effects on neuronal function, dependent on cell type and duration of treatment. Acute lithium administration has been shown to impact synaptic transmission using supraphysiological lithium concentrations (3-20mM) [57,87]. However, here we demonstrate that acute lithium exposure at therapeutic plasma levels did not yield significant alterations in GABAergic transmission in *Ank3* p.W1989R mice. This implies that chronic lithium exposure is necessary for neuronal activity modulation, rescue of synaptic dysfunction, restoration of long-term neuronal plasticity and neurotransmitter signaling [44-47,73,83,84,88,89]. The response to lithium treatment depends on exposure duration and the underlying phenotype [63,83], as indicated by varied effects in WT and *Ank3*-deficient mice [25,90]. Lithium's effects on proteins linked to neuronal plasticity, axonal transport, and neurotransmission vary between WT and *Ank3*-deficient mice, highlighting phenotype-dependent outcomes [25]. Interestingly, in another experimental model with Shank3 deficiency, lithium therapy can reverse overgrooming behavior and restore synaptic scaling while having no impact in WT conditions [83]. In this study chronic lithium treatment in *Ank3* p.W1989R mice positively impacts GABAergic neurotransmission, whereas WT mice show no significant differences. These findings suggest that the response to lithium therapy exhibits variability based on phenotypic or experimental model differences, possibly influenced by changes in *Ank3* expression, which could contribute to variable responses in BD patients [25,63,90]. This aligns with stem cell research, indicating that cells from lithium-responsive patients show reduced neuronal hyperactivity after lithium treatment, unlike cells from non-responsive patients [63].

As we show here, lithium therapy has positive effects by modulating neuronal excitability [63,83]. However, chronic application of lithium does not affect AP properties in stem cell cultures from patients suffering from BD [63] or in passive neuronal properties from layer II/III pyramidal neurons [83]. This is consistent with our observations where lithium treatment does not exert a significant influence on the individual AP characteristics of pyramidal neurons within brain slices of *Ank3* p.W1989R mice. A slight elevation in the threshold was observed in *Ank3* p.W1989R mice subjected to lithium treatment in contrast to their WT counterparts. However, this variation may be attributed to the inherent variability in response to lithium treatment, influenced by genotype.

Prior research consistently suggests that lithium treatment maintains neuronal excitability balance, achieving equilibrium in brain circuits through diverse mechanisms in various experimental models [25,59,83,89-93]. Lithium's influence on excitatory neurotransmission involves reducing the amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs), leading to synaptic downscaling [89]. Given the association of altered glutamatergic transmission and hyperactivity with BD pathology [94,95], these findings suggest that lithium effectively mitigates neuronal hyperexcitability by reducing excitatory neurotransmission. By contrast, lithium treatment enhances the amplitude of AMPAR-mediated mEPSCs, restoring synaptic scaling and reestablishing homeostatic plasticity in Shank3-deficient neurons. Such modulation of neuronal excitability is facilitated through GSK-3 inhibition [83]. Also, lithium and GSK-3β inhibitors enhances the surface expression of AMPA receptors, resulting in improvements in mania and depression-related behaviors [93]. Interestingly, in *Ank3*-deficient models undergoing lithium treatment, the effects on AMPA receptors mediated by GSK-3 inhibition are quite similar. *Ank3* haploinsufficiency, characterized by impaired kinesin-related axonal transport and glutamate signaling, is effectively mitigated by lithium treatment, which boosts kinesin-based transport and increases AMPA receptor availability [25]. Additionally, lithium and CHIR99021, a selective GSK-3β inhibitor, restores dendrite and spine density defects in AnkG-deficient neurons by GSK-3β signaling pathway [90]. This suggests that dendritic abnormalities observed in loss-of-function *ANK3* variants and BD patients may be alleviated by lithium treatment, however this study does not investigate its potential impact on neuronal excitability. Conversely, chronic exposure to lithium reduces intracellular calcium flux through mGlu5 receptor activation, resulting in reduced neuronal excitability, inhibition of GSK-3 activity, and alterations in synaptic function [59]. Overall, these studies are consistent with our hypothesis that chronic lithium treatment exerts its positive effects on neuronal modulation through GSK-3-β inhibition. Therefore, lithium therapy precisely fine-tunes neuronal excitability by simultaneously regulating both glutamatergic and GABAergic transmission, ultimately stabilizing intraneuronal signaling within neural networks. Essentially, lithium therapy re-establishes the

balance between excitatory and inhibitory processes, thus favorably shifting cells toward a state of normal neuronal excitability.

In summary, alterations in the BD-associated gene *ANK3* and its encoded protein, ankyrin-G, are implicated in the presentation of BD symptoms. Our findings demonstrate that chronic treatment with lithium or Tideglusib restores the deficit in GABAergic neurotransmission through GSK-3β inhibition, enhancing neuronal inhibitory transmission and reinstating pyramidal excitability. This study offers insights into the underlying pathophysiology of genetic variants in *ANK3* that confer susceptibility to BD. It also sheds light on how dysregulated functions in BD can be influenced by treatments aimed at alleviating their symptoms, providing valuable knowledge that may facilitate the development of more effective BD therapies, novel medications, and the selection of optimal treatment approaches.

MATERIALS AND METHODS

Animals

Ank3 p.W1989R heterozygous mice described previously [28], were crossed to generate respective genotypes and littermates were used for all experiments. Litters from the heterozygous breeders were weaned at postnatal day 21 (P21) and raised to P42-48. For mice that underwent lithium therapy, lithium chow was provided at P21, while control mice were fed with regular chow (vehicle). All experimental procedures were performed with the guidelines for animal care of the institutional animal care and use committee (IACUC), and university laboratory animal management (ULAM) at the University of Michigan.

Pharmacological treatments and measurement of plasma lithium levels

Littermates of both sexes were fed with 0.2% (g/kg chow) lithium carbonate diet (Harlan Teklad TD.170313/12) for 14 days followed by 7 days of 0.36% (g/kg chow) to achieve lithium therapeutic levels, as described previously [30]. After sacrifice the blood was collected in tissue plasminogen activator (TPA)/heparin (100 µL). Samples were sent to the University of Michigan clinical core to measure serum lithium concentration. For Tideglusib treatment; mice were administrated Tideglusib (Sigma-Aldrich, St. Louis, MO, USA) via subcutaneous injection every other day for 20 days (20 mg/kg body weight), or vehicle (corn oil; Sigma-Aldrich, St. Louis, MO, USA) [48].

***In vitro* electrophysiology recordings.**

Mice were anesthetized and sacrificed (by approved UM IACUC protocol PRO00010191). Then the brain was placed in 4°C solution containing (in mM) [1] 62.5 NaCl, 2.5 KCl, 1.25 KH_2PO_4, 26 $NaHCO_3$, 5 $MgCl_2$, 0.5 $CaCl_2$, 20 glucose and 100 sucrose (pH = 7.4 with O_2/CO_2, 95/5%, ~315mOsm). Coronal brain slices (~300µm thick) containing layers II/III cortex were cut with a microtome (VF-300, CompresstomeTM; Precisionary instruments, Natick MA). Brain slices were transferred to a chamber and maintained at room temperature in artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 2.5 KCl, 1.25 KH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, and 20 glucose, pH 7.4 and ~300 mOsm, for at least 1 hour before recording. Individual slices were transferred to a recording chamber perfused with ACSF. Recording micropipettes were pulled (P-97; Sutter Instruments, Novato, CA) from borosilicate glass capillaries (1.5 mm O.D.; Harvard Apparatus, Holliston, MA) for a final resistance of 5-7 MΩ and filled with solution containing (in mM) 135 K-Gluconate, 4 NaCl, 0.4 GTP, 2 Mg-ATP,0.5 $CaCl_2$, 5 EGTA and 10 HEPES, pH 7.25, ~290 mOsm. Signals were recorded with Axoclamp 700B amplifier (Axon Instruments, Union City, CA), low pass filtered at 10 kHz. Current clamp recordings were obtained from neurons in layers II/III of cortex; the cells were identified using a Nikon Eclipse FN-1 microscope with a 40X water-immersion objective and a DAGE-MTI IR-1000 video camera. The neurons were characterized electrophysiologically, by applying negative and positive current pulses of 10pA for 1000ms to calculate the rheobase and the maximum firing frequency response, and single positive pulses of 1ms to measure single AP properties. For GABAergic currents, the K-gluconate in the internal solution was replaced by CsCl, and the recordings were acquired at 2 kHz holding at -70mV. Spontaneous inhibitory postsynaptic currents (sIPSC) and miniature spontaneous inhibitory postsynaptic currents (mIPSC) were isolated pharmacologically with the AMPA/kainite receptor antagonist (CNQX,10µM) and NMDA receptor antagonist, (DL-AP-5, 100 µM). And for mIPSCs measurements, 1 µM tetrodotoxin (TTX) was added [28]. The frequency (Hz) and amplitude (pA) for synaptic events were analyzed automatically using Minianalysis software (Synaptosoft Inc.).

Immunocytochemistry of brain sections

After lithium treatment, were anesthetized using ketamine(80mg/kg)/xylazine (10mg/kg) mixture. Then sacrifice followed by cardiac perfusion was performed with PBS trailed by 4% paraformaldehyde fixation. Brains were removed and fixed overnight (4% paraformaldehyde). Next day, brains were processed using a standard single-day paraffin preparation protocol, including dehydration through ethanol and xylene. Paraffin sections were cut at 7µm thickness and placed on slides. Sections were deparaffinized and rehydrated through a protocol of washes. Antigen retrieval was performed by boiling the deparaffinized brain sections in 10µM sodium citrate in a microwave oven (20 minutes). Sections were then cooled, washed, and blocked with a blocking buffer (5% BSA, 0.2%, Tween 20 in PBS) for 1hr at room temperature. Primary antibodies were applied and incubated overnight (4°C) in a blocking buffer. Next day, slides were washed three times with PBS containing 0.2% Tween 20, incubated with secondary antibodies in blocking buffer for 1hr at room temperature, and washed three times. Finally, samples were mounted with Prolong Gold and imaged using a Zeiss LSM 880 with a 63X NA1.4 Oil/DIC Plan-Apochromat objective at 488, 561, and 647 nm lasers. AIS length and GABAergic synapse number were measured using maximum intensity projections of z-stacks. Only AISs entirely contained within the Z-stack bounds were quantified. The number of vGAT-positive puncta was quantified based on a consistent fluorescence intensity across all samples.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (Version 8.0) software. To calculate the significance of the differences between groups, Unpaired t-test, and one-way ANOVAs were applied, verifying that the results complied with the assumptions of normality and homogeneity of variances using the Shapiro-Wilk and Bartlett tests, respectively. For the cases where the Gaussian distribution criterion wasn't met, Kruskal-Wallis test with Dunn's post hoc was applied. When inequality of variances was detected, Welch's ANOVA was applied followed by the post hoc Dunnett's.

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AUTHOR CONTRIBUTIONS

RNFC and PMJ designed research. RNCF, AND, and PMJ performed experiments and analyzed data. LM performed the genotyping of the mice and participated in the editing and final design of the manuscript.

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CONFLICT OF INTEREST

The authors have nothing to disclose.

SUPPLEMENTARY MATERIALS

Figures S1 to S5

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